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SHORT REPORT

Single-step real-time PCR to quantify hepatitis B virus and distinguish genotype D from non-D genotypes

S. Amini-Bavil-Olyaee,[1,2] M. R. Pourkarim,[3,4] S. Schaefer,[5] F. Mahboudi,[2] M. Van Ranst,[3] A. Adeli,[2] C. Trautwein[1] and F. Tacke[1] [1]*Department of Medicine III, RWTH-University Hospital Aachen, Aachen, Germany;* [2]*Biotechnology Department, Pasteur Institute of Iran, Tehran, Iran;* [3]*Laboratory of Clinical Virology, Rega Institute for Medical Research, Leuven, Belgium;* [4]*Research Centre, Iranian Blood Transfusion Organization (IBTO), Tehran, Iran; and* [5]*Institut für Medizinische Mikrobiologie, Virologie und Hygiene, Universität Rostock, Schillingallee, Rostock, Germany*

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SUMMARY. Hepatitis B virus (HBV) viral load and its genotype play important roles in clinical outcome, management of disease and response to antiviral therapy. In many parts of the world such as Europe or the Middle East, distinguishing HBV genotype D from non-D is most relevant for treatment decisions, because genotype D-infected patients respond poorly to interferon-based therapeutic regimens. Here, we developed an in-house real-time PCR to concordantly assess HBV genotype (D *vs* non-D) based on melt curve analysis and quantify the viral load. Genotype distinction was established with control plasmids of all HBV genotypes and validated with 57 clinical samples from patients infected with six different HBV genotypes. Our in-house real-time PCR assay could discriminate HBV genotype D from non-D using single-step melt curve analysis with a 2 °C difference in the melt curve temperature in all samples tested. Viral load quantification was calibrated with the WHO HBV international standard, demonstrating an excellent correlation with a commercial kit ($r = 0.852$; $P < 0.0001$) in a linear range from 3.2×10^2 to 3.2×10^{10} IU/mL. In conclusion, we developed a rapid, simple and cost-effective method to simultaneously quantify and distinguish HBV genotypes D from non-D with a single-step PCR run and melt curve analysis. This assay should be a useful diagnostic alternative to aid clinical decisions about initiation and choice of antiviral therapy, especially in geographical regions with a high prevalence of HBV genotype D.

Keywords: copy number, genotype, hepatitis B virus, interferon, pegylated interferon, quantitative real-time PCR.

INTRODUCTION

The importance of hepatitis B virus (HBV) genotypes for disease progression and response to antiviral treatment has been well established during recent years [1]. Of particular clinical interest is the fact that the response to interferon-based treatments is much greater in patients infected with HBV genotype A and particularly low in patients infected with genotype D [2,3]. This observation has been recently confirmed in large trials testing pegylated interferon in HBeAg-positive [4–6] and HBeAg-negative patients [7]. HBV has been classified into eight genotypes (A-H) with a distinct geographical distribution and clinical outcome [1]. For instance, in Western, Central and Eastern Europe, HBV genotypes A and D are most prevalent, and each accounts for about 40–45% of cases [8,9], whereas in the entire Middle East genotype D is dominant with about 80–90% of the cases [10,11]. In such regions with a high prevalence of genotype D, i.e. Europe or Middle East [8,11], the differentiation between genotype D- and non-D-infected patients is highly desirable before deciding whether interferon- or nucleos(t)ide-based therapies should be started. However, at present, validated genotyping assays mostly involve direct sequencing of distinct regions of the HBV genome and post-hoc sequence alignment [12], which is a technically demanding and expensive methodology. In this study, we introduce a real-time PCR assay that is able to concomitantly differentiate genotype D from non-D and quantify the viral load, an important measure for treatment initiation as well as monitoring during therapy [13]. Our assay is carried out by a simple single-run real-time PCR and a single-step melt curve analysis. Therefore, this novel assay is a rapid, reliable, cost effective and technically straightforward

Abbreviation: HBV, hepatitis B virus.

Correspondence: Frank Tacke, MD, PhD, Department of Medicine III, RWTH-University Hospital Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany.
E-mail: frank.tacke@gmx.net

approach to distinguish HBV genotype D from non-D and quantify HBV viral load.

MATERIALS AND METHODS

Clinical specimens, positive, negative and assay controls

Plasmids harbouring the full-length HBV genome of the defined genotypes A1, B2, C2, D1, E, F3 and G were analysed [14–17]. Moreover, 57 HBV-positive sera from patients infected with different HBV genotypes (A, B, C, D, E and G) were tested blindly [18,19]. For the validation of viral load quantification, 11 HBV-positive samples (7 HBeAg-negative and 4 HBeAg-positive) with defined viral loads ranging from 5×10^3 to 1×10^9 copies/mL were used. Eight healthy individuals with no HBV infection markers served as negative controls. The HBV DNA working standard for nucleic acid amplification techniques, NIBSC code: 05/148 (NIBSC, Hertfordshire, UK) was employed in each PCR run to control the performance of the assay.

DNA extraction, sequencing and genotyping

HBV DNA was extracted from 200 μL of serum, WHO working standard and WHO international standard using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and eluted in 100 μL elution buffer. The final concentration of WHO working reagent was adjusted to 1000 IU/mL. HBV full-length genomes from 57 HBV-positive patients were amplified, fully sequenced and phylogenetically analysed to determine HBV genotypes as previously described [18,19].

Primer design

To locate conserved sequence regions for primer design, 150 HBV full-length genomes from genotype A through H were retrieved from GenBank and multiple-aligned using ClustalX software [20]. Among many primers designed and tested (data not shown), the primers F65: 5′-tgggtcaccatattcttggga-3′ (positions 2815–2835, genotype D) and R65: 5′-atgatc-gggaaagaatcccag-3′ (2880–2900) showed best results in terms of both differentiation of genotypes and quantification of viral load. These primers amplify an 86-bp (genotype D) or 119-bp (genotype non-D) fragment of the preS1 gene from all HBV genotypes.

HBV DNA quantification

The plasmid containing a full-length genome genotype A, subtype *adw2,* was used to generate the standard curve [21], after calibration against the WHO international standard [22]. As a reference method, HBV DNA was extracted by the QIAamp virus BioRobot 9604 kit (Qiagen), and viral load was measured using the Abbott RealTime HBV kit (Abbott Laboratories, Des Plaines, IL).

Real-time PCR and melt curve analysis

In each run, HBV plasmid controls with defined genotypes plus negative controls and WHO working standards were included. Tenfold serial dilutions of calibrated linear HBV DNA were used for quantification. Real-time PCR mixtures consisted of 1 × SYBR GreenER qPCR SuperMix containing hot-start Taq DNA polymerase, UDG/dUTP to prevent contamination carryover (Invitrogen, Carlsbad, CA, USA), 0.5 μM of each primers, 2 μL HBV DNA and appropriate volumes of RNase/DNase-free water in a total volume of 50 μL. After 2 min at 50 °C and 10 min at 95 °C, 40 cycle amplifications were performed (95 °C 15 s, 60 °C 1 min). Real-time PCR was performed on an ABI 7300 apparatus, and results were analysed by 7300 SDS Sequence Software (Applied Biosystems, Foster city, CA, USA). HBV real-time quantitative PCR

Table 1 Hepatitis B virus (HBV) genotype distinction by melting curve temperatures. Intra- and inter-assay variabilities of melt curve temperature (°C) were assessed using control plasmids with different defined HBV genotypes. For intra-assay variability, plasmid samples were measured 4 times within the same analysis in triplicate. For inter-assay variability assessment, 11 independent assays were performed at different time points, and individual measurements per assay were taken in triplicates. Last columns: Analysis of melting curve temperatures of 57 clinical samples from patients chronically infected by HBV from distinct genotypes. All clinical samples were measured in duplicates in three independent assays

HBV genotype (plasmid)	Intra-assay variability (°C)		Inter-assay variability (°C)		HBV genotype (patients)	Clinical samples (°C)	
	n	Mean ± SD	*n*	Mean ± SD		*n*	Mean ± SD
A	4	79.40 ± 0.17	11	79.37 ± 0.24	A	15	79.18 ± 0.35
B	4	79.00 ± 0.00	11	78.98 ± 0.22	B	7	79.53 ± 0.55
C	4	80.30 ± 0.00	11	80.20 ± 0.22	C	6	79.22 ± 0.28
D	4	77.60 ± 0.17	11	77.51 ± 0.38	D	21	77.25 ± 0.27
E	4	78.90 ± 0.17	11	78.87 ± 0.19	E	7	79.01 ± 0.16
F	4	80.20 ± 0.17	11	80.24 ± 0.22	F	–	
G	4	79.30 ± 0.00	11	79.58 ± 0.21	G	1	79.6

results were correlated with commercial test results using Pearson's test (GraphPad Prism, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Distinguishing genotype D from non-D by melt curve analysis in a single-step real-time PCR assay

Given the low response of patients chronically infected by HBV genotype D to interferon-based therapeutic regimens, the differentiation of genotype D from non-D is a clinically important factor in HBV-infected patients [5,23]. In this work, we developed a simple, rapid and cost-effective method to distinguish HBV genotype D from non-D based on a one-step real-time PCR followed by single melt curve analysis. Primers were constructed to bind outside the natural 33-bp deletion of the preS1 region of HBV genotype D. This generated amplicons that differ in size between genotype D and non-D, thereby resulting in different melting curve temperatures between the genotypes. The flanking of this region by primers was previously applied only by nonquantitative conventional PCR methods [10,24]. After testing different oligonucleotides (not shown), an optimal primer pair was identified that reliably resulted in a mean difference of melt curve temperatures for genotype D in comparison with non-D of 2.03 ± 0.19 °C (Table 1). This temperature difference was sufficient to differentiate the HBV genotype D from all other genotypes using plasmid constructs of defined genotypes (Fig. 1a).

The assay was then tested in a blinded fashion on 57 clinical samples with six different genotypes. After melt curve analysis, 21 cases were predicted to be genotype D based on a mean temperature of 77.25 ± 0.27 °C (range: 76.7–77.8 °C, Table 1). After decoding the samples, results of the melt curve analysis were in complete (100%) agreement with the results of the phylogenetic tree analysis based on full-length genome sequencing [18,19]. The temperature difference between genotype D and non-D among clinical samples was 1.98 ± 0.12 °C, thereby allowing reliable and unambiguous distinction between genotypes D and non-D (Fig. 1b).

Simultaneous quantification of HBV viral load

Quantification of viral load is important not only for determining the individual risk of the patient for disease progression and the decision for initiating antiviral treatment, but also during antiviral therapy to assess proper treatment responses [13]. Commercial assays are relatively expensive and not available in many developing countries. We therefore optimized our real-time PCR assay to also quantify HBV copy numbers in patient samples.

Tenfold serial dilutions of the linear full-length genome (genotype A) were employed to generate proper standard curves in each run. This standard was calibrated against 10-fold serial dilutions of the WHO HBV international standard with concentrations ranging from 2×10^2 to 2×10^6 IU/mL. The regression analysis from multiple real-time PCR assays with serial dilutions of the WHO standard yielded a correlation coefficient of 0.998 and y-intercept value of 43.41. The slope of −3.46 resulted in a PCR efficiency of 94.5% (Fig. 1c). This permitted the accurate calibration of the values for individual samples by comparison with the 'in-house standard' (linearized plasmid) to the WHO standard.

To determine the range of linearity, 10-fold serial dilutions of the calibrated linear HBV full-length genome were evaluated. A linear relation was observed over the range of 3.2×10^2 to 3.2×10^{10} IU/mL (Fig. 1d). The regression analysis of multiple independent experiments yielded a correlation coefficient of 0.998 and y-intercept value of 42.61. The slope of −3.34 indicated a PCR efficiency of 99.2% which is relatively close to the theoretical maximal amplification efficiency (Fig. 1d). Similar accuracies were achieved when plasmids with a genotype D background were serially diluted (data not shown). The specificity of the assay was 100%, as samples from eight volunteers without HBV markers were included in each run, with no amplification signal (data not shown). In each run of the PCR assay, a WHO standard with a defined concentration of 1000 IU/mL was used to control the performance of the assay. The mean quantification of this defined WHO standard sample in 12 independent assays was 1045.1 ± 188.6 IU/mL (range 785–1268 IU/mL), indicating a reliable calibration of the 'in-house standard' and accurate performance of the assay.

We next tested the accuracy of viral load quantification in a cohort of 11 chronically HBV-infected patients, in which the HBV load had been quantified using the commercial Abbott RealTime HBV kit and ranging from 5×10^3 to 1×10^9 copies/mL. From frozen serum samples of these patients, stored at −80 °C, viral DNA was freshly extracted and subjected to the in-house real-time PCR assay. The overall correlation of both assays was $r = 0.852$ ($P < 0.0001$), regardless of the HBV genotype ($n = 6$ HBV genotype D and $n = 5$ HBV genotype non-D) (Fig. 1e).

Using melt curve analysis as a method to genotype and quantify HBV was also reported by other investigators [25–27]. The first of these real-time PCR approaches was developed to distinguish genotype B and C, which are most prevalent in Asia [25]. Similar to our assay, this method uses a one-reaction real-time PCR and single-step melt curve analysis with only one pair of primers [25]. This underscores that such in-house assays can be specifically adapted to the local genotype prevalence, with assays distinguishing genotypes B and C in Asia and D from non-D in Europe/Middle East. A similar detection system for HBV genotypes B and C was introduced by Yeh *et al.* [26]; however, this group applied several primers and probes to distinguish genotype B and C, which makes their system relatively expensive and technically more complex. In 2006, Liu *et al.* [27]

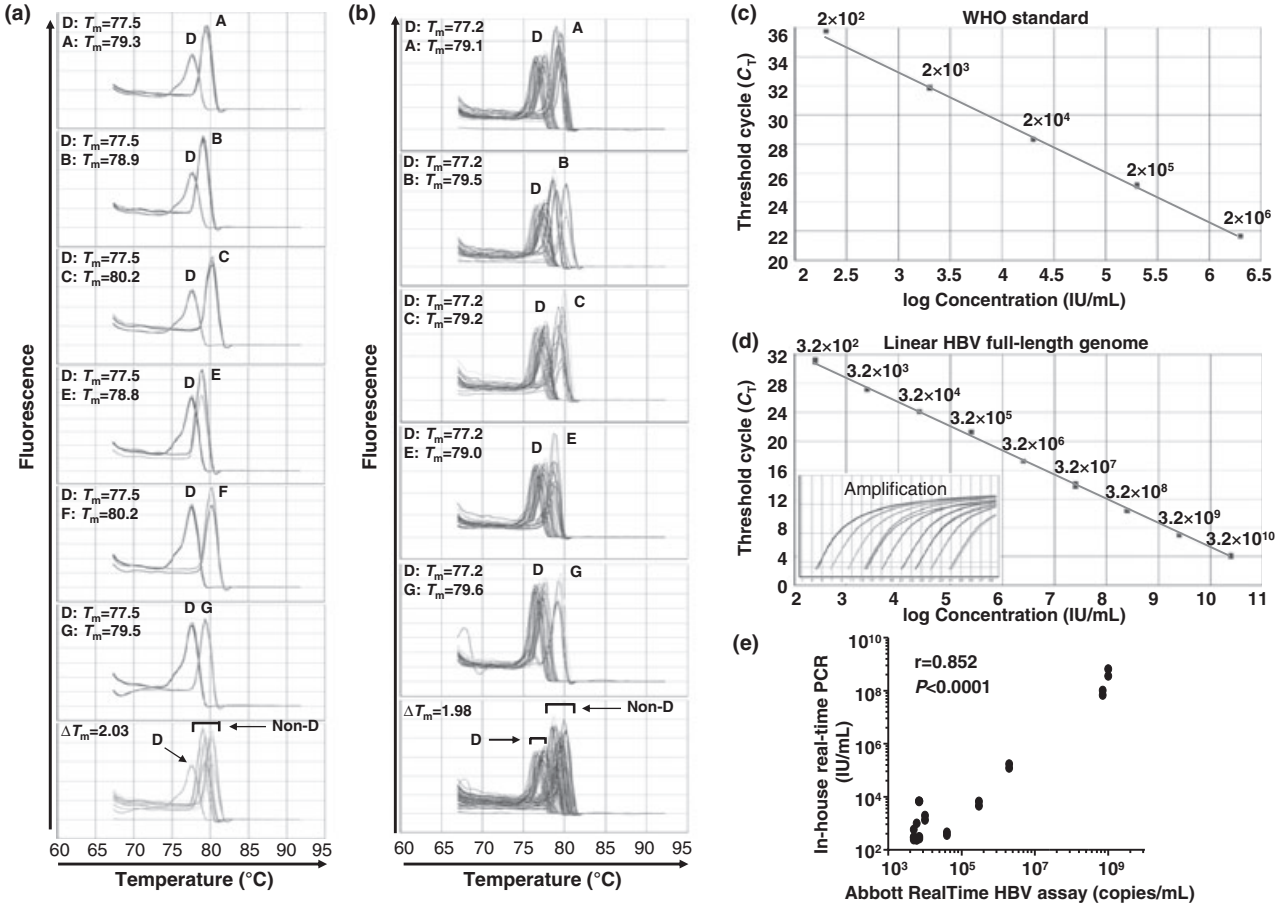

Fig. 1 Single-step real-time PCR assay for distinguishing hepatitis B virus (HBV) genotype D and non-D and quantification of viral load. (a) Representative melt curve analyses of our real-time PCR assay using plasmid constructs of genotype D and other genotypes, as indicated. Duplicate measurements are shown. The lowest plot displays the simultaneous measurement of all plasmids in one assay. The mean melting curve temperatures (T_m, in °C) are given in the plots. (b) Melting curve analyses of all 57 clinical samples that were tested by this assay are displayed. The lowest plot displays the simultaneous analysis of all samples in one assay. The mean melting curve temperatures (T_m, in °C) are given in the plots. (c) Tenfold serial dilutions of WHO HBV international standard. The standard curve is representative for three assays with WHO standards being run in triplicate. (d) Tenfold serial dilutions of linearized full-length HBV plasmid (genotype A) had been calibrated against the WHO standard and were then utilized to generate the real-time PCR standard curves, revealing linearity in a range from 3.2×10^2 to 3.2×10^{10} IU/mL. The standard curve is representative for >12 assays. Insert: Amplification curves (duplicates) of a representative assay. (e) HBV viral load was quantified from serum of 11 chronically HBV-infected patients by the commercial Abbott RealTime HBV kit. Thereafter, viral DNA was again extracted from the serum samples and quantified by the in-house real-time PCR in two independent assays each. Measurements from the commercial and the in-house assay revealed closely correlated viral load assessments.

introduced a real-time PCR-based method that could distinguish between all HBV genotypes. This method requires two sets of primers and probes and a two-step melt curve analysis to distinguish and to quantify all HBV genotypes concordantly [27]. In comparison with our assay, this method has the advantage of a clear distinction between all HBV genotypes, but the disadvantage of higher costs and considerably more complex handling with respect to several steps of PCR and post-PCR data analysis.

In conclusion, this study provides a rapid, simple and cost-effective method to simultaneously quantify and distinguish HBV genotypes D from non-D. This method may serve as a useful diagnostic alternative to aid clinical decisions about initiation and choice of antiviral therapy, especially in geographical regions with a high prevalence of HBV genotype D.

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