CLINICAL IMPLICATIONS OF QUANTITATIVE HEPATITIS B VIRUS DNA MEASUREMENTS

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KLINISCHE IMPLICATIES VAN KWANTITATIEVE HEPATITIS B VIRUS DNA METINGEN

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Voor Tim

Voor mijn ouders

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INTRODUCTION

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The hepatitis B virus (HBV) is a 42 nm viral particle and member of the hepadnaviridae family. The HBV genome is a relaxed circular, partially doublestranded DNA of approximately 3200 base pairs (1). Acute infection with HBV will resolve spontaneously in 90% of the adults; 10% become chronic carrier. Chronic HBV infection is defined as persistence of HBsAG for more than 6 months after the acute infection. Throughout the world, over 350 million people are chronic HBV carriers and complications of HBV infection leads to the death of around 1 million people each year (2). A known complication of chronic HBV infection is liver cirrhosis, which can lead to portal hypertension and liver failure. The second important complication is hepatocellular carcinoma which occurs in high association with HBV induced liver cirrhosis (1).

An important event in the natural history of chronic hepatitis B is the loss of HBeAg and seroconversion to anti-HBe. This HBeAg seroconversion is usually followed by normalization of serum transaminases and improvement of liver histology (3). Thus, HBeAg seroconversion usually represents a transition from chronic hepatitis B to an inactive HBsAg carrier state, in which there is little evidence of hepatitis and no or only low levels of HBV DNA in serum. This reflects also the major goal of antiviral therapy; to reach this status before advanced liver fibrosis or cirrhosis has occurred.

Not all patients who seroconvert have a sustained remission in disease. High levels of HBV DNA can be found in HBeAg negative patients due to the presence of mutations in the precore (G to A substitution at nucleotide 1896) and core promotor region (a dual mutation; $A_{1762}T$, $G_{1764}A$) (4).

Nowadays there are three registered treatment regimens against HBV: interferon-alpha (IFN-α), lamivudine and adefovir (2, 5, 6). Each treatment regiment has his limitations. The response rate to IFN-α in HBeAg-positive patients is approximately 30-40% and is sustained in over 70% of cases (7). Predictors of a positive response to IFN-α are elevated ALT levels and lower HBV DNA levels at baseline (6). However efficacy of IFN-α treatment is limited in HBeAg negative patients due to high number of post-treatment relapses. IFN-α treatment induced hepatitis flares may lead to decompensation in patients with cirrhosis, therefore only in highly selected cases this therapy should be initiated. Lamivudine has been shown to be effective in terms of HBV DNA suppression, normalization of transaminases and improvement in liver histology in both HBeAg positive and HBeAg negative patients (2, 8). However, treatment with lamivudine is associated with the development of YMDD variants. After 9 months of lamivudine therapy YMDD mutations emerge and this is associated with

the reappearance of HBV DNA and elevation of serum transaminases (9, 10). Adefovir provides effective antiviral therapy in both HBeAg positive and HBeAg negative patients (5, 11). It suppresses both wild-type and lamivudine-resistant HBV. Adefovir therapy was notable for the absence of major mutations in the HBV polymerase during treatment for up to 60 weeks (12). However, the durability of response after 72 weeks of therapy has not been studied extensively yet.

The development of amplification techniques, home-made and commercial has revolutionised the ability to detect the hepatitis B virus both qualitatively and quantitatively (13, 14). Potential applications of this new technology are described in table 1. The advent of DNA sequencing allowed the detection of precore mutants, YMDD mutants and the comparison of viral isolates on the genomic level (4, 15, 16). These new technologies enable the introduction of an individual patient disease management concept.

Table 1.

Potential application of HBV viral load measurements
Quantitative analysis
- assessment of viral replication in chronic HBsAg carriers
- to follow the course of a chronic infection in untreated patients to correlate biochemical activity to intermittent increase in viral replication
- determination of infectivity level
- selection of antiviral therapy: critical level 10^5 copies/ml
- to monitor treatment responses during antiviral therapy
- assessment of resistance to antiviral therapy (breakthrough HBV DNA)
Qualitative analysis
- determination of genotype
- detection of precore, core promoter mutants
- detection of nucleoside/nucleotide – resistant mutants
- genetic epidemiology

The objectives of the study were to describe the clinical implications of quantitative HBV DNA measurements. In this thesis we describe:

1. The predictive value of quantitative HBV DNA measurements in identifying non-responders to Interferon therapy (chapter 2.)
2. The use of quantitative HBV DNA measurements in monitoring antiviral therapy (chapter 2, 3, 4, 8).
3. The modelling of viral dynamics in response to antiviral therapy with the use of quantitative HBV DNA measurements (chapter 4).
4. The role of quantitative HBV DNA measurements in determining infectivity and the consequences for the management of chronic HBV infected Health Care Workers (chapter 5, 6).
5. The determination of HBV DNA levels in different body fluids and their potential infectivity (chapter 7).

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QUANTITATIVE HBV DNA LEVELS AS AN EARLY PREDICTOR OF NON-RESPONSE IN CHRONIC HBE-ANTIGEN POSITIVE HEPATITIS B PATIENTS TREATED WITH INTERFERON-ALPHA

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Submitted

Abstract

To reduce unnecessary exposure to treatment physicians must decide at an early stage whether continuation of treatment has a reasonable chance of success for the individual patient. The objectives of our study were to evaluate the previously described quantitative HBeAg measurements versus quantitative hepatitis B virus (HBV) DNA measurements for prediction of non-response and response in interferon (IFN)-α treated HBe-antigen positive chronic HBV patients.

Serum HBV DNA and HBeAg levels were assessed at baseline and weeks 8 and 12. For each test (HBV DNA level at baseline, HBV DNA decrease between baseline and weeks 8 and week 12, the combination of these two, HBeAg level at baseline, HBeAg decrease between baseline and weeks 8 and 12, and the combination of these two), we calculated the positive predictive value, negative predictive value, sensitivity and specificity.

Monitoring with quantitative HBV DNA levels (area under ROC 0.87) was superior to monitoring with quantitative HBeAg levels (0.76, $p<0.05$). Step-wise logistic regression identified HBV DNA at baseline and decrease in HBV DNA as independent predictors of response. The overall test performance of predicting non-response (predictive value 100%) was best for log HBV DNA testing at week 12 compared with testing at week 8 due to a better prediction of sustained response (SR) (46% versus 38%) and lower misidentification of non-response (NR) (39% versus 54%).

This study showed that quantitative HBV DNA testing at baseline in combination with a decrease between baseline and week 12 has a high predictive value for identifying patients who have virtually no chance of reaching a sustained response with IFN therapy.

Introduction

Interferon-alpha (IFN-α) is a powerful immune stimulatory drug used for the treatment of chronic hepatitis B patients. IFN-α treatment increases the spontaneous response rate and leads to loss of HBeAg in 15-40% of patients (1, 2). The most important predictive factors known for response to IFN-α are low baseline HBV DNA levels and elevated ALT levels (2) (3, 4). The most important adverse effects of treatment with IFN-α are flu-like syndrome with fever and myalgia, fatigue, asthenia, anorexia, depression and disturbed concentration (3).

To reduce unnecessary exposure to treatment and its potential side effects as well as to reduce costs, guidelines must be found that indicate at an early stage whether continuation of treatment has a reasonable chance of success for the individual patient.

We initiated a large-scale prospective, randomized controlled trial to investigate the efficacy of prolonging treatment for an additional 16 weeks for those patients who did not respond with HBeAg seroconversion during a standard 16-week IFN-α treatment course (3). A low level of HBV DNA (< 10 pg/mL) at randomization was found to be the only independent predictor of response (52% versus 0% P <0.001) for prolonged therapy, while a low HBV DNA level at entry tended toward significance (p=0.07). However, the HBV DNA assay used at that time , i.e. hybridization in solution (Genostics, Abbott Laboratories), has a lower limit of detection of 10^7-10^8 copies per milliliter. Meanwhile, more sensitive quantitative virology measurements have become available (5). The precise quantitative measurement of HBV DNA levels could further optimize response prediction by indicating an approximating response in the event of continuously decreasing serum HBV DNA.

Quantitative assessment of HBeAg in pretreatment serum and during therapy is considered an option in literature as an alternative for monitoring chronic HBV patients during therapy and has been used for the prediction of response of patients treated with IFN-α (6, 7). Since the availability of validated real-time-based quantitative polymerase chain reaction assays for the measurement of HBV DNA in serum, it is as yet not known which test should be used to monitor patients during treatment.

Early quantitative hepatitis C RNA measurements have been shown to predict sustained virologic response in chronic hepatitis C patients (8, 9). The current National Institute of Health Consensus limit is defined as 12 weeks of treatment with peginterferon-alpha and ribavirin. Chronic hepatitis C patients who fail to drop 2 log in viral load at week 12 should not be treated further after the 12 weeks (10). The question arose whether quantitative HBV DNA measurements at week 12 could be used to predict non-response for chronic hepatitis B

patients treated with IFN-α. Clinically our most relevant goals are a high predictive value for non-response without exclusion of potential sustained responders.

The objectives of our study were first to evaluate quantitative HBeAg measurements versus quantitative HBV DNA measurements for prediction of non-response and response for IFN-α treated patients. Secondly, we evaluated the value of precise quantitative HBV DNA measurements for predicting non-response and response of IFN-α treated patients.

Patients and methods

Patients

For this study data on patients treated in a large randomized controlled trial performed in 16 European centers (EUROHEP) were used (3). In this trial a standard 16-week treatment protocol (10 million units, three times a week) was compared with prolonged treatment for 32 weeks (10 million units, three times a week) All patients who were still HBeAg-positive at week 16 were randomized to undergo either no further treatment or prolonged treatment. For the present study, 29 patients were excluded for several reasons: discontinuation of therapy in an early phase (n=12) and insufficient serum (n=17). A total of 133 patients participated eventually in our study.

Virologic measurements:

HBV DNA assessments were performed routinely every 4 weeks during treatment and every 4-8 weeks during the follow-up period until week 52. If a week 0 sample was not available for assessment of HBV DNA at baseline, a sample from week –4 or week –8 was used. Quantitative HBeAg measurements were carried out using AxSYM HBe 2.0 Quantitative (Abbott Laboratories), as described previously (11). All HBV markers were assessed centrally.

Isolation of HBV DNA was performed using the MagnaPure LC isolation station (Roche Applied Science, Penzberg, Germany) with a modified protocol HBV-02 to perform a proteinase K digestion initially (5). The HBV DNA TaqMan assay, calibrated according to EUROHEP HBV DNA standards (12), was used for the quantitative measurement of HBV DNA in serum (5).

HBV genotyping was performed with a line probe assay (INNO-LiPA HBV DR; Innogenetics N.V., Ghent, Belgium). The INNO LiPA HBV DR assay was performed essentially as described previously (13). In case of a missing sample at baseline a sample taken at a timepoint close to baseline was used for genotyping.

Response criteria

For the definition of sustained response (SR) at week 52 we used the definition of virologic response recommended by the National Institute of Health workshop on chronic hepatitis B (14).

Sustained virologic response is defined as a loss of HBeAg, as indicated by AxSYM (<0.27 PEI U/ml; Abbott laboratories), together with a decrease in HBV DNA $<10^5$ copies/ml at week 52. All other patients were considered non-responders (NR).

The early virologic on-treatment responses were evaluated to determine their ability to predict response and non-response at week 52.

Statistical analyses

For each test (HBV DNA level at baseline, HBV DNA decrease between baseline and week 8 and week 12, the combination of these two, HBeAg level at baseline, HBeAg decrease between baseline and week 8 and 12, the combination of these two), we calculated the positive predictive value (%SR if the test is normal), its negative predictive value (%NR if test is abnormal), its sensitivity (%SR identified by test) and its specificity (%NR identified by test) using the 2 x 2 method. Because of its clinical relevance, we also calculated the reverse forms of the sensitivity and specificity i.e. the fraction of all SR not identified by the test and the fraction of NR not identified by the test. For all tests the areas under the ROC curves were calculated and compared according to the method described by Delong et al. (15). Backward stepwise logistic regression was used to determine the best set of independent predictors of non-response.

Chi-square testing was used for categorical variables. Statistical analysis of differences between groups was performed with the Mann-Whitney *U*-test. The Pearson correlation coefficient was calculated for log values of HBV DNA at 8 and 12 weeks. A *P*-value of <0.05 was considered significant. All calculations were performed with SPSS software (SPSS Inc, Chicago, IL USA).

Results

Demographic and clinical characteristics of patients are described in table 1.

Table 1. Patient characteristics at baseline and at time of randomization.

		All patients at baseline (n=133)	Randomized groups	
			Standard (n=52)	Prolonged (n=56)
Age (Years)*		34 (16-70)	32 (17-70)	34 (16-64)
Sex (Male:Female)		97:36	41:11	36:20
Race (%)	Caucasian	106 (80%)	42 (81%)	45 (80%)
	Asian	21 (16%)	5 (10%)	10 (18%)
	Other	6 (5%)	5 (10%)	1 (2%)
Cirrhosis (%)		21 (16%)	11 (21%)	7 (13%)
Log HBV DNA (copies/ml)*		8.7 (4.1-10.0)	9.1 (5.9-9.9)	8.8 (4.3-9.9)
AST in serum*#		1.5 (0.55-16.7)	1.2 (0.55-4.9)	1.5 (0.70-7.8)
Genotype (%)	A	61 (46%)	25 (48%)	23 (41%)
	B	6 (5%)	3 (6%)	2 (4%)
	C	16 (12%)	3 (6%)	9 (16%)
	D	42 (32%)	19 (37%)	18 (32%)
	other	8 (6%)	2 (4%)	4 (7%)

* Median (range)

 # Upper Limit of Normal (ULN)

Correlation between sustained response and pre-treatment serum HBV DNA levels, HBeAg levels and ALT levels

SR yielded significantly lower pre-treatment HBV DNA ($p<0.001$) and HBeAg levels ($p<0.001$) and significantly higher pre-treatment ALT levels ($p<0.05$) compared to NR.

Changes in serum HBV DNA levels in sustained responders versus non-responders

Retrospectively, SR yielded a significantly larger mean (standard deviation (=SD)) log decline in HBV DNA levels, 1.0 (SD 0.79), 1.7 (SD 0.98) and 2.5 (SD 1.38) at weeks 4, 8 and 12, respectively compared with NR, who showed a mean log HBV DNA decrease of 0.5 (SD 0.62), 0.8 (SD 0.89) and 1.0 (SD 1.27), respectively. $P<0.001$ for the change from baseline to

4, 8 and 12 weeks. Median log HBV DNA level at time of seroconversion to anti-HBe was 4.2 (range 2.8-7.6).

Correlation between decrease in HBV DNA and genotype

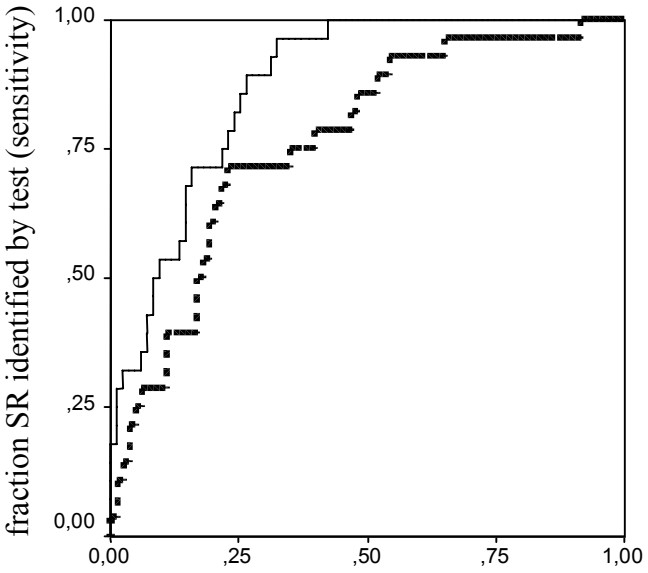
Of 133 patients 61 (46%) had genotype A, 42 (32%) genotype D, 16 (12%) genotype C and 6 (5%) genotype B. The study population consisted of 80% Caucasians, 16% Asians and 5% other. Caucasians were predominantly infected with genotype A (54%) or D (38%), Asians by B (24%) or C (57%). Chronic HBV patients infected with genotype A or D had a higher baseline serum HBV DNA (mean log HBV DNA level 8.6 (SD 1.0)) than chronic HBV patients infected with genotype B or C (mean log HBV DNA level 7.7 (SD 1.2), $p<0.01$. The decrease in log HBV DNA between baseline and week 16 for the different genotypes was 1.6 (SD 1.7) for genotype A, 1.2 (SD 1.4) for genotype D, 2.2 (SD 2.1) for genotype B and 2.3 (SD 1.8) for genotype C. If we compare the decrease in log HBV DNA in the first 16 weeks between genotype A and D (1.5 (SD 1.6)) and between B and C (2.3 (SD 1.8)) a significant stronger decline in log HBV DNA could be found for genotype B and C ($p<0.005$).

Quantitative HBeAg measurements versus quantitative HBV DNA measurements

The objectives of our study were first to evaluate quantitative HBeAg measurements versus quantitative HBV DNA measurements for the prediction of response and non-response in IFN-α treated patients. Receive operating characteristic (ROC) curves were used to compare the discriminative value between two diagnostics tests. Figure 1 shows the ROC curves for HBV DNA testing at baseline in combination with the HBV DNA decrease between baseline and week 12 and HBeAg testing at baseline in combination with the HBeAg decrease between baseline and week 12. ROC curves show the relationships per test between the chance of correctly identifying an eventual SR versus the chance of a false positive result. An optimal test would approach 100%, sensitivity 0% false positivity, while a test without discriminative value would only reach 100% sensitivity at 100% false positivity. Differences between curves were evaluated by comparing the area under the ROC curves. The area under the ROC curve was significantly higher for HBV DNA testing at baseline in combination with a HBV DNA decrease between baseline and week 12 (0.87) compared to HBeAg testing at baseline in combination with a HBeAg decrease between baseline and week 12 (0.76 $p<0.05$).

Figure 1 Value of HBV DNA at baseline in combination with HBV DNA decrease between baseline and week 12 (straight line) or HBeAg at baseline in combination with HBeAg decrease between baseline and week 12 (dotted line) for early discrimination between eventual sustained responders and non-responders.

The area under the ROC curve was significantly higher for HBV DNA at baseline in combination with HBV DNA decrease between baseline and week 12 than for HBeAg at baseline in combination with HBeAg decrease between baseline and week 12.



Prediction of response and non-response at week 12:

The second objective of our study was to evaluate the predictive value of precise quantitative HBV DNA measurements for the response and non-response of IFN-α treated patients.

The question was whether, as in chronic hepatitis C, quantitative measurements of HBV DNA could predict outcome of response. Stepwise logistic regression analysis identified HBV DNA at baseline and a decrease in HBV DNA as independent predictors of response. We therefore used the combination of these variables to develop a prediction model.

An ROC curve is a graph of the pairs of true positive (=sensitivity) and false positive rates (=1-specificity) that correspond to each possible cut-off for the diagnostic test result. Using the outcomes of the quantitative HBV DNA tests as end criteria for early treatment, the

clinically most relevant goals are a high predictive value for non-response and no exclusion of potential SR. We therefore selected the cut-off that maximized the true positive rate (=sensitivity of 100%) and used the corresponding HBV DNA value at baseline and HBV DNA decrease between baseline and week 12 to calculate the positive predictive value and the negative predictive value. Table 2 shows the results of this analysis. If patients had a log HBV DNA level at baseline above 8 in combination with a log decrease <1.0 log between baseline and week 12 the chance of non-response was 100%. If this test was used as a limiting criterion no SR would have been missed. If we compare this test with the test log HBV DNA at baseline above 9 in combination with 2.5 log decrease between baseline and week 12, the first test performance was better. This is due to a better prediction of SR (46% versus 38%) and lesser misidentification of NR (39% versus 54%).

Table 2. Predictive value, sensitivity and specificity of HBV DNA testing at baseline in combination with HBV DNA decrease between baseline and week 12.

Abnormal test	%NR if test is abnormal*	%SR if test is normal**	%NR not identified by test+	%SR not identified by test++	Odds ratio°	n
Log HBV DNA at baseline > 9 and < 2.5 log decrease between baseline and week 12	100%	38%	54%	0%	∞	112
Log HBV DNA at baseline > 8 and < 1.0 log decrease between baseline and week 12	100%	46%	39%	0%	∞	112

* Predictive value of an abnormal test for NR (treatment failure)

** Predictive value of a normal test for a sustained response (SR)

\+ 100% minus specificity (= %NR identified by test)

++ 100% minus sensitivity (= %SR identified by test)

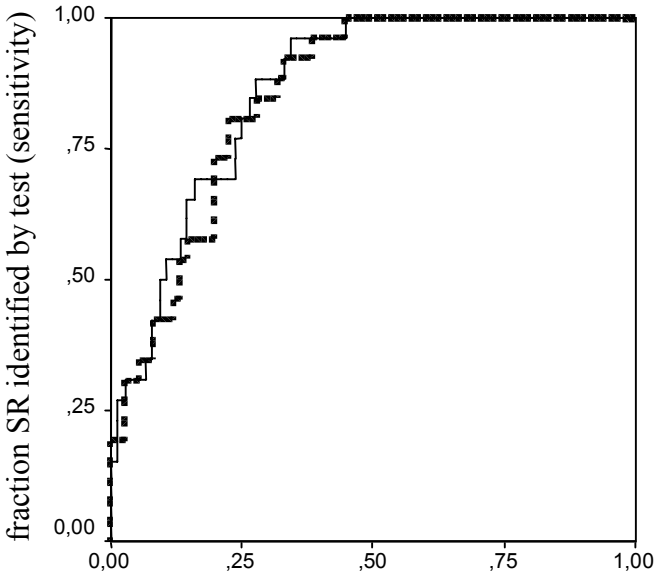
° Odds ratio: $p<0.001$

Prediction of non-response at week 8

We evaluated whether testing for HBV DNA before week 12 might lead to even greater benefits. The decrease in viral load 8 weeks after the first dose of IFN-α correlates with the decrease after 12 weeks of treatment. ($R=0.85$; $p<0.001$). Figure 2 shows the ROC curves for

log HBV DNA at baseline in combination with the HBV DNA decrease between baseline and week 12 versus log HBV DNA at baseline in combination with the log HBV DNA decrease between baseline and week 8. The areas under the ROC curves were not significantly different between the two tests (0.86 versus 0.85, p=0.60).

Figure 2 Value of HBV DNA at baseline in combination with HBV DNA decrease between baseline and week 12 (straight line) or HBV DNA at baseline in combination with HBV DNA decrease between baseline and week 8 (dotted line) for early discrimination between eventual sustained responders and non-responders.
The areas under the ROC curves were not significantly different between the two tests.
We calculated the positive predictive value and negative predictive value, using the values of log HBV DNA level at baseline and log HBV DNA decrease between baseline and week 8, which corresponded with the cut-off that maximized the true positive rate (=sensitivity of 100%) at week 8 (Table 3).



If we use the limit of log HBV DNA level at baseline above 8 in combination with a log decrease < 1 log between baseline and week 8, two out of 25 (7%) SR undergoing treatment would have been missed. The overall test performance of predicting non-response was best

for log HBV DNA testing at week 12 compared with testing at week 8 due to a better prediction of SR (46% versus 38%) and lesser misidentification of NR (39% versus 54%).
The predictive value of non-response does not change if we correct for duration of therapy. But we can improve the identification of NR by letting the cut off value depend on duration of therapy. For patients with standard therapy with a baseline log HBV DNA level >8 in combination with a viral load decrease between baseline and week 12 <2 log, the predictive value of non-response was 100% and only 33% of non-responders could not be identified. Prolonging therapy with 16 weeks in patients with standard therapy with a log HBV DNA baseline level >8 and viral load decrease between baseline and week 12 >1 but below 2 log would lead to response in 60% (3/5) of these patients.

Table 3. Predictive value, sensitivity and specificity of HBV DNA testing at baseline in combination with log HBV DNA decrease between baseline and week 8.

Abnormal test	%NR if test is abnormal*	%SR if test is normal**	%NR not identified by test+	%SR not identified by test++	Odds ratio°	n
Log HBV DNA at baseline > 9 and < 1.5 log decrease between baseline and week 8	100%	38%	54%	0%	∞	110
Log HBV DNA at baseline > 8 and < 1.0 log decrease between baseline and week 8	96%	43%	40%	7%	18.9	110

* Predictive value of an abnormal test for NR (treatment failure)

** Predictive value of a normal test for a sustained response (SR)

\+ 100% minus specificity (= %NR identified by test)

++ 100% minus sensitivity (= %SR identified by test)

° Odds ratio: $p<0.001$

Discussion

To reduce unnecessary exposure to treatment physicians must decide at an early stage whether continuation of treatment has a reasonable chance of success in the individual patient. The objectives of our study were to evaluate the assessment of quantitative HBeAg measurements versus the assessment of quantitative hepatitis B virus (HBV) DNA measurements for the use of prediction of non-response and response in IFN-α treated HBe-antigen positive chronic HBV patients.

Predictive factors for end of treatment response in chronic HBV patients treated with IFN-α have been studied in the past. In a randomised controlled trial of IFN-α, with or without prednisone priming, Perrillo et al. found that baseline serum HBV DNA level was the most important independent predictor of response (P=0.003) (2). Approximately 50% of the patients with baseline HBV DNA levels under 100 pg/ml (solution-hybridization assay,Abbott laboratories) responded to treatment with 5 million unites of IFN-α compared to only 7% of patients with HBV DNA levels at baseline above 200 pg/ml. A study investigating the post-treatment durability of HBeAg seroconversion following lamivudine, IFN monotherapy or IFN-lamivudine combination therapy also identified pre-treatment HBV DNA levels as the major predictor of sustained response (16). They also found a significant predictive value of pre-treatment ALT level for the durability of HBeAg seroconversion (higher baseline ALT – lower chance of relapse).

Midtreatment HBV DNA levels showed a significant correlation (P< 0.001) with response in Chinese adults with chronic HBV infection (17). Response was achieved in 53% of patients who had a HBV DNA level below 0.7 Meq/ml (branched DNA assay) at midtreatment, but in only 17% of those who remained HBV DNA positive. In a large prospective, randomized controlled trial investigating the efficacy of treatment prolongation with additional 16 weeks in those patients who did not respond with HBeAg seroconversion during a standard 16-week IFN-α course , a low level of HBV DNA (< 10 pg/mL) at randomization was found to be the only independent predictor of response (52% versus 0% P <0.001) during prolonged therapy, while a low HBV DNA level at entry tended toward significance (p=0.07) (3).

Early monitoring of HBeAg decrease during therapy with IFN-α showed that changes in the HBeAg level from the start of therapy to week 8 were significantly related with response at the end of follow up (6).

However, these known factors related to response are of limited value for the individual patient. This study has shown that quantitative HBV DNA measurements by validated real-

time pcr can be used for early identification of NR to IFN-α and that quantitative HBV DNA measurements by validated real-time pcr can be used for early identification of NR to IFN-α.

This study demonstrates that monitoring with sensitive quantitative HBV DNA measurements is superior to monitoring with quantitative HBeAg levels. Stepwise logistic regression identified HBV DNA at baseline and a decrease in HBV DNA as independent predictors of response; quantitative HBeAg levels did not add to the prognostic value. This is in contrast to earlier published studies where HBeAg levels in pre-treatment serum and the decrease in HBeAg level from the start of therapy to weeks 4 and 8 related significantly to response to therapy at week 16 (6). However, this study was performed using a HBV DNA assay based on hybridization in solution (Genostics, Abbott Laboratories), which generates a detection limit of only $10^7 – 10^8$ HBV copies per milliliter.

The overall test performance of predicting non-response was best for quantitative HBV DNA testing at baseline in combination with a decrease between baseline and week 12 compared with quantitative HBV DNA testing at baseline in combination with a decrease between baseline and week 8. We therefore suggest that a quantitative HBV DNA test at 12 weeks can be used as a management tool for the decision when to continue treatment and when to adjust it.

This study confirmed the significant relationship between lower pre-treatment HBV DNA levels and HBeAg levels and significantly higher pre-treatment ALT level with SR. This study shows that quantitative HBV DNA testing at baseline in combination with the decrease between baseline and week 12 has a high predictive value in identifying patients who have virtually no chance of reaching a sustained response with IFN-α therapy. Future research should explore whether this concept also holds for prolonged therapy (>/= 1 year), for PEG-interferon monotherapy and for patients who receive combination therapy of a nucleoside or nucleotide analogue and (PEG-) IFN.

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3

PERSISTENCE OF YMDD VARIANTS AFTER WITHDRAWAL OF LAMIVUDINE

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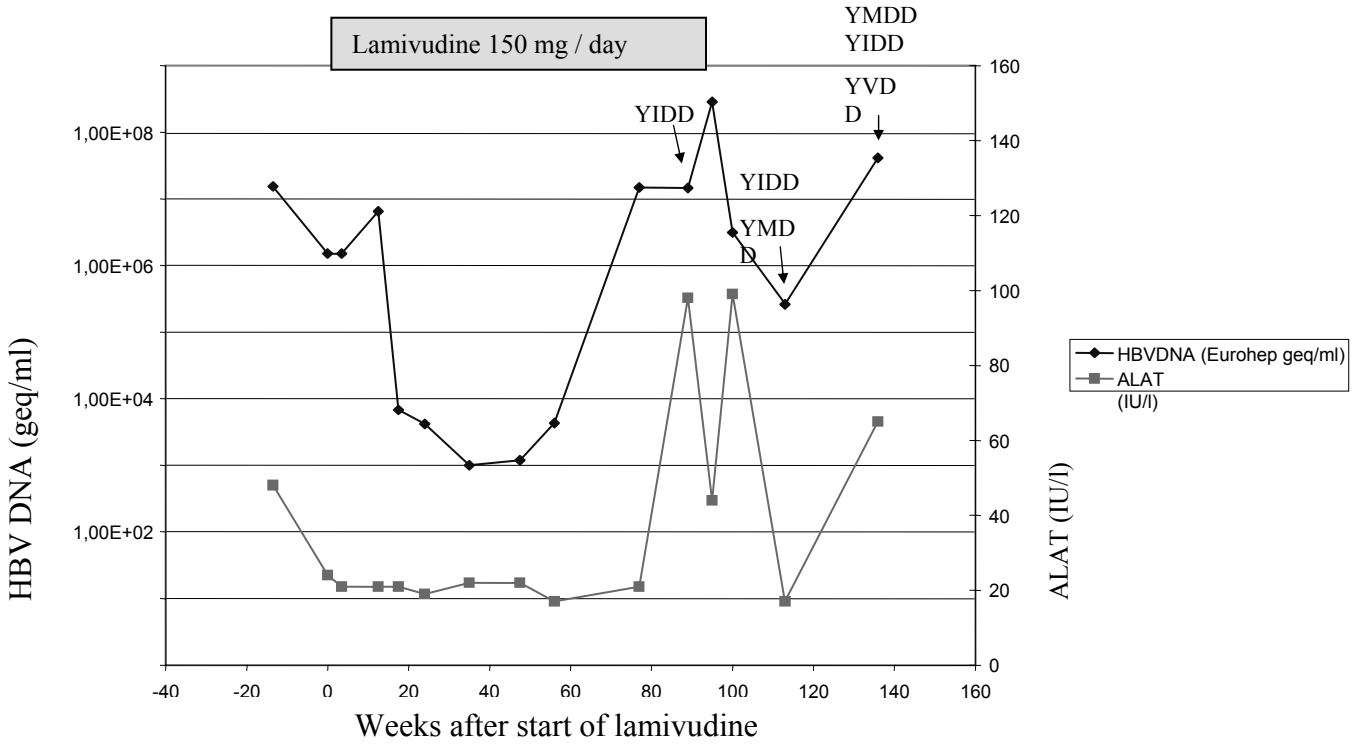
Lamivudine is a registered nucleoside analogue which has been found to suppress HBV replication. In chronic HBV carriers the decrease in serum HBV DNA – levels is followed by improvement in liver histology (1). The emergence of viral resistance however may compromise this. A 54-year-old woman with treatment naive chronic HBV infection (biopsy stage cirrhose, HBeAg negative, HBVDNA 1.53 E7 geq/ml (Digene Hybrid Capture II assay), ALT 48 IU/l (normal <31 IU/l) was treated with Lamivudine therapy for 95 weeks. Six weeks before therapy no variant viruses could be found. After 77 weeks of treatment HBV DNA-level levels increased 1.48 E7 geq/ml. Ten weeks later the ALT level increased to 106 IU/l. DNA sequence analyses were performed (Inno Lipa HBV- DR, Innogenetics Ghent Belgium) starting from week 89. Analyses of the YMDD motif of the HBV polymerase gene showed a methionine-to-isoleucine substitution; rtM204I (YIDD variant). After 95 weeks of treatment with lamivudine treatment was ceased. HBV DNA level at that time was 2.85 E8 geq/ml and ALT level 44 IU/l. Patient was closely monitored and HBV DNA, ALT and DNA sequence analyses were repeated. (Figure 1)

Figure 1: the emergence of YMDD variants during lamivudine therapy and persistence after withdrawal of therapy.



Although Lamivudine was withdrawn, while at week 18 post-dosing a mixture of wild type virus and YIDD variants were detected, at week 41 post-dosing a mixture of wild type-virus, YIDD and YVDD (rtM204V), was still detectable. Mutant detection dropped below the 5% range of the assay detection limit at week 54 post –dosing.

Viral resistance to Lamivudine with the emergence of YMDD variants during long-term Lamivudine therapy is well described and considered to be a problem for future optimal therapy (2-4). Also described is replacement of variant viruses by wild type virus 3 to 6 months after cessation of Lamivudine therapy (2, 3). This is however the first report of persistence of YMDD variants for at least 41 weeks. Our data strongly suggest that after withdrawal of Lamivudine it may take up to a year before the wild type virus replaces the treatment resistant population. This finding has immediate implications for the selection of optimal drug regimens in case of re-initiation in therapeutic studies, or eg. before and after liver transplantation.

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4

VIRAL DYNAMICS DURING TENOFOVIR THERAPY IN PATIENTS INFECTED WITH LAMIVUDINE-RESISTANT HEPATITIS B VIRUS MUTANTS

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Summary

Tenofovir, an anti-HIV drug, has activity against lamivudine-resistant Hepatitis B virus (HBV) mutants. To describe the efficacy of tenofovir in patients with lamivudine-resistant Hepatitis B we applied two investigative approaches based on mathematical models of viral dynamics: the individual non-linear fitting and the mixed-effect group fitting approaches.

Eleven chronic HBV patients on lamivudine for a median of 176 weeks (range: 72–382 weeks) with YMDD mutation-related HBV DNA breakthrough received "add-on" tenofovir 300 mg once-daily, while maintaining their existing therapy. Sequential sera were taken at day 1 (t=0 and t=8 hours), days 2, 4, 7, 10, 14, 21, 28 and every 4 weeks thereafter, and HBV DNA levels were assessed using a validated quantitative PCR assay.

Median baseline log HBV DNA was 8.62 (range 6.48–9.76 log HBV DNA). Tenofovir treatment resulted in a mean (±SD) log HBV DNA decline of 1.37±0.51 in the first phase, 2.54±0.91 after 4 weeks, and 4.95±0.90 log HBV DNA after 24 weeks. The median effectiveness of blocking viral replication in the individual fit model was 93% (range 73%–99%) for $\eta=0$ and 93% (range 59%–99%) for $\eta=1$. There was only a small difference between the efficacy parameter ε of the individual non-linear fitting and mixed effect group fitting on the bi-phasic exponential model.

These data show that tenofovir has good efficacy in blocking viral replication in HBV patients with lamivudine-induced drug-resistant HBV mutants, but effectiveness varies greatly among individuals. Both models can be used to describe viral decay during tenofovir therapy.

Introduction

Treatment of hepatitis B virus (HBV) infection with standard interferon alpha produces a durable response in one-fifth to one-third of patients but has undesirable side-effects and must be administered subcutaneously three times per week [1-3]. Although lamivudine treatment also produces a modest response rate with few side-effects, prolonged treatment is often necessary to prevent relapse on cessation of therapy, and continuous treatment can lead to the development of lamivudine resistance [4] [5]. Phenotypic lamivudine resistance, with the emergence of YMDD drug-resistant mutants in the polymerase gene of the hepatitis B virus (HBV), leads to an increase in serum HBV DNA levels. This suggests that there is a clinical need for new antiviral agents that adequately inhibit DNA-polymerase activity, both in wild-type and in mutant virus populations.

The search for drug-resistant mutants is usually initiated after an increase in serum HBV DNA load has been observed [6]. Studies have shown that the lamivudine mutations are localised in two major domains of the reverse transcriptase (rt) region of the polymerase gene [7, 8]. Analyses of the YMDD region of the C domain of the polymerase gene have shown that, in the case of resistance, methionine (rtM204) is replaced either by valine (rtM204V), isoleucine (rtM204I) or serine (rtM204S). The valine (rtM204V) variant is, in most cases, accompanied by another mutation (leucine to methionine; rtL180M) in the B domain [9]. A mixture of YMDD variants can exist in one individual.

Tenofovir disoproxil fumarate, an acyclic nucleotide analogue reverse transcriptase inhibitor, appears to be effective against the YMDD drug-resistant mutant population. In *in vitro* studies, tenofovir demonstrated a combination of low cytotoxicity and antiviral efficacy. It was equally effective at inhibiting wild-type HBV DNA replication and at inhibiting DNA replication in the YMDD variant rtM180V [10]. Clinical studies investigating the effect of tenofovir on HBV replication have shown that it has significant activity against lamivudine-resistant mutants both in chronic HBV patients and in human immunodeficiency virus (HIV)/HBV coinfected patients [11-17].

Mathematical modelling provides a tool for evaluating the effect of antiviral therapy. It can provide insight into the speed and variability in patterns of viral decay, which may be useful in the design of future treatment strategies. The decay curve of hepatitis B virus during therapy with nucleoside analogues exhibits a bi-phasic decline during the first four weeks of

treatment. Analysis of these viral kinetics can be used to calculate both the effectiveness of therapy in inhibiting viral production as well as the clearance of cells infected with hepatitis B virus. We have used two previously published models to describe viral decline during treatment in chronic hepatitis B patients and investigate the viral dynamics of hepatitis B virus replication after the addition of tenofovir to lamivudine therapy [18, 19].

Patients and methods

Patients

Eleven chronic hepatitis B patients (all liver-biopsy proven or HBsAg-positive for at least 6 months) with breakthrough HBV DNA on lamivudine therapy received tenofovir 300 mg once daily while maintaining their existing therapy, which included lamivudine. Five of these patients were coinfected with HIV.

Sequential sera, taken at day 1 (at t=0 and t=8 hours), days 2, 4, 7, 10, 14, 21, 28 and every 4 weeks thereafter, were quantitatively assessed for HBV DNA. The presence of YMDD mutants was determined at t=0 and t=28 days.

Virologic measurements

HBV DNA was isolated using the MagnaPure LC isolation station (Roche Applied Science, Penzberg, Germany) with a modified protocol HBV-02 in which the proteinase K digestion occurred first [20]. HBV DNA serum levels were quantitatively assessed using the HBV DNA TaqMan assay and calibrated using EUROHEP HBV DNA standards [21]. The Taqman assay enabled accurate quantitative determination to levels of 1000 copies per ml [20].

At days 1 and 28, HBV polymerase mutant analysis was performed on HBV DNA using a Line Probe assay (INNO-LiPA HBV DR; Innogenetics N.V., Ghent, Belgium) [22]. Where the INNO-LiPA assay was indeterminate, sequence analysis was used. A selected genome region of the polymerase gene was amplified and sequenced with particular primers described earlier [23].

Models for viral dynamics during the first 4 weeks of treatment

Mathematical modelling of viral decline was previously described by Neumann et al. [18] for hepatitis C and Nowak and Tsiang et al. [24] [25] for hepatitis B. We have used Neumann's bi-phasic-exponential model to describe the viral decay during the first 28 days of treatment in our patients:

$V(t)= V_0 \{A\exp [-\lambda_1 t] + (1-A) \exp [-\lambda_2 t]\}$, where

- V_0 = initial viral load
- λ_1 = slope of the first phase of viral decline
- λ_2 = slope of the second phase of viral decline
- A = $(\varepsilon c-\lambda_2)/(\lambda_1-\lambda_2)$
- $\lambda_{1,2}$ = $^1/_2\{(c+\delta)\pm[(c-\delta)^2+4(1-\varepsilon)(1-\eta)c\delta]^{1/2}\}$
- t = time
- δ = death rate of productively infected cells
- c = clearance rate of free virus
- ε = effectiveness of tenofovir in blocking virion production in infected cells
- η = effectiveness of tenofovir in blocking the *de novo* infection of uninfected cells

The first-phase decline reflects the clearance rate of free virus from plasma; the second-phase decline reflects the death rate of productively infected cells.

We used two different approaches to describe viral decay: individual non-linear fitting and mixed-effect group fitting. Mixed modelling implies a group-wise analysis while each patient retains his or her own subject-specific decline by introducing random effects on all parameters. All variables as well as all patient data are related; based on these data, group effects can be derived and compared. In the group fit approach, the random effect of λ_1 was set to zero, because of lack of variation between individuals. This indicates that λ_1 is stable and therefore justifies the choice of a fixed λ_1.

The non-linear modelling approach, which was used to fit the bi-phasic model, was conducted in the NLINMIX macro in SAS 8.02.

Neumann et al. [18] assumed that $\eta=0$ (there was no block of *de novo* infection of uninfected cells), while Tsiang [24] and Nowak et al. [25] assumed that $\eta=1$ (there was a complete block

of infection of uninfected cells). If $\eta=1$, then $\lambda_2=\delta$, and A reflects antiviral efficacy. We have explored both assumptions in both models (individual *versus* group fit) as a possibility and we report the mathematical efficacy for all four situations.

Models of viral dynamics during the first 24 weeks of treatment

Validated models of HBV viral kinetics are available only for the first 4 weeks of therapy and are unavailable for later viral kinetics. The viral kinetics patterns have been classified according to the definitions used by Neumann et al. for describing HBV DNA early kinetics in chronic hepatitis B patients treated with adefovir dipivoxil [26]. We modified these existing definitions to describe the first 24 weeks of kinetics, taking into account the availability of frequent quantitative HBV DNA measurements in the first four weeks of tenofovir treatment. First we investigated viral decay in the first week (first phase), then we examined decay in the following 23 weeks (second phase), which we further divided into two periods (up to 4 weeks, days 8–28 and up to 24 weeks, days 29–168).

Definitions of viral kinetic patterns in the first phase (day 1–7):

1. Rapid (R): decline of ≥ 1 log
2. Slow (S): decline between 0.5 and 1 log
3. Flat (F): decline of < 0.5 log

Definitions of viral kinetic patterns in the second phase (days 8–28 and 29–168):

1. Rapid: decline of > 1 log / 4 weeks
2. Slow: decline between 0.2 and 1 log / 4 weeks over 23 weeks
3. Flat: decline of < 0.2 log / 4 weeks over 23 weeks
4. Beyond Detection (bd): HBV DNA below the level of detection (< 1000 copies/ml)
5. Rebound (Rebound): a transient (only one time point) increase of > 1 log

The Wilcoxon Signed Rank test was used to assess change in log HBV DNA from baseline. Factors with a p-value < 0.05 were considered significant.

Results

Patient demographics

Eleven patients were evaluated for viral dynamics. In ten patients, viral decay was evaluated at the time points noted. In one patient, data were only available for the first ten days and then the patient was lost to follow up. Patient characteristics at baseline are described in table 1. Six patients were Asian and five were Caucasian.

Table 1. Patient characteristics at baseline.

Patient	Sex	Age (yrs)	Type of infection	Duration of lamivudine (weeks)	HBV DNA geq/ml	HBeAg status	YMDD variant	ALT IU/L
A	M	53	HIV/HBV	382	4.1×10^8	Positive	YVDD	165
B	M	39	HIV/HBV	282	5.8×10^9	Positive	YVDD	98
C	M	36	HIV/HBV	166	3.0×10^6	Positive	YVDD	53
D	M	40	HIV/HBV	91	4.8×10^9	Positive	YVDD	46
E	M	36	HIV/HBV	313	6.1×10^6	Positive	YVDD/YIDD	46
F	M	28	HBV	162	1.5×10^9	Positive	YVDD	37
G	M	26	HBV	72	4.1×10^8	Positive	YVDD	44
H	M	32	HBV	164	5.9×10^7	Negative	YVDD	781
I	M	41	HBV	274	4.2×10^8	Positive	YVDD	121
J	F	26	HBV	178	1.6×10^8	Positive	YSDD	14
K	F	26	HBV	176	4.3×10^7	Positive	YIDD	55

*Upper limit of normal for males = 41; upper limit of normal for females = 31.

HBV DNA levels

Mean (± SD) baseline log HBV DNA was 8.31 ± 1.07 (median 8.62; range 6.48–9.76 log HBV DNA). The use of tenofovir resulted in a mean log HBV DNA decline of 1.37 ± 0.51 in the first phase, 2.54 ± 0.91 (median 2.34; range 1.33–4.02 log HBV DNA) after 4 weeks of tenofovir treatment and a mean decline of 4.95 ± 0.90 log HBV DNA (median 5.05; range 3.64–5.94) after 24 weeks of treatment. The decline in HBV DNA was significant at the time points noted ($p=0.003$ for the change from baseline to the transition between the first and the second phase, $p=0.005$ for the change from baseline to 4 weeks and $p=0.005$ for the change from baseline to 24 weeks). In five patients, treatment achieved HBV DNA levels below the level of 1000 copies/ml. One patient had loss of HBeAg, without seroconversion to anti-HBe.

Lamivudine resistance and transaminase levels

HBV polymerase mutant analyses at day 28 showed the presence of baseline mutations in 9 patients; Patient F showed a mixed population of YVDD and YMDD variants. In one patient, the level of serum alanine aminotransferase (ALT) was > 1.1 Upper Limit Normal (ULN) after 24 weeks of treatment with tenofovir. In this patient, the ALT level after 24 weeks of treatment was higher than pretreatment ALT levels.

Safety and tolerability

Tenofovir disoproxil fumarate was generally well tolerated; none of the patients had abnormal renal function (data available for 10 patients) or phosphorous levels (n = 8).

Models of viral dynamics

Estimates of the parameters of efficacy, based on the bi-phasic model with individual non-linear fitting and mixed effect group fitting, are shown in table 2. The median effectiveness of blocking viral replication in the individual fit was 93% (range 73%–99%) for $\eta=0$ and 93% (range 59%–99%) for $\eta=1$. The half-life of free virus was 21.18 hours (median; range 16.23–47.34), the half-life of infected hepatocytes was 5.77 days (median; range 3.06–33.24) when assessed by the individual fit. Similarly, with the group fit, the half-life of free virus was 21.54 hours and the half-life of infected hepatocytes was 5.24 days.

On treatment with tenofovir, distinct patterns of response were observed. All patients showed a similar bi-phasic decline pattern in the first four weeks of treatment (Fig 1A.–1K). The combined data for the group fit for the data set clearly demonstrates bi-phasic decline pattern (Fig 2.).

Table 2. Parameter estimates based on the bi-phasic model with individual non-linear fitting and mixed-effect group fitting

	Individual fit median (range)	Group fit median (range subject specific fit)
Ln (initial viral load)	19.43 (15.25–22.50)	19.17 (15.65–22.87)
Clearance rate of free virus	0.79 (0.35–1.02)	0.76 (0.76–0.77)
δ (if η=0)	0.12 (0.02–0.23)	0.14 (0.073–0.22)
δ (if η=1)	0.11 (0.02–0.20)	0.13 (0.058–0.21)
ε (if η=0)	0.93 (0.73–0.99)	0.94 (0.81–0.97)
ε (if η=1)	0.92 (0.59–0.99)	0.91 (0.77–0.95)
Half-life (ln2/c)	21.18 hours (16.23–47.34)	21.54 hours (21.74–21.97)
Half-life (ln2/δ) (if η=0)	5.77 days (3.06–33.24)	5.24 days (3.16–9.52)

δ = death rate of productively infected cells

ε = effectiveness of tenofovir in blocking virion production in infected cells

η = effectiveness of tenofovir in blocking the *de novo* infection of uninfected cells

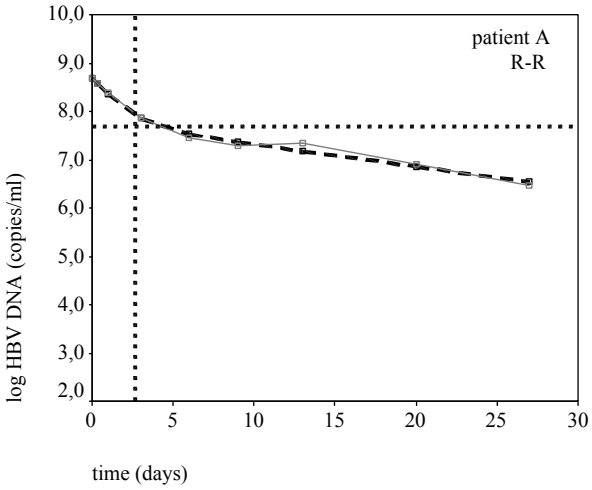
Figure 1 (A-K) Viral decline during the first 4 weeks of tenofovir therapy in 11 lamivudine-resistant patients. Each individual patient could be fitted using the bi-phasic model. The vertical straight dotted line represents the time of transition from the first to the second phase for each individual patient. When describing the different patterns of viral decay, the first week represents the first phase; the second phase begins at day 8.

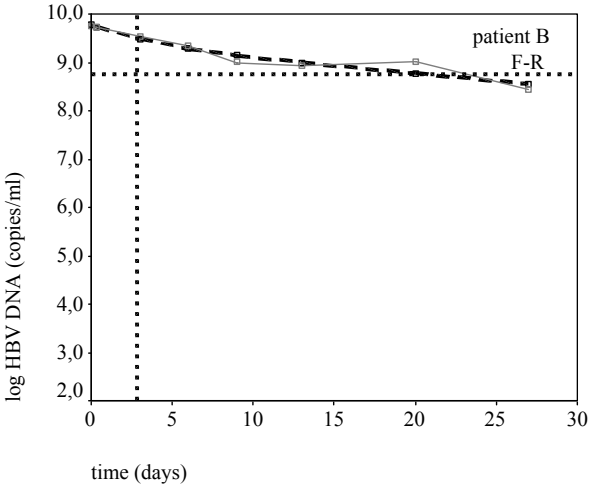
In this study, the first phase was categorised to one of the three patterns according to the rate of HBV DNA decline in the first seven days: rapid (R) with a decline of ≥ 1 log, slow (S) for a decline between 0.5 and 1 log, or flat (F) for a decline of < 0.5 log. The horizontal straight dotted line is placed 1 log below the initial viral load of the patient.

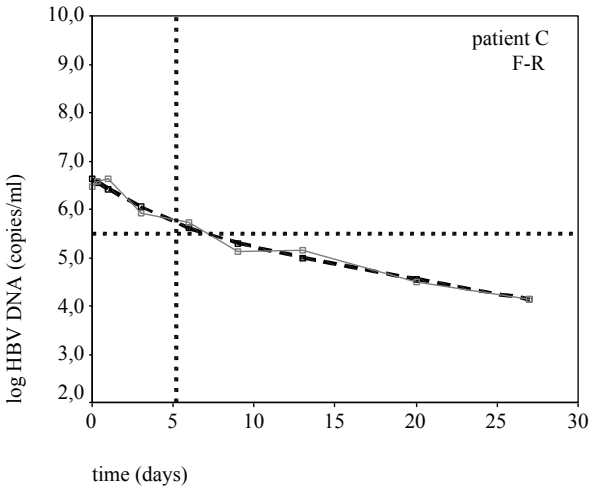
During the second phase, the pattern of viral decay was also categorised according to the rate of decline. The following definitions were used: R for rapid declines of > 1 log HBV DNA over the 4-week period, S for slow declines of between 0.2 and 1 log HBV DNA over 4 weeks, F for flat declines of < 0.2 log HBV DNA during the 4 weeks, and bd when the HBV DNA level fell below the level of detection.

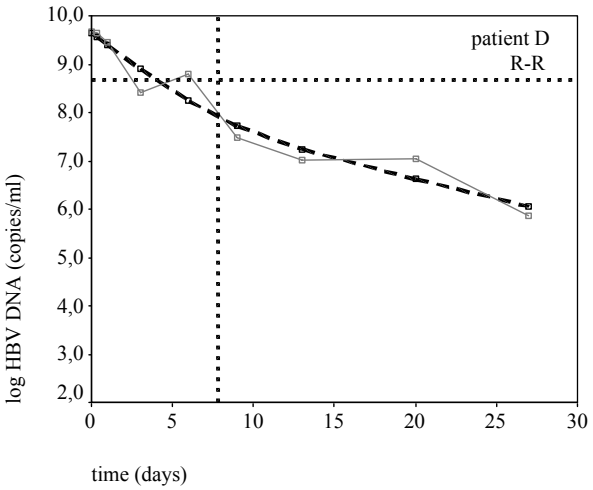
Straight grey line: observed HBV DNA data.

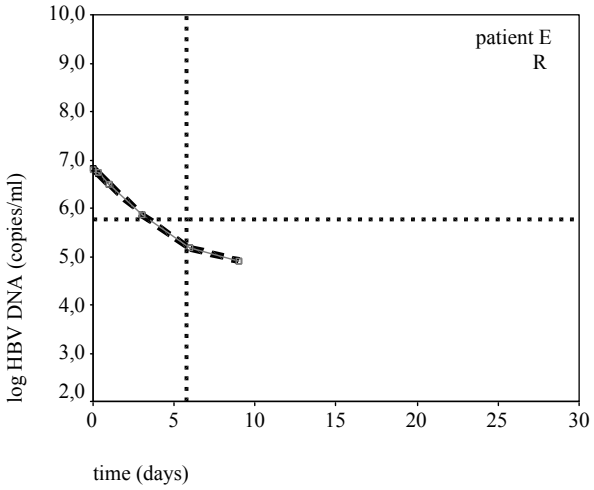
Black large dotted line: fitted HBV DNA data.

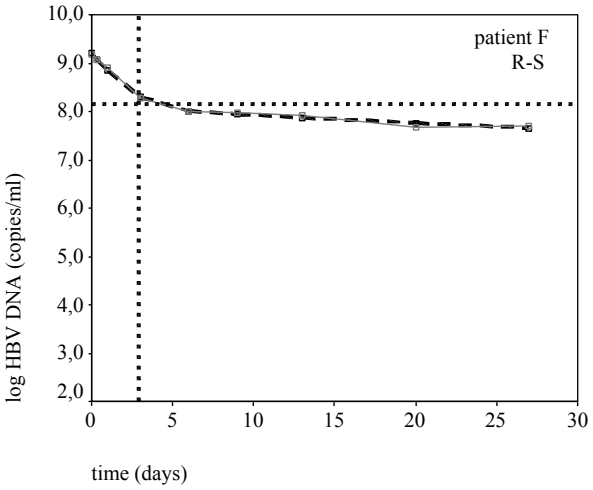


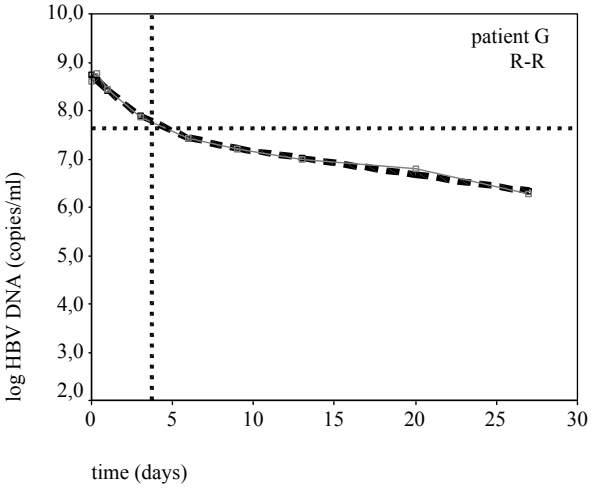


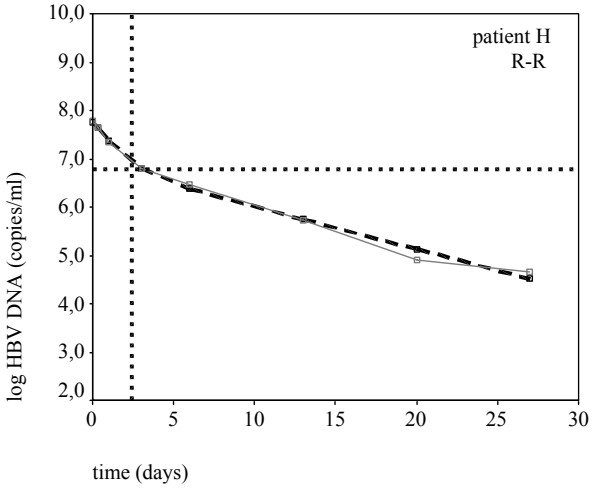


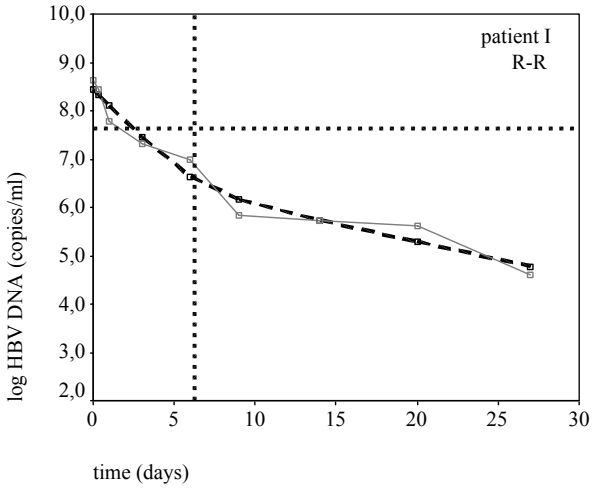


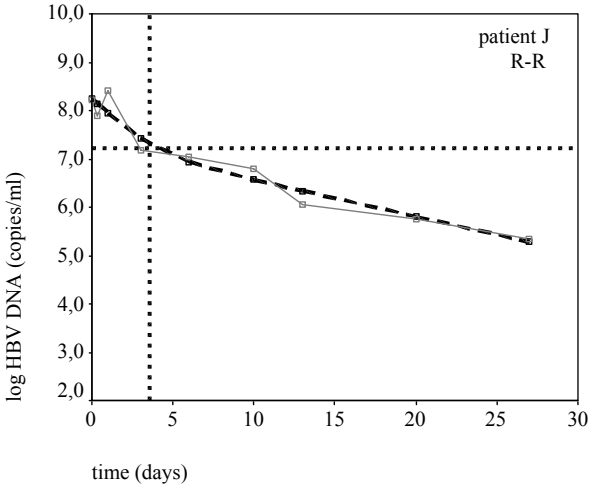





patient G
R-R
log HBV DNA (copies/ml)
time (days)


patient H
R-R
log HBV DNA (copies/ml)
time (days)


patient I
R-R
log HBV DNA (copies/ml)
time (days)


patient J
R-R
log HBV DNA (copies/ml)
time (days)

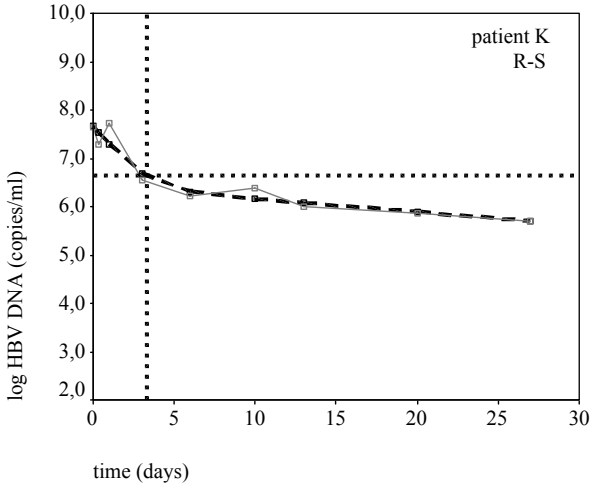
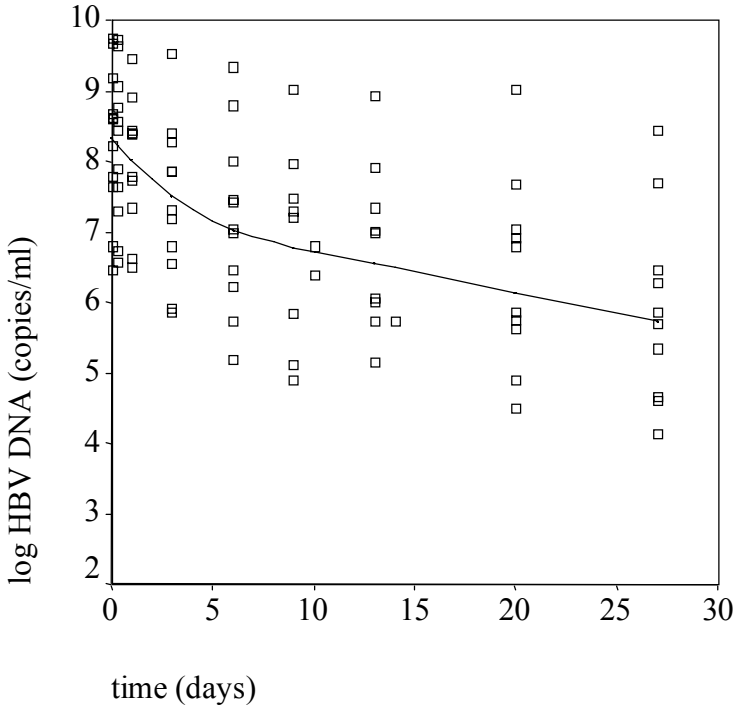

patient K
R-S
log HBV DNA (copies/ml)
time (days)

Figure 2 Overall viral decline during the first 4 weeks of tenofovir therapy in 11 lamivudine-resistant patients, by mixed-effect group fitting.
Open squares: observed HBV DNA data.
Straight black line: group fit HBV DNA data



In nine patients, the first-phase response was rapid (Fig 1A.–1K). Six of the nine patients followed this rapid first phase by an initially rapid second phase. However, in this study, the rate of viral decay in the first week of treatment did not appear to determine the rate in the following phase (the next three weeks of treatment). Some patients had rapid decay in the first phase, followed by slow decay in the second phase (patients F and K), others had 'flat' viral decay in the first phase followed by rapid decay in the second phase (patients B and C).

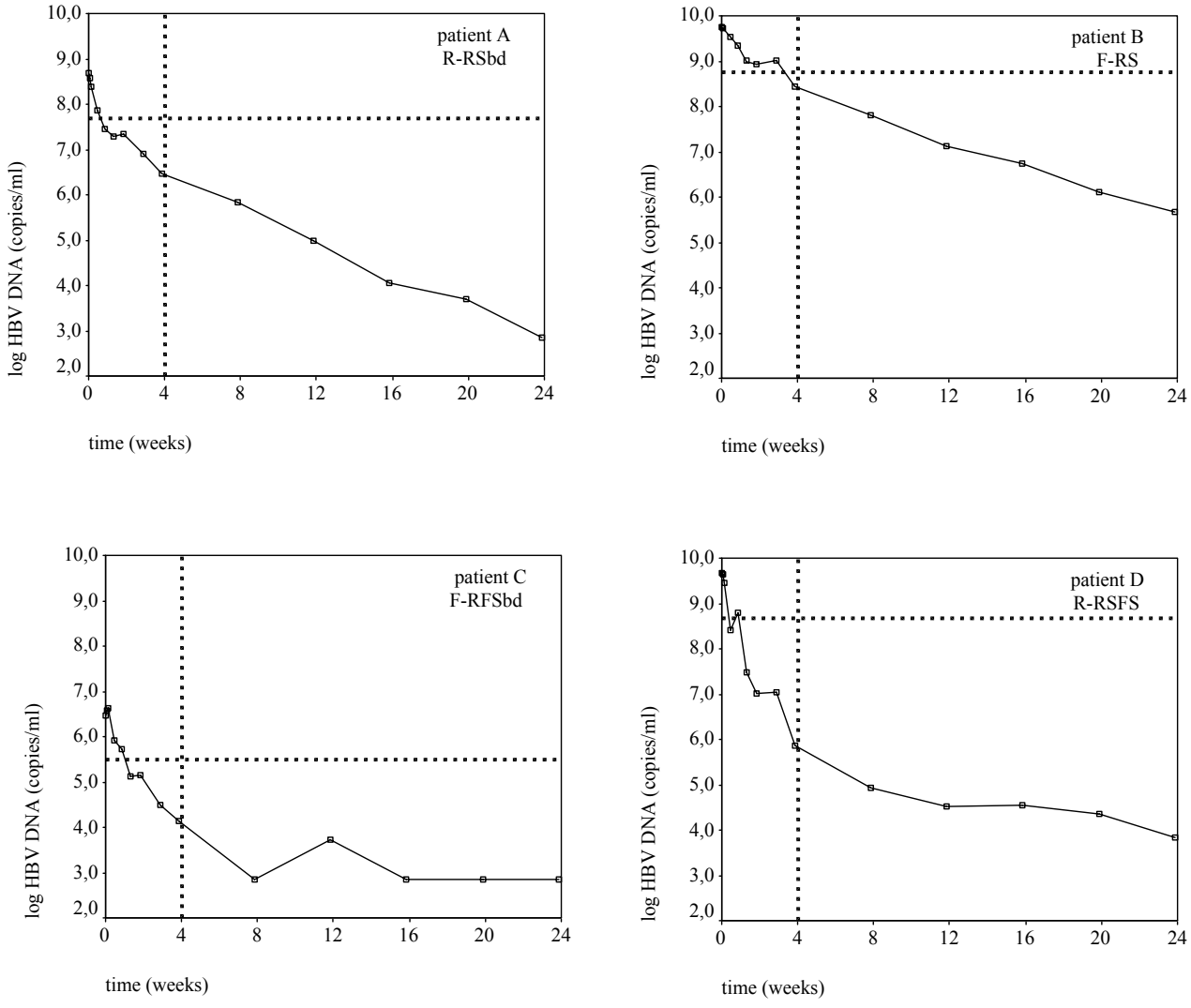
After the initial rapid decline in viral load of the first phase, the response in the following weeks was highly variable between the individual patients (Fig 3A.–3J.). The variability of response appeared to be due to the existence of complex multi-phasic decay patterns in some patients. Therefore, as for the early viral kinetics, the rate of viral decay in the first four weeks of treatment did not appear to determine the rate of viral decay in the following phase (the next 20 weeks of treatment).

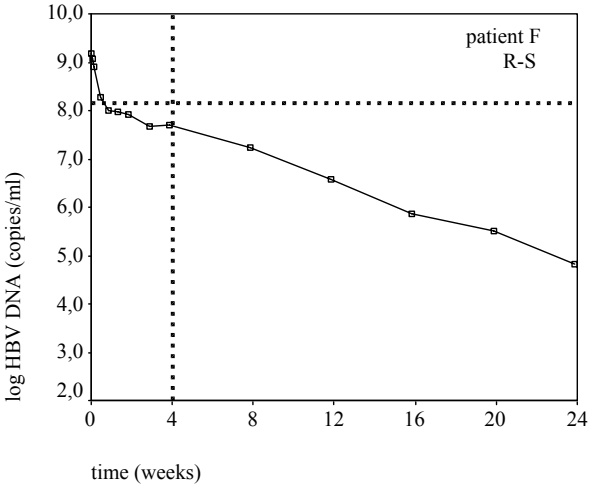
Figure 3 (A-J) Viral decline during 24 weeks of tenofovir therapy in 11 lamivudine-resistant patients who continued lamivudine treatment.

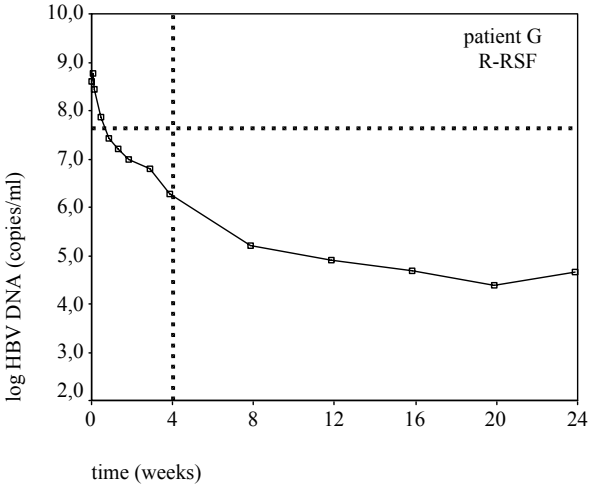
The first phase was categorised to one of three patterns, according to the rate of HBV DNA decline in the first 4 weeks of treatment: R for rapid declines of ≥ 1 log, S for slow declines between 0.5 and 1 log or F for flat declines of < 0.5 log. In the second phase, viral decay patterns were categorised according to the rate of decay over 4-weekly segments of the following 23-week period: R for rapid declines of > 1 log / 4 weeks over the 23-week period, S for slow declines of between 0.2 and 1 log / 4 weeks over 23 weeks, F for declines of < 0.2 log / 4 weeks over 23 weeks, bd for patients where the level of HBV DNA fell below the level of detection, and Rebound for where patients experienced a transient (only one time point) increase of > 1 log.

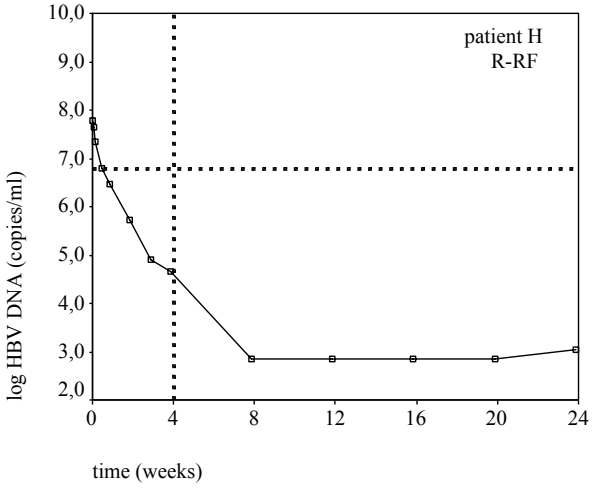
Because of lack of data, patient E is not described.

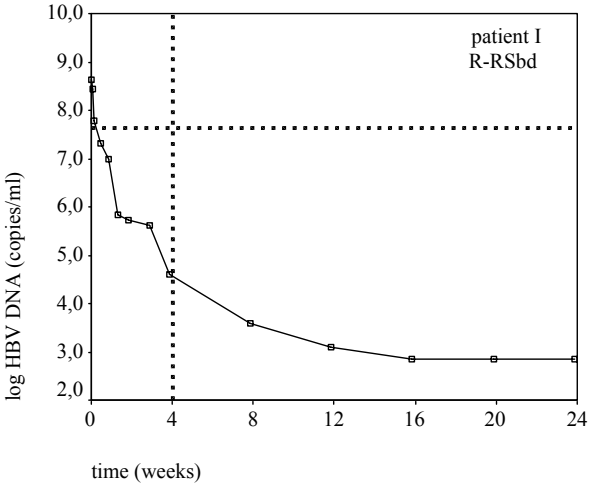
The horizontal straight dotted line is placed 1 log below the initial viral load of the patient; the vertical straight dotted line is placed at the 4-Week time point.

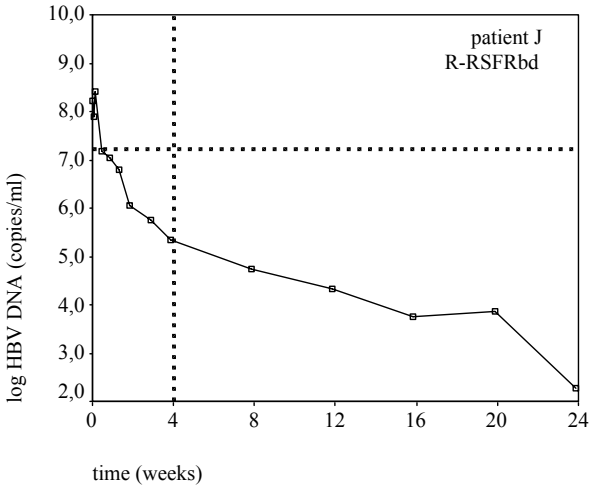


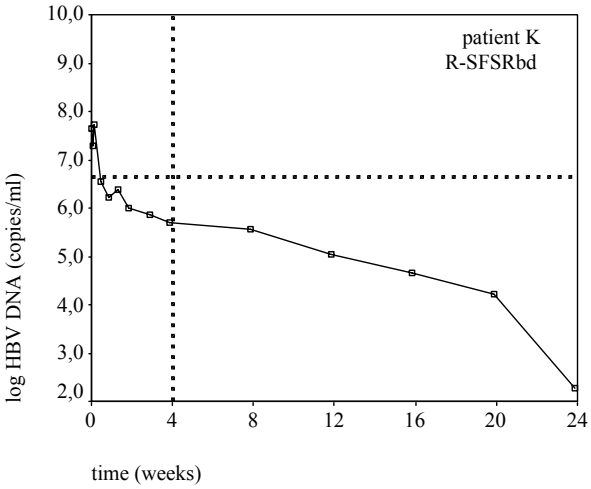

patient F
R-S
log HBV DNA (copies/ml)
time (weeks)


patient G
R-RSF
log HBV DNA (copies/ml)
time (weeks)


patient H
R-RF
log HBV DNA (copies/ml)
time (weeks)


patient I
R-RSbd
log HBV DNA (copies/ml)
time (weeks)


patient J
R-RSFRbd
log HBV DNA (copies/ml)
time (weeks)


patient K
R-SFSRbd
log HBV DNA (copies/ml)
time (weeks)

Discussion

This study provides the first detailed viral kinetic data following tenofovir treatment of patients with drug-resistant HBV mutants. Previous modelling studies in chronic infected HBV patients have demonstrated that a bi-phasic pattern of viral response occurs during the first 4 weeks of antiviral treatment with nucleoside analogues [19]. In the study reported here, the viral decay in patients treated with tenofovir showed a similar bi-phasic pattern of early viral response. However, after 4 weeks, treatment response was less predictable and a variety of patterns of viral decay were observed, a finding that is similar to the patterns of viral decay previously found following adefovir treatment [18].

The effectiveness of tenofovir, as calculated with the individual fit, was 0.926. This is much lower than the reported efficacy of adefovir in treatment-naïve patients, which was 0.993± 0.008 (mean standard error; median: 0.996) [24], but was comparable with the efficacy of 0.928 (± standard error 0.015) for lamivudine [19].

Also of note is that the duration of the first phase is less than 7 days, which means that the transition from the first to the second phase occurs in the first week. This is significant because Tsiang et al. [24] conducted the first HBV DNA measurement after 1 week, while we measured on day 1 (t=0 and t=8 hours), and on days 2, 4 and 7 during the first week.

Another difference in methodology is that Tsiang et al. [24] calculated the efficacy over a period of 12 weeks. By contrast, we calculated the efficacy, as in the study of Wolters et al. [19], over 28 days.

To determine the effects of different sampling frequency and of sampling over different periods of time, we applied individual non-linear fitting to the bi-phasic exponential model to describe the viral decay of the first 12 weeks in our tenofovir-treated patients. HBV DNA measurements were used from weeks 1, 2, 4, 8 and 12. A median efficacy of 0.996 was found (if $\eta=0$) and a median efficacy of 0.995 was found if $\eta=1$. These values are comparable with the values found in adefovir-treated patients (0.993) and show that outcome of the calculation depends on a combination of the sampling frequency and duration of the sampling period.

Tsiang et al. [24] assumed that generation of new productively infected cells during therapy is completely inhibited ($\eta=1$). By contrast, Neumann et al. [18] set $\eta=0$, based on the hypothesis

that the major effect of standard interferon alpha is to block viral production or release. Although we cannot rule out a possible effect of tenofovir on blocking infection (η varying between 0% and 100%), the viral kinetic data for tenofovir could be fitted assuming both effects (η=1 and η=0).

HBV DNA levels can fluctuate even in untreated patients. However, pre-treatment levels of HBV DNA in the patients in our study were similar to t=0. This suggests that the rapid decrease in HBV DNA levels after t=0 could be attributed to treatment with tenofovir, and was not the consequence of a spontaneous decrease.

In our study, 4 weeks after addition of tenofovir to the treatment regimen, a mean log HBV DNA decline of 2.54 ± 0.91 (median 2.34; range 1.33–4.02 log HBV DNA) could be observed. This is comparable with the 2.42 log HBV DNA decline found in a study with tenofovir in 5 HIV/HBV coinfected resistant patients [12], and is higher than the 0.9 log HBV DNA decline in a study performed in 12 HIV/HBV coinfected patients who were treated with tenofovir [11].

Taken together, the data which showed a mean log HBV DNA decline of 4.95 ± 0.90 log HBV DNA (median 5.05; range 3.64–5.94) after 24 weeks of tenofovir in our study and the data which showed a mean log decline of 3.4 copies/ml after 24 weeks treatment with adefovir in lamivudine-resistant HIV/HBV coinfected patients [27], suggests that tenofovir may have an important role to play in patients who experience breakthrough viraemia on lamivudine therapy.

The second-phase decline in viral levels reflects the death rate of productively infected cells. The death of these cells is thought to require a host immune response. A possible marker of the strength of host immune response is the level of ALT, which is an indicator of the level of cell damage and death.

Previously, authors have observed a positive correlation between the decay rate of infected cells and the pre-treatment ALT level among chronic HBV patients who were treated with lamivudine therapy [25]. Another study, which analysed the influence of lamivudine dose and baseline ALT on the viral dynamics of the HBV virus, confirmed that higher baseline ALT levels were significantly related to the slope of the second phase of viral decay [28].

Nevertheless, in another study, in which patients were treated with either lamivudine monotherapy or with a combination of lamivudine and famciclovir, the investigators found no association between the slope of the second phase and baseline ALT [29]. This is in agreement with our study, in which kinetic parameters λ_1, λ_2 and ε were not associated with the pre-treatment ALT levels. This discrepancy with some other studies may be explained by the selection of patients in our study, which included patients with only moderate elevation of ALT. We speculate that the ALT levels were too low to produce a detectable association with the slope of viral decay.

Our data demonstrate that direct comparison of the efficacies given by different mathematical models is not always possible. As we have demonstrated, variations between the models with respect to sampling frequencies and duration of follow up result in different outcomes.

In addition, our data show that tenofovir is capable of effectively blocking viral replication in patients with lamivudine-induced mutant viruses in both HBV and HBV/HIV co-infected patients. However, for effective treatment of patients, the first goal should be to totally inactivate disease by completely blocking virion production. In terms of modelling this will mean an antiviral efficacy ε equivalent to 1. Our results show that, in patients with lamivudine-induced drug-resistant mutants, we can reach an efficacy of 0.99. Therefore, despite the drug having an excellent effect, our data also show some low-grade viral replication remains. We suggest that the residual replication may present a risk for genotypic succession during tenofovir therapy.

Acknowledgement

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5

DOCTOR TO PATIENT TRANSMISSION OF HEPATITIS B VIRUS: IMPLICATIONS OF HBV DNA LEVELS AND POTENTIAL NEW SOLUTIONS

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Antiviral Research 2003; 60(2): 79-85

Summary

Hepatitis B virus (HBV) infected health care workers (HCWs) can infect patients undergoing exposure prone procedures. Until now reviews have focussed on the problem of the HBeAg-positive HCWs. After transmission of HBV by HBeAg-negative surgeons, the focus of Public Health policy in the UK and the Netherlands has changed from HBeAg-status to serum HBV-DNA level.

Viral load and the volume of blood transmitted determine the transmission risk of HBV. We have estimated the number of infectious particles transmitted by needlesticks, in comparison with those attributed in maternal-fetal transfusion. The blood-volume transmitted by needlestick is roughly 1-30% of that of delivery. As vertical transmission with maternal HBV-DNA levels below 10^7 geq/ml is rarely documented, HBV transmission by needlesticks is according to our assumptions unlikely to occur with HBV-DNA levels below 10^7 geq/ml.

Sera of transmitting HCWs contained HBV-DNA levels between $5.0x10^9$ and $6.35x10^4$ geq/ml, interpretation of these levels is hampered as the sera were taken at least 3 months after transmission. To prevent both loss of expertise and nosocomial transfer, highly viremic HCWs can be offered antiviral therapy. Lamivudine and alpha-interferon can now be complemented with adefovir, tenofovir and entecavir to provide effective new solutions for chronic HBV infected HCWs.

Introduction

Hepatitis B virus (HBV) is known to have been transmitted by infected health care workers (HCW) to patients undergoing exposure prone procedures (EPP). Worldwide 45 HCW, who transmitted HBV to their patients, have been identified since 1970 (1-8). These 45 cases of doctor-to-patient transmission resulted in 437 hepatitis B infected patients.

The CDC recommendation states that HCWs who are infected with HBV should not perform exposure-prone procedures unless they have sought counsel from an expert review panel and have been advised under what circumstances, if any, they may continue to perform these procedures. Such circumstances would include notifying prospective patients of the HCW's seropositivity before they undergo EPPs (9). Until 1997 all described cases of HBV transmission to patients involved hepatitis B e-antigen (HBeAg) positive HCWs. Therefore, to prevent patients from being infected, Public Health measures focussed on excluding HBeAg positive HCWs from performing EPPs.

Transmission by HBeAg negative surgeons was first described in 1997 (10). All described cases of HBeAg-negative HCWs, who infected patients, involved surgeons bearing precore mutants (6-8, 11). HBV carriers with this variant form of HBV do not produce HBeAg. After the identification of these transmitting HBeAg-negative HCWs a more direct measure of infectivity, based on HBV DNA levels, was required.

The 2000 NHS Health Service Circular defines criteria necessary for the conduct of exposure prone procedures by HBV carriers whose serum does not contain HBeAg. For a HBeAg negative HCW to be permitted to perform EPP their HBV DNA level must be below 10^3 geq/ml. The UK and Eire exclude all HBeAg positive HCW (12, 13). In the Netherlands a maximum HBV DNA level of 10^5 geq/ml is used to allow for the conduct of EPPs, irrespective of HBeAg status (14). In the US exclusion from performing EPPs is still based only on the presence of HBeAg (15).

In this paper we review doctor-to-patient transmission of HBV with main focus on quantitative HBV DNA levels.

Doctor-to-patient transmissions of hepatitis B virus

Since the early 1970s HBV infected HCWs have been identified to be the source of infection for patients who underwent surgical procedures. Retrospective investigations were performed to identify all possibly infected patients of HBV infected HCWs. Hasselhorn and Hofmann reported 40 cases of hepatitis B transmission by health care workers (6). We found that at

least five other cases of HBV transmission have been described, which are summarised here, pointing to a more widespread problem.

- 11 patients infected by a nurse (3)
- 7 infected patients and 4 cases of secondary transmission by a cardiac surgeon (5)
- 6 dental patients infected by a HBeAg positive dentist (4)
- 4 patients infected by a HBeAg positive oral surgeon (1)
- 2 patients infected by a HBeAg negative cardiothoracic surgeon bearing a precore mutant (7)

In our registry 45 described incidents of HBV transmission from HCW to patients resulted in 437 infected patients. The risk of transmission is proven and real, but still small. For example, in the Netherlands with a compulsory reporting of viral hepatitis, 3 cases of doctor to patient transmission have been described since the 1970s and about 500.000 surgical procedures are performed each year.

Risk of transmission of HBV

The risk for patients to become infected during surgical procedures depends on several factors (table 1). Most infections occur during high-risk procedures. Characteristics of procedures associated with higher risk of transmission include: blind digital palpation of a needle tip (16-18), digital guidance or handling of the needle tip while suturing (18), simultaneous presence of fingers and instrument in the operating area (19) and interrupted vision during a surgical procedure (20).

Table 1: Factors associated with the risk of hepatitis B virus transmission.

Factors associated with transmission risk:
• Serum HBV-DNA level (21, 23, 30)
• HBeAg positivity (20, 23, 27)
• Duration of surgery (20, 21, 27, 33)
• Volume of blood transmitted (21, 23, 30)
• Route of transmission: percutaneous vs. mucosal (23, 30)
• Skill and medical condition of HCW (20, 21)

Table 2: Estimation of the number of infectious HBV particles transmitted in needlestick accidents and delivery.

µl serum transmitted in different events		Exposure to infectious particles after transmission of serum with different HBV DNA levels*						
		10^3 geq/ml	10^4 geq/ml	10^5 geq/ml	10^6 geq/ml	10^7 geq/ml	10^8 geq/ml	10^9 geq/ml
Delivery: maternal-fetal transfusion (28)	0.64	<1	<1	6	64	640	6400	64.000
Needlestick with suture needle (24)								
▪ 0.33 mm needle, 2 mm penetration	~0.007	<1	<1	<1	<1	7	70	700
▪ 0.33 mm needle, 5 mm penetration	~0.03	<1	<1	<1	3	30	300	3.000
▪ 1.12 mm needle, 2 mm penetration	~0.06	<1	<1	<1	6	60	600	6.000
▪ 1.12 mm needle, 5 mm penetration	~0.23	<1	<1	2	23	230	2.300	23.000
Needlestick with hollow needle (24)								
▪ 1.07 mm needle, 2 mm penetration	~0.14	<1	<1	1	14	140	1400	14.000
▪ 1.07 mm needle, 5 mm penetration	~0.44	<1	<1	4	44	440	4.400	44.000

* Calculation infectious particles: volume of serum in ml (a) x HBV DNA concentration in geq/ml x 0.10 (b)

a) Percentage serum in whole blood is 0.64, assuming a hematocrite value of 0.36 (lower limit of normal in women).

Number of infectious HBV particles ≈ 10% of total number of HBV particles (29).

Although various routes of transmission of HBV have been described, most HBV infections are caused by contact with infected blood. Sharp injuries with needles or other sharp devices can occur during the treatment of patients. Percutaneous injuries occur in 6.9% of operative procedures, in 32% of the observed injuries to surgeons the sharp object recontacted the patient's open wound. The risk for contact between the HCW's and patient's blood is therefore 2.21% (21). A comparable risk for blood contact of 2.02% during operations was found by Tokars et al. (22).

The amount of infected blood transmitted affects the risk of transmission (21, 23). The volume of blood inoculated in a needlestick injury from a suture needle without the use of gloves varies from 11 nl (0.33mm needle, 2mm penetration) to 366 nl (1.12mm needle, 5mm penetration). The volume of blood inoculated in a needlestick with a phlebotomy needle is higher and varies between 133 nl (0.71mm needle, 2 mm penetration) and 683 nl (1.12mm needle, 5 mm penetration) (24). The volume of blood increases significantly with increasing depth of penetration, increasing needle diameter and the use of a phlebotomy needle instead of a suturing needle (24). Napoli found the mean volume of blood inoculated using a 0.71 mm (22 gauge) phlebotomy needle to be on the order of 1 µl (25). About 75% of sharp injuries is related to suturing (21-23). Injuries with solid-bore needles (suture needles) carry a lower risk of transmission than hollow-bore, blood-filled needles (26). Double gloving is effective in reducing the risk of inner glove puncture (27) and decreases the volume of blood transmitted by suture needles (24). During delivery infected maternal blood can be transmitted to the unborn child. Maternal-fetal transfusion during delivery with blood volumes greater than 1 µl is infrequent (28).

The number of HBV particles transmitted by a needlestick and during delivery depends on the viral load and the volume of blood transmitted. We have estimated the number of infectious HBV particles transmitted by maternal-fetal transfusion and needlesticks (table 2). Because HBV DNA levels are measured in serum, a factor of 0.64 has been used to calculate the volume of serum in the blood transmitted (assuming a hematocrite value of 0.36; the lower limit of normal in women). Multiplying the serum volume by the HBV DNA concentration gives an estimate of the number of viral particles in the serum volume. Heermann et al. stated that almost 10% of detected HBV particles is infectious (29). Therefore, the estimated number of viral particles in the serum volume has been devided by ten. The number of infectious particles transmitted during delivery and by needlesticks with HBV DNA levels ranging from 10^3 to 10^9 geq/ml is shown in table 2.

Implication of HBeAg status

HBeAg is considered a marker for viral replication and infectivity. High levels of HBV DNA usually correlate with the presence of HBeAg (30), serum of HBeAg positive persons is likely to contain up to 10^9 HBV particles per ml of serum (23, 27). The presence of anti-HBe is thought to indicate a low level or lack of viral replication and often the absence of virus in the blood (31). However, in HBV carriers with precore mutants HBeAg is not expressed despite continuing viral replication. The most common mutation, a G-to-A transition at nucleotide 1896 of codon 28 introduces a stop-codon, preventing expression of hepatitis B e-antigen. About half of all HBeAg negative, anti-HBe positive virus carriers carry a precore mutant. These mutants can be associated with highly productive infection in HBeAg negative HBV carriers (11, 12, 30, 31).

Martinot-Peignoux et al. performed a study to quantify HBV DNA levels in inactive HBsAg carriers using the Cobas Amplicor HBV Monitor (Roche) with a sensitivity of 200 geq/ml (32). The mean HBV DNA concentration in this group of patients was found to be 1300 geq/ml and 98% of sera of inactive HBeAg negative carriers contained HBV DNA levels below 10^5 geq/ml. Tedder et al. (2002) found evidence for fluctuations in HBV DNA levels in HBeAg negative HBV carriers. The variations in HBV DNA level of several orders of magnitude occurred over relative short time periods and indicate a dynamic host-parasite relationship (12).

HBeAg / anti-HBe status is often used as a marker of infectivity. However, serum HBeAg is at best an indirect measure of hepatitis B viremia because of possible mutations in the precore region. Measurement of both HBV DNA level and HBeAg status gives a more reliable estimate of infectivity (30).

HBV DNA concentration and transmission rate

The infection risk after exposure to HBV infected blood depends on the viral load. Although transmission of HBV from health care workers to patients has repeatedly been described since 1970, the viral load of the HCW involved has only been determined in five investigations (table 3). Sera of transmitting surgeons were found to contain HBV DNA levels between 5.0 x 10^9 and 6.35 x 10^4 geq/ml. The lowest measured HBV DNA level in serum from a transmitting surgeon was 4.0 x 10^4 geq/ml in a sample taken at least 3 months after transmission (11).

The proportion of patients infected with HBV after treatment by an infected HCW varies between 0.5 % and 13.1% in different investigations (6). The study performed by Spijkerman

et al. describes a retrospective analysis to identify infected patients (33). All patients operated on by the surgeon, sexual partners and household contacts of patients with evidence of HBV were offered serological testing for anti-HBc and HBsAg after the incubation period. Cases were considered confirmed cases if the patients' sera patients contained HBV markers and the same HBV DNA sequence as the surgeon. Probable cases were positive for anti-HBc and anti-HBs and provided clinical evidence of HBV infection within six months after surgery. Possible cases were positive for anti-HBc and anti-HBs and provided no epidemiological evidence of other sources of HBV infection. Using these definitions 8 confirmed cases, 2 probable cases and 18 possible cases were identified among 1564 tested patients. The proportion of infected patients (transmission rate) lies between 0.5% (confirmed cases only) and 1.8% (all cases). Because many different calculations and definitions have been used in the studies of doctor-patient transmissions the transmission rates were recalculated using the definitions stated by Spijkerman et al. (33).

The highest transmission rate is found by Harpaz et al., in this study 19 of 144 susceptible patients operated on were found to be infected by a surgeon (13.2%) (17). Sequence analysis confirmed infection in 9 cases. The total number of tested patients was actually 170, therefore the infection rate varies between 5.3% and 11.2%. In the study performed by Welch et al. (1989) a transmission rate of 8.9% was found (19). The transmission rate is based on 22 infected patients of 247 tested patients. In six patients with presence of HBsAg subtyping confirmed infection by the surgeon, resulting in a transmission rate between 2.4% and 8.9%. Prentice found that a surgical trainee infected 6.1% of patients treated (34). Two hundred eighty patients thought to be at risk were tested, 17 were found to have acquired HBV after the operation and 9 patients had the same HBV subtype as the surgeon. The transmission rate in study varies between 3.2% and 6.1%. The actual rate is probably lower because patients undergoing procedures with minimal risk of transmission were excluded from the study. Hadler (1981) found that 6 of 764 patients of a HBV infected dentist had signs of HBV infection (4). The proportion of infected patients lies between 0,3% and 0,8%. In two patients HBsAg subtyping could be performed and was found to be the same as the surgeon. In the studies performed by Molyneaux (2002), Sundkvist (1998), Haerem (1981) and Lettau (1986) all patients were found to have the same subtype as their surgeons (5, 7, 8, 18).

Table 3: Cases of doctor to patient transmission of HBV with HCW's profession, transmission rate and HBV DNA level of each case.

Author	HCW's profession	Published transmission rate[1]	Recalculated transmission rate[2]	HBV DNA (geq/ml)	Quantification technique	Time sample taken
(17)	Thoracic surgeon	13,1%	5,3 – 11,2 %	1.0×10^9	Semiquantitative PCR dot-blot hybridization, with serum containing 10^8 chimpanzee-infectious particles as comparison.	4 months after transmission
(10)	(1) General surgeon	N.A.[3]	N.A.	1.0×10^7	Liquid Hybridization and enzyme-linked oligonucleotide assay	12 weeks after transmission
		3,22%	1,08 – 3,22 %	4.4×10^6		Unknown
	(2) Gynaecologist	0,90%	0,90 %	5.5×10^6		Unknown
	(3) Gynaecologist	4,76%	4,76 %	2.5×10^5		12 weeks after transmission
	(4) General surgeon					
(7)	Surgeon	1.6 %	1,6 %	1.03×10^6	Lightcycler PCR	Unknown
(33)	Surgeon	0.5-1.8 %	0,5 – 1,8 %	5.0×10^9	Limited dilution PCR	1 year after identification first infected patient.
(11)	(1) Surgeon	N.A.	N.A.	1.12×10^8	*Chiron Quantiplex Branched DNA assay and Roche Amplicor HBV DNA monitor assay*	At least 3 months after transmission in all surgeons.
	(2) Surgeon	N.A.	N.A.	2.55×10^5		
	(3) Surgeon	N.A.	N.A.	6.72×10^5		
	(4) Surgeon	N.A.	N.A.	6.35×10^4		
	(5) Surgeon	N.A.	N.A.	4.20×10^8		
	(6) Surgeon	N.A.	N.A.	9.47×10^8		

[1] *Transmission rate: proportion of patients infected during medical procedures.*

[2] Recalculated transmission rate: transmission rates are recalculated according to one definition (33), [3] N.A.: data not available

Discussion

Public Health policy to prevent transmission of HBV to patients in different countries was based on serum HBeAg status. After incidents of transmission by HBeAg negative surgeons a more reliable estimate of non-infectivity was needed. Serum HBV DNA level may be more reliable to estimate non-infectivity than anti-HBe status alone. Current Public Health policy in the Netherlands does not allow the conduct of EPPs by HCWs with HBV DNA levels above 10^5 geq/ml, irrespective of HBeAg status (14). The use of this HBV DNA level of 10^5 geq/ml as a cut-off minimises the risk of transmission and allows most high-educated HBV infected HCWs to continue practice. Setting the cut off below this level would exclude the majority of HBeAg negative HCWs from performing EPPs in the Netherlands. The UK and Eire exclude all HBeAg positive HCW and a HBV DNA cut off level of 10^3 copies/ml is used for HBeAg negative HCWs (13). In the US excluding HCWs from performing EPPs is based on the presence of HBeAg only (15).

Transmission of HBV is not likely to occur with HBV DNA levels below 10^7 geq/ml according to vertical transmission studies. No cases of mother to child transmission were observed with maternal HBV DNA levels below 6,0 x 10^5 geq/ml, whereas 25% of children from women with HBV DNA levels above 10^7 geq/ml were infected (35). The maximum HBV DNA level of 10^5 geq/ml in the Netherlands is based on 10^7 geq/ml, below which vertical transmission is not likely to occur. A safety margin of 2 log is used to account for natural fluctuations in viral load and variations in the assay used for quantifying HBV DNA. In the UK the lower cut off level of 10^3 geq/ml is based on a safety margin of 3 log as the viral load is then unlikely to rise above 10^6 copies/ml.

Most percutaneous injuries during surgical procedures involve suture needles. As can be seen in table 2 the estimated number of particles transmitted with suture needles is less than the number transmitted by maternal-fetal transfusion. HBV transmission by needlesticks is according to our assumptions unlikely to occur with HBV DNA levels below 10^7 geq/ml.

Recent investigations of transmissions of HBV to patients involved determining HBV DNA levels of the HCWs. Transmission rate does not seem to depend on serum HBV DNA level only. Serum of the surgeon described by Harpaz et al. contained 1.0 x 10^9 geq/ml, associated with a maximum transmission rate of 11.2% (17). The surgeon described by Spijkerman et al. (2002) had a viral load of 5.0 x 10^9 geq/ml and infected 1.8% of his patients (33). Although these surgeons were both HBeAg positive and had comparable HBV DNA levels the transmission rates vary greatly. A relation between HBV DNA level and transmission rate was not found in the HBeAg-positive HCWs. A possible explanation could be that the

surgeon described by Harpaz et al. (1996), as a thoracic surgeon, performed more high risk procedures as the general surgeon described by Spijkerman et al. (2002).

The lowest serum HBV DNA level in a transmitting surgeon was found to be 4.0×10^4 geq/ml (11). This rises the question which HBV DNA level should be used to allow for the conduct of exposure prone procedures by HBV infected HCWs. However, in our opinion Public Health policy should not be based on the measurements of HBV DNA levels in these HCWs because all samples were taken at least 3 months after the actual transmission occurred. As described by Tedder et al. (2002), variations in HBV DNA levels in HBeAg negative carriers occur over relative short periods of time (12). Therefore, HBV DNA levels might actually have been higher at the time of transmission.

Doctor to patient transmission is a complex issue due to legal and ethical factors. These factors include the hospital's policy, federal discrimination laws and issues of informed consent and disclosure of HBV infection. Restriction of infected HCWs is also complicated by the definition of disability. A surgeon might be restricted in practice but possibly not qualify for disability insurance compensation (36).

HCWs not allowed to perform EPPs due to high HBV DNA levels can be offered antiviral therapy to prevent both exclusion from practice and transmission to patients. In most countries the registered treatment for HBV consists of alpha-interferon or lamivudine. In an experimental setting lamivudine is sometimes combined with interferon to accomplish higher HBeAg seroconversion rates, generally associated with low HBV DNA levels. Viral resistance to lamivudine with emergence of YMDD mutants in the c-region of the HBV polymerase gene during long-term lamivudine therapy is well described (37, 38). After 1 year in 15-30% and in up to 50% after 3 years of patients treated with lamivudine monotherapy a resistant virus emerges (39). Recently new nucleoside analogues tenofovir disoproxil fumarate (40), adefovir dipivoxil (41, 42) and entecavir (43, 44) have shown to be effective in suppressing both wild-type and YMDD-mutant HBV replication. Although these antiviral drugs have not yet been registered for the treatment of HBV in most countries, they can provide potential new solutions for the treatment of chronic hepatitis B infected health care workers.

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6

HEPATITIS B VIRUS (HBV) DNA LEVELS AND THE MANAGEMENT OF HBV INFECTED HEALTH CARE WORKERS

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Submitted

Abstract

Different guidelines exist for the management of HBV infected health care workers (HCWs). Various HBV DNA levels are used as a cut-off level to determine whether an HBV infected HCW is allowed to perform exposure prone procedures (EPPs) or not.

In this paper we discuss the implications of measuring HBV DNA levels in HCWs by comparing Dutch data with the available data of HBV infected HCWs in the United Kingdom. Dutch HCWs show relatively high HBV DNA levels in both HBeAg positive and negative persons. Applying a cut-off level of 10^5 copies/ml, significant more HCWs are to be excluded in the Netherlands (29%) than in the UK (11%) (p=0.001).

If assays for investigating the level of HBV DNA in the serum of HCWs are to be used to define acceptability for the conduct of EPPs, it is necessary to take into account at first, the variability in time of HBV DNA levels in HBV carriers and second the reliability and reproducibility of the molecular diagnostic test used.

The implementation of molecular diagnostic assays has made qualitative and quantitative detection of HBV relatively easy, but also requires the use and introduction of standardised materials as well as participation in international quality control programs. With the difference in HBV DNA levels found in our Dutch HCWs and those in the UK the issue of standardisation has to be addressed, before a universal, maximum level of viremia for EPP performing HCWs can be introduced.

Introduction

Since the early 1970's there have been over 45 reports of HBV transmission from health care workers (HCWs) to patients. These transmissions have resulted in more than 400 infected patients (1). The majority of the documented HBV transmissions have been associated with HCWs performing exposure prone procedures (EPPs). EPPs are those where there is a risk that injury to the worker may result in exposure of the patient's open tissues to the blood of the worker. In practice, EPPs are invasive procedures where the skin of the HCW may come into contact with sharp surgical instruments, needles or sharp tissues in body cavities or poorly visualised, confined body sites (2).

In the United States exclusion from performing EPPs is based on the HBeAg status only. The UK and Eire exclude all HBeAg-positive HCW from EPPs; in HBeAg-negative HCWs who perform EPPS the HBV DNA level must be below 10^3 copies per ml (3). In the Netherlands a maximum HBV DNA level of 10^5 copies/ml is used to allow for the conduct of EPPs, irrespective of the HBeAg status. This level was chosen based on a balance between exclusion rate and safety (4), considering that only in one incident the viraemia in the HCW involved was below 10^5 copies/ml (i.e. $6x10^4$ copies/ml) (5). Recently a European consensus group proposed a cut off level of 10^4 HBV DNA copies/ml (2). In addition it was recommended that all HBeAg-positive HCWs should be excluded from EPPs. However, the consensus group agreed that each country may determine it's own HBV DNA cut-off level for EPPs, balancing the risk to patients against the loss of valuable personnel.

The development of amplification techniques revolutionised the detection of HBV. The advent of DNA sequencing allowed the detection of precore mutants, YMDD mutants and enabled the comparison of viral isolates on the genomic level. In addition, new treatment strategies for chronic HBV infection have emerged.

In this paper we discuss the factors that determine HBV DNA levels. We present recent data on HBV infected HCWs in the Netherlands. We discuss the implications of different HBV DNA cut off levels for EPP performing HCWs, by comparing our data with the available data of HCWs in the United Kingdom.

Determinants of the HBV DNA level

Active replication of HBV is associated with the presence of HBeAg. Conversion from HBeAg to anti-HBe positivity in chronic HBV carriers, through treatment or spontaneously, is linked to a decrease in serum transaminases and HBV DNA levels. Hence the HBeAg/anti-HBe status is often used as a marker for infectivity, with HBeAg-positivity representing active

viral replication. Although HBV DNA levels are significantly higher in HBeAg-positive HBV carriers as compared to HBeAg negative carriers (5, 6), high HBV DNA levels can be found in HBeAg negative patients due to the presence of mutations in the precore and core promotor region of the HBV genome (7). A precore stop codon mutation abolishes HBeAg production and a dual mutation in the core promotor region down-regulates HBeAg production. Recently Chu et al. determined the prevalence of HBV precore/core promoter variants in 694 patients in the United States, showing that these variants were more common in HBeAg-negative than in HBeAg-positive patients (precore mutation in 38% of HBe-negative vs. 9% of HBe-positive; core promoter mutation in 51% of HBe-negative vs. 36% of HBe-positive patients; see Figure 2) (6). HBeAg negative patients with either core promoter or precore mutants had significantly higher HBV DNA levels compared with HBe-negative with wild-type sequence. In an earlier study a precore mutant was found in 52% of all HBeAg-negative/antiHBe-positive patients (8).

The risk to transmit HBV depends among others on the number of infectious particles involved. Assuming that HBV DNA levels reflect the number of infectious particles, the transmission risk thus is determined by the HBV DNA level and the volume of infectious fluid involved (9). To maintain HBeAg positivity as an exclusion criterion for EPPs will inevitably lead to ongoing conduct of EPPs by some highly viraemic, HBeAg negative HCWs. In addition, this policy unnecessarily may exclude some HBeAg positive, low viraemic HCWs. (As an additional exclusion criterion, the HBeAg-positivity criterion ensures compatibility with older regulations in some countries).

To some extent the HBV viral load is determined by the HBV genotype. HBV genotypes A and D show a higher baseline HBV DNA level in comparison with genotypes B and C (10). Asian patients are predominantly infected with genotype B and C, whereas Caucasian patients are predominantly infected with genotype A and D (10).

HBV DNA levels in HBV infected health care workers

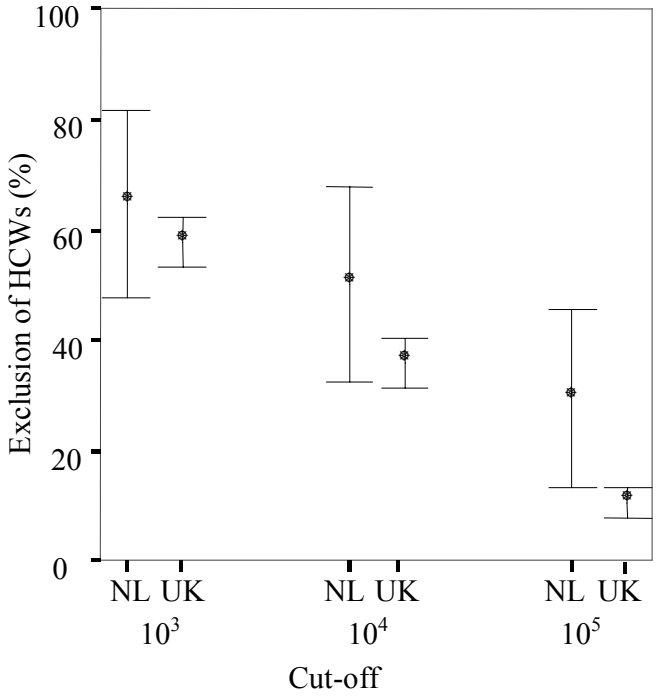
At the moment 47 HBV infected HCWs are known in the Netherlands. Table 1 and figure 1 show the HBV DNA levels and HBeAg status of these HCWs. Of 436 HBeAg-negative HCWs in the UK the HBV DNA level is known (2). HBV viraemia above 10^4 copies/ml in 36% of the HBeAg-negative HCWs seems illustrative for the UK population: a study examining the prevalence of precore variants in British HBV carriers also found HBV DNA levels above 10^4 copies/ml in 36% (82/228) of HBeAg negative carriers (11).

Table 1. HBV DNA levels and HBeAg status in HCWs in the Netherlands.

HBV DNA level (copies/mL)	No. of HBeAg positive patients	No. of HBeAg negative patients	Unknown eAg/anti-e status	Total no. of patients
$\leq 10^3$	0	12	3	15
$> 10^3$ and $\leq 10^4$	0	5	2	7
$> 10^4$ and $\leq 10^5$	0	7	1	8
$> 10^5$ and $\leq 10^6$	0	4	1	5
$> 10^6$ and$\leq 10^7$	0	6	0	6
$> 10^7$	5	0	1	6
Total	5	34	8	47

No. = number

Figure 1 Restriction of HBeAg negative HCWs (%; 95% confidence interval (CI)) with different HBV DNA cut-off levels. Significant more HCWs are excluded in the Netherlands than in the UK if a cut-off level of 10^5 copies/ml is used (p=0.001). No significant difference could be found in exclusion rate of HCWs between the 2 countries if a cut-off of 10^3 or 10^4 copies/ml is used (p=0.4 and p=0.1, respectively).



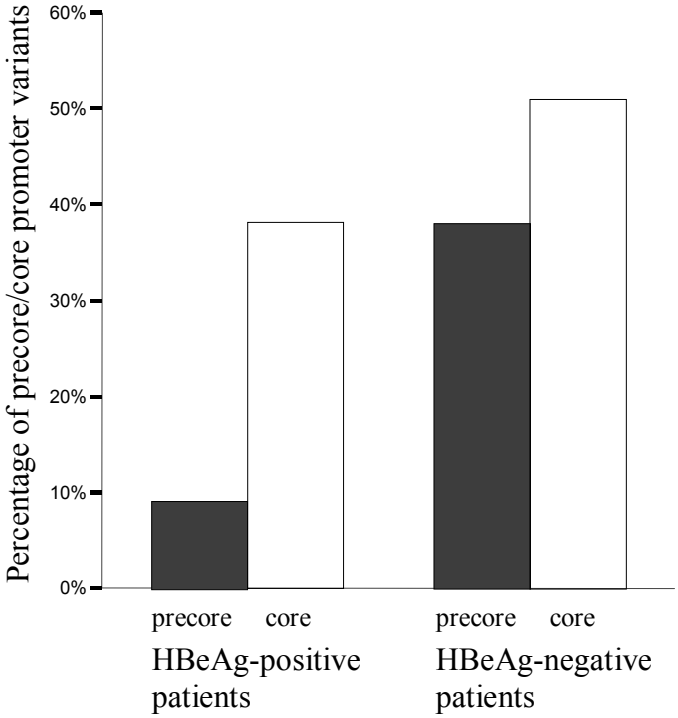
To demonstrate the consequence of different cut-off levels we constructed a table demonstrating the relation between cut-off level and exclusion rate for HBeAg-negative HCWs in the Netherlands and the UK (see Table 2). In the Netherlands, 29% (10/34) of HBeAg-negative HCWs were excluded from EPPs, while in the UK 58% (252/436) of HBeAg-negative HCWs were excluded. Due to the more lenient cut-off value in the Netherlands, 206 of the HCWs excluded in the UK are in theory allowed to perform EPPs in the Netherlands. Table 2 suggests that levels of HBV viraemia in Dutch HCWs are higher than in British personnel. Possibly in the UK more HBV infected HCWs originate from Asia, where HBV genotypes associated with lower levels of HBV DNA (i.e. genotypes B and C) are predominant. Furthermore, this difference raises questions about which assays and standards were used to quantify HBV DNA. The elevated levels of HBV DNA in both HBeAg positive and negative personnel in the Netherlands justifies not to consider the HBeAg status in the management of HBV infected, EPP performing personnel.

Table 2.

Restriction of HBeAg negative HCWs (%; 95% confidence interval (CI)) with different cut-off levels. Significant more HCWs are excluded in the Netherlands than in the UK if a cut-off level of 10^5 copies/ml is used (p=0.001). No significant difference could be found in exclusion rate of HCWs between the 2 countries if a cut-off of 10^3 or 10^4 copies/ml is used (p=0.4 and p=0.1, respectively).

	10^3	10^4	10^5
Netherlands	22/34 (65%; 48-82)	17/34 (50%; 32-68)	10/34 (29%; 13-46)
United Kingdom	252/436 (58%; 53-62)	156/436 (36%; 35-44)	46/436 (11%; 8-13)

Figure 2 The prevalence of HBV precore/core promoter variants in the United States in chronic HBeAg-positive and HBeAg-negative patients. Adapted from Chu CJ. et al. Prevalence of HBV precore/core promoter variants in the United States.



Implications of choosing a HBV DNA level as a cut-off level

If assays for investigating the level of HBV DNA in the serum of HCWs are to be used to define acceptability for the conduct of EPPs, it is necessary to take into account at first, the variability in time of HBV DNA levels in HBV carriers and second the reliability and reproducibility of the molecular diagnostic test used.

HBV DNA levels are known to fluctuate over time in a proportion of chronic HBV carriers. A one-year randomised placebo-controlled study evaluating the efficacy of famciclovir in chronic HBeAg-positive patients showed a median drop of 22% in HBV DNA levels in the placebo group (12). In a large placebo-controlled 48-week studies of adefovir, 53% of placebo patients had fluctuations of less than 0.5 $\log_{10}$ copies/ml, whereas the other 47% of placebo patients had a large (1-5 $\log_{10}$ copies/ml) oscillations in HBV DNA (10^4-10^9) (13). In a study of inactive HBsAg carriers (all anti-HBe positive) 98% of the patients had levels below 10^5 copies/ml and HBV DNA levels remained stable (1-6 years) in 97% of patients (14). Because of these naturally occurring fluctuations of HBV and assay variability a safety margin has to

be chosen. The margin safety needed to allow for fluctuation is balanced against the frequency at which it would be practicable to repeat HBV DNA tests. In the UK the Advisory Group of hepatitis B has recommended that infected HCWs should be restricted if their HBV DNA levels exceeds 10^3 copies/ml and that those with HBV DNA levels at or below this level should be re-tested yearly while they continue to perform exposure prone procedures (15). If exclusion of HCWs for performing EPPs is based on initial testing only, the use of a high safety margin taking into account all fluctuations appears reasonable. However, in combination with regular monitoring, a rise in HBV DNA can be detected early and necessary actions can be taken to minimise the risk of transmission, allowing for a more narrow safety margin (16). However, if HBV DNA elevations occur in the form of sudden flares, than more frequent testing will not significantly improve safety and cannot justify a smaller safety margin.

There are still a number of hurdles to be taken, which have to be resolved before implementation of a universal cut-off level for the exclusion of HBV infected HCWs to perform EPPs. The implementation of molecular diagnostic assays has made qualitative and quantitative detection of HBV relatively easy, but also requires the use and introduction of standardised materials as well as participation in international quality control programs (17).

With the introduction of quantitative assays, whether non-commercial or commercial, the need for internationally defined reference standards for appropriate calibration of these assays is greater than ever (18). Before December 7, 2003 the use of an internal calibration standard was not mandatory to standardise the non-commercial as well as commercial kits. Since this time, a standard is developed for HBV; each lot number has to be validated according to the standard (WHO 97/746). However, this standard is only developed for genotype A, assuming that assays are genotype independent. For each new version of an assay these data have to be provided. Standardisation is necessary to accurately and absolutely determine viral load and the possibility to compare data from different laboratories.

Well-defined quality control programs have been initiated and have shown that the need for more standardised material and for participation in this program is important (18, 19). False-positivity (due to contamination), but mainly false-negativity (due to the great difference in sensitivity of the different assays) are two important hurdles in molecular diagnostics (17). Furthermore, qualitative and quantitative assays must yield reproducible results. Inter- and intra-assay variability does occur and is more profound in samples with a low HBV DNA level (20). Internal control to monitor for loss of sample or inhibition is not implemented in most in-house developed quantification assays (17, 18). However, the use of an internal

control is imperative to monitor the quality of extraction and amplification. It provides both confidence in quantitative and negative test results.

With the difference in HBV DNA levels found in our Dutch HCWs and those in the UK the issue of standardisation has to be addressed, before a universal cut-off level for chronic HBV infected HCWs can be widely implemented.

Conclusion

The discussion on how to manage HBV infected HCWs continues. With comparable data available, different guidelines were devised in the UK, the USA, the Netherlands, and by a European consensus group. In the discussion three key elements play a role. Should HBV DNA be measured instead of HBeAg? Which level of HBV DNA is acceptable to prevent transmission of HBV from HCW to patient during EPPs? To what extent is the loss of valuable HCWs acceptable? In addition, officials may be confronted with HBV transmission from HCWs to patients, despite adherence to the guidelines; because transmission may sporadically occur during non-exposure-prone-procedures, or during EPPs through HCWs with HBV viraemia below an official cut-off value.

Vaccination against hepatitis B in HCWs is safe and should be mandatory for new employees (21). Therapy can be offered to high-viremic HCWs. Lamivudine and adefovir are powerful suppressors of HBV replication. However, it remains to be seen how long HBV viraemia can be controlled before escape mutant viruses emerge. Especially in HCWs who perform EPPs, a highly viraemic rebound phenomenon, caused by an emerging resistant HBV, may pose a significant threat to patients. Hence, frequent monitoring of the HBV DNA level is necessary in HCWs receiving HBV suppressive therapy. Nevertheless, each HCW who carries HBV must be referred to a hepatologist, because antiviral therapy may reduce the viral load sufficiently and thus may prevent unnecessary exclusion of valuable medical personnel.

Given our knowledge of HBV DNA levels in HBeAg-negative persons, exclusion of HCWs solely based on presence of HBeAg seems obsolete. Choosing a low HBV DNA cut-off level, it must be realised that the inter- and intra-assay variability is more profound in samples with low HBV DNA levels. Repeated testing of HCWs with a HBV DNA level of approximately 10^3 copies/ml will lead to a greater proportion of exclusion (20). In addition, differences in assay precision make it difficult to compare data from different laboratories, which emphasises the importance of standardisation (17).

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7A

PAIRED MEASUREMENTS OF QUANTITATIVE HEPATITIS B VIRUS DNA IN SALIVA AND SERUM OF CHRONIC HEPATITIS B PATIENTS: IMPLICATIONS FOR SALIVA AS INFECTIOUS AGENT

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Abstract

Background: After intensive source and contact tracing 20 % of acute Hepatitis B virus (HBV) infections remain unexplained. Saliva may be an unexpected vehicle of HBV DNA transmission. *Objective:*To further explore this hypothesis we evaluated the quantitative levels of HBV DNA in saliva and compared these with the HBV DNA levels measured in serum. *Study design:* Serum and saliva were collected from 27 chronic HBV patients attending our outpatient clinic. *Results:* There were 16 men and 11 women; 15 patients were HBeAg positive, anti-HBe negative and 11 patients were HBeAg negative, anti-HBe positive. One patient was HBeAg and anti-HBe negative. Samples of serum and saliva were collected on the same day. All saliva specimens were clear on inspection. HBV DNA in serum was measured by the Digene Hybrid Capture II microplate assay (Digene Diagnostics), the HBV Monitor assay (Roche Diagnostics) as well as an in-house developed HBV DNA TaqMan assay. The HBV DNA TaqMan assay was used for the quantitative measurement of HBV DNA in saliva. Median HBV DNA levels in serum were 2.10×10^5 geq/ml and ranged from 373 genome equivalents per ml (geq/ml) to 4.13×10^9 geq/ml; median HBV DNA levels in saliva were 2.27×10^4 geq/ml and ranged from 373 geq/ml to 9.25×10^6 geq/ml. A clear correlation was shown between HBV DNA in serum and saliva; log HBV DNA in saliva=1.01 + 0.56 x (log HBV DNA in serum).

Conclusions: this is the first report of precise quantitative measurements of HBV DNA levels in saliva and the relationship with HBV DNA levels in serum. Our findings show that saliva is a source of HBV DNA.This finding may have implications in selected patients for the infectivity of saliva and offer further insight in the routes of transmission of HBV infection.

Introduction

Transmission of Hepatitis B virus (HBV) in the absence of apparent parenteral, sexual, or perinatal exposure is common in highly endemic settings(1) and occurs within and between households (2,3). At least 20% of acute hepatitis B virus (HBV) infections remain unexplained after intensive source and contact tracing. HBsAg, HBeAg and anti-HBe are HBV markers which can be detected both in saliva and serum (4-7). HBV DNA, which indicates potential infectivity, has also been shown to be present in saliva, serum, urine and semen (5, 8-11) . In experimental studies using gibbons, saliva from HBsAg-positive and HBeAg-positive donors was administrated subcutaneously and orally (12, 13). The animals inoculated subcutaneously developed HBsAg followed by anti-HBs whereas none of the gibbons who were exposed orally developed evidence of HBV infection.

There is however no information regarding the precise amounts of HBV DNA in saliva and the relationship with HBV DNA levels in serum. A serum HBV DNA of 10^5 genome equivalents per ml (geq/ml) is considered as a level above which transmission of HBV may occur in surgical settings. If such levels can be detected in saliva this may emphasise the potential risk of saliva as a route of transmission. To further explore this hypothesis we evaluated the quantitative levels of HBV DNA in saliva and compared these with the levels measured in serum.

Patients and methods

Both serum and whole saliva were collected from 27 chronic HBsAg carriers attending our outpatient clinic. There were 16 men and 11 women; 15 patients were HBeAg positive anti-HBe negative and 11 patients were HBeAg negative. One patient was HBeAg and anti-HBe negative. Saliva samples of approximately of 1-2 ml were collected by asking the subjects to dribble into a sterile plastic container. All patients with gingival or buccal mucosa lesions or a tendency to bleeding gums were excluded from this study. All saliva samples were clear on visual inspection. Samples of serum and saliva were collected on the same day and stored at –20 ^{0}C until use.

For the accurate measurement of HBV DNA in serum both commercially available assays (Digene Hybrid Capture II microplate assay (Digene Diagnostics; dynamic range from 1,42 x 10^5 – 1,7 x 10^9 geq/ml), HBV Monitor assay (Roche Diagnostics; dynamic range from 1000 to 4 x 10^7 geq/ml) as well as an in-house developed HBV DNA TaqMan assay were used

(14). The TaqMan based assay enabled the accurate determination down to 373 geq/ml. All assays were calibrated using EUROHEP HBV DNA standards (15).

The Magnapure LC instrument (Roche Applied Science) was used to isolate HBV DNA from saliva, using the total nuclear acid isolation procedure. To monitor both loss of sample as well as inhibition, a universal interval consisting of a fixed number of PhHV-1-virus particles was added before the extraction procedure to the saliva sample (16). Corrections were made if necessary. The HBV DNA TaqMan assay was used for the quantitative measurement of HBV DNA in saliva as previously described (14).

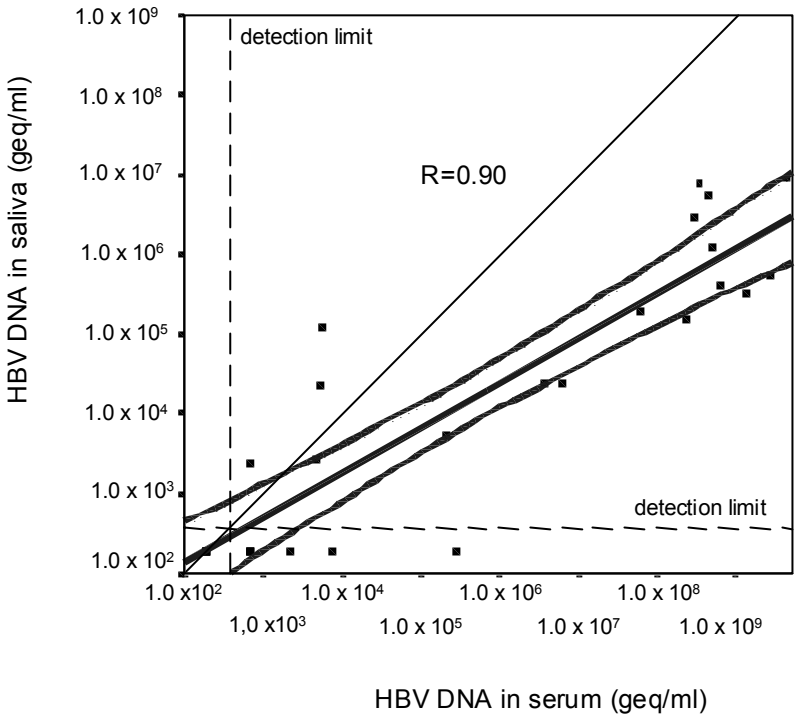
Statistical analysis was performed in SPSS-version 10. To test for correlation between HBV DNA in serum and HBV DNA in saliva we used Pearsons correlation and linear regression analysis with 95% confidence intervals.

Results

HBV DNA was detected in serum from 23 of 27 (85%) HBsAg-positive patients. Among the 15 HBeAg positive patients, HBV DNA in saliva was detected in 12 patients (80%) and in serum in 14 patients (93%). Among the 12 HBeAg-negative patients HBV DNA was detected in saliva in 5 patients (42%) and in serum in 9 patients (75%).

Two HBeAg-negative/anti-HBe-positive patients had a higher concentration of HBV DNA in their saliva samples than in their serum samples. Three patients who were HBeAg-negative/anti-HBe-positive and 2 patients who were HBeAg-positive/anti-HBe-negative had no detectable HBV DNA levels in saliva but HBV DNA could be detected in their serum samples. The patient who was both HBeAg and anti-HBe-negative had detectable HBV DNA levels in serum, but no HBV DNA could be detected in his saliva sample. Median HBV DNA level in serum was 2.10×10^5 geq/ml and ranged from 373 geq/ml to 4.13×10^9 geq/ml; median HBV DNA level in saliva was 2.27×10^4 geq/ml and ranged from undetectable geq/ml to 9.25×10^6 geq/ml. A high correlation was shown between HBV DNA in serum and saliva (Pearson correlation 0.90). The relation between saliva and serum HBV-DNA could be described by log HBV DNA in saliva = 1.01 + 0.56 x (log HBV DNA in serum) (figure 1.)

Figure 1: Association between HBV DNA in genome equivalents per ml (geq/ml) in serum and saliva. The skewed lines represent the 95% confidence bands of the regression line.



Discussion

This is the first report of precise quantitative measurements of HBV DNA levels in saliva and the relationship with quantitative HBV DNA levels in serum. We used whole saliva which was clear on visual inspection, because in everyday situations when transmission can occur whole saliva is the medium that is transmitting the HBV virus.

We have described two samples in which the HBV DNA levels in saliva are higher than in serum. These results are quite unexpected one of the possible explanations could be a possible role of salivary glands as a site of HBV replication. This hypothesis might explain the higher level reached in saliva.

In daily practice, saliva is not considered as an important mode of HBV transmission nowadays. However, transmission of HBV following a bite or a spit in the eye is described (17-20) and our findings suggest that saliva can be a source of considerable amounts of HBV DNA. In patients with high viremia this finding may have implications for the infectivity of saliva and give insight in the possible routes of transmission of HBV. Saliva as a vehicle of transmission of HBV may be another way of transmitting hepatitis B virus. Further research is

needed to gain more insight in the viremia of body fluids such as saliva and in the transmission risk for each log level of viremia. This might allow clinicians and public health workers to provide patients with better information on potential infectivity of body fluids and alternative transmission routes.

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7B

PAIRED MEASUREMENTS OF QUANTITATIVE HEPATITIS B VIRUS DNA IN SALIVA, URINE AND SERUM OF CHRONIC HEPATITIS B PATIENTS

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Submitted

Abstract

Objectives: Despite an abundance of epidemiological evidence for horizontal transmission of Hepatitis B Virus (HBV), the transmission route remains to be fully elucidated. In a new approach, we evaluated quantitative HBV DNA content in serum, saliva and urine as a first step in exploring possible modes of horizontal transmission.

Methods: In an outpatient setting of an academic hospital, paired serum, saliva and urine samples were collected from 150 chronically infected Hepatitis B virus patients. A validated HBV DNA TaqMan assay was used to quantitatively measure HBV DNA.

Results: Mean log HBV DNA in serum was 5.8 (range 2.3–10.0 log HBV DNA), 50% of the patients had HBV DNA above 10^5 copies/ml in serum. Mean log HBV DNA level in saliva was 3.2 (range 2.3–7.5), 15% had a HBV DNA above 10^5 copies/ml in saliva. Mean log HBV DNA level in urine was 2.6 (range 2.3–5.4) and 1% had a HBV DNA above 10^5 copies/ml in urine. A high, non-linear correlation was shown between HBV DNA in serum and saliva (Spearman's rho 0.82) and between serum and urine (Spearman's rho 0.74).

Conclusions: The significant amounts of HBV DNA found in saliva and urine in chronic HBV patients with high viraemia in serum may have implications for the understanding of hepatitis B epidemiology. The potential infectivity of these body fluids may provide and explanation for the 20% of cases of infection obtained through horizontal transmission for which the origin of infection is yet unknown.

Introduction

Hepatitis B virus (HBV) infection is a major cause of chronic hepatitis. Carriers of the virus are at increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (1). The most common modes of transmission of the virus are through parenteral, sexual or perinatal exposure to the virus. In addition, epidemiological studies of HBV show a steep rise in the prevalence of HBV in childhood, which indicates the importance of horizontal transmission as route of infection (2-5). Despite abundant epidemiological evidence for horizontal transmission of HBV, the exact mechanism of viral spread in patients who are not infected at birth and who are not yet sexually active remains to be elucidated.

With respect to the potential infectivity of body fluids, a reference point is provided by the US Public Health Service guidelines for the management of occupational exposure to HBV, hepatitis C virus (HCV) and human immunodeficiency virus (HIV), and its recommendations for post exposure prophylaxis (6). In the guidelines, blood, body fluids containing visible blood, semen and vaginal secretions are considered potentially infectious. Other potentially infectious fluids include: cerebrospinal, synovial, pleural, peritoneal, pericardial and amniotic fluids. In addition, the report states that faeces, nasal secretions, vomitus, tears, sputum, saliva and urine are not considered potentially infectious unless they contain blood. Furthermore, it notes that the risk for transmission of HBV, HCV and HIV infection from these fluids and materials is extremely low.

Nevertheless, the presence of HBV DNA in body fluids may indicate the potential for transmission of infection and thereby infectivity (7). When it is considered that HBV DNA has previously been detected in body fluids other than serum, including in saliva and urine, this indicates that these fluids may be infectious (8-12). This suggests that urine or saliva could inoculate HBV through cutaneous scratches, abrasions, burns, other lesions or on mucosal surfaces, which is supported by previous reports of HBV transmission following a human bite or being spat in the eye by an infected individual (13-16). In our department's experience, we have seen a patient whose only known route of infection was through kissing an infected individual, which prompted us to study the infectivity of saliva in more detail.

The development of molecular diagnostic assays has revolutionised the ability to detect viruses both qualitatively and quantitatively. Studies have shown that, following needle stick injuries and after delivery of babies from HBsAg positive mothers, infectivity increases with

increasing levels of HBV DNA (17, 18). Therefore, more information regarding the precise amounts in various body fluids would provide insight in the potential infectivity of these fluids. Our study describes the results of paired, quantitative HBV DNA measurements in serum, saliva and urine in 150 chronic HBV patients and the development of a model showing the correlation between HBV DNA levels in these fluids. It is the first study to use the TaqMan technology to assay HBV DNA in urine and saliva and, as such, aims to provide new insights into the potential importance of these fluids in the mode of horizontal transmission of HBV. The model's correlations between HBV DNA levels in various fluids will help subsequent epidemiological field studies to test the hypothesis that particular groups of patients are at risk of horizontal HBV transmissions.

Patients and methods

Selection of patients

Eligible patients included both men and women attending our outpatient clinic who were chronic HBsAg carriers above 18 years. Patients were excluded if they were known to be co-infected with HCV, the hepatitis D virus or HIV. Treatment for chronic HBV infection was not an exclusion criterium. All patients with gingival or buccal mucosa lesions or a tendency to bleeding gums were excluded. To develop the model, patients were pre-selected based on a previous quantitative HBV DNA test and thus provided a range of serum HBV DNA levels.

Study design

Paired serum, saliva and urine samples were collected from 150 pre-selected chronic Hepatitis B virus patients. Unstimulated saliva samples of approximately of 1–2 ml were collected by asking the subjects to dribble into a sterile plastic container. Saliva samples were clear of blood on visual inspection. Serum, saliva and urine samples from individual patients were collected on the same day and stored at –20 ^{0}C until use.

This study was performed in accordance with the principles of Good Clinical Practice. The Medical Ethics Committee of the participating centre approved the protocol and all patients gave written, informed consent.

Patients in the transition population used to test the model

For comparison we collected serum, urine and saliva samples from 237 consecutive unselected chronic HBsAg carriers who visited the municipal health service in Rotterdam, the

Netherlands. These patients were identified from a regular source and contact tracing study and provided a 'transition' population with which to test the model because they were expected to have HBV DNA levels representing the general population with chronic HBV.

Assays

Isolation of HBV DNA was performed using the Magnapure LC isolation station (Roche Applied Science, Penzberg, Germany) with a modified protocol HBV-02 that included and initial proteinase K digestion (19).

To monitor both loss and inhibition of the sample, before the extraction procedure a universal internal control consisting of a known number of Phocid herpesvirus type 1 (PhHV-1-virus) particles was added to the saliva and urine sample (20). Corrections were made if necessary.

HBV DNA in serum, saliva and urine was quantitatively measured using the HBV DNA TaqMan assay, as previously described (19, 21). This assay enabled the accurate determination to a level of 373 genome equivalents per ml. All assays were carried out in duplicate with negative control samples and were calibrated using EUROHEP HBV DNA standards (22).

Urine analyses

Nephur^{6} test strips® (Roche Diagnostics) were used to measure urine pH and to detect leucocytes, glucose, nitrate protein and blood (erythrocytes, haemoglobin) in urine.

Statistics

Statistical analysis was performed using SPSS-version 10.1. To test for correlation between HBV DNA in serum and in saliva and between HBV DNA in serum and in urine, we used the Spearman correlation coefficient (rho). This is measures the strength of association between two variables.

To describe the associations between log HBV DNA in serum and saliva or urine, linear and non-linear regressions (exponential, quadratic and cubic) were applied. For statistical reasons, samples negative by PCR were calculated as 186.5 (a figure of half of the detectable range of 0–373 copies/ml). Samples positive by PCR were calculated as 686.5, which is the value

between 373 and 1000 copies/ml. These calculations compensated for instances where the signal was barely detectable and where it was not possible to accurately quantify the signal.

Association between HBV DNA in saliva and either age or HBeAg status was tested by univariate analyses (t-test or one-way analysis of variance for group data and linear regression analysis for continuous data).

Likewise, associations between HBV DNA in urine and sex, HBeAg-status, pH of the urine sample, the presence of leucocytes, glucose, nitrate, protein and blood in urine were tested univariately (t-test or one-way analysis of variance for group data and linear regression analysis for continuous data). Factors with a p-value <0.05 were considered significant.

The derived quadratic model was then used to test for association between log HBV DNA levels in urine and, individually, the significant factors: sex, HBeAg and urine leucocytes. It was also used to account for the high correlation between log HBV DNA in serum and urine. Multivariate analyses with sex, HBeAg and leucocytes completed the analyses.

Results

HBV DNA in serum and saliva

A total of 94 men (63%) and 56 (37%) women were pre-selected to participate in the study. We collected 141 paired serum and saliva samples and 145 paired serum and urine samples. For the purposes of the calculations, the missing cases were excluded with their paired samples.

Mean log HBV DNA in serum was 5.8 (range 2.3–10.0 log HBV DNA); mean log HBV DNA level in saliva was 3.2 (range 2.3–7.5 log HBV DNA) and the level in urine was 2.6 (range 2.3–5.4 log HBV DNA). A high correlation was shown between HBV DNA in serum and saliva (Spearman's rho 0.82) and between serum and urine (Spearman's rho 0.74).

Of the 147 patients positive for HBV DNA in serum, 69 (47%) also had detectable HBV DNA in saliva and 47 (32%) had detectable HBV DNA in urine. None of the patients who were negative for HBV DNA in serum were positive for HBV DNA in saliva or urine. Table 1 shows the percentage of patients who were positive for HBV DNA in saliva and urine for

each log level of HBV DNA measured in serum. The missing cases were excluded with their paired samples.

Table 1. The proportion of patients who were positive for HBV DNA in saliva and urine for each log level of HBV DNA in genome equivalents per ml measured in serum.

Log HBV DNA in serum (copies/ml)	N	Positive for HBV DNA in saliva	Positive for HBV DNA in urine
≤ 2.27	8	0 (0%)	0 (0%)
> 2.27 – ≤ 2.84	22	3 (14%)	1 (5%)
> 2.84 – ≤ 4	21	1 (5%)	0 (0%)
> 4 – ≤ 5	22	4 (18%)	1 (5%)
> 5 – ≤ 6	8	2 (25%)	0 (0%)
> 6 – ≤ 7	11	7 (64%)	2 (18%)
> 7 – ≤ 8	15	14 (93%)	8 (53%)
> 8 – ≤ 9	17	17 (100%)	12 (71%)
> 9 – ≤ 10	22	20 (91%)	22 (100%)
> 10	1	1 (100%)	1 (100%)
Total	147	69 (47%)	47 (32%)

The likelihood of transmitting hepatitis B is dependent on several factors, including the number of infectious particles transmitted. Assuming that the HBV DNA level reflects the number of infectious particles, infectivity is thus determined by HBV DNA level and volume of infectious fluid. Below an HBV DNA level of 10^5 copies/ml, virus transmission via needlestick or mucosal scratch is highly unlikely (23). Of the selected patients in this study, 50% had levels of HBV DNA of $\geq 10^5$ copies/ml in serum, 15% had HBV DNA levels of $\geq 10^5$ copies/ml in saliva and 1% had $\geq 10^5$ copies/ml in urine.

HBV DNA levels in the transition population

In the 237 unselected patients who visited the municipal health service, mean log HBV DNA level in serum was 4.0 (range 2.3–10.3). Seventeen per cent of the patients had HBV DNA levels of $\geq 10^5$ copies/ml in serum, 11% had serum HBV DNA levels of $> 10^7$ copies/ml and 7% had serum levels of $> 10^9$ copies/ml.

Model development

We used the Lowess (locally weighted scatterplot smoothing) curve to describe the data. The Lowess fit uses an iterative locally weighted curve to a set of points and is ideal for modelling complex processes for which there is no prior knowledge about the shape or form of the curve. Of the linear, exponential, cubic and quadratic models, the quadratic one best fitted the Lowess curve. Therefore, the relationships between quantitative HBV DNA levels in serum and saliva and between HBV DNA levels in serum and urine could be described by using a quadratic model.

The following quadratic equation was used to describe the relationship between log HBV DNA in serum and the log HBV DNA level found in saliva and urine:

$Y = b_0 + (b_1 \times t) + (b_2 \times t^2)$

Where : $Y =$ log HBV DNA in saliva or urine

b_0, b_1 and b_2 = constant

$t =$ log HBV DNA in serum

The following fit (95% confidence interval) for saliva was obtained from the quadratic equation:

Y=log HBV DNA in saliva

$b_0 = 3.45$ (2.64;4.24) ($p<0.001$)

$b_1 = -0.65$ (-0.96;0.35) ($p<0.001$)

$b_2 = 0.090$ (0.065;0.12) ($p<0.001$)

t = log HBV DNA in serum

Thus the relationship between HBV DNA in serum and saliva was fitted by:

Log HBV DNA in saliva =

3.45 + (-0.65 x log HBV DNA in serum) + (0.090 x [log HBV DNA in serum]^2) (fig. 1A)

For urine, the fit (95% C.I.) was:

Y = log HBV DNA in urine

$b_0 = 2.99$ (2.58;3.41) ($p<0.001$)

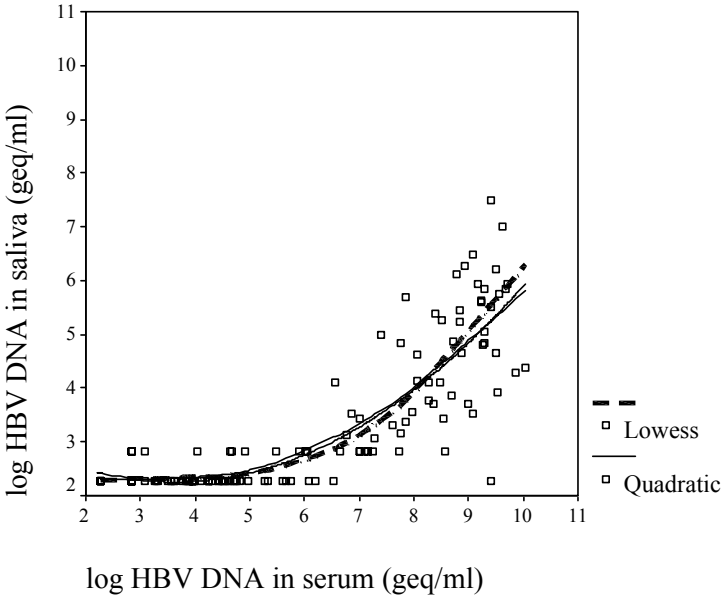
$b_1 = -0.36$ (-0.52;-0.20) ($p<0.001$)

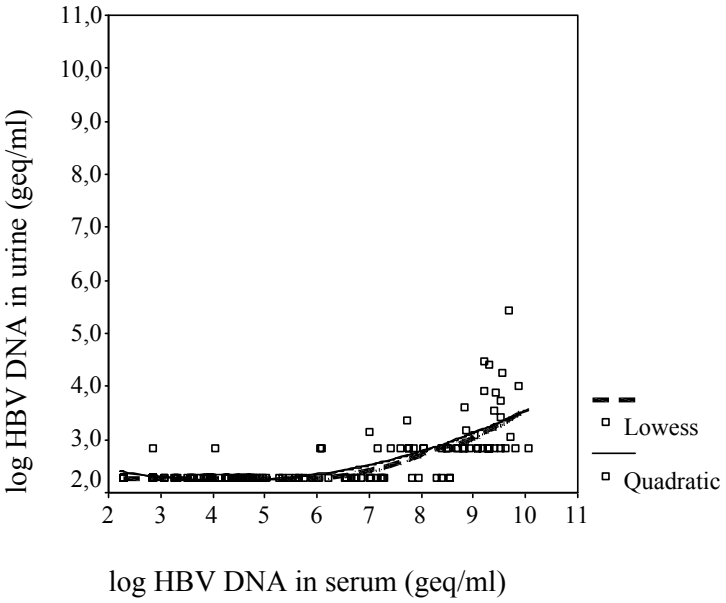
$b_2 = 0.042$ (0.029;0.054) ($p<0.001$)

t = log HBV DNA in serum

Thus the relationship between HBV DNA in serum and urine was fitted by:
Log HBV DNA in urine =
2.99 + (-0.36 x log HBV DNA in serum) + (0.042 x [log HBV DNA in serum]^2) (fig. 1B)

Figure 1A+B Individuals with low viraemia (less than 10^5 copies/ml) had undetectable or very low levels of HBV DNA in saliva or urine. High virus levels (more than 10^5 copies/ml) in saliva were found in individuals with serum HBV DNA of 10^7 copies/ml or more. High virus levels in urine were only found in individuals with serum HBV DNA of 10^9 copies/ml or more.
(1A.) Association between HBV DNA (copies/ml) in serum and saliva. The dotted line represents the Lowess fit; the black line represents the fitted curve of the quadratic model.
(1B.) Association between HBV DNA (copies/ml) in serum and urine. The dotted line represents the Lowess fit; the black line represents the fitted curve of the quadratic model.





Determinants of HBV DNA in saliva and urine

The HBeAg status was determined for 148 pre-selected patients; 65 patients (44%) were HBeAg positive, 82 (55%) were HBeAg negative and one patient (1%) was HBeAg borderline. All HBeAg positive patients had detectable HBV DNA levels in their serum. HBV DNA was detectable in saliva in 47 of 62 HBeAg positive patients (76%) and in urine in 39 of 65 HBeAg positive patients (60%). Seventy-three of 81 HBeAg negative patients (90%) had detectable HBV DNA in serum. In saliva, HBV DNA was detectable in 23 of 79 (29%) and, in urine, 8 of 80 HBeAg negative patients (10%) had detectable HBV DNA.

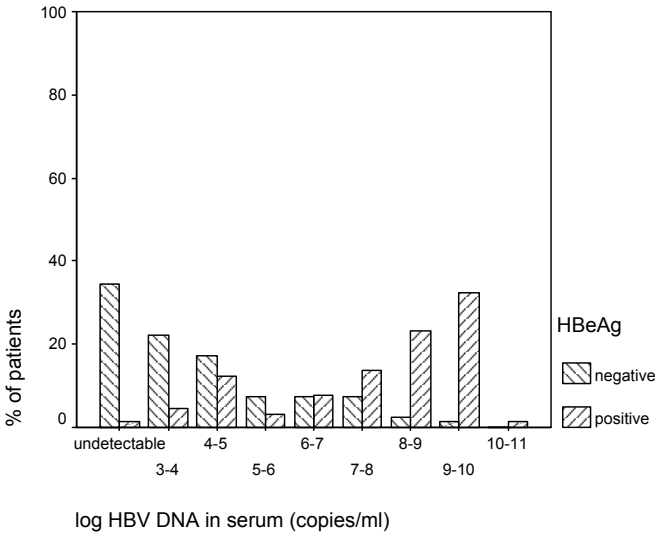
Figures 2A, 2B and 2C show quantitative HBV DNA levels in serum, saliva and urine versus HBeAg status of the patient.

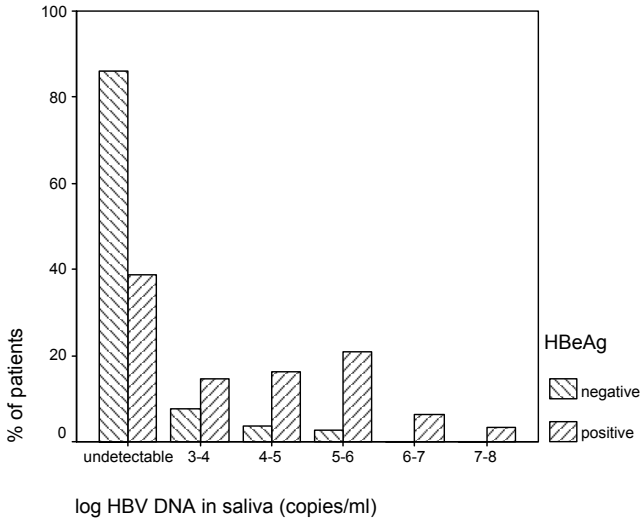
Figure 2

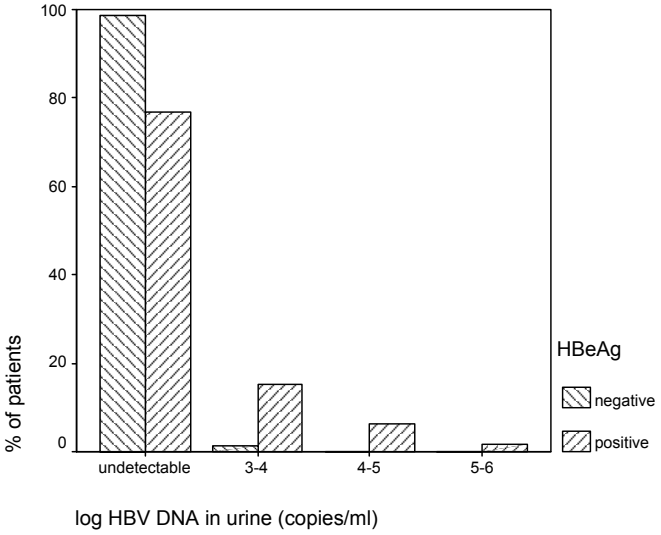
(2A.) Quantitative log HBV DNA levels (copies/ml) in serum versus HBeAg status of the patient.

(2B.) Quantitative log HBV DNA levels (copies/ml) in saliva versus HBeAg status of the patient.

(2C.) Quantitative log HBV DNA levels (copies/ml) in urine versus HBeAg status of the patient.







In the univariate analyses, the level of HBV DNA in serum and serum HBeAg status were significantly related to the level of HBV DNA in saliva ($p<0.001$). By contrast, using the quadratic model, HBeAg shows no significant relation ($p=0.26$) with the level of HBV DNA in saliva. This discrepancy can be explained by the high correlation between the level of HBV DNA in serum and HBeAg status (Spearman rho: 0.65).

In addition to the level of HBV DNA in serum ($p<0.001$), the following factors were significantly related to the level of HBV DNA in urine by univariate analyses: sex ($p<0.002$), HBeAg status of the patient ($p<0.001$) and the presence of leucocytes in urine ($p<0.001$). Thirteen of 94 male patients (14%) compared with 28 of 54 female patients (52%) ($p<0.001$) showed the presence of leucocytes in their urine. When the quadratic model was used

individually with each of these factors, no association was found between levels of HBV DNA in urine and HBeAg status (p=0.65), a borderline significant association was found with gender of the patient (p=0.054) and a significant association was found with the presence of leucocytes in urine (p<0.03).

Including all these factors in the multivariate analyses resulted in over-parameterisation (sex, p=0.20; HBeAg status of the patient, p=0.90; and the presence of leucocytes in urine, p=0.18), as a result of the high correlation between: sex and the presence of leucocytes (Spearman rho=0.41), sex and HBeAg (Spearman rho=0.22), and serum HBV DNA and HBeAg status (Spearman rho=0.65).

Discussion

In this large study on paired, quantitative HBV DNA measurements in serum, saliva and urine, we found a non-linear correlation between the level of HBV DNA in serum and the level of HBV DNA in saliva or urine. HBV DNA levels above 10^5 copies/ml were found in 15% of saliva and 1% of urine samples. None of the samples showed a higher HBV DNA level in saliva or urine than in the paired serum sample. This agrees with an earlier study by Knuttson et al. (11), which found a difference in end-point titration PCR between serum and urine averaging 10^3.

In this study there was a statistically significant relationship between serum HBV DNA and detection of saliva HBV DNA. A similar observation has also been documented between plasma HCV RNA and saliva HCV RNA (24). Possible explanations for the presence of HBV DNA in saliva of chronic HBV carriers may include: contamination of saliva with blood, the presence of peripheral blood mononuclear cells (PBMCs), a possible role for salivary glands as sanctuary site for HBV, or the presence of oral mucosal transudate (25).

The CDC report states that saliva is not to be considered potentially infectious unless it contains blood and another study shows that an HBV DNA level of 10^5 copies/ml is a level below which virus transmission via needlestick or mucosal scratch is highly unlikely (6, 23). The simplistic view is that a clear saliva sample is unlikely to be infectious. However, in both HBeAg-positive and HBeAg-negative patients, our results demonstrated HBV DNA levels of up to 10^7 copies/ml in clear saliva samples. In patients with high virus levels (HBV DNA levels above 10^7 copies/ml) in serum, we therefore postulate a potential role for saliva in

transmitting HBV. This is based on previous experimental studies in which gibbons inoculated subcutaneously with saliva from HBsAg-positive donors developed HBsAg followed by anti-Hbs (7, 26). However, in these studies, none of the animals that were administrated saliva orally developed evidence of HBV infection. These experiments show that the HBV DNA particles in saliva may remain infectious under particular conditions.

A previous study found a significant female predominance among the positive urine samples ($p<0.05$) (11). In our study, the association between sex and HBV DNA level in urine was only of borderline significance. The explanation for this difference may lie in the presence of leucocytes in the urine. PBMCs, such as leucocytes, have been shown to harbour HBV (27). HBV DNA in urine is significantly related to the presence of leucocytes, and these cells are more frequently found in the urine of women than of men. Urine is considered infectious only if it is contaminated with blood (28). However, our data supports the suggestion by Knuttson et al. (11) that there may be other circumstances under which urine is potentially infectious.

Saliva HBV DNA levels of above 10^5 copies/ml were found in patients with serum HBV DNA levels of above 10^7 copies/ml and, in urine, similarly high levels were found in patients with HBV DNA levels above 10^9 copies/ml in serum. In a hospital population of selected patients, 31% of the chronic HBV patients reached HBV DNA levels in serum above 10^7 copies/ml compared with 11% of the (unselected) patients who were tested by the Municipal Health Service.

In this study, the selected group of patients, with an expected broad spread of serum HBV DNA levels (log transformed HBV DNA values to achieve a normal distribution), were used to develop a model to estimate HBV DNA levels in saliva and urine. Using this model, quadratic curves best approximated to the relationship between HBV DNA levels in serum and those in saliva or urine.

To test the model we also examined an unselected population of apparent chronic HBsAg carriers in a public health setting. In these patients, the mean log HBV DNA level in serum was 4.0 (range 2.3–10.3). Given this range, the model suggests that at least some of these patients are likely to have HBV DNA saliva and urine and therefore contact with these fluids may result in infection.

Conclusions

Estimates suggest that approximately 10% of HBV particles detected in serum are infectious (22). The proportion of infectious HBV particles in saliva and urine are unknown. In our study HBV DNA in urine and saliva reached levels above those which, in serum, would be considered infectious. The potential infectivity of these body fluids may provide and explanation for the 20% of cases of infection obtained through horizontal transmission for which the origin of infection is yet unknown. However, we advise transmission studies in appropriate models to establish the transmission risk for each log level of viraemia for the various body fluids before further translating our findings in public health advisory practice.

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8

DISCUSSION: CLINICAL SIGNIFICANCE OF QUANTITATIVE TESTING OF VIRAL LOAD IN HEPATITIS B

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Introduction

Virologic diagnosis and monitoring of Hepatitis B virus (HBV) infection are based on serologic assays detecting specific HBV antigens and antibodies and assays that can detect or quantify HBV DNA (1, 2). The development of amplification techniques was a great step forward and it became possible to study the viral load in relation to disease management.

Therapy of chronic HBV infection has evolved rapidly during the last years. Nowadays there are three registered treatment regimens for chronic HBV: interferon-alpha (IFN-α), lamivudine and adefovir (3-5). A number of other compounds are currently under investigation in phase II/III trials and include pegylated IFN (PEG-IFN), emtricitabine, clevudine, entecavir and others. Other studies are assessing the efficacy of combining anti-HBV therapies. To identify parameters that accurately reflect the clinical efficacy of these treatment regiments in chronic HBV patients is not easy, due to the heterogeneity of the disease. The final goal in HBV therapy is to prevent complications of decompensated liver disease leading to morbidity (ascites, infections) and mortality (variceal bleeding, the development of hepatocellular carcinoma). However, due to the slow course of chronic HBV, these complications cannot serve as an endpoint in clinical trials and therefore other endpoints are searched for to assess the efficacy of therapeutic interventions over shorter periods of time. Especially by the FDA, liver histology was adopted as the primary efficacy endpoint. Nowadays the definitions of treatment endpoints as described by the EASL International Consensus Conference On Hepatitis B has included a HBV DNA level in their definition of virologic response (6).

The presence of HBV DNA in peripheral blood reflects active viral replication in the liver. HBV DNA quantification can be used to monitor viral replication kinetics to better understand the mechanism of infection, to monitor the effect of therapy or the emergence of drug-resistant variants (7-10). Furthermore, a statistically significant correlation between serum HBV DNA levels and histological grading at the end of treatment has been found (10). Thereby making the decrease in HBV DNA a possible substitute marker for histology, although not yet adapted by the FDA (registration authorities).

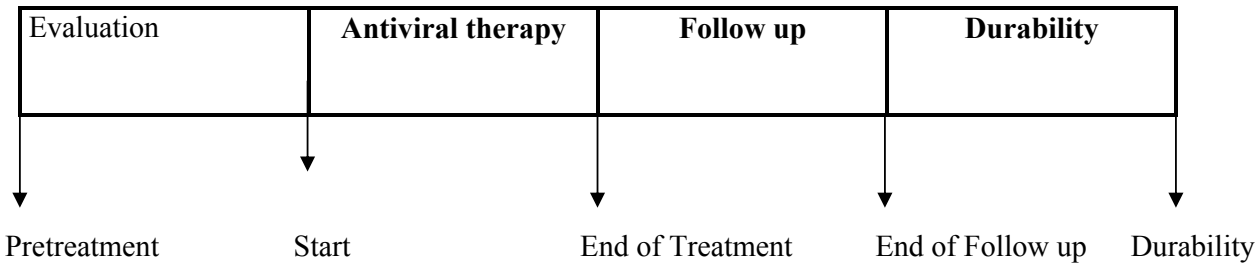
The advent of DNA sequencing allowed the detection of precore mutants, YMDD mutants and the comparison of viral isolates on the genomic level (11-13). These new technologies enable the introduction of an individual patient disease management concept.

The EASL International Consensus Conference On Hepatitis B recommended HBV DNA testing at different time points (figure 1.):

1. before therapy
2. during treatment
3. at the end of therapy (EOT) and at the end of follow up (EOF)
4. to evaluate durability of response

We discuss the clinical relevance of quantitative HBV DNA measurements at these specific time points that – in all likelihood – were the bases for recommendations of the consensus panel.

Figure 1. Definitions to describe a course of a chronic HBV patient.



Clinical significance of quantitative HBV DNA measurements before therapy

HBV DNA quantification in untreated HBsAg carriers enables the physician to assess viral replication and to follow the course of infection.

HBV DNA levels are known to fluctuate over time in a proportion of chronic HBV carriers. A one year randomised placebo-controlled study evaluating the efficacy of famciclovir in chronic HBeAg-positive patients showed a median drop of 22% in HBV DNA levels in the placebo group (14). This confirms the observation that the natural course of HBV in patients with stable liver disease shows a tendency towards biochemical and virological remission. In a large placebo-controlled 48-week study of adefovir, 53% of placebo patients had fluctuations of less than 0.5 $\log_{10}$ copies/ml, whereas the other 47% of placebo patients had a large (1-5 $\log_{10}$ copies/ml) oscillations in HBV DNA (10^4-10^9) (3).

With the knowledge that HBV DNA levels can fluctuate in the individual patient, serial determinations of viral load are recommended to ascertain HBV replication status after the first diagnosis of a chronic HBV infection has been made (3, 14, 15).

Active viral replication is associated with the presence of HBeAg. Seroconversion from HBeAg to anti-HBe in chronic HBV carriers either through treatment or spontaneously is linked to a decrease in serum transaminases and HBV DNA levels. Therefore the HBeAg/anti-HBe status is often used as a marker for infectivity, with HBeAg-positivity representing active viral replication (16, 17). However, high HBV DNA levels can be found in HBeAg negative patients due to the presence of mutations in the precore (G to A substitution at nucleotide 1896) and core promotor region (a dual mutation; $A_{1762}T$, $G_{1764}A$) (12). Differentiation between HBeAg – negative chronic HBV and the inactive HBsAg carrier state may be difficult and require HBV DNA testing for 6-12 months.

For the selection of optimal therapy in the individual patient HBV DNA levels can be used. Predictive factors for end of treatment response in chronic HBV patients treated with IFN-α have been studied in the past. In a randomised controlled trial of IFN-α, with or without prednisone priming, Perrillo et al. found that baseline serum HBV DNA level was the most important independent predictor of response (P=0.003) (5). Approximately 50% of the patients with baseline HBV DNA levels under 100 pg/ml (solution-hybridization assay, Abbott laboratories) responded to treatment with 5 million unites of IFN-α compared to only 7% of patients with HBV DNA levels at baseline above 200 pg/ml. A study investigating the post-treatment durability of HBeAg seroconversion following lamivudine, IFN monotherapy or IFN-lamivudine combination therapy also identified pre-treatment HBV DNA levels as the major predictor of sustained response (18).

HBV DNA quantification also enables the clinician to interfere in a pre-clinical phase of disease development. Vertical transmission of HBV can occur despite vaccination of the child. Failure of neonatal HBV vaccination has been associated with high maternal viremia. In a Dutch study the protection rate of vaccination was 100% if maternal HBV DNA was ≤ 1.2×10^9 geq/ml, but only 68% if HBV DNA levels were above this level (19). Treatment of high-viremic mothers (HBV DNA level above 1.2×10^9) with lamivudine during the last month of pregnancy reduced HBV DNA levels significantly and improved the chance of successful vaccination of the child (20).

Clinical significance of quantitative HBV DNA measurements during therapy

Quantification of HBV DNA enabled physicians to monitor clinical situations such as the effect of antiviral therapies, the emergence of drug-resistant mutants and the evaluation of treatment compliance (3, 4, 9).

Frequent HBV DNA measurements at the start of therapy gives insight into the speed and variability of patterns of viral decay. This viral decay can be modelled with a mathematical approach and subsequently provides a tool for evaluating the effect of antiviral therapy (7, 8, 21, 22).

Midtreatment HBV DNA levels during IFN-α therapy showed a significant correlation ($P< 0.001$) with response in Chinese adults with chronic HBV infection (23). Response was achieved in 53% of patients who had a HBV DNA level below 0.7 Meq/ml (branched DNA assay) at midtreatment, but in only 17% of those who remained HBV DNA positive.

A study evaluating quantitative HBeAg measurements versus quantitative HBV DNA measurements for prediction of non-response and response in interferon (IFN)-α treated HBe-antigen positive chronic HBV patients showed that monitoring with quantitative HBV DNA levels was superior to monitoring with quantitative HBeAg levels. This study also showed that quantitative HBV DNA testing at baseline in combination with an absence of decrease between baseline and week 12 has a high predictive value for identifying patients who have virtually no chance of reaching a sustained response with IFN therapy (24). If patients had a log HBV DNA level at baseline above 8 in combination with a log decrease <1.0 log between baseline and week 12 the chance of non-response was 100%. Sixty-one % of non-responders could be identified in this way, with no exclusion of potential responders.

If a rise in HBV DNA levels is observed during nucleoside treatment, the compliance of the patient has to be evaluated at all times. The alternative explanation is viral resistance to lamivudine with the emergence of YMDD drug resistance variant (25). Mixture of YMDD variants can exist next to each other and they are mainly identified after an increase in the HBV DNA load in serum is observed (9). Resistant variants have been well characterized and are localised in two major domains of the reverse transcriptase (rt) region of the polymerase gene (13, 26). Analyses of the YMDD region of the C domain of the polymerase gene showed in case of resistance a replacement of methionine (rtM204) by either valine (rtM204V), isoleucine (rtM204I) or serine (rtM204S). The valine (rtM204V) variant is in most cases accompanied by a mutation of leucine into methionine (rtL180M) in the B domain (27). The incidence of arising resistant variants increases with a longer duration of therapy. After 1 year in 15-30% of patients and in up to 50% after 3 years of lamivudine monotherapy a resistant virus emerges (4, 28, 29). Buti et al. performed a study to determine whether measuring quantitative HBV DNA early during lamivudine therapy in chronic Hepatitis B is useful in predicting maintenance of response to therapy and the emergence of YMDD variants (30). Quantitative HBV DNA testing at month 3 of therapy that shows a negative result (below

1000 copies/ml) has a sensitivity of 73% and a specificity of 88% for the early prediction of maintained response to lamivudine therapy (=undetectable serum HBV DNA throughout the therapy period). The HBV DNA levels at month 3 were lower among responders than among non-responders, but no significance differences were detected in HBV DNA decline during the first 3 months of therapy between those with or without YMDD variants at year 1. Another study performed by Puchhammer- Stöckl et al. did found a statistically significant difference in HBV DNA levels after 3 months of therapy with lamivudine between patients who developed resistant HBV strains during treatment and those who not (31). All patients in whom later drug-resistant virus emerged still had detectable virus levels of 10^3-10^5 HBV copies/ml of serum after 3 months of therapy, whereas patients who did not develop lamivudine-resistant HBV variants during follow-up had no detectable level of virus in serum by PCR (detection limit 1000 copies/ml) at that time. Early detection of HBV DNA breakthrough and viral resistance permits earlier intervention, eg treatment modification from lamivudine to adefovir, tenofovir or entecavir.

Clinical significance of quantitative HBV DNA measurements at the end of therapy until the end of follow up

HBV DNA measurements at the end of treatment can be used to determine the effect of therapy in an individual patient and to compare the efficacy of different treatment regiments (3, 7, 32). The development of different amplification techniques has shifted the lower limit of detection of HBV DNA over the last few years (1, 2, 33). In patients, who previously were considered responders because of negative results by non-PCR assays, nowadays low levels of persisting viral replication can be detected. This has changed the definitions of treatment responses and a HBV DNA level of 10^5 copies/ml has been proposed as a cut-off below which hepatitis B is considered inactive (34, 35).

The EASL international consensus conference on hepatitis B used the following definitions for treatment endpoints, without indicating a specific type/time point to be adopted as a primary efficacy end point (35). A biochemical response is a fall in serum aminotransferase levels to the normal range. A histological response is defined as a pre-determined decrease in histological activity score with no worsening in fibrosis, as assessed by dual observers. A 2-point decrease in HAI score has been the most commonly used end-point. A virological response implies that HBV DNA falls below 10^5 copies/ml. In addition HBeAg becomes undetectable when present initially (35).

The cut-off level of 10^5 copies/ml, which is proposed as a level below which HBV is inactive and non-progressive, is the limit of detection in the non-PCR-based commercial assays used in many past clinical studies. In HBeAg-positive chronic hepatitis B patients the most important goal of therapy is to obtain seroconversion to anti-HBe. The level of HBV DNA, which is associated with seroconversion, is still unknown. In a study of 24 patients, who received lamivudine therapy, 6 of 12 patients with dramatic decreases in HBV DNA level (below 10^4 copies/ml) seroconverted to anti-HBe, versus none of 11 patients whose serum HBV DNA remained above 10^4 copies/ml (36). A study performed by Chu et al. showed a wide range in HBV DNA levels (10^3-10^7 copies/ml) at the time HBeAg first became undetectable (37). This observation was true for spontaneous as well as IFN-related HBeAg clearance/ seroconversion. A further decline in serum HBV DNA levels was observed in patients with sustained HBeAg loss. All of the inactive carriers had a HBV DNA persistently below 10^5 copies/ml. With the availability of more sensitive quantification methods the cut-off level of 10^5 copies/ml needs to be evaluated further in large prospective clinical trials.

A statistically significant correlation between serum HBV DNA levels and histological grading at the end of treatment has been found (10). However, a major reservation of this study is the use of median (or mean) values instead of individual patient data. Also many different HBV DNA assays and hatches were used. The finding of a correlation between serum HBV DNA levels and histological grading at the end of treatment does not prove true surrogacy. To implementate HBV DNA as the primary surrogate endpoint of efficacy for new therapies appears therefore premature.

The HBV DNA level at the end of therapy can be an important guide for further therapy. In a large prospective, randomised controlled trial investigating the efficacy of treatment prolongation with additional 16 weeks in those patients who did not respond with HBeAg seroconversion during a standard 16-week IFN-α course, a low level of HBV DNA (< 10 pg/mL) at randomization was found to be the only independent predictor of response (52% versus 0% P <0.001) during prolonged therapy, while a low HBV DNA level at entry tended toward significance (p=0.07) (38).

Most clinical studies include a 6-month follow up for the assessment of sustained response; the international consensus conference advises a minimum of 12 months post-treatment follow-up.

Clinical significance of quantitative HBV DNA measurements to evaluate the durability of response

In the first year of follow up, durability for virologic response should be monitored every 1-3 months in patients treated with nucleoside analogues and at least every 6 months in patients who received IFN-α. The occurrence of relapse after stopping of therapy is discussed below.

Because chronic HBV is a disease with variable long-terms outcomes, durability of response has to be assessed after stopping of therapy. The EASL international consensus conference on hepatitis B recommends after 1 year of follow up monitoring at least every 6 months to assess durability of response.

Interferon-alpha (IFN-α) is known as a powerful immune stimulatory drug and IFN-α treatment increases the spontaneous response rate and leads to loss of HBeAg in 15-40% of patients (39). IFN-α induced HBeAg seroconversion in HBeAg-positive patients is sustained in approximately 70% after three years of follow-up and significantly reduces morbidity and mortality (18). But the sustained response rate is much lower in HBeAg-negative patients who were treated with IFN-α. A study performed by Manesis et al. in HBeAg-negative patients showed that after 7 years of median follow up, 39 patients (one third of the 117 initial responders) were still in biochemical and virological remission after a single IFN course (40). After discontinuation of IFN-α therapy, 65 of the 117 initial responders (56%) relapsed during the first year and another 13 patients relapsed after the first treatment year within a median of 20.6 (range, 13.7-32.2) months (40). Prolonging of IFN-α treatment in HBeAg-negative patients seems to increase long-term efficacy. A study of 24-months interferon therapy in HBeAg-negative patients showed in 30 of the 46 responders (65%) a sustained response (i.e., normal ALT level and undetectable serum HBV DNA level by non-PCR methods) for a median of 71 months (27-101) after stopping treatment (41).

After one year of lamivudine monotherapy HBeAg seroconversion occurs in approximately 20% of patients. Disappearance of HBeAg can be observed more frequently in patients with high ALT levels (42). Prolongation of therapy leads to a greater proportion of patients losing HBV DNA and HBeAg. However, durability of HBeAg seroconversion after lamivudine monotherapy is limited: HBeAg relapse can be observed in 42% after one year and in up to 54% of patients after three years of follow-up (18).

The use of adefovir dipivoxil for chronic hepatitis B infection has been reported to be effective in HBeAg-positive and HBeAg-negative HBV carriers (3, 43). It provides effective antiviral therapy in both wildtype and lamivudine-resistant HBV infected patients (32, 44). Adefovir therapy is notable for the absence of major mutations in the HBV polymerase during

treatment for up to 60 weeks (45). The incidence of resistance after 2 years is estimated at 2%. The rate of sustained response to adefovir is still unknown.
Monitoring of HBV DNA after 1 year of follow-up is still useful in determining long-term durability of sustained response.

Discussion

HBV DNA quantification gives insight in the level of viral replication in the untreated patient and is a helpful tool in the assessment of response, the selection of drug-resistant mutants and the evaluation of treatment compliance in treated patients. The frequency of assessment of viral load is different for each individual patient, but guidelines have been developed. Based on our experiences and on the guidelines given by the EASL international consensus conference on hepatitis B, we want to recommend quantitative HBV DNA assessments at the following timepoints:

Before start of therapy:

Monitoring for 3-6 months with 1-3 monthly determination of HBV DNA before start of treatment; especially important for differentiating inactive carriers from patients with HBeAg-negative chronic hepatitis. Also important is detecting patients with ALT flares.

During therapy:

At the start of therapy a HBV DNA determination is useful as a baseline level to assess the effect of treatment later in time. To assess virological response, to guide decisions on when to stop treatment and to detect virological breakthrough, HBV DNA levels should be measured during treatment at least every 3-6 months.

After stopping of therapy:

Durability of virologic response should be monitored every 1-3 months during the first 12 months after cessation of therapy and every 6-12 months thereafter.

The use of quantification assays for HBV has improved our understanding of the pathogenesis and natural history of HBV infection. The improved sensitivity of assays enabled physicians to determine if continued treatment is associated with a further decrease in HBV DNA levels as well as earlier detection of a rise in HBV DNA levels. However, there are still questions that need to be answered.
The implementation of molecular diagnostic assays has made the detection of qualitative and quantitative HBV relatively easy, but also warrants the use and introduction of standardised materials as well as participation in international quality control programs (46).

Standardisation is necessary to accurately and absolutely determine viral load and the possibility to compare data from different laboratories (2).

To reduce unnecessary exposure to treatment and to reduce costs physicians must decide at an early stage whether continuation of that treatment has a reasonable chance of success for the individual patient. More studies evaluating the use of quantitative HBV DNA measurements in the prediction of response and non-response have to be performed. Clinically the most relevant goals are a high predictive value for non-response without exclusion of potential sustained responders.

Finally, the cut-off level of 10^5 copies/ml, which is proposed as a level below which HBV is inactive and non-progressive needs to be re-evaluated in large clinical trials. Which reduction in HBV DNA level is needed before HBeAg-seroconversion occurs? The importance of continuous low-level replication with normal ALT levels is not clear and has to be evaluated in long-term follow up studies.

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9

Summary

The development of amplification techniques, home-made and commercial has revolutionised the ability to detect the hepatitis B virus both qualitatively and quantitatively. The objectives of this thesis were to describe the clinical implications of quantitative HBV DNA measurements.

In chapter 2 we describe the predictive value of quantitative HBV DNA measurements in identifying non-responders to Interferon-alpha therapy. To reduce unnecessary exposure to treatment physicians must decide at an early stage whether continuation of treatment has a reasonable chance of success for the individual patient. The clinical value of surrogate tests proposed for the assessment of disease activity or viral replication such as HBeAg must be assessed. The objectives of our study were first to evaluate quantitative HBeAg measurements versus quantitative HBV DNA measurements for prediction of non-response and response for IFN-α treated patients. Secondly, we evaluated the value of precise quantitative HBV DNA measurements for predicting non-response and response of IFN-α treated patients. Monitoring with quantitative HBV DNA levels was superior to monitoring with quantitative HBeAg levels. This study also showed that quantitative HBV DNA testing at baseline in combination with a decrease between baseline and week 12 has a high predictive value for identifying patients who have virtually no chance of reaching a sustained response with IFN therapy.

Quantification of HBV DNA enabled clinicians to monitor clinical situations such as the effect of therapy and the emergence of drug-resistant variants. These mutations are mainly identified after an increase in the HBV DNA load in serum is observed. We describe a patient who developed a lamivudine-resistant mutation during long-term lamivudine therapy (Chapter 3). Our report showed persistence of YMDD variants for at least 41 weeks after withdrawal of lamivudine. This finding has immediate implications for the selection of optimal drug regimens in case of re-initiation in therapeutic studies, or eg. before and after liver transplantation.

Because of the emergence of mutations during long-term lamivudine therapy there is a need to explore new therapies. A mathematical model has been applied to describe hepatitis C viral decline in hepatitis C. In chapter 4 we use this same model to describe viral decline in lamivudine-resistant patients with HBV DNA breakthrough during therapy with tenofovir. We applied two investigative approaches based on mathematical models of viral dynamics: the individual non-linear fitting and the mixed-effect group fitting approaches. There was only a small difference between the efficacy parameter ε of the individual non-linear fitting and mixed effect group fitting on the bi-phasic exponential model. Our data showed that

tenofovir has good efficacy in blocking viral replication in HBV patients with lamivudine-induced drug-resistant HBV mutants, but effectiveness varies greatly among individuals. Both models can be used to describe viral decay during tenofovir therapy.

The possibility to transmit hepatitis B is dependent among others on the number of infectious particles transmitted. Assuming that HBV DNA level reflects the number of infectious particles, it is thus determined by HBV DNA level and volume of infectious fluid. Public Health policy to prevent transmission of HBV to patients in different countries was based on serum HBeAg status. After incidents of transmission by HBeAg negative surgeons a more reliable estimate of non-infectivity was needed. Serum HBV DNA level may be more reliable to estimate non-infectivity than anti-HBe status alone. In chapter 5 we review doctor-to-patient transmission of HBV with main focus on quantitative HBV DNA levels. To prevent both loss of expertise and nosocomial transfer, highly viremic HCWs can be offered antiviral therapy.

Each country in Europe has it's own rules and guidelines for the management of HBV-infected HCWs. In chapter 6 we discuss the implications of measuring HBV DNA levels in HCWs as well as the consequences for the management of this group of patients by comparing our data of Dutch HCWs with the available data of HCWs in the United Kingdom.

HBV DNA, which indicates potentially infectivity, has been shown to be present in other body fluids than serum such as saliva and urine. More information regarding the precise amounts in the different body fluids provides insight in the potential infectivity of these fluids. We have described the results of paired quantitative HBV DNA measurements in serum, saliva and urine in chronic HBV patients (Chapter 7). We have found a non-linear correlation between the level of HBV DNA in serum and the level of HBV DNA in saliva or urine. Furthermore, we found a significant amounts of HBV DNA in saliva and urine in chronic HBV patients with high viremia in serum and this could have implications for the understanding of hepatitis B epidemiology, as the origin of infections especially in horizontal transmission remains unknown in up to 20% of cases.

In conclusion, the advent of DNA sequencing allowed the detection of precore mutants, YMDD mutants and the comparison of viral isolates on the genomic level. HBV DNA quantification can be used to determine infectivity, to monitor viral replication kinetics and to monitor the effect of therapy or the emergence of drug-resistant variants. These new technologies enabled the introduction of an individual patient disease management concept.

Samenvatting

De ontwikkeling van amplificatie technieken, in eigen huis ontwikkeld en commercieel, heeft de mogelijkheid om het hepatitis B virus zowel kwalitatief als kwantitatief te detecteren, gerevolutioniseerd. De doelstellingen van dit proefschrift waren om de klinische implicaties van kwantitatieve HBV DNA metingen te beschrijven.

In **hoofdstuk 2** beschrijven we de predictieve waarde van kwantitatieve HBV DNA metingen in het identificeren van non-responders van Interferon-alfa (IFN-α) therapie. Om onnodige blootstelling aan de behandeling te reduceren moeten artsen in een vroeg stadium beslissen of het continueren van een behandeling een redelijke kans op succes heeft voor de individuele patiënt. De klinische waarde van plaatsvervangende testen, die zijn voorgesteld voor het vaststellen van ziekte activiteit of virale replicatie, zoals HBeAg, moet worden vastgesteld. De doelstellingen van onze studie waren ten eerste evaluatie van kwantitatieve HBeAg metingen versus kwantitatieve HBV DNA metingen voor de predictie van non-respons en respons in IFN-α behandelde patiënten. Ten tweede hebben we de waarde geëvalueerd van precieze kwantitatieve HBV DNA metingen voor de predictie van non-respons en respons van IFN-α behandelde patiënten. Monitoren met kwantitatieve HBV DNA metingen was superieur ten opzichte van monitoren met kwantitatieve HBeAg metingen. Deze studie heeft ook aangetoond dat kwantitatief testen op HBV DNA niveau op baseline in combinatie met het meten van daling van HBV DNA tussen baseline en week 12 een hoge predictieve waarde heeft voor het identificeren van patiënten, die eigenlijk geen kans hebben op respons met IFN-α therapie.

Kwantificeren van HBV DNA maakte het voor clinici mogelijk om klinische situaties, zoals het effect van therapie en het voorkomen van resistente variant virussen te monitoren. Deze mutaties worden voornamelijk opgemerkt, nadat een stijging in HBV DNA niveau is waargenomen. Wij beschrijven een patiënt, die een lamivudine-resistente mutant ontwikkeld, tijdens langdurig gebruik van lamivudine (**hoofdstuk 3**). Onze studie toont de blijvende aanwezigheid van YMDD varianten aan voor ten minste 41 weken na stoppen van lamivudine. Dit gegeven heeft onmiddellijk implicaties voor de selectie van het optimale geneesmiddel in het geval van re-initiatie in studies, of bijv. voor en na levertransplantatie.

Vanwege het optreden van mutaties tijdens langdurige therapie met lamivudine is het noodzakelijk om nieuwe therapieën te ontwikkelen. Een wiskundig model is gebruikt om de afname van virus in hepatitis C patiënten te beschrijven. In **hoofdstuk 4** maken we gebruik van dit zelfde model om de afname van virus te beschrijven in lamivudine-resistente patiënten, die een doorbraak van HBV DNA vertonen gedurende therapie met tenofovir.

We hebben twee methodes toegepast gebaseerd op mathematische modellen van virale dynamiek: de individuele non-lineaire “fit” en de “mixed-effect” groepsgewijze “fit”. Er was maar een klein verschil tussen parameter ε van de individuele non-lineaire “fit” en de “mixed-effect” groepsgewijze “fit” op het bifasische model. Onze data heeft laten zien, dat tenofovir effectief virusreplicatie blokkeert in HBV patiënten met lamivudine-geïnduceerde HBV mutanten, maar de effectiviteit varieert enorm tussen individuen. Beide modellen kunnen gebruikt worden om daling van het virus te beschrijven gedurende therapie met tenofovir.

De mogelijkheid om het hepatitis B virus over te dragen is onder meer afhankelijk van het aantal infectieuze partikels, dat wordt overgedragen. Aangenomen dat de hoogte van HBV DNA niveau het aantal infectieuze deeltjes weergeeft, wordt het dus bepaalt door de hoogte van het HBV DNA en de hoeveelheid infectieuze vloeistof. Het openbare gezondheidszorg beleid om transmissie van HBV naar patiënten te voorkomen was in verschillende landen gebaseerd op HBeAg-status. Na incidenten waarbij transmissie van HBeAg negatieve chirurgen heeft plaatsgevonden was er een meer betrouwbare schatting nodig om niet-infectiviteit vast te stellen. Serum HBV DNA niveau zal meer betrouwbaar zijn om niet-infectiviteit in te schatten, dan HBeAg-status alleen. In **hoofdstuk 5** geven we een overzicht van dokter-patient transmissie van HBV met het belangrijkste focus op kwantitatieve HBV DNA niveaus. Om verlies van expertise en nosocomiale transmissie te voorkomen kan aan hoog-viremische HCWs antivirale therapie aangeboden worden.

Elk land in Europa heeft zijn eigen regels en leidraden voor de management van HBV-geïnfecteerde HCWs. In **hoofdstuk 6** bespreken we zowel de implicaties van het meten van HBV DNA niveaus in HCWs als de consequenties voor de management van deze groep patiënten door onze data van Nederlandse HCWs te vergelijken met de beschikbare data van de HCWs van het Verenigd Koninkrijk.

HBV DNA, dat duidt op potentiële infectiviteit, is aangetoond in andere lichaamsvloeistoffen dan serum, zoals speeksel en urine. Meer informatie betreffende de precieze hoeveelheid in de verschillende lichaamsvloeistoffen geeft inzicht in de potentiële infectiviteit van deze vloeistoffen. We hebben de resultaten beschreven van gepaarde kwantitatieve HBV DNA metingen in serum, speeksel en urine in chronische HBV patiënten (**Hoofdstuk 7**). We hebben een niet-lineaire correlatie gevonden tussen het niveau van HBV DNA in serum en het niveau van HBV DNA in speeksel of urine. Bovendien vonden we significante hoeveelheden HBV DNA in speeksel en urine in chronische HBV patiënten met hoge virus niveaus in serum en dit gegeven kan implicaties hebben voor het begrijpen van hepatitis B epidemiologie, daar de origine van de infectie onbekend is tot in 20% van de gevallen.

Concluderend, de komst van DNA sequensing maakte de detectie mogelijk van precore mutanten, YMDD mutanten en het vergelijken van virus isolaties op het niveau van het genoom. Kwantificatie van HBV DNA kan gebruikt worden om de infectiviteit te bepalen, om virale kinetiek te bestuderen en om het effect van therapie of de opkomst van resistente varianten tegen het geneesmiddel te monitoren. Deze nieuwe technologieën maakte de introductie mogelijk van een individuele patiënt management concept.

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Curriculum Vitae

De auteur van dit proefschrift werd op 20 augustus 1975 in Krimpen aan den IJssel geboren. Na het V.W.O.-eindexamen aan het IJsselcollege te Capelle aan den IJssel werd in 1994 gestart met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Tijdens haar studie heeft zij in de periode 1996-1998 onderzoek verricht naar o.a. chronische pijn bij kinderen aan het instituut Huisartsgeneeskunde van dezelfde universiteit. Tevens heeft zij als docente pathologie en verloskunde van de opleiding tot verpleegkundige aan het Albeda college te Rotterdam gewerkt. Na het behalen van haar artsexamen in 2000 heeft zij een half jaar als arts-assistent Interne Geneeskunde in het Ikazia ziekenhuis te Rotterdam gewerkt. Van februari 2001 tot januari 2004 was zij werkzaam als arts-onderzoeker op de afdeling Maag-, darm- en leverziekten van het Erasmus MC te Rotterdam. Tijdens deze periode werd onder begeleiding van Prof.dr. S.W. Schalm en Dr. R.A. de Man onderzoek verricht naar de klinische implicaties van kwantitatieve hepatitis B virus DNA metingen uiteindelijk leidend tot de totstandkoming van dit proefschrift. Sinds januari 2004 is zij werkzaam als arts-onderzoeker op de afdeling Virologie van het Erasmus MC Rotterdam onder leiding van Prof.dr. A.D.M.E. Osterhaus. De opleiding tot arts-microbioloog zal gestart worden op 1 januari 2005 in het Erasmus MC te Rotterdam.

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Submitted

Annemiek A. van der Eijk, Robert A. de Man, Hubert G.M. Niesters, Solko W. Schalm, Hans L. Zaaijer
Hepatitis B virus (HBV) DNA levels and the management of HBV infected health care workers
Submitted

Annemiek A. van der Eijk, Hubert G.M. Niesters, Bettina E. Hansen, Suzan D. Pas, Jan H. Richardus, Marijke Mostert, Harry L.A. Janssen, Solko W. Schalm, Robert A. de Man
Paired measurements of quantitative Hepatitis B virus DNA in saliva, urine and serum of chronic hepatitis B patients
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Abbrevations

AL(A)T	alanine aminotransferase
anti-HBe	antibody against HBeAg
AS(A)T	aspartate aminotransferase
AUC	area under curve
BMI	body mass index
CccDNA	covalently closed circular DNA
DNA	deoxyribonucleic acid
DR	drug resistant
EPP	exposure prone procedures
Geq	genome equivalents
HBV	hepatitis B virus
HBeAg	hepatitis B envelop antigen
HBsAg	hepatits B surface antigen
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCWs	health care workers
HIV	human immunodeficiency virus
IFN-α	interferon-alpha
IU	international units
PCR	polymerase chain reaction
PEG	pegylated
Pg	picogram
RNA	ribonucleic acid
ROC curves	receive operating characteristic curves
RT	reverse transcriptase
SD	standard deviation
ULN	upper limit of normal
YIDD	thyrosine isoleucine aspartate aspartate
YMDD	thyrosine methionine aspartate aspartate
YSDD	thyrosine serine aspartate aspartate
YVDD	thyrosine valine aspartate aspartate