

Quantitative Detection of the M204V Hepatitis B Virus Minor Variants by Amplification Refractory Mutation System Real-Time PCR Combined with Molecular Beacon Technology[∇]

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Mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif are frequently associated with resistance to antivirals and represent a major concern in the treatment of hepatitis B virus (HBV) infection. Conventional methods fail to detect minority populations of drug-resistant viral quasispecies if they represent less than 25% of the total sample virus population. The amplification refractory mutation system real-time PCR (ARMS RT-PCR) was combined with molecular beacon technology using the LightCycler system. The samples from HBV patients selected for assay evaluation included (i) 57 samples from treatment-naïve patients for biological discriminatory ability (cutoff) estimation, (ii) 12 samples from patients with treatment failure that were M204V positive by sequencing, and (iii) 13 samples from patients with treatment failure that were negative for mutation at codon 204 by sequencing. The discriminatory ability of the assay was 0.25% when tested with laboratory-synthesized DNA target sequences. The median mutant-to-wild-type ratio for samples from naive patients tested positive for the wild type and for mutant variants was 0.01% (5th and 95th percentiles = 0.0001 and 0.04%, respectively). A value of 0.04% was selected as the biological cutoff of the assay of clinical samples. In all samples M204V positive by sequencing (12/12), the mutant variant was detected as the predominant population (range, 82.76 to 99.43%). Interestingly, in 5 (38%) of 13 samples negative by sequencing, the M204V variant was detected at a ratio above the biological cutoff (0.05 to 28%). The assay represents an efficient technique for the early detection and quantification of M204V variants before mutant strains emerge to dominate the population.

Nucleoside analogs inhibit hepadnavirus replication by terminating viral DNA synthesis and have been used in the treatment of patients with hepatitis B virus (HBV) infection. Antiviral treatment has been shown to rapidly reduce serum HBV DNA to levels below the detection limit of standard commercial assays and to be well tolerated without major adverse events (23). However, as with other antivirals, resistance may occur at a rate mainly dependent on the nucleoside/nucleotide analogue used. Taking lamivudine as an example, resistance increases with longer durations of treatment, from 24% at year 1 to 65 to 70% of treated patients at year 5 (17).

Resistance to lamivudine has been frequently associated with mutations that reside in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the C domain (M204I, M204V) and secondarily with mutations residing outside the C domain but mainly in the B domain (V173L, L180M) of the HBV polymerase region (Pol/RT). The M204V mutation has also been associated with resistance to other antivirals such as entecavir and emtricitabine; selection of M204I/V mutants affects future treatment options (4, 16). In vitro studies have demonstrated that mutant (Mut) viral variants exhibit a diminished replication capacity compared with

that of wild-type (WT) strains (21). However, under the selective pressure of antiviral drug treatment that prevents replication of the WT variant, Mut variants are able to outcompete the WT virus, as they appear to be sufficiently fit (8).

In general, the development of HBV resistance is detected by nucleotide sequencing of PCR products. This genotypic technique is unable to detect and quantify minorities of viral quasispecies below 25% (5, 19, 22). Clinical observations suggest that minor viral variants can frequently emerge as the major viral population. Due to the limitations of the currently used techniques, it has been difficult to establish the exact time and mode of emergence of the Mut virus.

The aim of our study was to develop a sensitive, reproducible, and accurate method for the quantification of viral populations with the M204V mutation, even at concentrations below the currently used thresholds, combining the amplification refractory mutation system real-time PCR (ARMS RT-PCR) assay with molecular beacon biotechnology.

MATERIALS AND METHODS

Experimental target design. Sixty-seven full-length HBV sequences representing all of the available human HBV genotypes were downloaded from the GenBank sequence database (<http://www.ncbi.nlm.nih.gov>) in order to create a reference alignment for the design of target sequences; we included sequences from genotype A (M57663, AJ309370, AJ344115, AF297622, AF418677, AY161142, X70185, X51970), genotype B (D23678, D23679, D23677, AB010289, AB073838, AB010290, AB010291, AB010292, AB073854, AB073853), genotype C (D50489, D23680, M38636, AB026811, AB042282, AB033556, AB074047, V00867, Y18855), genotype D (AF418679, X59795, X72702, AY161150, AF121239,

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AF151735, AB090270, AF043593, AB033558), genotype E (AB032431, AB091255, AB091256, AB106564, X75664, X75657), genotype F (AB036906, AB036907, AB036908, AB036905, AB036909, AB036911, AB036910, AF223962, AF223964, AF223963), genotype G (AB064313, AB056514, AB064310, AB064311, AF160501, AF405706, AB056513, AB056515), and genotype H (AB064315, AY090460, AB059659, AB059660, AY090454, AY090457, AB064312). Sequences were aligned by using the CLUSTAL W program (version 1.81) (31) and manually corrected.

Based on the HBV reference sequence alignment, a single-stranded DNA target sequence containing 95 bases was designed so as to include codon 204 (MEGA version 3.1) (13). The nomenclature based on HBV reference sequence X02496 has been used in order to define the primers and molecular beacon position on the target sequences (30). Due to viral strain nucleotide polymorphisms, the target sequence was chosen to be designed at the least polymorphic site possible. Based on the design of the HBV WT target sequence containing the triplet ATG (encoding methionine) in codon 204, the Mut target sequence was constructed in such a way that the point mutational triplet GTG (encoding valine) would be included. Single-stranded target sequence synthesis and high-purity clearance were performed by Invitrogen Laboratories (www.invitrogen.com).

Primer design. According to the ARMS technique, the reverse primer specifically designed for the WT and Mut target sequences were refractory to PCR on Mut and WT template DNA sequences, respectively. An additional mismatch at the n-1 position next to the 3'-end base of the Mut primer decreased the amplification efficiency of the WT target sequence to a greater extent than that of the Mut target sequence (20, 28). The primers used were 5'-CTCYTGGCT CAGTTTACTAGTGC-3' (forward primer, positions 535 to 557), 5'-CCCAA ACCAVATMATCCAT-3' (reverse WT primer, positions 610 to 629), and 5'-C CCAAWACCAVATMATCCGC-3' (reverse Mut primer, positions 610 to 629). Due to polymorphisms in the viral strains, ambiguity codes had to be included in the primer design. Primers were designed according to the following standard requirements: (i) to avoid primer-dimers and (ii) to ensure that the melting temperature (T_m) of the primers would be similar to or at least 5 to 10°C lower than the T_m of the molecular beacon. The software tool used for assessment of the T_m calculations was from the Virtual Genome Center (<http://www.yeastgenome.org/cgi-bin/web-primer>). Primers were synthesized by Invitrogen Laboratories.

Molecular beacon design. Molecular beacon, a single-stranded oligonucleotide hybridization probe that forms a stem-and-loop structure, was used for RT detection of amplification without interfering with the reaction (<http://www.molecular-beacons.org/>) (27, 29, 32). Molecular beacon consisted of 27 nucleotides and was 5' labeled with 6-carboxyfluorescein (detected at 530 nm, channel 1 for the LightCycler and LightCycler 2.0 systems) and 3' labeled with Black Hole Quencher 1. An additional CG terminal was added to both sides of the required sequence in order to form the stem. The molecular beacon used was 5'-CGCGGCGGAAAGCCCKRCGMACCACTGAACAAAGCCGCG-3' (positions 560 to 586). Synthesis of the molecular beacon was performed by Biologio Laboratories (www.biologio.com). Ambiguity codes were again included in the molecular beacon design due to polymorphisms in the viral strains.

Generation and quantification of the WT and Mut double-stranded DNA (dsDNA) target sequences. WT and Mut target sequences were amplified and converted to dsDNA forms by PCR. The cycling conditions used were 94°C for 2 min; 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30s; and a final extension of 72°C for 7 min. Thereafter, the Quant-iT PicoGreen dsDNA reagent, an ultrasensitive fluorescent nucleic acid stain for quantitating dsDNA in solution, was used to quantitate the PCR amplification procedure under RT conditions with the LightCycler 2.0 system (www.invitrogen.com, www.roche-applied-science.com).

LightCycler RT-PCR optimization and amplification. The LightCycler system was used for the RT-PCR (www.roche-applied-science.com). Multiple reactions with different concentrations of the master mix (MM) components were performed to define the optimum concentrations for an efficient and accurate amplification reaction. The MM final concentrations were set as follows: 10× LC FastStart DNA Master HybProbe, 30 μM each of the forward and reverse set of primers appropriate for the target sequence, 25 mM MgCl₂, 10 μM of the molecular beacon, and 1 μl of heat-labile uracil-DNA glycosylase. Twenty-microliter PCR capillary tubes were used for the mixture of 10.5 μl of MM with 10 μl of the HBV DNA target sequences. Each reaction mixture contained only one set of primers; for different targets, different reactions were needed (not a multiplex reaction). However, as the LightCycler has 32 possible positions, both WT and Mut targets were quantified within the same experiment.

Melting curves were determined to estimate the T_m of the molecular beacon. Different annealing temperatures were tested to optimize the reaction. Due to

the difference between the annealing temperatures of the primers and the molecular beacon, we decided to enter a slope in the cycling protocol to ameliorate the assay; annealing was performed at 60°C, while fluorescence was measured at 52°C. The final cycling conditions used were an initial denaturation at 94°C for 10 min; 50 cycles of 94°C for 0 min and annealing at 60°C for 0.05 min; and a final extension of 72°C for 0.1 min.

Preparation of the DNA standard curve. (i) Pure targets. Serial dilutions were performed for WT and Mut target sequences, starting at the initially identified concentration and ending at a concentration of 10 copies per reaction mixture, in order to construct the DNA standard curve. The two different sets of primers (one specifically designed for the WT target and the other for the Mut target) were used with the different DNA concentrations of both targets for the evaluation of the ARMS RT-PCR assay with molecular beacon technology.

(ii) Mixtures. After evaluation of the assay with pure experimental targets, multiple reaction mixtures with both primer sets were prepared with both WT and Mut experimental target sequences, resembling a clinical sample. In the first mixture, a standard concentration of 10⁶ copies per reaction mixture was prepared for the WT target sequence, while a gradient of 10⁶ to 10 copies per reaction mixture was used for the Mut target. The second mixture contained the opposite ratio of WT and Mut target sequences.

Evaluation of the ARMS RT-PCR assay with molecular beacon technology with DNA extracted from clinical samples. Viral DNA was extracted from 500 μl of serum by using the QIAamp DNA Blood Mini Kit (www.qiagen.com) according to the standard protocol. DNA was eluted in 60 μl (final volume) of DNase-free water.

Biological discriminatory ability (cutoff) estimation. Fifty-seven samples from treatment-naïve patients with viral loads of more than 10⁵ copies/ml were randomly selected to be measured by the ARMS RT-PCR assay with molecular beacon technology. Patients were not exposed to any nucleoside/nucleotide analogue. Each sample was measured with both WT and Mut (forward-reverse) sets of primers. The results were elaborated to estimate the discriminatory ability (cutoff) of the assay with clinical samples.

Use with clinical samples. Samples from chronically infected HBV patients known to have failed antiviral treatment (mainly lamivudine and adefovir) were selected to be included for evaluation of the ARMS RT-PCR assay with molecular beacon technology. Samples from 12 patients who had developed the M204V mutation, as well as samples from 13 patients with no mutations at codon position 204, tested by sequencing, were used to evaluate the ARMS RT-PCR assay.

Bioethical approval. The Bioethical Committee of the Medical School, Athens University, approved this study (reference no. 37/26-4-06).

Statistical analysis. Pure target sequences were measured in 20 repeats with the ARMS RT-PCR assay with molecular beacon technology to evaluate the analytical sensitivity of the assay. The coefficient of variation (CV) was calculated for samples quantified in repeated measurements. Pearson's correlation coefficient was used to assess the strength of the linear association between the expected concentrations of the WT and Mut target sequence and those observed by the ARMS RT-PCR assay with molecular beacon technology. The fitted regression line of expected versus observed concentrations was compared with the line of equality by testing the two-tailed hypotheses of slope = 1 and intercept = 0.

To determine the biological cutoff of the assay, we obtained the distribution of Mut/WT ratios in 57 treatment-naïve patients. The biological cutoff was defined as the 95th percentile of that distribution, i.e., the ratio below which 95% of the observations were found. Our choice of the 95th percentile for determining the cutoff was based on the fact that in a normally distributed characteristic, the normal limits are usually chosen in such a way that 95% of the healthy population has values within these limits. Furthermore, taking into consideration publications reporting that the presence of the M204V mutation affects future treatment options, we would opt for a less relaxed cutoff value, i.e., 0.04% (95th percentile) rather than 0.22% (99th percentile), to avoid cross-resistance with other antivirals in patients falsely identified as not having the M204 variant population.

RESULTS

Repeated measurements of the WT and Mut target sequences with the relevant (forward-reverse) sets of primers were performed to evaluate the ARMS RT-PCR assay with molecular beacon technology in pure experimental targets, as well as in mixtures with known concentrations of both WT and Mut target sequences.

TABLE 1. Statistics after 20 repeated measurements of each target sequence with specifically designed primers

Target sequence and concn ^a	Mean \pm SD of exptl values ^b	CV (%) of exptl values ^b	Analytical sensitivity ^c
Mut			
10 ⁶	6.058 \pm 0.146	2.4	20/20
10 ⁵	4.931 \pm 0.151	3.1	20/20
10 ⁴	4.015 \pm 0.125	3.1	20/20
10 ³	2.915 \pm 0.271	9.3	20/20
10 ²	2.106 \pm 0.269	12.8	20/20
10	1.345 \pm 0.496	36.9	19/20
WT			
10 ⁶	6.280 \pm 0.263	4.2	20/20
10 ⁵	4.698 \pm 0.522	11.1	20/20
10 ⁴	4.046 \pm 0.289	7.1	20/20
10 ³	2.904 \pm 0.244	8.4	20/20
10 ²	1.902 \pm 0.322	16.9	20/20
10	1.045 \pm 0.422	40.4	20/20

^a The ratio is the number of copies per reaction mixture.

^b In log₁₀-transformed values.

^c Number of tests positive/total.

Pure target sequences. Multiple repeats of the experiments revealed that the ARMS RT-PCR assay with molecular beacon technology showed a sensitivity of 10 copies per reaction mixture obtained after measurements of pure viral WT or Mut target sequences with the relevant (forward-reverse) set of primers. Concentrations as low as 10 copies per reaction mixture for the WT and Mut target sequence were quantitatively detected by the ARMS RT-PCR assay at frequencies of 100% (20/20) and 95% (19/20), respectively, as shown in Table 1. The expected concentrations of the WT and Mut targets were highly correlated with those observed with the ARMS RT-PCR assay with molecular beacon technology (Pearson's $r = 0.998$ for both WT and Mut target sequences). The equality line of the expected concentrations versus those observed with the assay fell within the 95% confidence interval of the corresponding fitted regression line for both WT (Fig. 1A) and Mut (Fig. 1B) target sequences when detected by the relevant set of primers. The variability of the assay, as measured by the CV, increased as the expected concentration was decreasing (Table 1). The linearity of the ARMS RT-PCR assay with molecular beacon technology was sustained at higher concentrations of both WT and Mut target sequences.

Mixtures of both WT and Mut target sequences. From the experiments performed with mixtures of both target sequences with the appropriate set of primers, we have seen that the observed measured concentrations were reaching a threshold of quantification beyond which any further gradient of the minor target sequence resulted in the same value observed regardless of the concentration of the minor target sequence. Further analysis was performed by using mixtures with 10⁶ copies of the WT target sequence per reaction mixture and gradient concentrations of the Mut target sequence, with the addition of intermitted concentrations of 5×10^3 , 2.5×10^3 , and 1.25×10^3 copies per reaction mixture detected with the relevant set of primers for the Mut target sequence.

The WT target sequence at a concentration of 10⁶ copies per reaction mixture was detected with both the relevant WT primers and the Mut primers to determine the discriminatory ability

of the ARMS RT-PCR assay with molecular beacon technology. While the WT target was detected in the 23rd cycle with the relevant set of primers, it was detected in the 34th cycle with the Mut primers. This 11-cycle window was defined as the discriminatory ability of the assay, suggesting that reliable Mut target detection can be performed within this window. As shown in Fig. 2, the discriminatory ability of the ARMS RT-PCR assay combined with the molecular beacon was 0.25% in repeated measurements, regardless of the initial concentrations of the fixed targets. The only important factor was the ratio of the Mut and WT target concentrations. The same concentrations of WT and Mut targets were detected at the same cycle when the relevant set of primers was used.

The variability of the ARMS RT-PCR assay with molecular beacon technology was evaluated by repeated measurements of the Mut target sequence in mixtures with known concentrations of WT and Mut targets in predetermined proportions with the relevant set of primers for Mut detection (Table 2).

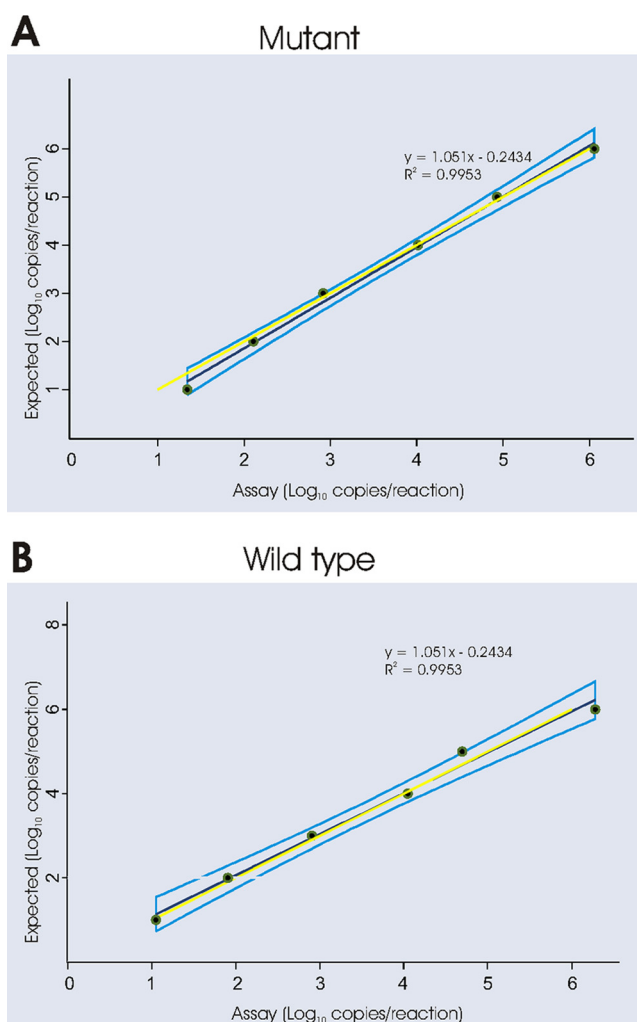


FIG. 1. Fitted regression line of the concentrations obtained with the ARMS RT-PCR assay with molecular beacon technology for the Mut target sequence (A) and the WT target sequence (B) versus the expected concentrations (black line), the equality line (yellow line), and the 95% confidence interval of the fitted regression line (blue line).

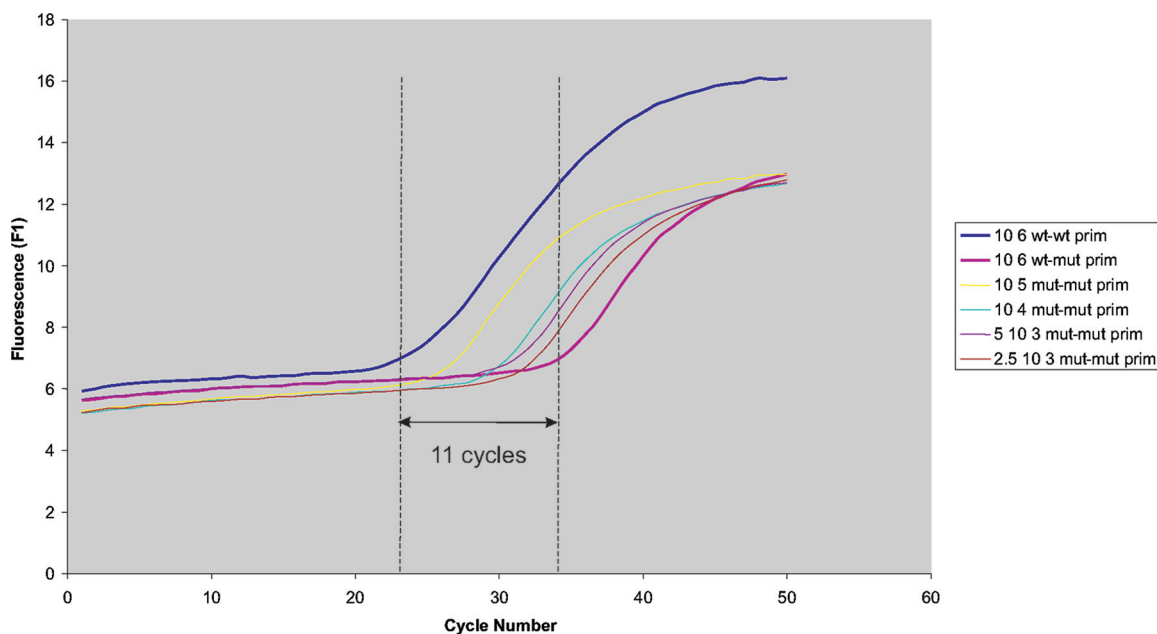


FIG. 2. Discriminatory ability of the ARMS RT-PCR assay with molecular beacon technology. The concentrations shown are 10^6 copies per reaction mixture of the WT target sequence determined with the set of primers (prim) specifically designed for detection of the WT (blue line), 10^6 copies per reaction mixture of the WT target sequence determined with the set of primers specifically designed for detection of the Mut (pink line), 10^5 copies per reaction mixture of the Mut target sequence determined with the set of primers specifically designed for detection of the Mut (yellow line), 10^4 copies per reaction mixture of the Mut target sequence determined with the set of primers specifically designed for detection of the Mut (light blue line), 5×10^3 copies per reaction mixture of the Mut target sequence determined with the set of primers specifically designed for detection of the Mut (purple line), and 2.5×10^3 copies per reaction mixture of the Mut target sequence determined with the set of primers specifically designed for detection of the Mut (brown line). The two-headed arrow shows the limits of the discriminatory ability of the assay.

The same procedure was also used with the Mut target sequence as the major viral population (Table 2).

Biological discriminatory ability (cutoff) with clinical samples. All 57 samples from treatment-naïve patients were analyzed with the ARMS RT-PCR assay with molecular beacon technology. It was possible to determine a Mut/WT ratio for 36

(63.2%) of the 57 samples; in the remaining 21 samples, the Mut variant was not detected. The range of Mut/WT ratios in the 36 samples was 1.81×10^{-7} to 2.23×10^{-3} . The median value of the Mut/WT ratio based on the samples tested positive for Mut and WT variants was 0.01% (5th and 95th percentiles = 0.0001 and 0.04%). The 95th percentile of the Mut/WT ratio, i.e., 0.04%, was defined as the biological cutoff of the assay.

Use with clinical samples. In all (12/12) of the samples from chronic HBV patients who had failed antiviral treatment (M204V mutation detectable by DNA sequencing), the Mut variant was quantified as the dominant viral population, representing 82.8 to 99.4% of the total viral population (Table 3). Interestingly, among 13 samples derived from chronic HBV patients who had failed antiviral treatment and tested WT at codon 204 by sequencing, 5 (38%) had a detectable M204V Mut variant when quantitated by the ARMS RT-PCR assay (Table 3). In five samples, the M204 Mut variant was detected at a ratio above the predetermined biological cutoff, ranging between 0.05 and 28%, as shown in Table 3.

TABLE 2. Statistics after repeated measurements of target sequences with primers specifically designed for WT or Mut target detection

No. of measurements, target, and target concn ratio ^a	Mean \pm SD of exptl values ^b	CV
8, WT		
$10^6/10^6$	6.448 ± 0.166	2.6
$10^6/10^5$	5.510 ± 0.141	2.6
$10^6/10^4$	4.487 ± 0.172	3.8
$10^6/10^3$	3.832 ± 0.201	5.3
$10^6/10^2$	3.696 ± 0.365	9.9
$10^6/10$	3.660 ± 0.354	9.7
12, Mut		
$10^6/10^6$	6.485 ± 0.393	6.1
$10^6/10^5$	5.215 ± 0.285	5.5
$10^6/10^4$	4.352 ± 0.229	5.3
$10^6/10^3$	3.622 ± 0.281	7.8
$10^6/10^2$	3.567 ± 0.241	6.8
$10^6/10$	3.528 ± 0.175	5.0

^a The ratio is the number of copies per reaction mixture. For the WT target, the WT/Mut target ratios are shown, and for the Mut target, the Mut/WT target ratios used are shown.

^b In \log_{10} -transformed values.

DISCUSSION

Nucleoside analogs have revolutionized the treatment of chronic HBV infection and have offered therapeutic options for the management of all types of HBV patients. However, long-term therapy is associated with selection of HBV polymerase mutants and emergence of resistance (7, 11, 14, 15, 33). Resistance to antiviral treatment (including lamivudine) has

TABLE 3. Results after measurement of samples from HBV patients who had failed antiviral treatment

Sample no.	No. of copies/reaction mixture		Mut/WT ratio	% Mut population	Sequencing result
	WT	Mut			
1	51,290,000	246,200,000	4.8	82.8	M204V
2	6,795	39,380	5.8	85.3	M204V
3	2,387	415,700	174.2	99.4	M204V
4	33,330,000	329,300,000	9.9	90.8	M204V
5	3,069,000	25,220,000	8.2	89.2	M204V
6	1,253	193,300	154.3	99.4	M204V
7	2,974	178,600	60.1	98.4	M204V
8	339.6	24,030	70.8	98.6	M204V
9	25,260,000	252,800,000	10	90.9	M204V
10	541,000,000	9,252,000,000	17.1	94.5	M204V
11	184,500	2,372,000	12.9	92.8	M204V
12	7,845	281,600	35.9	97.3	M204V
13	503,300	115.3	<BC ^a	NC ^b	No M204V, no M204I
14	219.5			NC	No M204V, no M204I
15	151.5			NC	No M204V, no M204I
16	16,170,000	889.9	<BC	NC	No M204V, no M204I
17	171,000,000	15,520	<BC	NC	No M204V, no M204I
18	1,466,000	408,200	0.2784	NC	No M204V, no M204I
19	1,645			NC	No M204V, no M204I
20	263,300	136.4	0.0005	NC	No M204V, no M204I
21	30,730	23.4	0.0008	NC	No M204V, no M204I
22	80,300,000	10,320	<BC	NC	No M204V, no M204I
23	196,100	53.1	<BC	NC	No M204V, no M204I
24	804,000	6,080	0.0076	NC	No M204V, no M204I
25	109,800	10,070	0.0917	NC	No M204V, no M204I

^a <BC, below biological cutoff.

^b NC, not calculated.

frequently been associated with mutations residing in the highly conserved YMDD motif (24, 25).

Various methods have been used to detect YMDD mutant populations of viruses (among others, nested PCR, cloning, and sequence analysis). These methods showed several faults, and subsequently novel methods had to be used. Lack of sensitivity and a failure to adequately detect mixtures of Mut and WT viral populations were the more important disadvantages of the methods. False-positive results could be encountered in nested PCR assays due to Taq polymerase fidelity. Moreover, most of these methods were qualitative and not quantitative and showed no cost effectiveness.

Assays based on restriction fragment length polymorphism (RFLP), fluorescence polarization, and reverse hybridization have been contributing to the understanding of the occurrence of the Mut HBV strains. RFLP assays have certain advantages over DNA sequencing, such as a higher throughput capacity for sample analysis, greater sensitivity for the detection of mixed populations of WT and variant HBV strains, and the ability to quantitatively detect mixtures of WT and Mut strains after PCR amplification even at low virus concentrations (3). However, such assays are time consuming and labor intensive and are not suitable for screening large number of samples, as they require multiple DNA amplifications and enzyme digestions or complex hybridization steps (6, 12, 34).

RT assays for the detection and quantitation of HBV Mut variants using molecular beacons have already been described. However, the developed beacons were not as sensitive as other techniques, including RFLP, and could only detect the variant strain later in the PCR when used with samples from two patients (26). Other assays were based on allele-specific PCR

extension for primer design, which may show a reduced ability to discriminate between the WT virus and Mut viral variants compared with that of the ARMS RT-PCR technique. The difference between allele-specific PCR extension and ARMS is that in the ARMS technique there is an insertion of a mismatch at the n-1 position of the 3' end of the primer to increase the specificity of the assay.

The INNO-LiPA DR assay has also been described for the detection of the presence of different genetic variants of HBV containing mutations which confer resistance to lamivudine (18). As previously reported, the line probe assay shows a higher sensitivity for detecting minor viral populations compared to sequencing (1, 9). However, the line probe assays are only capable of detecting the presence of the commonly occurring known mutations and the established HBV genotypes, a limitation that also exists for the ARMS RT-PCR assay with molecular beacon technology. In contrast, polymorphisms within the region of the probes can prevent annealing and yield indeterminate results with the line probe assays, while in the ARMS RT-PCR assay these polymorphisms have already been included in the design of the primers and the molecular beacon (10). Moreover, the detection limit of the line probe assay shows an ability to detect a minor viral population when this makes up 10% of the total viral load, while the ARMS RT-PCR assay with molecular beacon technology has a much higher discriminatory ability (26).

Pyrosequencing, a DNA sequencing technology based on the sequencing-by-synthesis principle, has recently come onto the market in the field of quantification of allelic frequency in populations. Pyrosequencing has the potential advantages of accuracy, flexibility, and parallel processing and can be easily

automated. An inherent problem with the method is de novo sequencing of polymorphic regions in heterogeneous DNA material. In most cases, it will be possible to detect the polymorphism, as in the case of polymorphism following substitution or deletion/insertion of the same kind as the adjacent nucleotide on the DNA template. In the case of a deletion/insertion of another type, the sequencing reaction can become out of phase, making the interpretation of the subsequent sequence difficult. Moreover, only one analysis can be run at a time and the light signal becomes diluted (2).

The ARMS RT-PCR assay with molecular beacon technology represent a sensitive, reproducible, and accurate assay for the quantification of the M204V Mut viral populations even at concentrations below the currently used thresholds. WT and Mut targets from all HBV genotypes can be detected, as polymorphisms have been incorporated in the primer design. The ARMS RT-PCR assay with molecular beacon technology shows good discriminatory ability even at concentrations lower than those previously described for other RT assays. Moreover, the assay is adaptable to the detection of other mutations with different sets of primers. It represents an RT-PCR system that does not require restriction enzyme digestion, gel electrophoresis, or analysis of PCR product sequences. The combination of the ARMS RT-PCR with molecular beacon biotechnology enables the monitoring of results in RT. A limitation of the assay is that novel polymorphisms not yet registered in the GenBank sequence database are not incorporated in the primer design. As combination therapy is used and more mutations are described, laboratories will need to run many reactions and testing will get very complex, as will primer and probe design, a limitation that is common to all base-specific quantitative assays.

Results obtained with the ARMS RT-PCR assay with molecular beacon technology and clinical samples were comparable to those obtained with experimental targets. The discriminatory ability of the assay was 0.25% when tested with laboratory-synthesized DNA target sequences. However, when the assay was used with clinical samples, the biological cutoff was determined to be 0.04%. The discriminatory ability of the assay was defined as the ability to distinguish between the two different target sequences (WT and Mut) at predetermined concentrations when the Mut target sequence represented the minor population. The biological cutoff was statistically calculated when samples from naïve patients supposed not to contain Mut variants were quantified by using both the WT and Mut primers. The Mut/WT viral population ratio for each sample was used to create a curve. The upper 95th limit of the curve was selected to represent the biological cutoff of the assay. These two parameters should result in similar values if the Mut and WT targets were identical. Due to natural genetic variations in clinical samples, the discriminatory ability and biological cutoff of the assay differ. The ARMS RT-PCR assay is very sensitive, and even minor changes in the target may alter the PCR kinetics. This hypothesis is further supported by our finding that the Mut/WT concentration ratio was consistently lower in clinical samples than in laboratory-synthesized DNA target sequences. In most of the previously published studies, discriminatory ability was estimated on the basis of plasmids or synthesized targets and not clinical samples.

The ARMS RT-PCR assay with molecular beacon technol-

ogy has the potential to be an efficient technique for the early and accurate quantitative detection of minor viral populations, especially when these are related to resistance, before they emerge as major viral populations that may influence the natural history of the HBV infection. Moreover, the assay successfully detected the Mut variants as the predominant population in all samples originating from patients who had failed antiviral treatment and were proven to have the viral population with the M204V mutation when the sample was tested by sequencing. Interestingly, in almost one-third of the samples proven to contain WT viral population by sequencing, the ARMS RT-PCR assay with molecular beacon technology succeeded in quantitatively detecting minor viral populations with the M204V mutation conferring resistance to antiviral treatment. The utility of ultrasensitive assays like the ARMS RT-PCR assay with molecular beacon technology is expected to provide a better understanding of HBV resistance kinetics in treated populations and a prognostic tool with value in detecting minor resistant viral populations affecting the response to treatment with antiviral agents. Full validation (of aspects of specificity, sensitivity, and reproducibility) is need before implementation in clinical practice.

In conclusion, the ARMS RT-PCR assay with molecular beacon technology represents a simple but efficient method for the identification of minor variants in mixed viral populations and may provide a helpful tool in decision making when treating patients with HBV infection.

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