Detection of anti-Hepatitis B drug resistance

FIELD OF THE INVENTION

The present invention relates to the field of Hepatitis B virus (HBV) diagnosis. More particularly, the present invention relates to the field of genetic monitoring of anti-HBV drug resistance during HBV therapy.

BACKGROUND OF THE INVENTION

Hepatitis B virus is a small, enveloped DNA virus of approximately 3200 bp long that belongs to the Hepadnaviridae, characterized by a significant hepatotropism and species specificity. The HBV genome is of complex nature having a partially double stranded DNA structure with overlapping open reading frames encoding surface, core, polymerase and X genes. HBV replicates via an RNA intermediate and utilizes reverse transcription in its replication strategy (Summers and Mason, 1982). HBV causes major medical problems, such as chronic liver disease and hepatocellular carcinoma (Schroder and Zentgraf, 1990).

In chronic Hepatitis B virus (HBV)-infected patients with active wild-type virus replication, successful antiviral therapy is characterized by clearance of HBV-DNA from the blood circulation, followed by clearance of Hepatitis B e antigen (HBeAg), and seroconversion to anti-HBe antibodies. Unfortunately, disappearance of HBV-DNA is not always followed by HBeAg seroconversion, and quantitative HBeAg measurements were suggested to have predictive value for the outcome of antiviral therapy (Heijtink et al., 1997).

Currently, interferon-alpha (IFN∀) and lamivudine (3TC) are the two licensed agents for treatment of chronic Hepatitis B. IFN-∀ has antiviral and immunomodulatory properties. Lamivudine (3TC) is a (-) enantiomer of 3' thiacytidine, a 2' 3'-dideoxynucleoside analogue, and is known to be a potent inhibitor of HBV replication

through inhibition of the reverse transcriptase (RT) activity of the HBV polymerase. Lamivudine treatment can result in histological improvements in chronic hepatitis patients, and, when given pre- and post-liver transplantation, it can prevent graft re-infection (Honkoop et al., 1995; Naoumov et al., 1995). Both drugs may be given in mono-therapy or in combination therapy (Lai et al., 1998; Mutimer et al., 1998). Another compound with direct antiviral effect, famciclovir (9-[4-hydroxy-3 hydroxymethyl-but-1-yl] guanine), is currently in phase III clinical evaluation. Other antiviral compounds, lobucavir (Colacino and Staschke, 1998) and adefovir, (Heathcote et al., 1998) have been shown to be safe and are effectively inhibiting the virus replication, but are also still in clinical phase. Penciclovir has been shown to inhibit the reverse transcriptase activity of the HBV polymerase (Shaw et al., 1996).

In some patients, however, a hepatitis flare-up is observed during or after treatment, ALT is elevated and HBV DNA becomes detectable again. This HBV DNA rebound is associated with a new quasi species equilibrium. Several independent reports illustrated the development of famciclovir- and lamivudine-resistant HBV strains (reviewed in Bartholmeusz et al., 1998). The exact nature of this resistance has been ascribed to the accumulation of mutations in the RT part of the polymerase. Treatment schedules with antiviral compounds such as lamivudine and famciclovir have resulted in the accumulation of a variety of mutations in the HBV polymerase. Sequence analysis revealed the emergence of a specific mutation in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene, whereby the methionine is replaced by either an isoleucine or a valine (Ling et al., 1996; Tipples et al., 1996). A similar mechanism in the HIV RT polymerase has been found, where upon lamivudine treatment, mutations accumulate in the YMDD motif (Gao et al., 1993). Another important mutation site is located 24 amino acids upstream from the YMDD motif, replacing a leucine (L) for a methionine (M) (WO 98/21317 to Locarnini et al.). The V555I (X199 in HBsAg) mutation might also be clinically relevant. Pichoud et al. (1999) showed that the V555I mutant has a decreased replication capacity, does not produce HBsAg, and is resistant to penciclovir but sensitive to lamivudine. An overview of the mutations observed heretofore after lamivudine or famciclovir treatment and the consequences of viral fitness is currently available (Ling et al., 1996; Tipples et al., 1996; Honkoop et al., 1997; Bartholomeusz et al., 1998; Buri et al., 1998; Chayama et al., 1998; Melagari et al., 1998; Mutimer et

al., 1998; Niesters et al., 1998; Pillay et al., 1998; Hunt et al., 2000). Mutations in the HBV polymerase are also detected upon treatment with penciclovir and other anti-HBV drugs.

As these mutations are generally considered as the cause of viral non-responsiveness and treatment failure, the detection of these mutations is of clinical importance for the monitoring of anti-HBV drug resistance during HBV therapy. Therefore, a rapid, reliable and very specific method for the detection of these mutations is needed. This will allow a fast and precise monitoring of HBV drug resistance and provide a quicker and more efficient design of anti-HBV treatment strategies.

AIMS OF THE INVENTION

It is an aim of the present invention to provide a rapid, reliable and precise method for the monitoring of anti-HBV drug resistance in a patient.

It is another aim of the present invention to provide a rapid, reliable and precise method for the monitoring of anti-HBV drug resistance in a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a rapid, reliable and precise method for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a rapid, reliable and precise method for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain present in a biological sample of a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a rapid, reliable and precise method for the simultaneous detection of at least the mutations L528M and M552V/I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a rapid, reliable and precise method for the simultaneous detection of at least the mutations L528M and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a rapid, reliable and precise method for the simultaneous detection of at least the mutations M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a rapid, reliable and precise method for the simultaneous detection of at least the mutations L528M, M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a rapid, reliable and precise method for the monitoring of lamivudine resistance in a patient.

It is another aim of the present invention to provide a rapid, reliable and precise method for the monitoring of famciclovir resistance in a patient.

It is another aim of the present invention to provide a rapid, reliable and precise method for the monitoring of penciclovir resistance in a patient.

It is another aim of the present invention to provide one or more probes for use in a method as described above.

It is another aim of the present invention to provide a composition comprising at least one of the probes as described above.

It is an aim of the present invention to provide a diagnostic kit for the monitoring of anti-HBV drug resistance in a patient.

It is another aim of the present invention to provide diagnostic kit for the monitoring of anti-HBV drug resistance in a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a diagnostic kit for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a diagnostic kit for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain present in a biological sample of a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a diagnostic kit for the simultaneous detection of at least the mutations L528M and M552V/I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a diagnostic kit for the simultaneous detection of at least the mutations L528M and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a diagnostic kit for the simultaneous detection of at least the mutations M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a diagnostic kit for the simultaneous detection of at least the mutations L528M, M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a diagnostic kit for the monitoring of lamivudine resistance in a patient.

It is another aim of the present invention to provide a diagnostic kit for the monitoring of famciclovir resistance in a patient.

It is another aim of the present invention to provide a diagnostic kit for the monitoring of penciclovir resistance in a patient.

It is an aim of the present invention to provide a Line Probe Assay for the monitoring of anti-HBV drug resistance in a patient.

It is another aim of the present invention to provide a Line Probe Assay for the monitoring of anti-HBV drug resistance in a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a Line Probe Assay for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a Line Probe Assay for the genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of a HBV strain present in a biological sample of a patient receiving anti-HBV treatment.

It is another aim of the present invention to provide a Line Probe Assay for the simultaneous detection of at least the mutations L528M and M552V/I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a Line Probe Assay for the simultaneous detection of at least the mutations L528M and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a Line Probe Assay for the simultaneous detection of at least the mutations M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a Line Probe Assay for the simultaneous detection of at least the mutations L528M, M552V/I and V/L/M555I in the DNA polymerase of a HBV strain.

It is another aim of the present invention to provide a Line Probe Assay for the monitoring of lamivudine resistance in a patient.

It is another aim of the present invention to provide a Line Probe Assay for the monitoring of famciclovir resistance in a patient.

It is another aim of the present invention to provide a Line Probe Assay for the monitoring of penciclovir resistance in a patient.

It is an aim of the present invention to provide new sequences of the HBV DNA polymerase gene.

FIGURE LEGENDS

Figure 1. Alignment of new HBV DNA polymerase sequences from serum samples obtained from patients before and during HBV therapy, as described in example 1. Target sequences that can be used for probe design are boxed.

Figure 2. Design of a HBV drug resistance LiPA strip. Conj.cont.: conjugate control; Amp.cont.: amplification control. The strip contains a total of 19 probe lines with a total of 38 probes; n probes equals the total amount of probes on each line. The specific probes applied on each line are indicated in table 6. Interpretation: Polym. or HBsAg: amino acid for the polymerase or HBsAg open reading frame, applied on this particular probe line.

Figure 3. HBV drug resistance LiPA strip results illustrating reactivity of each independent probe line. Results are obtained with 16 recombinant clones of the reference panel. Reactive lines are indicated with their number. A strip showing the relative position of all lines and the codons and amino acids detected on that line, is indicated on the right. Interpretation per codon (cd) is given below each strip.

Figure 4. Monitoring of two HBV infected patients with anti-HBV drug resistance. Left: patient A; right: patient C. Days of follow-up are indicated in X-axis. The interpretation of the reactivity pattern on each strip is given for the three codons (indicated as cd). Treatment schedules are shown on top of the graphs.

Figure 5. Patient treatment schedule with viral load and ALT data as described in example 4. This graph shows the follow up samples analyzed. Underneath the data is the genetic information for codon 528, 552 and 555 given. Cd: codon; Seq: data from sequence analysis; LiPA: data obtained from LiPA analysis. Amino acids are indicated with the one letter code. Mixtures are indicated in small letters: lm = leucine + methionine, mvi = methionine + valine + isoleucine, mi = methionine + isoleucine, vm = valine + methionine. 3TC = lamivudine; LT: Liver transplantation; HBIg: Hepatitis B immuno globulin.

Figure 6. LiPA analysis of the patients over 1259 days of follow up as described in example 4. Sampling days are indicated above each strip. Treatment schedules are also indicated: 3TC: lamivudine, Fam: famciclovir, HBIg: Hepatitis B immuno globulin. Conj cont: conjugate control; Ampl. Cont.: HBV amplification control.

Figure 7. Detailed analysis of selected amplicons on individual LiPA probes. Days of follow up are indicated above each strip. Left panel: samples taken during lamivudine treatment; right panel: samples taken after withdrawal of 3TC therapy; middle panel: genetic composition of the relevant sequences applied on the indicated position. At day 524, a weak hybridization is also observed on a probe containing TAA (stop codon) at codon 551. Probes for codon 552I and codon 555 were not applied. Control lines are not shown.

Figure 8. Analysis of the HBV polymerase variability at three time points (X-axis). The number of clones at each time point is indicated on the Y-axis. The genetic composition of the clones is indicated in the legend.

TABLES

Table 1. Position of HBV DNA polymerase drug resistance codons with respect to the different HBV genotypes.

Wild type	Genotype				Mutant
	A	B, C, and F	D	E	
L	528	526	515	525	M
M	552	550	539	549	V/I
V/M/L	555	553	542	552	I

Table 2. Probes used for the genetic detection of the L528M mutation in the HBV DNA polymerase.

Reference	Sequence	SEQ ID NO
HBPr270	5'-CCGTTTCTCTTGGCTC-3'	1
HBPr293	5'-CCGTTTCTCCTGGCTCA-3'	2
HBPr294	5'-CGTTTCTCTTAGCTCAG-3	3
HBPr412	5'-CGTTTCTCCTAGCTCAG-3	4
HBPr274	5'-CGTTTCTCATGGCTCA-3'	5
HBPr355	5'-CGTTTCTCATGGCCCAG-3'	6
HBPr415	5'-GTTTCTTATGGCTCAG-3'	7
HBPr461	5'-GTTTCTCATGACTCAG-3'	8
HBPr468	5'-TTTCCCATGGCTCAG-3'	9
HBPr468-1	5'-TTTCCCATGGCTCAGTTT-3'	10

Table 3. Probes used for the genetic detection of the M552V/I mutation in the HBV DNA polymerase.

Reference	Sequence	SEQ ID NO
HBPr308	5'-CTTTCAGCTATATGGATGA-3'	11
HBPr322	5'-CTTTCAGTTATATGGATGAT-3'	12
HBPr349	5'-GTGGCTTTTAGTTATATGGAT-3'	13
HBPr478	5'-GCTTTCAGCCATATGGATG-3'	14
HBPr309	5'-TTTCAGCTATGTGGATGAT-3'	15
HBPr318	5'-TTTCAGCTATGTGGACGA-3'	16
HBPr426	5'-TTTCAGTTATGTGGATGAT-3'	17
HBPr427	5'-TTTTAGTTATGTGGATGAT-3'	18
HBPr463	5'-TTTCAGTCATGTGGATGA-3'	19
HBPr315	5'-CTTTCAGCTAAGTGGATGA-3'	20
HBPr363-1	5'-GCTTTCAGCTATATAGATGA-3'	21
HBPr407	5'-GCTTTCAGCTATATTGATG-3'	22
HBPr488	5'-TTTCAGCTATATCGATGAT-3'	23
HBPr433	5'-CTTTCAGTTATATTGATGA-3'	24
HBPr433-1	5'-CTTTCAGTTATATTGATGAT-3'	25
HBPr465	5'-CTCTCAGTTATATTGATGA-3'	26
HBPr456	5'-TGGTTTTCAGTTATATTGAT-3'	27
HBPr380	5'-GCTTTTAGTTATATCGATG-3'	28
HBPr453	5'-CTTTCAGTTACATTGATGA-3'	29
HBPr485	5'-TTCGGTTATGTGGATGAT-3'	97
HBPr486	5'-TTTCGGTTATGTGGATGA-3'	98
HBPr487	5'-CTTTCGGTTATGTGGAT-3'	99
HBPr488	5'-GCTTTCGGTTATGTGG-3'	100

Table 4. Probes used for the genetic detection of the V/L/M5551 mutation in the HBV DNA polymerase.

Reference	Sequence	SEQ ID NO
HBPr279	5'-GATGATGTGGTATTGGG-3'	30
HBPr338	5'-GGACGATGTGGTATTGG-3'	31
HBPr341	5'-TGATGTGGTACTGGGGG-3'	32
HBPr345	5'-TGATGTGGTTTTGGGGG-3'	33
HBPr474	5'-GATGTGGTGTTGGGGG-3'	34
HBPr332	5'-GATGATGTGATATTGGGGG-3'	35
HBPr328	5'-GATGATCTGGTATTGGGGG-3'	36
HBPr385	5'-ATGATATTGTATTGGGGGC-3'	37
HBPr289	5'-GATGATATAGTACTGGGG-3'	38
HBPr299	5'-ATGATATAGTATTGGGGGC-3'	39
HBPr419	5'-GATGATATGGTATTGGG-3'	40
HBPr490	5'-GATGACGTGGTATTGGGGC-3'	101
HBPr491	5'-ATGACGTGGTATTGGGGCC-3'	102
HBPr492	5'-TGACGTGGTATTGGGGCC-3'	103
HBPr494	5'-GATGATTTGGTATTGGGG-3'C	104
HBPr495	5'-ATGATTTGGTATTGGGGCC-3'	105
HBPr496	5'-ATGATTTGGTATTGGGGCCA-3'	106

Table 5. Primers used for amplification of the HBV DNA polymerase gene or part thereof.

Reference	Sequence	Sense/antisense	SEQ ID NO
HBPr134	5'-TGCTGCTATGCCTCATCTTC-3'	sense	41
HBPr135	5'-CA(G/A)AGACAAAAGAAAATTGG-3'	antisense	42
HBPr135A	5'-CAGAGACAAAAGAAAATTGG-3'	antisense	43
HBPr135B	5'-CAAAGACAAAAGAAAATTGG-3'	antisense	44
HBPr75	5'-CAAGGTATGTTGCCCGTTTGTCC-3'	sense	45
HBPr94	5'-GGTA(A/T)AAAGGGACTCA(C/A)GATG-3'	antisense	46
HBPr94A	5'-GGTATAAAGGGACTCACGATG-3'	antisense	47
HBPr94B	5'-GGTATAAAGGGACTCAAGATG-3'	antisense	48
HBPr94C	5'-GGTAAAAAGGGACTCAAGATG-3'	antisense	49
HBPr105	5'-GG(T/C)A(A/T)AAAGGGACTCA(C/A)GATG-3'	antisense	50

Table 6. INNO-LiPA strip set up for simultaneous detection of mutations in codon 528, 552 and 555 of the HBV DNA polymerase. De amino acids detected in the indicated HBV DNA polymerase codon and in the corresponding HBsAg codons are indicated. LiPA strip production and use are explained in example 2.

Line	Probes	Codon in DNA polymerase	Codons in HBsAg	
1	HBPr 270, 293	L528	S171	W172
2	HBPr 294, 412	L528	S171	X172
3	HBPr 274, 355	M528	S171	W172
4	HBPr 415	M528	L171	W172
5	HBPr 461	M528	S171	X172
6	HBPr 468-1	M528	P171	W172
7	HBPr 308, 322, 349, 478	M552	I195	W196
8	HBPr 309, 318, 426, 427, 463	V552	M195	W196
9	HBPr 315	V552	K195	W196
10	HBPr 363-1	I552	I195	X196
11	HBPr 407, 433-1, 465,456	I552	I195	L196
12	HBPr 380, 488	I552	I195	S196
13	HBPr 453	I552	T195	L196
14	HBPr 279, 338, 341, 345, 474	V555	M198	W199
15	HBPr 332	V555	M198	X199
16	HBPr 328	L555	I198	W199
17	HBPr 419	M555	I198	W199
18	HBPr 385	I555	I198	L199
19	HBPr 289, 299	I555	I198	X199

Table 7. Prevalence of mutants in codons 528, 552 and/or 555 in two patients at different time points during HBV therapy.

Day	Total number of clones	Number of clones with codon combination 528/552/555				
		L/M/V	L/M/I	L/V/V	M/M/V	M/V/V
Patient A						
1	9	9	0	0	0	0
360	8	4	4	0	0	0
420	9	3	6	0	0	0
510	18	18	0	0	0	0
570	55	23	32	0	0	0
630	46	2	1	1	2	40
750	13	0	0	0	0	13
Total	158	59	43	1	2	53
Patient C						
1	5	5	0	0	0	0
148	6	6	0	0	0	0
325	11	1	0	0	10	0
407	9	0	0	0	9	0
450	12	1	0	0	11	0
519	23	0	0	0	3	20
542	6	0	0	0	0	6
Total	72	13	0	0	33	26

Table 8. Analysis of mutation at codon 528 of HBV polymerase. Comparision of data obtained by LiPA versus data obtained by sequencing.

	Sequencing		
LiPA	**M (mutant)**	**L (wild type)**	**Total**
M (mutant)	23		23
L (wild type)		44	44
L/M (mix)	15	1	16
Total	38	45	83

Table 9. Analysis of mutation at codon 552 of HBV polymerase. Comparison of data obtained by LiPA versus data obtained by sequencing.

	Sequencing			
LiPA	**I (mutant)**	**V (mutant)**	**M (wild type)**	**Total**
I (mutant)	8			8
V (mutant)		17		17
M (wild type)			45	45
V/I (mutant)		2		2
M/I (mix)	4			4
M/V (mix)		1	2	3
Total	12	20	47	79

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for the monitoring of anti-HBV drug resistance in a patient by genetic detection of at least one of the mutation L528M, M552V/I and/or V/L/M555I in the DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following steps:

(i) if needed, release, isolation and/or concentration of the polynucleic acids present in said biological sample;

(ii) if needed, amplification of the HBV DNA polymerase gene or part thereof in said biological sample with at least one suitable primer pair;

(iii) hybridization of the polynucleic acids obtained in steps (i) and/or (ii) with at least one probe capable of specifically hybridizing with a target sequence in the HBV DNA polymerase gene or the complementary, said target sequence being selected from the target sequences shown in figure 1;

(iv) detection of the hybrids formed in step (iii);

(v) inference, from the hybridization signal obtained in step (iv), on the presence or absence of the L528M, M552V/I and/or V/L/M555I mutation in the DNA polymerase and on possible anti-HBV drug resistance of the HBV strains present in said biological sample.

The method described above allows to determine whether a HBV strain is susceptible or resistant to a certain anti-HBV drug by the genetic detection of a mutation in at least one of the codons 528, 552 and/or 555 of the HBV DNA polymerase gene. The isolation of a large number of new HBV DNA polymerase gene sequences has allowed the inventors to develop a reference panel of target sequences on which base a very specific and very sensitive hybridization assay for detection of the above mentioned mutations, could be developed.

Throughout the present application, the codon numbering for the HBV polymerase gene was adopted from genotype A. An overview of the corresponding numbering of the codon positions 528, 552 and 555 in the other genotypes is shown in table 1.

The mutation L528M means that in codon 528 of the HBV DNA polymerase gene the genetic code for leucine is substituted by the genetic code for methionine. The mutation M552V/I means that in codon 552 of the HBV DNA polymerase gene the genetic code for methionine is substituted by the genetic code for valine or isoleucine.

The mutation V/L/M555I means that in codon 555 of the HBV DNA polymerase gene the genetic code for valine, leucine or methionine is substituted by the genetic code for isoleucine.

The term "genetic detection of a mutation" as used in the present invention means that a mutation in an amino acid sequence is detected by determination of the corresponding nucleic acid sequence.

In the method of the present invention, the mutations in codons 528, 552 and/or 555 of the HBV DNA polymerase are detected by hybridization of the nucleic acids present in the patients biological sample, with one or more probes that are capable of specifically hybridizing with a target sequence in the HBV DNA polymerase gene as shown in Figure 1. The term "to hybridize specifically" means that said probe forms a duplex with part of its target sequence or with the entire target sequence under the experimental conditions used, and that under those conditions said probe does not form a duplex with other sequences of the polynucleic acids present in the sample to be analyzed.

The term "target sequence" of a probe, according to the present invention, is a sequence within the HBV DNA polymerase gene that comprises a mutated or a wild type nucleic acid sequence of the codon encoding amino acid 528, 552 and/or 555 of the HBV DNA polymerase and to which the probe is completely complementary or partially complementary (i.e. with up to 20%, more preferably 15%, more preferably 10% or most preferably 5% mismatches). It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases. The target sequences depicted in figure 1 were obtained from serum and plasma samples of various patients in follow up studies and cross sectional studies, and have not been previously disclosed. By use of these novel polymorphic nucleotide sequences of the HBV DNA polymerase gene, which also form part of the present invention, it became possible to design new probes that allow a very specific and sensitive detection of anti-HBV drug resistance. It should be understood that probes that are designed to specifically hybridize to a target sequence of a nucleic acid, may fall within said target sequence or may to a large extent overlap with said target sequence (i.e. form a duplex with nucleotides outside as well as within said target sequence).

The term "probe" refers to a single stranded sequence-specific oligonucleotide that has a sequence that is complementary to the target sequence of the HBV DNA polymerase gene. Preferably, the probe is about 5 to 50 nucleotides long, more preferably from about

10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides used in the probes of the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine, or nucleotides containing modified groups that do not essentially alter their hybridization characteristics.

In a specific embodiment, the probe used in a method of the invention is selected from tables 2, 3 and/or 4, wherein:

- the probes specifically hybridizing with the L528M target sequences are selected from the following list: HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461, HBPr468, HBPr468-1 (SEQ ID NO 1 to SEQ ID NO 10);
- the probes specifically hybridizing with the M552V/I target sequences are selected from the following list: HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433, HBPr433-1, HBPr465, HBPr456, HBPr380, HBPr453, HBPr485, HBPr486, HBPr487, HBPr488 (SEQ ID NO 11 to SEQ ID NO 29, SEQ ID NO 97 to SEQ ID NO 100);
- the probes specifically hybridizing with the V/L/M555I target sequences are selected from the following list: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299, HBPr419, HBPr 490, HBPr 491, HBPr 492, HBPr 494, HBPr 495, HBPr 496 (SEQ ID NO 30 to SEQ ID NO 40, SEQ ID 101 to SEQ ID NO 106).

The mutations at codon 528, 552 and/or 555 are detected by hybridization with at least one probe, preferably at least 2, more preferably at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 14, 15, 16, 17, 18, 19 or more oligonucleotide probes.

In a preferred embodiment the present invention relates to a method as indicated above, further characterized in that said probes are optimized for simultaneous hybridization to their target regions under the same hybridization and wash conditions (for instance in a LiPA format, see below) allowing the detection of a number of polymorphic regions at the same time.

More specifically, the present invention relates to a method as described above characterized further in that the probes used in step (iii) are at least one of the following combination of probes:

- for the detection of the L528M mutation, probes: HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461 and HBPr468-1 (SEQ ID NO 1 to SEQ ID NO 10);
- for the detection of the M552V/I mutation, probes: HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433-1, HBPr465, HBPr456, HBPr380 and HBPr453 (SEQ ID NO 11 to SEQ ID NO 29);
- for the detection of the V/L/M555I mutation, probes: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299 and HBPr419 (SEQ ID NO 30 to SEQ ID NO 40).

The present invention also relates to the oligonucleotides used as probes to perform any method as described above.

In a preferred embodiment, the present invention relates to the oligonucleotides as depicted in tables 2, 3 and 4.

Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides form the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

Since the current application requires the detection of single base pair mismatches, stringent conditions for hybridization of probes are required, allowing only hybridization of exactly complementary sequences. However, it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence might be allowable towards the extremities of the probe when longer probe sequences are used. When other hybridization conditions would be preferred, probes may be adapted accordingly by adding or deleting one or more nucleotides at their extremities. It should be understood that these concomitant adaptations should give rise to the same results, namely that the probes still hybridize specifically to their respective type-specific target sequences. Such adaptations may also be necessary if the amplified material is RNA and not DNA as is the case in the NASBA system. Said deviations and variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics as the exactly complementary probes.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied. Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions are explained further herein:

- The stability of the [probe: target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the hybrids with G:C base pairs, and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be more stable at higher temperatures.
- Conditions such as ionic strength and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that the degree of hybridization will increase as the ionic strength of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strength. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohol's, that disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.
- It is desirable to have probes that hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form. Hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a

hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

- The length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another that differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide probes of this invention are between about 5 to 50 (more particularly 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.
- Regions in the target DNA or RNA that are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self-complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.
- Standard hybridization and wash conditions are disclosed in the Materials & Methods section of the Examples. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C. Other solutions (SSPE (Sodium saline phosphate EDTA), TMAC (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the

specificity and sensitivity of the probes is maintained. When needed, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

The probes according to the invention can be prepared by cloning recombinant plasmids containing inserts including the corresponding nucleotide sequence, if need be by excision of the latter from the cloned plasmids by use of the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

The term "biological sample" as used in the present invention refers to any biological material (tissue or fluid) taken either directly form the infected human being, or after culturing (enrichment) and containing HBV nucleic acid sequences. Biological material may be e.g. expectoration's of any kind, broncheolavages, blood, skin tissue, biopsies, sperm, lymphocyte blood culture material, colonies, liquid cultures, fecal samples, urine, hepatocytes, etc. More particularly "biological sample" refers to blood serum or plasma samples.

The nucleic acids are released, concentrated and/or isolated from the biological sample by any method known in the art. Currently, various commercial kits are available such as the QIAamp Blood Kit from Qiagen (Hilden, Germany) for the isolation of nucleic acids from blood samples and the 'High pure PCR template preparation Kit' (Roche Diagnostics, Brussels, Belgium). Other well-known procedures for isolation of DNA or RNA from a biological sample are available (Maniatis et al., 1989).

The nucleic acids in the sample to be analyzed may be either DNA or RNA, e.g. genomic DNA, messenger RNA, viral RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

The HBV DNA polymerase gene or part thereof, present in said biological sample, can be amplified by polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990) or amplification by means of Qß replicase (Lomeli et al., 1989) or by any other suitable method known in the art, that allows the amplification of nucleic acid molecules. Also TMA (Guatelli et al., 1990)

or bDNA (Sanchez-Pescador et al., 1988; Urdea et al., 1991) techniques can be used in the method of the present invention.

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product or amplification product that is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably, the length of the primer is about 5-50 nucleotides. More preferably, the length of the primer is about 10-30 nucleotides. Most preferably, the length of the primers is about 20-25 nucleotides. Specific length and sequence will depend on the complexity of the required DNA or RNA target, as well as on the conditions at which the primer is used, such as temperature and ionic strength.

The expression "primer set" refers to a pair of primers allowing the amplification of the HBV DNA polymerase gene or part thereof. A primer set always consists of a forward primer (sense primer or 5' primer) and a reverse primer (antisense primer or 3' primer).

In a preferred embodiment, the present invention relates to a method as described above, characterized further in that at least one of primers used in step (ii) is selected from table 5 (HBPr134, HBPr135, HBPr135A, HBPr135B, HBPr75, HBPr94, HBPr94A, HBPr94B, HBPr94C and/or HBPr105; SEQ ID NO 41 to SEQ ID NO 50).

More particularly, the present invention relates to a method as described above characterized further in that the set of primers consists of the following 2 primers:

- HBPr134 as forward primer and HBPr135 as reverse primer; and/or
- HBPr75 as forward primers and HBPr94 as reverse primer; and/or
- HBPr75 as forward primers and HBPr 105 as reverse primers.

The skilled man will understand that these primers (SEQ ID NOs 41 to 50) may be adapted by addition or deletion of one or more nucleotides at their extremities. Such adaptations may be required, for instance, if the conditions of amplification are changed, if the amplified material is RNA instead of DNA, as is the case, for example, in the NASBA system. The fact that amplification primers do not have to match exactly with the corresponding target sequence in the template to warrant proper amplification is amply documented in the literature (Kwok et al., 1990). However, when the primers are not completely complementary to their target sequence, it should

be taken into account that the amplified fragments will have the sequence of the primers and not of the target sequence.

The primers and/or probes of the invention may be labeled. Labeling may be carried out by any method known to the person skilled in the art. The nature of the label may be isotopic (^{32}P, ^{35}S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

The oligonucleotides used as primers or probes may also contain or consist of nucleotide analogues such as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain intercalating agents (Asseline et al., 1984). The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

As most other variations or modifications introduced into the original DNA sequences of primers and probes, these variations will necessitate adaptations with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. The eventual results of the priming or hybridization with these modified oligonucleotides, however, should be essentially the same as those obtained with the unmodified oligonucleotides.

In a preferred embodiment, the present invention relates to a method, as indicated above, further characterized in that said probes are capable of simultaneously hybridizing to their respective target regions under appropriate hybridization conditions and wash conditions allowing the detection of more than one wild type codon and/or mutated codon at the same time.

More specifically, the present invention relates to a method as described above characterized further in that both mutations L528M and M552V/I are detected in one step.

More specifically, the present invention also relates to a method as described above, characterized further in that both mutations L528M and V/L/M555I are detected in one step.

More specifically, the present invention relates to a method as described above characterized further in that both mutations M552V/I and V/L/M555I are detected in one step.

More specifically, the present invention relates to a method as described above characterized further in that the three mutations L528M, M552V/I and V/L/M555I are detected in one step.

The method of the present invention can be used to screen for mutations in codon 528, 552 and/or 555 present before initiating anti-HBV therapy and/or arising during the course of anti-HBV drug therapy (i.e. monitoring of drug therapy) and conferring resistance to lamivudine, famciclovir and/or penciclovir. Accordingly, the present invention relates to a method characterized further in that the HBV strain present in the biological sample shows resistance to lamivudine, famciclovir and/or penciclovir.

The method of the invention may also be used to determine resistance to anti-HBV drugs other than the above-mentioned drugs, provided that resistance to these other drugs is linked to one or more of the three mutations that are detected by this method. The term "anti-HBV drug" refers to any anti-HBV nucleoside analog or any other DNA polymerase inhibitor that causes a reduction of the viral DNA in the patient. Other anti-HBV drugs include but are not limited to adefovir, BMS 200475, foscarnet, fiacitabine, fialuridine, (-)-FTC, ganciclivor, GEM 132, interferon, L-FMAU, lobucavir, n-docosanol, ribavirin, sorivudine, vidarabine or compounds mentioned in WO 98/18818. The method of the invention can also be used in combination with a method for the detection of one or more other mutations that possibly confer resistance to other anti-HBV drugs. Thus, also probes that allow the detection of other mutations can be added in the method of the invention.

The present invention also relates to a composition, comprising at least one probe of the invention. The term "composition" as used in the present invention relates to a mixture of probes in an appropriate buffer used to carry out the method of the invention. The present invention also relates to the use of the probes and the composition as defined above, for in vitro monitoring of anti-HBV drug resistance in a patient.

The present invention also relates to a diagnostic kit for the monitoring of anti-HBV drug resistance in a patient by genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the HBV DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following components:

(i) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said biological sample;

(ii) when appropriate, at least one suitable primer pair;

(iii) at least one probe as indicated above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

(vii) when appropriate, a means for attaching said probe to a known location on a solid support.

The term "hybridization buffer" means a buffer allowing a hybridization reaction between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropriate stringency conditions.

The term "wash solution" means a solution enabling washing of the hybrids formed under the appropriate stringency conditions.

Any assay method that relies on the formation of a hybrid between the nucleic acids of the biological sample and the oligonucleotide probes according to the present invention, may be used. For example, the hybridization can be accomplished using a Southern blot, Northern blot or dot blot format, the unlabelled amplified sample being bound to a membrane, the membrane being incorporated with at least one labeled probe under suitable hybridization and wash conditions, and the presence of bound probe being monitored. An alternative is a "reverse" format, in which the amplified sequence contains a label. In this format, the selected probes are immobilized to certain locations on a solid support and the amplified polynucleic acids are labeled in order to enable the detection of the hybrids formed. The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate (e.g. in the DEIA technique), a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead) or a chip. Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin, haptens or proteins.

Accordingly, the present invention relates to a line probe assay for the monitoring of antiviral drug resistance in a patient by the genetic detection of at least one of the

mutations L528M, M552V/I and/or V/L/M555I in the HBV DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following components:

(i) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in a biological sample of the patient;

(ii) when appropriate, at least one suitable primer pair;

(iii) at least one of the probes specifically hybridizing with the L528M target sequences shown in figure 1 and selected from HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461 and HBPr468, fixed to a solid support; and/or

(iv) at least one of the probes specifically hybridizing with the M552V/I target sequences shown in figure 1 and selected from HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433-1, HBPr465, HBPr456, HBPr380, HBPr453, HBPr485, HBPr486, HBPr487 and HBPr488, fixed to a solid support; and/or

(v) at least one of the probes specifically hybridizing with the V/L/M555I target sequences shown in figure 1 and selected from: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299, HBPr419, HBPr490, HBPr491, HBPr492, HBPr494, HBPr495 and HBPr496, fixed to a solid support.

(vi) a hybridization buffer, or components necessary for producing said buffer;

(vii) a wash solution, or components necessary for producing said solution;

(viii) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

In this embodiment, the selected set of probes is immobilized to a membrane strip in a line fashion. Said probes may be immobilized individually or as mixtures to the delineated locations. The amplified HBV DNA polymerase polynucleic acids or part thereof can be labeled with biotin, and the hybrid can then, via a biotin-streptavidine coupling, be detected with a non-radioactive color developing system.

The present invention also relates to novel sequences as depicted in figure 1 (SEQ ID NOs 51-86 and SEQ ID NOs 107 to 109), or a fragment thereof, wherein said fragment consists of at least 10, preferably 15, even more preferably 20 contiguous nucleotides as shown in figure 1 and said fragment contains one of the codons

encoding the wild type or mutated amino acid 528, 552 and/or 555 of the HBV DNA polymerase. Accordingly, the present invention relates to an isolated nucleic acid consisting of or comprising a nucleotide sequence as indicated above. The term "nucleic acid" refers to a single stranded or double stranded nucleic acid sequence, which may contain from 10 nucleotides to the complete nucleotide sequence as shown in figure 1. A nucleic acid may consist of deoxyribonucleotides, ribonucleotides, nucleotide analogues or modified nucleotides. It is to be understood that also the complement of the above mentioned nucleic acids forms part of the present invention.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of stated integers or steps but not to the exclusion of any other integer or step or group of integers or steps.

EXAMPLES

Example 1: Isolation of new HBV DNA polymerase gene sequences

a. Patients

Serum or plasma samples were collected from 41 patients during follow up studies and from 80 patients in a cross sectional study. The patients in the follow up studies were as follows: 18 patients in a follow up study by Dr. F. Zoulim (INSERM, Lyon, France), 5 patients in a follow up study by Dr. D. Pillay (Public Health Laboratory Service, Birmingham, UK), 3 patients in a follow up study by Dr. G. Leroux (University Hospital, Gent, Belgium) and 15 patients in a follow up study by Dr. D. Lau (NIH, Bethesda, MD, US). The cross sectional study was carried out by Dr. J. Lau (Schering Plough, Madison, NJ, US).

b. HBV DNA purification and amplification

HBV DNA was isolated from the serum or plasma samples by using the commercially available >High pure PCR template preparation Kit' (Boehringer-Mannheim, Brussels, Belgium). Purified DNA was amplified for the HBV polymerase region using a nested PCR approach: 10 µl purified DNA was mixed with 5 µl 10 x buffer, 0.4 µl 10 mM dXTPs, 10 pmol of the sense primer, 10 pmol of antisense primer, 1 Unit Taq polymerase (Stratagene Europe, Amsterdam, The Netherlands), and completed to 50 µl with HPLC-grade H_2O. PCR consisted of annealing at 45°C, extension at 72°C, and denaturation at 94°C, each time for 30 sec. An outer PCR contained 40 cycles; the nested reaction contained 35 cycles. The HBV polymerase region was amplified with the following primer combination: outer sense HBPr134: 5'-TGCTGCTATGCCTCATCTTC-3' (SEQ ID NO 41); outer anti-sense HBPr135: 5'-CAG/AAGACAAAAGAAAATTGG -3' (SEQ ID NO 42); nested sense HBPr75: 5'-CAAGGTATGTTGCCCGTTTGTCC -3' (SEQ ID NO 45); nested anti-sense HBPr 94: 5'-GG(T/C)A(A/T)AAAGGGACTCA(C/A)GATG -3' (SEQ ID NO 46). Nested amplification products were (primers included) 341 bp long, analyzed on a 2%

agarose gel, and visualized by ethidium bromide. In the case of LiPA experiments, primers were provided at their 5' ends with a biotin group.

c. Plasmid cloning and DNA purification

Two µl of the amplification product was mixed with 1 µl pretreated EcoRV cut pGemT vector (Promega, Leiden, The Netherlands) and ligated by means of the >Ready to Go= T4 ligase (Pharmacia, Leusden, The Netherlands). After transformation in competent *E. coli* strains, single recombinant clones were selected, and plasmid DNA was purified with the >High Pure Plasmid Isolation Kit (Boehringer Mannheim, Brussels, Belgium) or the 'Qia prep 96 Turbo Bio Robot kit (Qiagen, Hilden, Germany). Inserts from recombinant clones were PCR-amplified by means of either plasmid-derived primers or the nested HBV primers.

d. Development of the reference panel and probe design

A reference panel for probe design was developed simultaneously with the evaluation of the probes. In a primary phase, a few probes, specific for the detection of the presence or absence of the mutations L528M, M552V/I and/or V/L/M555I were designed and obtained after considering parameters of percentages of GC, probe length, ionic strength of the hybridization buffer, and temperature of incubation. These specific probes were evaluated by applying them to nitrocellulose membranes followed by reverse hybridization of the biotinylated PCR fragments generated from the plasma or serum samples (in a LiPA format), streptavidine-alkaline phosphatase incubation, and color development. Details on the probe optimization phase, LiPA strip production and reverse hybridization are described in Stuyver et al. (1996), Stuyver et al. (1997) and Van Geyt et al. (1998).

Upon analyses of the serum and plasma samples with this primary phase LiPA strip, many of their PCR products were not reactive with these primary phase selected probes. Sequence analysis of these non-reactive PCR products revealed new motifs for which the corresponding probes were designed. Including these newly designed probes on the LiPA strip resulted in a decrease of non-reactivity of the samples.

Subsequently, in further analysis of the reactivity of the new set of probes with the plasma and serum samples, all PCR products that were non-reactive with the selected set of probes were sequenced, the new motifs were added to the reference panel, and new probes were designed. Finally, a total of 35 selected clones were this way retained as reference panel and sequenced. Double-stranded sequences were obtained from biotinylated PCR products or, in case of recombinant clones, by using vector-derived sequencing primers as described in Stuyver et al. (1996). The newly obtained sequences are shown in figure 1 (SEQ ID NOs 51 to 86 and SEQ ID NOs 107 to 109).

Example 2: Design and testing of a LiPA for monitoring drug-resistance in HBV-infected patients

a. Design of a LiPA for monitoring drug-resistance

By use of the sequence in the reference panel, specific probes for codon positions 528, 552, and 555, covering both the wild-type and mutant motifs, and for the different genotypes and polymorphism's were designed and validated. Probes were pooled according to their ability to detect the different wild type and mutant codons in the HBV DNA polymerase, but also according to relevant information from the overlapping HBsAg reading frame, and applied on a strip. Several probes designed for different nucleotide polymorphisms but not introducing an amino acid change were pooled together and applied on one line. This finally resulted in a strip with 19 different probe lines with in total 38 specific probes (Figure 2; Table 6). Details on the probe optimization phase and LiPA strip production are described in Stuyver et al. (1996), Stuyver et al. (1997) and Van Geyt et al. (1998).

b. Testing of the LiPA with clones from the reference panel

The LIPA strip was then incubated with biotinylated PCR products obtained from the recombinant clones in the reference panel. Details on the reverse hybridization are described in Stuyver et al. (1996), Stuyver et al. (1997) and Van Geyt et al. (1998). Figure 3 shows the specific reactivity of some of the PCR products with the probes on

the strip. The selection of probes was very specific in detecting the corresponding amplicon. Simultaneous detection at codon 528, 552 and 555 of wild type or mutant codon was possible in one single experiment.

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Example 3: Use of the LiPA for monitoring anti-HBV drug resistance in two patients receiving HBV therapy

a. Analysis of patient follow-up samples with the HBV LiPA for drug resistance

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The LiPA was used to test follow up samples of 2 patients taken before the start of therapy, during therapy and during therapy but with viral rebound. Evidence of treatment failure was shown by viral load and ALT levels (Figure 4). Patient A had been infected with HBV genotype A, patient C with genotype C. The LiPA reactivity
15 obtained with these follow up samples is also shown in figure 4. In patient A, treated with lamivudine, there was a mixture of V555I (line 14 and 19) transiently present at day 360, 420, 570 and weakly at day 630. As can be deduced from figure 2, a reactivity at line 19 correlates with a translational stop at HBsAg codon 199. This motif disappeared after the emergence of a mutant at codon 552 and 528. Also on day
20 630, mixtures on both codon 528 (lines 1+3) and 552 (lines 7+8) were observed. Wild type motifs L528 and M552 disappeared at day 750 at the time of viral breakthrough. In patient C, treated with famciclovir followed by lamivudine, a sequential selection of a M528 mutant followed by the double M528+V552 mutant was observed. Wild type L528 (line 1) co-existed with a mutant M528 (line 3) at day 325, but from day
25 407 this wild type motif was no longer detected. Progressive take-over of mutant virus (line 8) over wild type virus (line 7) was observed at codon 552 from day 450. From day 542 onwards, a pure double mutant M528 + V552 was present. In patient C, no codon changes at codon 555 were observed. The current selection of probes appeared useful for monitoring the emergence of drug resistance during antiviral treatment.

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b. Clonal analysis of follow up samples

From patient A, a total of 7 plasma samples was available at different time points spread over the 750-day period of antiviral treatment. Amplification products were

cloned and 158 recombinant clones were retained for LiPA analysis (table 7). Except for I555, there was no evidence for selection of mutations at codons 528 and 552 between day 1 and day 570 in a total of 99 clones. At day 630, the majority of the clones belonged already to the group of the double mutant M528+V552. However, single mutants L/V/V and M/M/V were marginally present, which could be interpreted as the remnants of the intermediate forms for double-mutant selection. This shows that the emergence of resistance occurred within a period of 60 days (between day 570 and 630).

In patient C, another 72 clones were analyzed, spread over 7 different time points during HBV treatment. Due to the treatment schedule with famciclovir, the single mutant M528 existed as the majority population at day 325. Only after introduction of lamivudine (day 325) a M528 + V552 mutant was detected as the major population (Figure 4 and table 7). The selection of the M552 mutant occurred in a period of at maximum 69 days (between day 450 and 519). The pressure created by lamivudine therapy on a single M528 mutant virus (from day 325) resulted in a rapid selection of the double mutant at day 450.

Example 4: Use of the LiPA for monitoring the dynamics of emergence and disappearance of lamivudine-associated mutations in Hepatitis B virus.

a. Patient history

A 52-year-old Caucasian male was first diagnosed with chronic hepatitis B in 1985 when he was found to have abnormal serum aminotransferase (ALT) levels during routine laboratory testing. The patient remained a-symptomatic but sought medical attention begin 1995. At that time, he was found to have slightly elevated ALT levels. Serum HBV DNA was 0.7 pg/ml as measured by liquid-phase hybridization assay (Abbott Diagnostics, Chicago, ILL, USA). The patient had thrombocytopenia (platelets = 64000) and evidence of mild hepatic synthetic dysfunction with decreased serum albumin (3.1 g/dl) and a slightly prolonged prothrombin time (17.1 secs). The patient was closely monitored for several months with regular testing of his HBV serologic profile. In december 1995, he became highly positive for HBV DNA, and

showed abnormal serum ALT levels (Figure 5). The patient began lamivudine (3TC) therapy (150 mg daily) (day 331) which resulted in the rapid disappearance of HBV DNA, and normalization of serum ALT activity. At day 633 (after 302 days of 3TC therapy), the serum ALT and viral load became sharply elevated and HBV DNA was again detectable. Lamivudine therapy was discontinued at day 663 and replaced with famciclovir (FCV) (500 mg tid). This therapy had no effect on HBV DNA levels and was discontinued at day 779. Three months later (day 856), the patient developed ascites, worsening coagulopathy, and variceal hemorrhage. He was listed for liver transplantation in august 1997 (approx. day 950). While awaiting transplant the patient received no antiviral therapy, but experienced a spontaneous, transient loss of HBV DNA. When HBV reactivated (day 1105), 3TC was restarted which resulted in a rapid drop of HBV DNA to undetectable levels. The patient received a donor liver at day 1223 and treatment with Hepatitis B immuno globulin (HBIg) therapy to prevent reinfection was started. At day 1259, HBV DNA levels were undetectable by conventional assays (not PCR), and ALT levels normalized.

b. DNA manipulations

HBV purification and amplification. HBV DNA was extracted from 50 µl of plasma using the Tri-Reagent LS protocol for DNA. Nested PCR was used to amplify the HBV polymerase regions A-E. First round PCR combined: 3 µl of the purified DNA template, 10 µl 10X buffer with $MgCl_2$, 2 µl dNTP (120 mM stock), 1 µl each of forward (HBV-8For 5'-CATCAGGATTCCTAGGACC-3'; SEQ ID NO 87) and reverse (HBV-9Rev 5'-ATACTTTCCAATCAATAGGCC-3'; SEQ ID NO 88) primer (30 pmol/µl stock), 0.5 µl Taq DNA polymerase and brought to 100 µl with HPLC-grade H_2O. The amplification program consisted of: denaturation at 94°C (45 sec), annealing at 49.8°C (1 min), extension at 72°C (2 min) for 40 cycles. The 810 bp product was purified with the Qiagen PCR Purification Kit and eluted into 30-50 µl H_2O. Nested PCR reaction combined 5 µl of first round PCR product, 5 µl 10X buffer II, 3.7 µl $MgCl_2$, 1 µl dNTP (10 mM stock), 1 µl each of forward (HBV-2For 5'-CGCTGGATGTGTCTGCGGCG-3'; SEQ ID NO 89) and reverse (HBV-R3 5'-CCAACTTCCAATTACATAACCC-3'; SEQ ID NO 90) primers (30 pmol/µl stocks), 0.5 µl Taq DNA polymerase and brought to 50 µl with HPLC-grade H_2O.

Amplification program consisted of: denaturation at 94°C (45 sec), annealing at 55°C (1 min) and extension at 72°C (2 min) for 35 cycles. Final fragment size is 533bp.

Sequencing. Pharmacia (Upsala, Sweden) A.L.F. sequencing was performed using a collection of fluorescein-labelled primers: HBV-8ALF (5'-CGTTCCACCAAACTCTTCAAG-3'; SEQ ID NO 91), HBV-10ALF (5'-CAAGGTATGTTGCCCGTTTGTCCTC-3'; SEQ ID NO 92), HBV-12ALF (5'-CCCATCCCATCATCTTGGGC-3'; SEQ ID NO 93), HBV-14ALF (5'-GGGTATGTAATTGGAAGTTGG-3'; SEQ ID NO 94), HBV-3ALF (5'-GCACTAGTAAACTGAGCCA-3'; SEQ ID NO 95) or HBV-6ALF (5'-AGTCTAGACTCGTGGTGGAC-3'; SEQ ID NO 96). Standard A.L.F. sequencing protocols were followed and the A.L.F. analytical program was set to scan for sequence mutations at the highest stringency (homologous).

Clonal analysis. Two µl of the HBPr75-94 amplification product (see example 1) was ligated into the pretreated pGem plasmid vector (Promega, Leusden, The Netherlands) and transformed into competent *E. coil* cells. Single recombinants were selected and the plasmid DNA purified with the High Pure Plasmid Isolation Kit (Boehringer Mannheim, Brussels, Belgium). Inserts from recombinant clones were PCR amplified with either the plasmid-derived primers or the nested HBV primers. All products were analysed by means of LiPA.

c. Variability at the HBV DNA polymerase drug resistant codons 528, 552 and 555

Samples selected before, during and after (day 331, 338, 345, 633, 715, 726, 738, 752, 779, and 802) lamivudine therapy were sequenced over the HBV polymerase region between amino acid (aa) 473 and 561. This region included domain B (HBV polymerase aa 508 to 530) and domain C (HBV polymerase aa 548 to 558). Compared to the day 331 sequence, variability was observed from day 633 onwards, with the detection of M528 and V552, but from day 779 onwards, wild type motifs L528 and M552 re-appeared (Figure 5). There were no other amino acid changes observed in these follow up samples.

All available samples were also analyzed on LiPA (Figures 5 and 6). Lamivudine resistance mutations were detected as complex mixtures as early as day 524 (L and M at 528; M, V and I at 552). The I552 variant could not be further detected at day 548, but all other mixtures remained. From day 633 pure mutant was detected. This corresponded with an increase in viral load. Until day 835, mixtures of wild type and mutant variants at both codon positions were detected. Finally, at day 1041 only pure wild type virus was apparent. Re-initiation of lamivudine therapy occurred at day 1105, and a 139 days later, a complex mixture of wild types and mutant strains at codon 552 were present (nearly comparable with day 524). But since M528 could not be detected, L528 + M552 and L528 + I552 combinations are the most likely combinations selected (day 1244 and day 1259).

d. Virus evolution during antiviral therapy: an analysis of the quasispecies

In order to better understand the evolution of the virus at the codon positions that showed early nucleotide changes during the therapy (Figure 6), another hybridization experiment was performed. Therefore, LiPA probes that were otherwise pooled (as was previously described in example 2) were now applied individually on strip and incubated with the biotinylated amplicons obtained from the plasma virus (Figure 7). There were two remarkable observations: (i) the selection of the M528 and V552 variant occurred over an intermediate nucleotide sequence with no consequences at the aa level; and (ii) the disappearance of the mutant virus goes over the same intermediate forms. For codon 528: the wild type L(TTG) evolves over L(CTG), finally resulting in the resistant variant M(ATG). After discontinuation of 3TC therapy, reversal from mutant M(ATG) over L(CTG) to L(TTG) was observed. For codon 552: intermediate forms were observed at codon 550 with the presence of a change for serine (AGC to AGT). The latter however, disappeared when V552 (GTG) emerged. Comparable to the observation for codon 528 and following discontinuation of 3TC therapy, reversal to wild type occurred at codon 552, with again a temporary detection of the codon 550 (AGT) variability.

To further illustrate these intermediate forms, HBV amplicons from day 442, 468, and 524 were cloned in plasmids, and a total of 54 individual clones were further analyzed (Figure 8). Six kinds of clones were detected in which different combinations of

motifs at codon 528, 550 and 552 were present. The clones with L528 (CTG) + M552 (with AGT on 550) were present as an important population prior to the selection of resistance motifs. Furthermore, two clones with M528 + M552 were found, indicating that this single mutant population is also an intermediate form for the double mutant. However, the single mutant I552 could not be found in the 19 clones isolated from the day 524 amplicon. Nevertheless this aberration in the cloning results, it was clearly present in the population, as visualized in figure 6 (line 11, day 524).

Example 5: Comparison of data obtained with the LiPA for monitoring drug-resistance versus data obtained by sequencing.

a. Patient samples

Plasma or serum samples were collected at two centers: Prof. Dr. S. Locarnini, Research and Molecular Development, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Australia (9 patients) and Prof. Dr. A. S. F. Lok, University of Michigan Medical Center, Ann Arbor, MI, USA (9 patients). All patients were HBV-infected individuals under lamivudine treatment. The first sample to be included had to correspond with the 'baseline' (time point just before lamivudine treatment started). The second and third sample had to correspond respectively with a time point before and after redectable viral load levels. For some patients also additional sequential samples were obtained.

b. DNA manipulations

HBV isolation and amplification was done as described above. Sequencing was carried out by standard methods (Sambrook et al., 1989). The LiPA was used as described in example 2.

c. Results

The analysis by LiPA and by sequencing of a possible mutation at codon 528, is shown in table 8. For most samples there was a concordance between the data

obtained by the LiPA versus the data obtained by sequencing: 44 samples showed the presence of the wild type (L) virus and 23 samples showed the presence of the mutant (M) virus. In 15 samples, the LiPA detected the mixture wild type/mutant (L/M), while sequencing only detected the mutant (M). In 1 sample the LiPA showed a mixture wild type/mutant (L/M), while sequencing only detected the wild type (L).

The analysis by LiPA and by sequencing of a possible mutation at codon 552, is shown in table 9. For most samples there was a concordance between the data obtained by the LiPA versus the data obtained by sequencing: 45 samples showed the presence of the wild type (M) virus, 17 samples showed the presence of the mutant (V) virus, and 8 samples showed the presence of the mutant (I) virus. In 1 sample, the LiPA detected the mixture wild type/mutant (M/V), while sequencing only detected the mutant (V). In 2 samples the LiPA detected a mixture wild type/mutant (M/V), while sequencing only detected the wild type (M). In 4 samples, the LiPA detected the mixture wild type/mutant (M/I), while sequencing only detected the mutant (I). In 2 samples, the LiPA detected the mutants V/I while sequencing only detected the mutant V.

Analysis by LiPA and by sequencing of a possible mutation at codon 555, showed the presence of the wild type codon V555 in all samples.

From this study it is clear that the data obtained by the LiPA for monitoring drug resistance coincide with the data obtained by sequencing. In addition, the detection of a higher number of mix sequences at codon 528 and/or 552 by the LiPA while these mixtures were not detected by sequencing, strongly indicates that a higher sensitivity is obtained by the LiPA.

REFERENCES

Asseline U, Delarue M, Lancelot G, Toulme F and Thuong N (1984) Nucleic acid-binding molecules with high affinity and base sequence specificity: intercalating agents covalently linked to oligodeoxynucleotides. Proc Natl Acad Sci USA 81: 3297-3301.

Barany F (1991) The ligase chain reaction in a PCR world. PCR Methods Appl 1: 5-16.

Bartholmeusz A, Schinazi RF and Locarnini SA (1998) Significance of mutations in the Hepatitis B virus polymerase selected by nucleoside analogues and implications for controlling chronic disease. Viral Hep Rev 4: 167-187.

Buri M, Jardi R, Cotrina M, Rodriguez-Frias F, Esteban R and Guardia J (1998) Transient emergence of Hepatitis B variants in a patient with chronic Hepatitis B resistant to lamivudine. J Hepatol 28: 510-513.

Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K and Kumada H (1998) Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 27: 1711-1716.

Colacino JM and Staschke KA (1998) The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. Prog Drug Res 50: 259-322.

Compton J (1991) Nucleic acid sequence-based amplification. Nature 350: 91-92.

Duck P (1990) Probe amplifier system based on chimeric cycling oligonucleotides. Biotechniques 9: 142-147.

Gao Q, Gu Z, Parniak M, Cameron I, Cammack N, Boucher C and Wainberg M (1993) The same mutation that encodes low-level human immunodeficiency virus type-1 resistance to 2', 3'-dideoxyinosine and 2',3'-dideoxycytidine confers high level resistance to the (-) enantiomer of 2',3'-dideoxy-3'-thiacytidine. Antimicrob Agents Chemother 37: 1390-1392.

Guatelli J, Whitfield K, Kwoh D, Barringer K, Richman D and Gengeras T (1990) Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 87: 1874-1878.

Heathcote EJ, Jeffers L, Wright T et al. (1998) Loss of serum HBV DNA and HBeAg and seroconversion following short-term (12 weeks) adefovir dipivocil therapy in chronic hepatitis B: two placebo-controled phase II studies. Hepatology 28: A260.

Heijtink RA, Kruining J, Honkoop P, Kuhns MC, Hop WC, Osterhaus AD and Schalm SW (1997) Serum HBeAg quantitation during antiviral therapy for chronic Hepatitis B. J Med Virol 53: 282-287.

Honkoop P, de Man R, Zondervan P, Niesters H and Schalm S (1995) Histological improvement in patients with chronic Hepatitis B virus infection treated with lavimudine is associated with a decrease in HBV-DNA by PCR. Hepatology 22: abstract 887.

Honkoop P, Niesters HGM, de Man RAM, Osterhaus ADME and Schalm SW (1997). Lamivudine resistance in immunocompetent chronic Hepatitis B. J Hepatology 26: 1393-1395.

Hunt C, Mc Gill J, Allen M and Condeay L (2000) Clinical relevance of Hepatitis B viral mutations. Hepatology 31: 1037-1044.

Kwoh D, Davis G, Whitfield K, Chappelle H, Dimichele L and Gingeras T (1989) Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 86: 1173-1177.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C and Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18: 999-1005.

Lai CL, Chien RN, Leung NWY, Chang TT, Guan R, Tai DI, Ng K-Y, Wu PC, Dent JC, Barber J, Stephenson SL and Gray DF (1998) One year trial of lamivudine for chronic Hepatitis B. New Engl J Med 339: 61-68.

Landgren U, Kaiser R, Sanders J and Hood L (1988) A ligase-mediated gene detection technique. Science 241: 1077-1080.

Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM and Harrison TJ (1996) Selection of mutations in the Hepatitis B Virus polymerase during therapy of transplant recipients with lamivudune. Hepatology 24: 711-713.

Lomeli H, Tyagi S, Pritchard C, Lisardi P and Kramer F (1989) Quantitative assays based on the use of replicatable hybridization probes. Clin Chem 35: 1826-1831.

Manitatis (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press.

Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen J and Broder S (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc Natl Acad Sci USA 84: 7706-7710.

Melagari M, Scaglioni PP and Wands JR (1998) Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology 27: 628-633.

Miller P, Yano J, Yano E, Carroll C, Jayaram K and Ts'o P (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. Biochemistry 18: 5134-5143.

Mutimer D, Naoumov N, Honkoop P, Marinos G, Ahmed M, de Man R, McPhillips P, Johnson M, Williams R, Elias E, and Schalm S (1998) Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic Hepatitis B infection: results of a pilot study. J Hepatol 28: 923-929.

Naoumov N, Perillo R, Chokshi S, Dienstag ., Vicary C, Brown N and Williams R (1995) Reduction in Hepatitis B virus quasispecies during lamivudine treatment is associated with enhanced virus replication and hepatocytolisis. Hepatol 22: abstract 885.

Nielsen P, Egholm M, Berg R and Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254: 1497-1500.

Nielsen P, Egholm M, Berg R, Buchardt O (1993) Sequence specific inhibition of DNA restriction enzyme cleavage by PNA. Nucleic-Acids-Res 21: 197-200.

Niesters HGM, Honkoop P, Haagsma EB, de Man RA, Schalm SW, and Osterhaus ADME (1998) Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine treatment. J Infect Dis 177: 1382-1385.

Pichoud C, Seigneres B, Wang Z, Trépo C and Zoulim F (1999) Transient selection of a hepatitis B virus polymerase gene mutant associated with a decreased replication capacity and famciclovir resistance. Hepatology 29: 230-237.

Pillay D, Bartholomeusz A, Cane P, Mutimer D, Schinazi RF, and Locarnini S (1998) Mutations in the Hepatitis B virus DNA polymerase associated with antiviral resistance. Int Antiviral News 6: 167-169.

Saiki RK, Gelfland DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, and Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sambrook J, Fritsch E and Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sanchez-Pescador R, Stempien MS, Urdea MS (1988) Rapid chemiluminescent nucleic acid assays for detection of TEM-1 beta-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria. J Clin Microbiol 26: 1934-1938.

Schroder CH and Zentgraf H (1990) Hepatitis B virus related hepatocellular carcinoma chronocity of infection the opening to different pathways of malignant transformation. Biochem Biophys Acta 1032:137-156.

Shaw T, Mok SS, Locarnini SA (1996) Inhibition of Hepatitis B virus DNA polymerase by enantiomers of penciclovir triphosphate and metabolic basis for selective inhibition of HBV replication by penciclovir. Hepatology 24: 996-1002.

Stuyver L, Wyseur A, van Arnhem W, Hernandez F and Maertens G (1996) A second generation line probe assay for hepatitis C virus. J Clin Microbiol 34: 2259-2266.

Stuyver L, Wyseur A, Rombout A, Louwagie J, Scarcez T, Verhofstede C, Rimland D, Schinazi RF, and Rossau R (1997) Line probe assay (LiPA) for the rapid detection of drug-selected mutations in the HIV-1 reverse transcriptase gene. Antimicrob Agents Chemother 41: 284-291.

Summers J and Mason WS (1982) Replication of the genome of a Hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29: 403-415.

Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM and Tyrell DLJ (1996) Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 24: 714-717.

Urdea MS, Horn T, Fultz TJ, Anderson M, Running JA, Hamren S, Ahle D, Chang CA (1991) Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. Nucleic Acids Symp Ser 24:197-200.

Van Geyt C, De Gendt S, Rombaut A, Wyseur A, Maertens G, Rossau R and Stuyver L (1998) A line probe assay for hepatitis B virus genotypes. In: R.F. Schinazi, J.P. Sommadossi, and H. Thomas (eds.). Therapies of viral hepatitis. International Medical Press, London, UK, pp. 139-145.

Wu D and Wallace B (1989) The ligation amplification reaction (LAR) - amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4: 560-569.

CLAIMS

1. Method for the monitoring of anti-HBV drug resistance in a patient by genetic detection at least one of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following steps:
   (i) if needed, release, isolation and/or concentration of the polynucleic acids present in said biological sample;
   (ii) if needed, amplification of the HBV DNA polymerase gene or part thereof in said biological sample with at least one suitable primer pair;
   (iii) hybridization of the polynucleic acids obtained in steps (i) and/or (ii) with at least one probe capable of specifically hybridizing with a target sequence in the HBV DNA polymerase gene or the complementary, said target sequence being selected from the target sequences shown in figure 1;
   (iv) detection of the hybrids formed in step (iii);
   (v) inference, from the hybridization signal obtained in step (iv), on the presence or absence of the L528M, M552V/I and/or V/L/M555I mutation in the DNA polymerase and on possible anti-HBV drug resistance of the HBV strains present in said biological sample.

2. Method according to claim 1 further characterized in that the probe used in step (iii) is selected from tables 1, 2 and/or 3, wherein:
   - the probes specifically hybridizing with the L528M target sequences are selected from the following list: HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461, HBPr468, HBPr468-1 (SEQ ID NO 1 to SEQ ID NO 10);
   - the probes specifically hybridizing with the M552V/I target sequences are selected from the following list: HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433, HBPr433-1, HBPr465, HBPr456, HBPr380, HBPr453, HBPr485, HBPr486, HBPr487, HBPr488 (SEQ ID NO 11 to SEQ ID NO 29 or SEQ ID NO 97 to SEQ ID NO 100);

- the probes specifically hybridizing with the V/L/M555I target sequences are selected from the following list: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299, HBPr419, HBPr490, HBPr491, HBPr492, HBPr494, HBPr495, HBPr496 (SEQ ID NO 30 to SEQ ID NO 40 or SEQ ID NO 101 to SEQ ID NO 106).

3. Method according to any of claims 1 to 2 characterized further in that the probes used in step (iii) are at least one of the following combination of probes:
   - for the detection of the L528M mutation, probes: HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461, HBPr468 and HBPr468-1;
   - for the detection of the M552V/I mutation, probes: HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433, HBPr433-1, HBPr465, HBPr456, HBPr380 and HBPr453;
   - for the detection of the V/L/M555I mutation, probes: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299 and HBPr419.

4. Method according to any of claims 1 or 3 characterized further in that the set of primers used in step (ii) is selected from the following (table 5): HBPr134, HBPr135, HBPr135A, HBPr135B, HBPr75, HBPr94, HBPr94A, HBPr94B, HBPr94C and/or HBPr105 (SEQ ID NO 41 to SEQ ID NO 50).

5. Method according to claim 4 characterized further in that the set of primers consist of the following 2 primers:
   - HBPr134 as forward primer and HBPr135 as reverse primer; and/or
   - HBPr75 as forward primers and HBPr94 as reverse primer; and/or
   - HBPr75 as forward primers and HBPr 105 as reverse primers.

6. Method according to any of claims 1 to 5 characterized further in that both mutations L528M and M552V/I are detected in one step.

* C L M S *

7. Method according to any of claims 1 to 5 characterized further in that both mutations L528M and V/L/M555I are detected in one step.

8. Method according to any of claims 1 to 5 characterized further in that both mutations M552V/I and V/L/M555I are detected in one step.

9. Method according to any of claims 1 to 5 characterized further in that the three mutations L528M, M552V/I and V/L/M555I are detected in one step.

10. Method according to any of claims 1 to 9 characterized further in that the HBV strain present in the biological sample shows resistance to lamivudine, famciclovir and/or penciclovir.

11. A probe as defined in any of claims 1 to 10, for use in the monitoring of antiviral drug resistance in a patient and in the genetic detection of the mutations L528M, M552V/I and/or V/L/M555I in the DNA polymerase of HBV strains present in a biological sample of said patient.

12. A composition comprising at least one probe as defined in claim 11.

13. The use of a composition of probes according to claim 12 for in vitro monitoring of antiviral drug resistance in a patient.

14. A diagnostic kit for the monitoring of antiviral drug resistance in a patient by genetic detection of at least one of the mutations L528M, M552V/I and/or V/L/M555I in the HBV DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following components:

(i) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said biological sample;

(ii) when appropriate, at least one suitable primer pair;

(iii) at least one probe according to claim 11, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

* C L M S *

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

(vii) when appropriate, a means for attaching said probe to a known location on a solid support.

15. A line probe assay for the monitoring of antiviral drug resistance in a patient by the genetic detection of the mutations L528M, M552V/I and/or V/L/M555I in the HBV DNA polymerase of the HBV strains present in a biological sample of said patient, comprising the following components:

(i) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in a biological sample of the patient;

(ii) when appropriate, at least one suitable primer pair;

(iii) at least one of the probes specifically hybridizing with the L528M target sequences shown in figure 1 and selected from HBPr270, HBPr293, HBPr294, HBPr412, HBPr274, HBPr355, HBPr415, HBPr461, HBPr468, fixed to a solid support; and/or

(iv) at least one of the probes specifically hybridizing with the M552V/I target sequences shown in figure 1 and selected from HBPr308, HBPr322, HBPr349, HBPr478, HBPr309, HBPr318, HBPr426, HBPr427, HBPr463, HBPr315, HBPr363-1, HBPr407, HBPr488, HBPr433-1, HBPr465, HBPr456, HBPr380, HBPr453, HBPr485, HBPr486, HBPr487, HBPr488, fixed to a solid support; and/or

(v) at least one of the probes specifically hybridizing with the V/L/M555I target sequences shown in figure 1 and selected from: HBPr279, HBPr338, HBPr341, HBPr345, HBPr474, HBPr332, HBPr328, HBPr385, HBPr289, HBPr299, HBPr419, HBPr490, HBPr491, HBPr492, HBPr494, HBPr495, HBPr496, fixed to a solid support.

(vi) a hybridization buffer, or components necessary for producing said buffer;

(vii) a wash solution, or components necessary for producing said solution;

(viii) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

16. An isolated nucleic acid consisting of or comprising a sequences as depicted in figure 1 (SEQ ID NOs 50-86 and SEQ ID NOs 107 to 109), or a fragment thereof,

wherein said fragment consists of at least 10 contiguous nucleotides as shown in figure 1 and contains one of the codons encoding amino acid 528, 552 or 555 of the HBV DNA polymerase.

