JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2006, p. 1094–1097 Vol. 44, No. 3
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.3.1094–1097.2006

Sensitive Line Probe Assay That Simultaneously Detects Mutations Conveying Resistance to Lamivudine and Adefovir

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Received 28 November 2005/Returned for modification 2 December 2005/Accepted 8 December 2005

The INNO-LiPA HBV DR v2 assay is designed to detect hepatitis B virus mutations conveying resistance to lamivudine and adefovir. Our study confirms that this assay can simultaneously detect the presence of lamivudine and adefovir resistance mutations in clinical samples, has a high degree of concordance with sequencing, and can detect mutants earlier.

Hepatitis B virus (HBV) mutations conveying resistance to antivirals are a major problem in the treatment of chronic hepatitis B. Direct sequencing is the most accurate method of characterizing drug resistance mutations, but it is tedious. The development of rapid, simple, and sensitive assays that can detect low levels of emerging antiviral-resistant HBV mutants is important for monitoring the response to treatment and permitting early institution of rescue therapy. The aims of this study were (i) to assess the accuracy of a new version of the line probe assay, INNO-LiPA HBV DR v2 (Innogenetics NV, Ghent, Belgium), by comparing the results of this assay with those of direct sequencing for concordance; and (ii) to determine if the emergence of lamivudine- and adefovir-resistant HBV mutants can be detected earlier with the DR v2 assay than with sequencing.

Fifty-six chronic hepatitis B patients who had breakthrough infections during lamivudine and/or adefovir treatment were studied. The use of human sera was approved by our Institutional Review Boards, and written consent was obtained from all patients. A total of 104 serum samples were analyzed for the presence of antiviral resistance mutations, using both the line probe assay and direct sequencing.

HBV DNA was extracted from serum (1) and amplified in a nested PCR assay using *Taq* PCR master mix (QIAGEN, Inc., Chatsworth, CA) and previously described primers (2). Samples with detectable HBV DNA were purified using a QIAquick PCR purification kit (QIAGEN, Inc., Chatsworth, CA) and sequenced at the DNA sequencing core facility of the University of Michigan Medical Center, using second-round primers.

For the line probe assay, PCR was performed using 10 μl DNA and the biotinylated primers HBPr950 (5′-CGTGGTGGACTT CTCTCAATTTTC-3′; HBV nucleotides [nt] 255 to 278) and HBPr952 (5′-AGAAAGGCCTTGTAAGTTGGCGA-3′; HBV nt 1121 to 1099) with *Taq* PCR master mix. An annealing temperature of 55°C for 50 cycles was used. Samples with undetectable DNA after first-round PCR (24%) were subjected to nested PCR using primers HBPr 977 (5′-TGGCCAAAATTTGCAGTC CC-3′; HBV nt 301 to 320) and SASI (2). All samples with detectable HBV DNA after PCR were subjected to reverse hybridization by the INNO-LiPA HBV DR v2 assay according to the manufacturer's instructions. Briefly, 10 μl of amplified product was denatured and hybridized to specific oligonucleotide probe-coated reaction strips. These probes can differentiate wild-type from mutant sequences at codons 80, 173, 180, and 204 and at codons 181 and 236 of HBV reverse transcriptase, which are known to be associated with lamivudine and adefovir resistance, respectively.

Results were considered concordant if both direct sequencing and the line probe assay showed the wild-type sequence, if both tests showed a mutant sequence, or if one test showed a mutant sequence and the other test showed a mixture of wild-type and mutant sequences. Results were considered discordant if one test showed a mixed or mutant sequence and the other showed the wild-type sequence only.

Of the 624 amino acid positions analyzed, complete concordance between DR v2 and sequencing results was observed for 587 (94%) positions (Tables 1 and 2). Among the 37 discordant cases, the DR v2 assay detected mutants in 15 cases and a mixture of the wild type and mutants in 20 cases, while sequencing revealed the wild-type sequence only (Table 2). For 30 of these 35 samples, the presence of mutants was confirmed in follow-up samples by both assays, with the DR v2 assay detecting mutants earlier than sequencing by a mean of 6.7 months (range, 3 to 42 months). Figure 1 shows an example for one such patient, where the DR v2 assay detected the rtL180M and rtM204V changes at month 9, while sequencing did not detect these changes until month 15. At month 21, the DR v2 assay detected the rtV173L change, whereas sequencing did not detect that change until month 42. The detection of both of these mutations by the DR v2 assay was associated with an increase in HBV DNA levels.

Of the two discordant cases, sequencing of one detected a mutant sequence at position 180, while the DR v2 assay detected the wild-type sequence at that position; the DR v2 assay and sequencing of follow-up samples collected 6 months later confirmed the presence of the mutant sequence. Complete discordance was observed in only one case, where the DR v2

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TABLE 1. Comparison of results obtained by INNO-LiPA HBV DR v2 assay and direct sequencing[a]

Amino acid	INNO-LiPA result	Direct sequencing result at indicated amino acid																							
		L 80	V 80	I 80	L+I 80	80 stop	V 173	L 173	V+L 173	L 180	L+M 180	M 180	A 181	V 181	A+V 181	T 181	A+T 181	M 204	I 204	V 204	M+V 204	N 236	T 236	N+T 236
L80	76	75				**1**																		
V80	13	**1**	12																					
I80	7			7																				
L+I 80	5	**2**		2	1																			
L+V 80	3	**2**	1																					
V173	97						97																	
L173	4						**2**	2																
V+L173	3						**2**		1															
L180	59									58		**1**												
M180	35									**5**	6	24												
L+M180	9									**4**	4	1												
A181	97												97											
V181	2													2										
A+V181	3													1	2									
A+T181	2															1	1							
M204	34																	34						
M+I204	5																	**5**						
I204	40																	**3**	37					
V204	22																	**4**		17	1			
M+V204	3																	**2**		1				
N236	96																					96		
T236	1																						1	
N+T236	7																					**3**		4

[a] Numbers of positions with results obtained by the DR v2 assay (vertical) and by direct sequencing (horizontal) are shown. Numbers of samples across the diagonal represent concordance between the DR v2 assay and sequencing. Discordant results are shown in bold.

assay showed the wild-type sequence at amino acid position 80, whereas sequencing showed a nucleotide change from CTG to ATG, resulting in a stop codon at that position. Since the DR v2 assay does not have any probe to detect a stop codon at position 80, this change went undetected by the line probe assay.

For three patients, the DR v2 assay detected lamivudine and adefovir resistance mutations simultaneously. The presence of mutations to both antiviral agents was confirmed by sequencing for all three patients. Figure 2 shows an example for one such patient. This patient had evidence of the lamivudine resistance mutation rtM204I 22 months after the onset of lamivudine therapy, and adefovir was added with good viral suppression. Seven months later, lamivudine was stopped and the patient continued on adefovir. Thirty-two months after the patient was switched to adefovir, the resistance mutation rtN236T was detected by the DR v2 assay, and it was detected 2 months later by sequencing. Lamivudine was reintroduced, and serial testing revealed a reemergence of lamivudine resistance mutations rtL180M and rtM204V and the replacement of rtN236T by rtA181V 6 months after the reintroduction of lamivudine.

TABLE 2. Concordance between sequencing and INNO-LiPA HBV DR v2 results

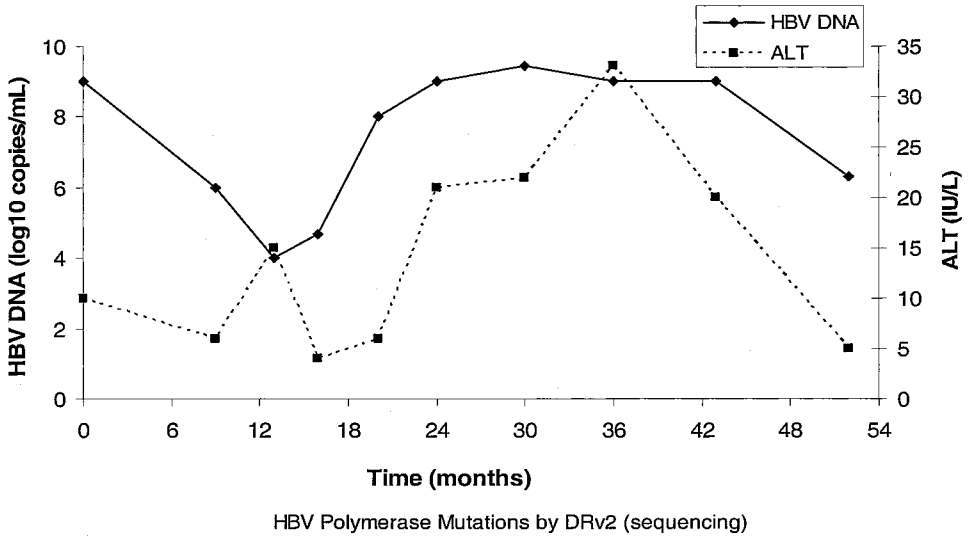
DRv2 assay result (no. of sequences)	No. of sequences by sequencing[a]		
	WT	WT plus mutant	Mutant
WT (425)	423	0	**2**
WT plus mutant (40)	**20**	13	7
Mutant (159)	**15**	7	137

[a] Discordant results are shown in bold.

The concordance between the DR v2 assay and direct sequencing was found to be >99%. As reported previously (3, 5), the line probe assay is more sensitive in detecting mutations than direct sequencing, thus permitting earlier detection of newly selected antiviral resistance and prompt initiation of rescue therapy (4, 6, 7). The DR v2 assay allows simultaneous detection of lamivudine and adefovir resistance mutations. This will be important as more antiviral therapies become available and multidrug resistance mutations become increasingly frequent. Compared to sequencing, the DR v2 assay is technically simple and rapid, enabling it to be applied in clinical diagnostic or research laboratories where a large number of samples need to be tested.

The line probe assay has some limitations. (i) It is only capable of detecting the presence of known mutations. As novel mutations to new antiviral therapies are characterized, new probes have to be designed and the assay has to be constantly upgraded. (ii) Polymorphisms within the region of the probes can prevent annealing and yield indeterminate results. (iii) The assay is unable to characterize samples with novel mutations and deletions in the region of the probes.

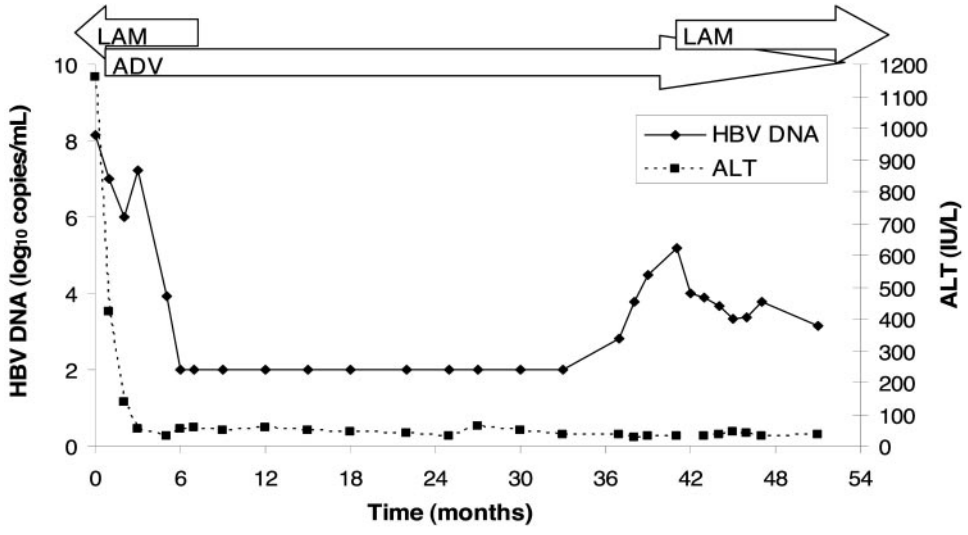
In conclusion, we have demonstrated that the INNO-LiPA HBV DR v2 assay is accurate, can detect lamivudine and adefovir resistance mutations simultaneously, and is more sen-



HBV Polymerase Mutations by DRv2 (sequencing)

rt80	L (L)	L (L)	L (L)	L (L)	L (L)	L (L)	L (L)	L (L)	L (L)
rt173	V (V)	V (V)	V (V)	V (V)	L (V)	L (V)	L (V)	**L (L)**	**L (L)**
rt180	L (L)	M (L)	M (L)	**M (M)**	**M (M)**	**M (M)**	**M (M)**	**M (M)**	**M (M)**
rt181	A (A)	A (A)	A (A)	A (A)	A (A)	A (A)	A (A)	A (A)	A (A)
rt204	M (M)	**V (M)**	**V (M)**	**V (V)**	**V (V)**	**V (V)**	**V (V)**	**V (V)**	**V (V)**
rt236	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)	N (N)

FIG. 1. The DR v2 assay detected lamivudine resistance mutations earlier than direct sequencing. HBV polymerase sequence results, as obtained by the DR v2 assay, are shown below the graph. Results obtained by sequencing are shown in parentheses. Mutations are indicated in bold. At months 9 and 12, mutations at positions rt180 and rt204 were detected by the DR v2 assay but not by sequencing. Similarly, the DR v2 assay detected the mutation rtV173L at months 21, 30, and 36, whereas sequencing indicated the wild-type sequence at this position in the same samples. ALT, alanine aminotransferase.



HBV Polymerase Mutations by DR v2 (sequencing)

rt80	L (L)	L (L)	L (L)	L (L)	L (L)	L (L)
rt173	V (V)	V (V)	V (V)	V (V)	V (V)	V (V)
rt180	L (L)	L (L)	L (L)	L (L)	**M (M)**	**M (M)**
rt181	A (A)	A (A)	A (A)	A (A)	A (A)	**V (V)**
rt204	**I (I)**	**M+I** (M)	M (M)	M (M)	**V (V)**	**V (V)**
rt236	N (N)	N (N)	**N+T** (N)	**T (T)**	N (N)	N (N)

FIG. 2. Simultaneous detection of lamivudine and adefovir resistance mutations. HBV polymerase sequence results, as obtained by the DR v2 assay, are shown below the graph. Results obtained by sequencing are shown in parentheses. Mutations are indicated in bold. The DR v2 assay detected the mutations rtM204I and rtN236T at months 6 and 36, respectively, whereas sequencing indicated the wild-type sequence at these positions. Dual drug resistance mutations rtL180M, rtM204V, and A181V were found at month 48 by both DR v2 assay and sequencing. ALT, alanine aminotransferase.

sitive than direct sequencing for the detection of antiviral resistance mutations, thereby allowing prompt initiation of rescue therapy.

The U.S. HBV Liver Transplant Study is supported by National Institutes of Health grant U01-DK57577 (A.S.L.). The primers, hybridization reagents, and strips were provided by Erwin Sablon (InnoGenetics, Ghent, Belgium). S. Fung was supported by grants from the Canadian Institutes of Health Research, the Canadian Association for the Study of the Liver, and Hepatitis Foundation International. A. S. F. Lok is a paid consultant and has received research support from GlaxoSmithKline, Bristol-Myers Squibb, Gilead, Idenix, Roche, and InnoGenetics.

Laboratory supplies for Carmela Cursaro and Pietro Andreone were provided by RME (Associazione per la Ricerca sulle Malattie Epatiche), Bologna, Italy.

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