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Development and validation of an oligonucleotide ligation assay to detect lamivudine resistance in hepatitis B virus

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Abstract

Treatment of chronic hepatitis B virus (HBV) infection with lamivudine-monootherapy rapidly selects mutant variants in a high proportion of individuals. Monitoring lamivudine resistance by consensus sequencing is costly and insensitive for detection of minority variants. An oligonucleotide ligation assay (OLA) for HBV lamivudine-resistance was developed and compared to consensus sequencing. Both assays detected drug resistance mutations in 35/64 (54.7%) specimens evaluated, and OLA detected minority mutants in an additional six (9.4%). OLA may offer a sensitive and inexpensive alternative to consensus sequencing for detection of HBV drug resistance in resource-limited settings.

Keywords

HBV drug resistance; Oligonucleotide ligation assay; Lamivudine resistance; HBV treatment

An estimated 248 million people worldwide are chronically infected with hepatitis B virus (HBV), with >5% prevalence in sub-Saharan Africa and East Asia (Schweitzer et al., 2015). Chronic or lifelong infection can result in hepatic cirrhosis, end stage liver disease and/or hepatocellular carcinoma. Nucleoside analogs, such as lamivudine (3TC), congener emtricitabine (FTC), telbivudine, or entecavir, or nucleotide analogs such as tenofovir disoproxil fumarate (TDF) are used to inhibit viral replication. This treatment is not curative but diminishes the risk of liver cirrhosis and cancer (Liaw et al., 2012).

Currently, TDF or entecavir is the preferred option for treatment of chronic HBV infection due to their high barrier to resistance and few side effects (WHO, 2015). Long term studies have shown no clinical resistance in patients on TDF for up to 6 years (Kitrinos et al., 2014).

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In human immunodeficiency virus type-1 (**HIV**)/HBV co-infected individuals initiating antiretroviral treatment (**ART**), a simplified regimen of TDF plus 3TC (or FTC) plus efavirenz is recommended, which can suppress both HIV and HBV replication and reduce the risk of liver disease (Manosuthi et al., 2015; WHO, 2015). However, prior to TDF use in low- and middle-income countries, widespread use of 3TC-containing ART exposed many individuals with chronic HBV to 3TC-monotherapy, especially in Asian settings where prevalence of HBV infection is high (Chasombat et al., 2009).

3TC-monotherapy of HBV rapidly selects drug resistant variants with mutations at codons rt80, rt173, rt180, and rt204 (Iacomi et al., 2009; Libbrecht et al., 2007; Thibault et al., 1999); occurring in ~15% of treated individuals after 1 year and up to 80% after 3 years (Benhamou et al., 1999; Hoofnagle et al., 2007). Also, after 3TC-monotherapy, the diagnosis of HBV infection may be obscured by selection of mutants not detected by hepatitis B surface antigen (**HBsAg**) assays (Torresi et al., 2002; Yeh, 2010). Individuals harboring these mutants may spread HBV that is resistant to HBV-vaccine-induced immune responses (Kamili et al., 2009; Locarnini and Yuen, 2010). Importantly, lamivudine resistance mutations also confer cross resistance to telbivudine and entecavir but not TDF (Seifer et al., 2009).

HBV resistance to 3TC can be monitored by consensus sequencing (**CS**) or by reverse hybridization, which are costly and insensitive for detection of minority variants (<20–50%) in the viral population (Lok et al., 2007; Margeridon-Thermet et al., 2009; Xiao et al., 2006). Massive parallel sequencing, a highly sensitive but technically complex and costly method, has demonstrated a high frequency of 3TC resistant HBV mutants, including many below the limit of detection by CS (Margeridon-Thermet et al., 2009).

An oligonucleotide ligation assay (**OLA**) has been used by our group and others to evaluate drug resistance in HIV (Beck et al., 2002; Ellis et al., 2004; Jourdain et al., 2010; Micek et al., 2010; Soria et al., 2012). OLA is more sensitive than CS, with detection of mutant populations at 2% of the quasispecies (Beck et al., 2014, 2002; Ellis et al., 2004). The simplicity of the assay combined with its low cost, and the potential to detect mutant variants that test HBsAg-negative, suggests that the OLA may improve care of a select subset of HIV/HBV co-infected individuals, such as individuals intolerant of TDF or in areas of low TDF coverage, and in children for whom clinicians may be reluctant to give TDF. Here we describe an OLA developed to identify HBV 3TC-resistance, and a comparison of the OLA to CS.

The OLA was evaluated by testing plasma from individuals known to be chronically HBV infected and treated with 3TC for 1–8.5 years who were enrolled in the multicenter HIV Prevention and Treatment cohort ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00433030) NCT00433030) in Thailand (Khamduang et al., 2013, 2012). DNA was extracted from participants' plasma using an automatic sample extraction system (Abbott M2000sp). Ten microliters of DNA were amplified by nested PCR of a region spanning the reverse transcriptase domain (nucleotide positions 251–1058) (Khamduang et al., 2013; Villeneuve et al., 2003). HBV PCR amplicons were “blindly” tested by OLA for 3TC resistance mutations, and subsequently

compared to CS of the same amplicons (BigDye Terminator Mix V.1.1; Applied Biosystems, Foster City, CA).

OLA probes to detect mutations encoding rtL180M and rtM204V/I were designed to accommodate common genetic variations using alignments of HBV sequences from subjects residing in Thailand (Khamduang et al., 2013, 2012) (Table 1). Wild-type and mutant plasmids, generated by cloning subjects' amplicons (TOPO® TA Cloning® Kit for Sequencing, Invitrogen, Carlsbad, CA), were mixed at 2, 5, 10, 25, 50, 75, 90, and 95% mutant, and used to quantify the proportion of mutants in the clinical specimens.

The OLA was performed as previously described (Beck et al., 2014), with each ligation reaction containing 0.67 pmol of the mutant-specific and common probes, and 0.17 pmol of the wild-type probe. Because the two mutations evaluated at codon rt204 occur at different positions within this codon (M204V: ATG → GTG; M204I: ATG → ATY), these were tested in separate assays to prevent competition of overlapping OLA probes. All subjects' amplicons and controls were analyzed in duplicate.

Drug resistance was detected by both OLA and CS in 35 of 64 (54.7%) specimens evaluated (Table 2). While OLA and CS identically categorized individuals as harboring resistant or wild-type HBV, mixtures of wild-type and mutant virus were detected by OLA in 8 of 64 (12.5%) specimens and by CS in only 2 of 64 (3.1%; $p < 0.05$, two-tailed, Chi-square). Mixtures of mutant variants rt204V/I were detected by OLA in a few subjects; although one variant usually dominated the viral population and the other was present at very low levels (Subjects 5, 14 and 43 in Table 2). The minority mutant variant in mixtures of rtM204I/V could not be accurately quantified within a specimen, as the other variant's mutant base interfered with ligation of the OLA probes, leading to low wild-type OD or indeterminate results. All indeterminate results occurred because of these competing mutant genotypes, and all specimens were evaluable at the two codons tested.

The OLA we developed to detect 3TC-resistance mutations in HBV compared favorably with CS to discriminate specimens with versus without HBV resistance. In this cohort, testing for rtL180M and rtM204V/I by OLA detected 3TC-resistance at exactly the same rate as CS that also evaluated rtV173. This suggests that the addition of this mutant to the OLA is unlikely to increase detection of HBV-3TC-resistance. The OLA detected mutations at concentrations below the sensitivity of CS, and while this did not increase the prevalence of HBV-resistance in this study, this added sensitivity could be relevant in other populations, perhaps patients who received a short duration of 3TC-monotherapy of HBV. OLA could be used to detect early resistance to 3TC.

The first step of OLA is to PCR-amplify the HBV DNA, which detects plasma viremia, and thus could detect occult infections if not detected by HBsAg assays due to mutations, including those selected by 3TC (Torresi et al., 2002; Yeh, 2010). After DNA extraction and PCR, the OLA can be completed in 4 h, requiring only a thermocycler and plate reader for simple read out and interpretation of results. In the USA laboratory, the cost of the OLA reagents and supplies for detection of HBV-3TC-resistance were \$14. The low cost and

relative simplicity of the OLA could facilitate drug resistance testing in laboratories without access to sequencing facilities.

Limitations of this study include: (1) the evaluation of a relatively small number of specimens, (2) testing of only individuals residing in Thailand where HBV genotypes C and B are circulating, (3) no evaluation of the OLA sensitivity to detect occult HBV infections, and (4) no quantification of input of HBV DNA templates into the assay to help determine the sensitivity to detect minor variants.

The OLA probes can easily be adapted for A, D or G genotypes and to other drug resistance mutations. This inexpensive OLA could serve as a sensitive alternative to CS to detect lamivudine- as well as entecavir- or telbivudine-associated resistance mutations in HBV, and may have added value by detecting viruses that are negative by HBsAg testing, including infants born to mothers with HBV infection and previous or ongoing 3TC-monotherapy.

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Abbreviations

HBV	hepatitis B virus
3TC	lamivudine
FTC	emtricitabine
TDF	tenofovir disoproxil fumarate
HIV	human immunodeficiency virus type-1
ART	antiretroviral treatment
HBsAg	hepatitis B surface antigen
CS	consensus sequencing
OLA	oligonucleotide ligation assay

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Table 1

Oligonucleotide ligation assay probes for detection of lamivudine/emtricitabine resistance mutations in HBV.

Mutation	Probe	Sequence (5' -> 3')
L180M	Wild-Type	Dig/TGGGCCTCAGTCCGTTTCTC <u>Y</u>
	Mutant	FAM/TGGGCCTCAGTCCGTTTCTC <u>A</u>
	Common	Phos/ TGG CCTCAGTTTACTAGTGCCATTTG/Bio
M204V	Wild-Type	Dig/CCACTGTYTGGCTTTCAGTTAT <u>A</u>
	Mutant	FAM/CCACTGTYTGGCTTTCAGTTAT <u>G</u>
	Common	Phos/ TGG ATGATGTGGTATTGGGGGC/Bio
M204I	Wild-Type	Dig/CCACTGTYTGGCTTTCAGTTAT <u>ATG</u>
	Mutant	FAM/CCACTGTYTGGCTTTCAGTTAT <u>ATY</u>
	Common	Phos/GATGATGTGGTATTGGGGGCCAAGT/Bio'

Dig = digoxigenin; FAM = fluorescein; Phos = phosphate; Bio = biotin.

Underline indicates the wild-type or mutant base of the probe; bold type font indicates the bases comprising the codon of interest.

Table 2

Comparison of HBV lamivudine resistance detected by OLA and consensus sequencing in Thai patients.

Subject Number	Time on 3TC (months)	HBV viral load (IU/mL)	Genotype	Mutations evaluated by OLA			Mutations detected by CS			
				rtL180M	rtM204V	rtM204I	rtL180M	rtM204V	rtM204I	Other
1	0	4,881,704	B	wt	wt	wt	wt	wt	wt	
2	66	29,799,100	C	MUT	MUT	IND	MUT	MUT	wt	
3	70	7,780,072	C	MUT	IND	MUT	MUT	wt	MUT	
4	90	41,843,616	B	43%	IND	MUT	MIX	wt	MUT	
5	0	152,832,300	C	wt	wt	wt	wt	wt	wt	
	90	117,253,455		MUT	4%	MUT	MUT	wt	MUT	
6	60	1,235,082	C	MUT	MUT	wt	MUT	MUT	wt	
7	83	25,668,815	C	MUT	MUT	IND	MUT	MUT	wt	
8	0	12,964,700	B	wt	wt	wt	wt	wt	wt	
9	0	144,489,427	B	wt	wt	wt	wt	wt	wt	
10	38	262	C	wt	wt	wt	wt	wt	wt	
11	0	35,251,946	B	wt	wt	wt	wt	wt	wt	
12	0	31,225,330		wt	wt	wt	wt	wt	wt	
13	0	202,722	C	wt	wt	wt	wt	wt	wt	
14	0	49,649,570	B	wt	wt	wt	wt	wt	wt	
	36	45,049,720		93%	MUT	7%	MUT	MUT	wt	
	53	155,807,976		96%	MUT	5%	MUT	MUT	wt	
	77	172,378,269		97%	MUT	<2%	MUT	MUT	wt	
15	50	36,668	C	MUT	IND	MUT	MUT	wt	MUT	
	66	31,593,805		MUT	IND	MUT	MUT	wt	MUT	
	81	33,724,285		MUT	IND	MUT	MUT	wt	MUT	
16	0	3,206,685	B	wt	wt	wt	wt	wt	wt	
17	0	913,491	C	wt	wt	wt	wt	wt	wt	
18	0	37,322,595	C	wt	wt	wt	wt	wt	wt	
19	0	148	B	wt	wt	wt	wt	Wt	wt	
20	0	113,543,830	C	wt	wt	wt	wt	wt	wt	

Subject Number	Time on 3TC (months)	HBV viral load (IU/mL)	Genotype	Mutations evaluated by OLA			Mutations detected by CS			
				rtL180M	rtM204V	rtM204I	rtL180M	rtM204V	rtM204I	Other
21	36	78,179,730		MUT	IND	MUT	MUT	wt	MUT	rtV173L
	65	112,906,968		MUT	IND	MUT	MUT	wt	MUT	rtV173L
	81	137,808,813		MUT	IND	MUT	MUT	wt	MUT	rtV173L
	87	122,272,900		MUT	IND	MUT	MUT	wt	MUT	rtV173L
22	0	9,553,023	C	wt	wt	wt	wt	wt	wt	
23	0	33,295,747	C	wt	wt	wt	wt	wt	wt	
24	0	125,462,341	C	wt	wt	wt	wt	wt	wt	
25	0	88,077,696	C	wt	wt	wt	wt	wt	wt	
26	0	274,186,700	G	wt	wt	wt	wt	wt	wt	
27	0	3,171,499	C	wt	wt	wt	wt	wt	wt	
28	0	359,454,600	C	wt	IND	wt	wt	wt	wt	
29	103	26,637,070	C	MUT	IND	MUT	MUT	wt	MUT	
30	88	82,809,200	B	MUT	IND	MUT	MUT	wt	MUT	
31	70	230,119,600	C	MUT	MUT	wt	MUT	MUT	wt	rtV173L
32	0	280,650,966	C	wt	wt	wt	wt	wt	wt	
33	60	86,904,055	B	MUT	MUT	wt	MUT	MUT	wt	
34	83	276,867,500	B	MUT	MUT	IND	MUT	MUT	wt	
35	84	56,782,943	C	MUT	MUT	wt	MUT	MUT	wt	
36	23	315,770,970	B	MUT	MUT	wt	MUT	MUT	wt	
37	35	389,773,518		MUT	MUT	wt	MUT	MUT	wt	
38	79	244,424,705	C	MUT	IND	MUT	MUT	wt	MUT	rtV173LV
39	0	195,100	B	wt	wt	wt	wt	wt	wt	
40	0	2,802,580	C	wt	wt	wt	wt	wt	wt	
41	12	187,733,860	C	wt	wt	wt	wt	wt	wt	
40	37	13,820	C	48%	wt	68%	MIX	wt	MIX	
	64	26,685,591		MUT	IND	MUT	MUT	wt	MUT	
	70	2,539		MUT	IND	MUT	MUT	wt	MUT	
	16	88,981,654		MUT	MUT	IND	MUT	MUT	wt	

Subject Number	Time on 3TC (months)	HBV viral load (IU/mL)	Genotype	Mutations evaluated by OLA			Mutations detected by CS			
				rtL180M	rtM204V	rtM204I	rtL180M	rtM204V	rtM204I	Other
42	0	12,000,615	C	wt	wt	wt	wt	wt	wt	
43	45	36,603,930	C	MUT	MUT	5%	MUT	MUT	wt	rtV173L
44	0	403,333,028	C	wt	wt	wt	wt	wt	wt	
45	79	36,194,425	C	91%	IND	MUT	MUT	wt		
46	22	57,653	B	wt	wt	wt	wt	wt	wt	
47	0	16,545	B	wt	wt	wt	wt	wt	wt	
48	24	80,450,850	C	MUT	MUT	wt	MUT	MUT	wt	rtV173L
	35	120,143,260		MUT	MUT	wt	MUT	MUT	wt	rtV173L
	41	41,887,487		MUT	MUT	wt	MUT	MUT	wt	rtV173L
49	87	394,689	C	MUT	MUT	wt	MUT	MUT	wt	

Key of genotype:

MUT: Mutant genotype detected by OLA as a pure population (> 99%) or by consensus sequencing (CS).

MIX: Mixture of mutant and wild-type variants detected (numbers indicate % mutant detected by OLA).

wt: „wild type“, no mutant detected.

IND: Negative reaction in OLA for both mutant and wild-type for this genotype.