Ultrasensitive Genotypic Detection of Antiviral Resistance in Hepatitis B Virus Clinical Isolates^{∇†}

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Amino acid substitutions that confer reduced susceptibility to antivirals arise spontaneously through error-prone viral polymerases and are selected as a result of antiviral therapy. Resistance substitutions first emerge in a fraction of the circulating virus population, below the limit of detection by nucleotide sequencing of either the population or limited sets of cloned isolates. These variants can expand under drug pressure to dominate the circulating virus population. To enhance detection of these viruses in clinical samples, we established a highly sensitive quantitative, real-time allele-specific PCR assay for hepatitis B virus (HBV) DNA. Sensitivity was accomplished using a high-fidelity DNA polymerase and oligonucleotide primers containing locked nucleic acid bases. Quantitative measurement of resistant and wild-type variants was accomplished using sequence-matched standards. Detection methodology that was not reliant on hybridization probes, and assay modifications, minimized the effect of patient-specific sequence polymorphisms. The method was validated using samples from patients chronically infected with HBV through parallel sequencing of large numbers of cloned isolates. Viruses with resistance to lamivudine and other L-nucleoside analogs and entecavir, involving 17 different nucleotide substitutions, were reliably detected at levels at or below 0.1% of the total population. The method worked across HBV genotypes. Longitudinal analysis of patient samples showed earlier emergence of resistance on therapy than was seen with sequencing methodologies, including some cases of resistance that existed prior to treatment. In summary, we established and validated an ultrasensitive method for measuring resistant HBV variants in clinical specimens, which enabled earlier, quantitative measurement of resistance to therapy.

Nearly 400 million people are chronically infected with hepatitis B virus (HBV) worldwide (25). After prolonged infection, often lasting decades, patients frequently develop severe liver disease that can lead to cirrhosis and hepatocellular carcinoma (reviewed in reference 13). Current therapies for chronic HBV include regimens of type I interferons and the nucleoside/nucleotide analogs lamivudine, adefovir-dipivoxil, entecavir, telbivudine, and tenofovir-disoproxil fumarate (13). Long-term antiviral therapy with these nucleosides/nucleotides often results in the emergence of drug-resistant variants, which can result in a loss of treatment benefits and impair the effectiveness of subsequent therapies. Although resistant viruses are often growth impaired, additional changes emerging under continued drug selection enable the expansion of adapted, resistant variants, which come to dominate the viral population (20). Detection of resistance early, either prior to or during therapy, can positively impact patient management and treatment selection. For example, resistance to lamivudine, from substitutions in the HBV reverse transcriptase (RT) YMDD active site motif, results in complete in vitro cross-resistance to

other L-nucleoside analog therapies, telbivudine, emtricitabine, and clevudine (3, 6, 11, 22).

The mechanistic genesis of antiviral resistance has recently been reviewed by Soriano et al. (27). Resistance in HBV is believed to arise through high rates of error-prone HBV polymerase reverse transcription and DNA synthesis and a lack of proofreading (18). Coupled with the production of more than 10¹² virions per day (33), this results in the probability of every nucleotide of the HBV genome being substituted many times over each day. The fact that antiviral therapy for HBV is effective at all demonstrates that other factors are important, including greater growth capacity of the resident wild-type (WT) virus than the resistant variants, long-lived WT-infected hepatocyte reservoirs, and immune clearance of viruses. In addition, the pressure of retaining the HBV surface antigen reading frame that overlaps with that of the polymerase also serves to reduce the resistance rate.

While early detection of resistance is important, conventional nucleotide sequencing generally detects only mutations that are present in approximately 20% of the circulating virus population (reviewed in reference 23). However, resistant viruses are often only a minor fraction of the total circulating virus population, due to their impaired replication capacity, and especially when drug pressure has been removed prior to testing. Nevertheless, resistant viruses rapidly reemerge as the dominant species upon the reinitiation of therapy (9).

In our studies of HBV resistance, we identified a small fraction of patients with evidence of viral resistance at baseline,

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FIG. 1. Steps in amplification and analysis of serum-derived HBV DNA. DNA was extracted from patient serum and quantified by quantitative PCR for the HBV RT, as described in Materials and Methods. Three steps of amplification were performed, first to increase the amount of template (step 1), then the region containing the resistance substitution (step 2), and finally, quantitative real-time PCRs for the wild-type and mutant DNAs in the mixed patient isolate (step 3). Step 1 was omitted unless additional material for other applications was needed. SNP, single nucleotide polymorphism.

using population-based nucleotide sequencing (5, 30). These included substitutions associated with lamivudine resistance, M204I or -V substitutions in the YMDD motif of the HBV RT, in a small fraction of nucleoside treatment-naïve patients (4/663, 0.6%). Additionally, in studies of patients with preexisting lamivudine resistance, entecavir resistance (ETVr) substitutions at T184A, -C, -F, -G, -I, -L, -M, or -S; S202C, -I, or -G; or M250I, -L, or -V (2) were found in a fraction of patients (10/187, 5%) (28). Other patients exhibited emerging resistance on therapy. To facilitate detection of lower levels of resistant viruses, we developed a single nucleotide polymorphism, allele-specific, real-time PCR approach (AS-PCR) for the detection of lamivudine resistance and ETVr substitutions in HBV DNA from patient serum.

MATERIALS AND METHODS

Study samples. HBV isolates were derived from the serum of nucleoside treatment-naïve or lamivudine-experienced patients enrolled in clinical studies designed to test the safety and efficacy of entecavir (5, 30). In general, WT isolates were derived from studies with nucleoside treatment-naïve patients and lamivudine-resistant and entecavir-resistant isolates were derived from studies with lamivudine-experienced patients. Detailed patient criteria and study protocols are described elsewhere (4, 29, 30). Nucleoside-naïve patients were included only if they had not used antiviral agents with activity against HBV within 24 weeks before randomization or had ≤ 12 weeks of prior lamivudine therapy. All study protocols conformed to the 1975 Declaration of Helsinki and were approved by appropriate institutional review boards, and all patients provided written informed consent.

PCR amplification. Herculase II Fusion DNA polymerase (Stratagene) was used for PCR amplifications, except for AS-PCR. Oligonucleotide primers containing locked nucleic acid (LNA) bases were obtained from Integrated DNA Technologies, Inc. (Coralville, IA), or Sigma-Genosys (The Woodlands, TX). Primer melting temperatures (T_m s) were calculated using the Exiqon online tool (<http://lna-tm.com>) (21). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the size and integrity of each amplicon were confirmed using agarose gel electrophoresis. All DNAs were diluted in 10 mM Tris-HCl buffer (pH 8.0).

Serum HBV DNA extraction and quantitation. HBV DNA was extracted from patient serum using the QIAamp MinElute virus kit (Qiagen), followed by real-time PCR quantitation using primers for the HBV RT region (see Table S1 in the supplemental material) under standard real-time PCR cycle conditions (1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min).

Quantification of the HBV DNA extracted from serum by real-time PCR specifically for the HBV RT was important as the Roche Cobas Amplicor PCR quantities from unextracted serum often did not correlate with those following extraction, presumably due to sample loss and variation.

Initial amplification of full-length HBV RT DNA. Initial amplification of full-length HBV RT DNA (step 1 in Fig. 1) could be omitted if only allele-specific analysis, and not other downstream applications, needed to be performed. For most analyses, the method began at step 2 of Fig. 1. For step 1, the full-length HBV RT amplicons were amplified from patient serum HBV DNA (10^6 copies or the maximum amount available when the total amount of DNA was less than 10^6 copies) and WT and resistant variant control plasmids (each 10^6 copies) using primers PCR1f and PCR1r (see Table S1 in the supplemental material). The DNAs were denatured initially at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s. After 40 cycles, reactions were extended at 72°C for an additional 2 min.

Amplification of smaller amplicons. Amplification of smaller amplicons, containing resistance changes (Fig. 1, step 2), was performed using primers specific for each amino acid position where resistance emerges (see Table S1 in the supplemental material). The primers contained two LNA bases at their 3' termini (7). One primer mapped close to the resistance substitution, with a 3' terminus ~ 3 bases upstream of the resistant variant nucleotide to be assayed. The primer sequence matched the resistant patient isolate population sequence. Primer T_m s were ~ 68 to 72°C . DNAs (10^6 copies) from PCR1, including samples and standards, were amplified using reaction conditions that were similar to those above, except that during the 40 reaction cycles, the templates were annealed at 60°C for 30 s and extended at 72°C for 30 s. In cases where the serum HBV DNA was abundant, the smaller amplicon (PCR2) was amplified directly from serum HBV DNA and PCR1 was omitted.

Preparation of standards and unknowns for AS-PCR. WT and resistant variant DNA plasmid standards were derived from corresponding patient samples. These standard DNAs were amplified in parallel with the unknown samples to be tested, in PCR1 and PCR2. WT standard DNAs were 10-fold serially diluted from 2×10^7 to 2×10^3 copies/ μl . The resistant variant standard DNAs were 10-fold serially diluted from 2×10^6 copies/ μl in WT DNA standards at a constant amount of 2×10^6 total copies/ μl . This resulted in 100%, 10%, 1%, 0.1%, etc., resistant variants in WT ratios. Unknown patient samples to be tested were diluted to 2×10^6 copies/ μl prior to AS-PCR (below).

AS-PCR. The AS-PCR was composed of two separate PCRs, performed in parallel, to quantify WT and resistant variant DNAs. Each PCR paired a universal primer with a primer specific to either WT or resistant variant. Results from defined WT and resistant standards were used to generate standard curves for quantification of patient samples. The AS-PCR mixtures were assayed in duplicate, and the mean values from at least three independent experiments are presented. The primers used in AS-PCR were designed to have T_m s of $\sim 60^\circ\text{C}$, unless indicated otherwise in Table S1 in the supplemental material. The 5' ends of the primers were ~ 6 bases nested from the PCR2 primers, and the 3' termini were ~ 3 bases downstream from the 3' ends of the PCR2 primer. The remaining

FIG. 2. HBV AS-PCR performance. (A) The dynamic range of HBV AS-PCR of standards and defined mixtures using oligonucleotide primers entirely composed of natural DNA bases and primers containing 3' LNA bases. Symbols: (—x—), pure S202G variant DNA with an LNA primer; (—●—), S202G variant DNA in the presence of WT DNA using an LNA primer; (—○—), S202G variant DNA in the presence of WT DNA using an entirely natural DNA base primer. (B) M204V resistant DNA standards amplified using Platinum *Taq* high-fidelity or Herculanase II Fusion DNA polymerase and subsequently assayed by the HBV AS-PCR method.

sequences overlapped with the PCR2 primers. The primers specific to WT and resistant variants contained a single LNA base at the 3' termini corresponding to the WT and resistant nucleotides, respectively (12). Real-time PCRs were performed using the Power SYBR green PCR Master Mix and contained conventional *Taq* DNA polymerase in a 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). The AS-PCR mixtures contained 300 nM each of forward and reverse primers (see Table S1 in the supplemental material), 1× Power SYBR reaction buffer/enzyme mixture, and 5 μ l of diluted DNA in a final volume of 20 μ l. All AS-PCRs were performed using the same conditions as for the quantitation of HBV DNA from serum, except for resistance substitution M204I (codon ATA), where the 40 amplification cycles were extended at 65°C instead of 60°C.

Data analysis. Quantitation of WT and resistant variants in samples was based on the WT and resistant variant DNA standard curves, using SDS 2.2.2 software (Applied Biosystems, Inc.). Only the quantities that yielded results within the linear range were used. The data were expressed as the percent resistant variant, the resistant/WT ratio, and the resistant variant signal over the WT background.

RESULTS

We developed a sensitive method based on real-time PCR (Fig. 1) to detect the nucleotide substitutions that give rise to HBV antiviral resistance. The method has similarity to other AS-PCR assays but contains several innovative applications of new technology and approaches to improve sensitivity and reliability and reduce the incidence of false-positive results. Primer sets were developed to detect a wide range of HBV RT amino acid substitutions, including those associated with lamivudine and telbivudine resistance, M204I or -V, and ETVr

substitutions T184A, -C, -F, -G, -I, -L, -M, or -S; S202C, -I, or -G; or M250I, -L, or -V, which occur in addition to the lamivudine resistance changes (2).

LNA-enhanced primers. Primers containing a single LNA base at the 3' termini were used to increase the specificity of amplification (12). Figure 2A (resistant variant standard curve) shows that the LNA primers yielded greater sensitivity than did unmodified oligonucleotide primers. In the absence of WT DNA, the resistant variant standard curve was completely linear with respect to DNA copy number. In the presence of added WT DNA, using a DNA primer for the resistant variant, the standard curve remained linear to ~1% resistant variant (r^2 value, 0.999). At lower levels of the resistant variant DNA, the standard curve generated with standard primers began to deviate from linearity. This resulted from cross-reaction of the resistant variant primer with WT DNA through mispriming, generating a very low level of false amplification (background). The actual level of background depends on the amount of WT DNA present. This background level determines the limit of resistant variant detection, while the linearity of the standard curve indicates the range of accurate quantitation of resistant variant in the presence of the abundant WT population. Comparatively, the LNA primers produced a lower level of background and a longer range of linearity in the standard curve and enabled detection of the variant at levels of <0.1% (r^2

FIG. 3. Quantitation is consistent over a wide range of DNA concentrations. Amplified WT S202 and variant S202G standard DNAs were mixed at 1%, 0.1%, and 0% in WT DNA, with total DNA in the HBV AS-PCR assay ranging from 1,000 to 10⁸ copies. The absolute numbers of WT and variant DNA copies were determined, the percent variant was calculated, and the results were plotted and compared to theoretical values. The results represent the means ± SDs for three independent experiments.

value, 0.998). Dilution of the variant DNA below 0.01% resulted in a complete overlap in detection of the resistant and WT DNAs, at the WT background level, suggesting a limit of quantitation above 0.01% for this primer set.

DNA polymerase. Routinely, patient isolate DNAs must be amplified by PCR to provide sufficient material for analytical procedures. Herculanase II Fusion DNA polymerase (Stratagene) exhibits higher 3'-to-5' exonuclease proofreading activity and greater fidelity than Platinum *Taq* DNA high-fidelity polymerase (Invitrogen), which resulted in a lower background and an increased sensitivity. This effect is illustrated in Fig. 2B, where M204V resistant variant DNA was analyzed following amplification using Herculanase II Fusion polymerase or Platinum *Taq* DNA high-fidelity polymerase. The standard curve for the mutant variant using Herculanase II-amplified DNA was linear and sensitive to beyond 0.1% (variant/WT + variant DNA), while the one using Platinum *Taq* DNA high-fidelity polymerase was linear only to 1.0%.

Quantification over a wide range of DNA concentrations. Figure 3 shows that quantification of low levels of variants was consistent over a wide range of HBV DNA concentrations. Resistant variants at 1% and 0.1% in WT DNA gave consistent results; even when as few as 10 copies of the resistant variant were present in the assay. In both cases, the signal from the resistant variant was at least 21-fold higher than the signal from the WT background (illustrated in Fig. 2A). All patient data reported below used 10⁷ copies of DNA in the AS-PCR assay, which ensured that resistant variants at 0.001% levels would be represented with at least 100 copies in the assay.

Panel of resistance mutations. Primer sets to detect HBV variants with resistance to lamivudine at M204 (YMDD motif) and at positions involved in ETVr (positions T184, S202, and M250) (2) were developed and optimized (see Table S1 in the supplemental material). The sensitivity level of each primer set was limited by the background signal from the WT population

(example shown in Fig. 2A) and was represented by % mutant in WT DNA, determined empirically using the WT and variant standard curves generated in parallel (Table 1). Detection of resistant variants needed to be above these levels to be considered positive. Each primer set exhibited a different level of sensitivity, which is listed in Table 1. The median sensitivity for the entire collection of primers was $9.9 \times 10^{-4}\%$. The limit of detection (WT background level) ranged from as low as 1/22,936,630 ($4.4 \times 10^{-6}\%$) for T184G to as high as 1/2,017 ($5.0 \times 10^{-2}\%$) for M204I (ATT codon). The most common clinical ETVr genotypes, involving M204V, S202G, and T184L substitutions (29), were detected with high sensitivity, at 1/5,929 (M204V), 1/18,120 (S202G), and 1/293,609 (T184L). The M250 ETVr residue is located in a highly polymorphic region near the 3' end of the RT. Consequently, patient-specific M250 primer sets had to be designed for each patient isolate and could have considerable sequence variation between patients. Furthermore, even when the M250 resistance codon was the same, the detection sensitivity occasionally varied more than 10-fold across individual patients. This was the only substitution where the limit of detection sensitivity above background sometimes fell above 0.1%. The highly heterogeneous sequence around M250 presents a challenge to sensitivity for all AS-PCR-based methods. These findings suggest that this method is not as robust for detecting substitutions at the HBV RT M250 position, or other positions with a high degree of inherent polymorphisms. However, very few patients develop M250 ETVr substitutions (30).

Despite the varied sensitivities of the different resistance primer sets, we established a conservative cutoff of 0.1% variant (1 variant DNA/1000 WT DNAs) as the limit of detection for all substitutions when reporting positive detection of resistance in patients. This level was chosen because 0.1% was the lowest level of detection where conventional sequencing of individual clones might reasonably be utilized to validate the

TABLE 1. Resistant variant detection primers and sensitivities

Resistance type	WT residue	Substitution	Nucleotide change(s)	3'-end nucleotide	Background ^a /sensitivity	
					Resistant/WT	% Resistant/ (resistant + WT) (% ± SD)
Lamivudine	M204 (ATG)	M204I (ATA)	G→A	1 LNA	1/2,017	0.050 ± 0.026
		M204I (ATT)	G→T	1 LNA	1/23,503	0.0043 ± 0.0032
		M204V (GTG)	A→G	1 LNA	1/5,929	0.017 ± 0.0052
Entecavir	T184 (ACT)	T184A (GCT)	A→G	1 LNA	1/12,672	0.0079 ± 0.0030
		T184C (TGT)	A→T	1 LNA	1/2,396,028	0.000042 ± 0.000017
		T184F (TTT)	AC→TT	DNA	1/277,777	0.00036 ± 0.00016
		T184G (GGT)	AC→GG	DNA	1/22,936,630	0.0000044 ± 0.0000028
		T184I (ATT)	C→T	1 LNA	1/8,425	0.012 ± 0.0082
		T184L (CTT)	AC→CT	DNA	1/293,609	0.00034 ± 0.00010
		T184M (ATG)	CT→TG	DNA	1/1,803,428	0.000055 ± 0.000031
		T184S (AGT)	C→G	1 LNA	1/177,391	0.00056 ± 0.000090
		T184S (TCT)	A→T	1 LNA	1/394,592	0.00025 ± 0.00013
		S202 (AGT)	S202C (TGT)	1 LNA	1/189,550	0.00053 ± 0.00014
	S202 (AGT)	S202G (GGC)	A→G, T→C	1 LNA	1/269,046	0.00037 ± 0.000071
		S202G (GGT)	A→G	1 LNA	1/18,120	0.0055 ± 0.0016
		S202I (ATT)	G→T	1 LNA	1/101,147	0.00099 ± 0.00020
		M250 (ATG)	M250L ^b (CTG)	1 LNA	1/3,506	0.029 ± 0.007
	M250 (ATG)	M250V ^b (GTG)	A→G	1 LNA	1/136	0.73 ± 0.098
				1 LNA	1/3,074	0.033 ± 0.002
				1 LNA	1/486	0.21 ± 0.023

^a Variant background detected from amplified WT DNA.^b Results varied according to patient/isolate; the results of both are presented.

AS-PCR results, as 1,000 clones would theoretically be needed to identify a variant clone. Sensitivities below the 0.1% level are valid for most primer sets (Table 1) but would be difficult to routinely verify.

Validation through nucleotide sequencing of individual clones. The sensitivity of the AS-PCR assay was validated through quantitation of defined mixtures of DNA amplified from WT and resistant variant clones derived from the corresponding patient (Fig. 2). Further validation was obtained by nucleotide sequencing of clones derived from patient samples (Tables 2 and 3). Table 2 shows the results of AS-PCR and clonal analysis of baseline samples from four patients. More than 1,000 clones were sequenced for the baseline patient samples 6, 43, and 23. Samples that were both positive and negative by the AS-PCR method were found. For patient 6

baseline, AS-PCR detection of both the lamivudine-resistant variants M204I and M204V was 0.23% while the sequence of 1,103 clones yielded 0.27% for both these mutations (Table 2). For patients 23 and 43, baseline AS-PCR and sequencing of 1,064 and 1,521 clones, respectively, were in agreement in that no resistant variants were detected. These results were important because most other methods failed in our hands due to false-positive detection of resistance (data not shown). Patient 16 had a complex resistance profile, with lamivudine resistance (M204I) at baseline by sequencing. Resistance was estimated from the sequencing chromatograph to be ~30% of the total isolate population, consistent with the AS-PCR finding of 34%. The M204V change, however, was also detected at baseline by AS-PCR at 0.92% but was below levels detectable by sequencing, as expected. Interestingly, this patient showed the emer-

TABLE 2. Validation of AS-PCR detection of resistance by cloning and sequencing

Patient no.	Lamivudine resistance substitution(s) at M204	Detection by population sequencing at baseline (estimated %)	% by AS-PCR (% ± SD)	% by sequencing clones (no. of variant clones)	No. of clones sequenced
6	M204V (GTG)	— ^a	0.22 ± 0.038	0.27 (3)	1,103
	M204I (ATA)	—	0.23 ± 0.038	0.27 (3)	
	M204I (ATT)	—	0	0	
43 ^b	M204V/I	—	0	0	1,521
23	M204V/I	—	0	0	1,064
16	M204V (GTG)	—	0.92 ± 0.014	ND ^c	ND
	M204I (ATT)	30	34 ± 0.024		
	M204I (ATA)	—	ND		

^a —, negative.^b Serum HBV DNA quantity available for AS-PCR testing was <260 copies, limiting the maximum sensitivity of detection to 0.38%.^c ND, not done.

TABLE 3. Longitudinal AS-PCR analysis of resistance development

Patient	Time point (wk)	Lamivudine resistance and ETVr substitution (codon)	Detection by population sequencing	% by AS-PCR (% $\pm$ SD)	% by sequencing clones (no. of variant clones)	Total no. of clones sequenced
3	0 (baseline)	M204V/I	— ^a	—	0	24
		M204I (ATA)	—	0.41 $\pm$ 0.081	0	23
	24	M204I (ATT)	—		—	0
		M204V (GTG)	—		—	0
		S202G (GTG)	—		—	0
		M204I (ATA)	+ ^b	6.6 $\pm$ 0.57	4.1 (1)	24
		M204I (ATT)	—		—	0
		M204V (GTG)	—		—	0
		S202G (GTG)	—		—	0
		M204I (ATA)	—	0.38 $\pm$ 0.094	0	22
	124	M204I (ATT)	—		—	0
		M204V (GTG)	+	98.0 $\pm$ 0.069	100 (22)	
		S202G (GGT)	+	98.3 $\pm$ 0.56	100 (22)	
		M204I (ATA)	—	0.42 $\pm$ 0.086	0	23
	144	M204I (ATT)	—		—	0
		M204V (GTG)	+	99.9 $\pm$ 0.0085	100 (23)	
		S202G (GGT)	+	99.9 $\pm$ 0.022	100 (23)	
44	0	M204V/I	—	—	ND ^d	
		S202G (GGT)	—	—	ND	
	112 ^e	M204V/I	—	—	ND	
		S202G (GGT)	—	—	ND	
	124 ^f	M204V	—	2.4 $\pm$ 0.23	ND	
		S202G (GGT)	—	3.2 $\pm$ 0.041	ND	
		M204V (GTG)	+	44 $\pm$ 4.4	33.3 (8)	24
	139	S202G (GGT)	+	46 $\pm$ 2.8	33.3 (8)	

^a —, negative.^b +, positive.^c Amount of serum DNA used in AS-PCR analysis was <100 copies; therefore, the limit of detection of resistant variants was >1%.^d ND, not done.^e Total, 95 copies of serum HBV DNA after extraction.^f Total, 375 copies of serum HBV DNA after extraction.

gence of an M204V, L180M, S202G isolate sequence on therapy (5).

Table 3 shows validation of the method for following the emergence of lamivudine resistance and ETVr in longitudinal analyses. Patient 3 showed the emergence of lamivudine resistance and ETVr. Neither clone sequencing nor AS-PCR analysis detected substitutions at baseline, but the presence of M204I (ATA) was detected at 0.41% at week 24 by AS-PCR. This substitution was not detected by sequencing of 23 clones, as expected. M204I increased to 6.6% by AS-PCR and in 1 of 24 clones (4.1%) at week 100, while other substitutions remained negative. This substitution remained positive at <1% levels at weeks 124 and 144, below the detection limit by population sequencing, and was absent in 22 sequenced clones. At weeks 124 to 144, clones from patient 3 all contained the lamivudine-resistant M204V (GTG), L180M, and the entecavir-resistant S202G (GGT) changes. AS-PCR detection of these two substitutions ranged from 98 to 99%, similar to the results of cloning and sequencing. In this patient, there was a shift, on continued therapy, of the M204I substitution to the M204V change, as seen in many other patients with resistance on lamivudine (unpublished observations). In patient 44, M204V, L180M, and S202G were detected by sequencing only after week 139. Overall, the results obtained by cloning and sequencing correlated well with the AS-PCR results and verified that the method could be used to show the early appearance of resistance.

Detection of baseline lamivudine resistance in nucleoside-naïve patient cohorts. AS-PCR was used to determine if resistance that emerged in patient population sequences on treatment was actually present in baseline samples collected prior to therapy. Table 4 shows the results from studies of 663 nucleoside treatment-naïve patients through year 5 in which resistance was detected on therapy (29). In four cases (53, 1, 3, and 16), population sequencing revealed lamivudine resistance substitutions at baseline. In nine others, resistance substitutions appeared to emerge on therapy. However, AS-PCR analysis of baseline isolates from these patients showed that, in all but four cases, resistant variants existed at baseline at levels that were unable to be detected by population sequencing.

Detection of ETVr substitutions at baseline in lamivudine-refractory patient cohorts. ETVr is rare in nucleoside-naïve patients but occurs at higher levels in patients who showed continued viremia or resistance on lamivudine therapy. Table 5 shows the lamivudine-refractory patient cohort that had evidence of ETVr during our study (4). Fifty-seven lamivudine-refractory patients with ETVr at baseline or emerging on therapy were tested in the AS-PCR assay for various ETVr changes. Approximately 5% (10/187) of these lamivudine-refractory patients also had ETVr substitution sequences at baseline, prior to entecavir therapy (Table 5). In some other patients, ETVr changes emerged during entecavir therapy. AS-PCR analysis of baseline isolates from the patients with emerging resistance revealed that a number had ETVr substitutions

TABLE 4. Detection of lamivudine resistance at baseline in nucleoside-naïve patients

Patient no.	Phylogenetic genotype	Prior treatment with lamivudine (no. of days)	Baseline M204 resistance by population sequencing	Emerging M204 resistance by population sequencing (wk)	Baseline resistance by AS-PCR (substitution) (% $\pm$ SD)
53	D	NR ^a	M204 M/I	NC ^b	NT ^c
1	G	NR	M204I	NC	NT
3	D	NR	M204I	NC	NT
16	D	NR	M204M/I	M204V (48)	NT
6	A	31	— ^d	M204V (48)	0.21 $\pm$ 0.038 (M204V [GTG]), 0.21 $\pm$ 0.038 (M204I [ATA])
14	D	NR	—	M204I (ATT)(48)	0.20 $\pm$ 0.038 (M204I [ATA])
13	A/C ^e	NR	—	M204V (48)	0.19 $\pm$ 0.038 (M204I [ATA])
17	A	NR	—	M204V (96)	0.12 $\pm$ 0.033 (M204I [ATA])
74	D	202	—	M204I (ATT) (104)	0.234 $\pm$ 0.022 (M204I [ATT])
43	D	NR	—	M204I (53)	ND, ^f LC ^g
23	D	NR	—	M204V (84)	ND
44	A	NR	—	M204V (139)	ND
76	B	NR	—	M204V (246)	ND

^a NR, not reported.^b NC, no change on treatment detected from baseline lamivudine resistance substitutions.^c NT, not tested.^d —, negative.^e Patient isolate was genotype A at baseline and genotype C on therapy.^f ND, not detected above 0.1% (1:1,000).^g LC, low copies; only 260 copies of serum HBV DNA available to be tested.

at low levels prior to entecavir therapy. Interestingly, 64% (7/11) of patients with emerging ETVr detected in the first year of therapy had baseline resistance that was detected by AS-PCR at levels of $\geq 1/1,000$ (0.1%). This frequency decreased over time as only 25% (3/12) and 6.7% (1/15) of patients with ETVr emerging in years 2 and 3, respectively, had AS-PCR-detectable resistance above 1/1,000 at baseline. None of the patients with emerging ETVr in year 4 (Table 5; 0/7 patients) and year 5 (0/2 patients, data not shown) had AS-PCR-detectable ETVr at baseline. The patients in Tables 4 and 5 were infected with a variety of phylogenetic genotypes, showing that the AS-PCR method worked across HBV genotypes.

Compiled results of patient samples in context with detection limits. Figure 4 shows the results of testing samples from all patients, both lamivudine refractory and nucleoside naïve, for the presence of the different resistance changes. The figure shows the empirically determined background or limit of detection levels for each primer set ($\pm$ standard deviation [SD]). Many patient samples showed results that were near the WT background level, while others were strongly positive. Many samples were above the 1/1,000 (0.1%) conservative cutoff level for the M204V, M204I (ATT codon), S202C, S202G, and all the T184 substitutions. The M204I (ATA) lamivudine resistance substitution cutoff was only modestly above the 0.1% level. However, patient 6 (Fig. 4A) with M204I (ATA) illustrates the detection level of a positive sample that was verified by sequencing of 1,103 cloned isolates at 0.27%, closely matching the 0.23% result of the AS-PCR assay (Table 2). Therefore, results that were near, but still above, the detection limit are confirmed positive. As mentioned above, the M250L and -V substitutions were difficult to detect below 0.1% levels in some cases, even with patient-specific primers. Figure 4D shows that the detection sensitivity reached 1/3,000 for three patients with M250V and one patient with M250L (patient 1). However, detection sensitivity for M250L in samples from patient 2 and for M250V in patient 5 (with two different isolate subpopula-

tions with M250V) was poor because the WT background control yielded the same signal as did a cloned resistant variant control at nearly 1.0% abundance (Fig. 4D). Overall, the more conserved regions of the HBV RT, however, containing resistance mutations at residues 184, 202, and 204, were amenable to more sensitive resistance detection by AS-PCR.

DISCUSSION

The emergence of resistant variants is the primary virologic basis for antiviral treatment failure. Determining the presence of preexisting and emerging resistant strains has significant utility in the setting of clinical therapy, in the selection of antiviral treatment regimens. Population sequencing of PCR-amplified viral DNA is routinely used to identify resistant variants (24). However, it is often insufficiently sensitive to detect variants when they emerge at low levels or decline after cessation of therapy. Other methods, such as Inno LiPa line probe analysis (14), have increased sensitivity to $\sim 5\%$ to 10%, but often variants are present below these levels. The discriminatory PCR assays with peptide-linked nucleic acids (16) and ligase chain reaction (17) methods were less successful in our studies. Methods based on mass spectrometric detection are limited by sequence polymorphisms surrounding ETVr changes and lower reported sensitivity (8). Even ultradeep sequencing methods are limited to 1.0% sensitivity, regardless of the number of clones sequenced, by the relatively high error rate of the polymerase (26). We developed a new real-time AS-PCR approach, containing several innovative applications of new technology and procedures, for sensitive, reliable, and quantitative detection of single nucleotide polymorphisms within DNA. The application of this method for patient samples with mixed isolates resulted in robust detection of a wide range of HBV substitutions involved in resistance to lamivudine and other L-nucleoside analogs (3, 6, 11, 22) and entecavir (30). This method could prove useful in the sensitive quanti-

TABLE 5. Detection of ETVr at baseline in lamivudine-resistant patients

Yr of ETVr emergence (population sequencing)	Patient	Phylogenetic genotype	Baseline ETVr by sequencing	Baseline ETVr by AS-PCR (% $\pm$ SD)	% ETVr of $\geq 0.1\%$
0 (baseline)	27	D	L180M, T184S, M204V	ND ^a	100 by sequencing
	60	D	L180M, S202C, M204V	ND	
	1	D	L180M, T184T/S, M204V	ND	
	94	B	L180L/M, M204I/V, M250M/L	ND	
	51	D	L80L/I, V173V/L, L180M, T184T/S, M204I/V	ND	
	82	B	L180L/M, M204I/V, M250M/L	ND	
	45	C	L180M, T184T/S, M204I/V	ND	
	22	C	L80L/V, L180M, T184T/I, M204M/V	ND	
	49	B	M204I, M250M/I,	ND	
	97	A	L180L/M, M204I/V, M250M/L	ND	
1	69	C		S202G, 0.13 $\pm$ 0.0048	63.4 by AS-PCR
	56	A		T184A, 0.036 $\pm$ 0.0029; T184S, 0.23 $\pm$ 0.026	
	52	C		T184I, 0.99 $\pm$ 0.044	
	16	B		M250L, 2.8 $\pm$ 0.28	
	93	C		T184S, 2.3 $\pm$ 0.069	
	4	C		T184I, 0.14 $\pm$ 0.030	
	13	D		UD ^b	
	41	D		S202G, 4.7 $\pm$ 0.40	
	30	C		UD	
	74	B		UD	
	7	D		T184A, 0.045 $\pm$ 0.0020	
2	6	C		UD	25 by AS-PCR (12)
	63	D		T184S, 0.54 $\pm$ 0.024	
	35	D		UD	
	62	D		UD	
	86	D		UD	
	36	C		S202G, 0.2 $\pm$ 0.011	
	31	A		T184S, 0.0056 $\pm$ 0.0011; S202G, 0.041 $\pm$ 0.0024	
	22	D		UD	
	96	C		UD	
	24	A		S202G, 0.038 $\pm$ 0.0047; T184F, 0.026 $\pm$ 0.0066; T184S (AGT), 0.024 $\pm$ 0.0046; T184S (TCT), 0.21 $\pm$ 0.13	
	61	C		UD	
3	2	D		UD	6.70 by AS-PCR
	28	A		UD	
	3	C		T184F, 0.037 $\pm$ 0.012	
	6	D		UD	
	8	C		T184S, 8.3 $\pm$ 0.27	
	53	C		T184S (TCT), 0.0030 $\pm$ 0.00068; T184S (AGT), 0.0018 $\pm$ 0.00032	
	29	F		S202G, 0.033 $\pm$ 0.0017	
	65	IND ^c		S202G, 0.02 $\pm$ 0.0018; T184F, 0.0057 $\pm$ 0.0012	
	55	F		UD	
	100	C		UD	
	13	D		UD	
4	31	A		UD	0 by AS-PCR
	10	C		T184F, 0.015 $\pm$ 0.0018	
	99	D		UD	
	20	D		T184L, 0.0019 $\pm$ 0.000038; T184M, 0.00096 $\pm$ 0.000070; T184S (TCT), 0.0064 $\pm$ 0.00039	
	95	B		T184S (TCT), 0.0056 $\pm$ 0.00099	
	51	D		UD	
	30	C		T184L, 0.0018 $\pm$ 0.00021	
	5	A		S202G, 0.053 $\pm$ 0.0013	
	60	A		UD	
	71	C		UD	
	75	B		UD	
	32	A		UD	

^a ND, not done.^b UD, not detected above negative controls.^c IND, indeterminate.

tative detection of single nucleotide polymorphisms which are associated with early breakthrough on therapy, allowing a change to a drug with a different resistance profile.

Sensitivity and reliability are key aspects of any assay to detect resistance substitutions. Traditional AS-PCR methods

use the intentional addition of an artificial mismatch into primers to enhance the differential amplification of resistant versus WT DNAs (15, 19, 32). To reduce complications from minor populations containing polymorphic nucleotides matching or interfering with the introduced artificial mutation, we instead

FIG. 4. Compiled results of patient samples analyzed using HBV AS-PCR. The results of all samples and substitutions tested by AS-PCR are shown. The individual circles represent the mean results $\pm$ SDs of individual patient samples, from at least three independent experiments (some error bars are too small to be seen). The shaded region shows the results using the WT background DNA tested in parallel, shown as the SD, calculated using Microsoft Excel-Fit. The numbers of WT determinations are shown below the graphs (WT $n = x$). (A) M204 lamivudine resistance; (B) S202 ETVr; (C) T184 ETVr; (D) M250 ETVr. For the M250V substitution, patient 5 had two subpopulations of isolates with M250V (5A and 5B) tested separately. The triangular symbol in panel A indicates the results from patient no. 6, which was verified by clone sequencing (Table 2).

developed our method using primers terminated with LNA bases. This enhanced the differential amplification of WT and mutant without artificial mutations in the primers. The sensitivity that we routinely obtained for each lamivudine-resistant and entecavir-resistant variant that we tested is shown in Table 1, and median sensitivity for the entire collection of primers was $9.9 \times 10^{-4}\%$ (range, 1/22,936,630 [$4.4 \times 10^{-6}\%$] to 1/2,017 [$5.0 \times 10^{-2}\%$]). In some cases, resistance mutations involved two nucleotide changes. In these instances, both LNA base primers, as well as unmodified base primers containing both mutations, could be used (data not shown). The application of two consecutive LNA bases at the 3' termini of primers for amplifying DNA prior to analysis was employed since these primers were completely resistant to the 3'-5' exonuclease activity of the proofreading DNA polymerase (7), thus preventing primer degradation through proofreading and subsequent nonspecific amplification.

Another key aspect of our assay was the use of a high-fidelity DNA polymerase, Herculanase II Fusion, with a high level of proofreading activity. Platinum *Taq* DNA high-fidelity polymerase (Invitrogen), on the other hand, gave an unacceptable level of background and reduced sensitivity. With Herculanase II Fusion, we successfully amplified full-length HBV RT from

serum by using as few as 20 copies of serum HBV DNA. We were unable to amplify HBV RT DNA directly from patient serum extracts using other high-fidelity DNA polymerases such as PFX (Invitrogen) and PFU (Stratagene) DNA polymerases. The rate of successful amplification of HBV DNA from patient samples was also increased by the use of LNA-terminated oligonucleotide primers (data not shown).

Quantification within the real-time PCR assay used the SYBR green fluorescent DNA dye reporter, rather than sequence-specific fluorescent hybridization probes or molecular beacons. The sequence-independent reporter dye provides greater flexibility for detecting resistant variants within highly heterogeneous viral sequences by eliminating the need for a detection probe that hybridizes to an absolutely conserved region. SYBR green also offered the ability to detect resistant variants by using PCR from either the forward or the reverse direction, as sensitivity often differed between the two.

An additional improvement that enhanced the AS-PCR specificity was the use of a smaller amplicon (PCR2) that contained the resistance changes, in order to eliminate sequences that could cause nonspecific mispriming. The sequence overlap between primers for this small amplicon (PCR2) and the AS-PCR (15) was another improvement in

that it ensured that the smaller amplicon of PCR2 would have homologous primer binding sites for the AS-PCR primers. We extended the single nonoverlapping base in these primers, described by Lu et al. (15), to ~3 bases to maintain a high specificity of the AS-PCR.

Our method for quantitation of resistant variants is based on absolute quantitation using WT and resistant variant standard curves. There are similar AS-PCR studies using the threshold cycle ($\Delta\Delta C_T$) method; however, to use the $\Delta\Delta C_T$ method, either the variant and total DNA PCRs must have equivalent amplification efficiencies or the amount of total DNA in all samples and standards needs to be kept constant. Otherwise, the $\Delta\Delta C_T$ value will vary depending on the amount of template DNA within the reaction and could result in errors (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf [page 52]). Furthermore, introducing an artificial mismatch into primers of typical AS-PCR often affects the mutant and WT PCR efficiencies differently. If the objective is to detect the presence of multiple resistance changes in clinical samples, the absolute quantitative method described in this paper would be best to accurately measure all substitutions in different samples.

The AS-PCR analysis described here was used to analyze HBV isolates from before and during entecavir therapy. A fraction of patients had evidence of resistance existing prior to therapy that could be detected through population sequencing (5, 30). Other reports have documented lamivudine resistance in patients without evidence of prior therapy, as reviewed by Tsubota (31). In our studies of 663 nucleoside treatment-naïve patients, only four had detectable lamivudine resistance at baseline by sequencing and five others with emerging lamivudine resistance on therapy actually had very low levels of resistance at baseline detectable by AS-PCR. Thus, 9/663 patients (1.4%) had detectable lamivudine resistance at baseline, far below the levels reported in other studies with smaller numbers of patients (1, 10, 16).

Resistance was found by AS-PCR in the baseline samples of several lamivudine-refractory patients where sequence evidence of ETVr emerged during entecavir therapy. This could be predicted based on population sequencing results indicating ETVr in 10 (5%) of lamivudine-refractory patients at baseline prior to entecavir therapy. The ETVr substitutions that emerged after several years of therapy were least likely to be detected by AS-PCR at baseline, suggesting that they preexisted at levels below AS-PCR detection but that they could still emerge in the setting of prolonged selective pressure from therapy. Overall, when baseline samples from patients with emerging resistance (to lamivudine or entecavir) were tested using the AS-PCR method, 27 patients with apparent emerging resistance were found to have resistance prior to therapy.

In summary, we established an ultrasensitive method for resistance detection in clinical HBV samples. Methods to detect 17 different resistance substitutions, encompassing lamivudine, telbivudine, emtricitabine, clevudine, and entecavir resistance, were developed. The method was validated using clinical samples and used to show that resistance can often be detected at early time points, prior to detection using conventional nucleotide sequencing and even prior to therapy in a fraction of cases. This method can be applied broadly to detect

any low-abundance polymorphism in the populations of other viruses or other DNA templates.

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