Rapid quantification of hepatitis B virus DNA by real-time PCR using fluorescent hybridization probes

Stephen K. N. Ho,¹ Wing-Cheong Yam,² Eric T. K. Leung,² Lei-Po Wong,² Jack K. H. Leung,¹ Kar-Neng Lai¹ and Tak-Mao Chan¹

Departments of Medicine¹ and Microbiology², Queen Mary Hospital, The University of Hong Kong, Hong Kong, China

Correspondence
Tak-Mao Chan
dtmchan@hku.hk

A highly sensitive and rapid assay has been developed to quantify hepatitis B virus (HBV) DNA, based on the fluorescence resonance energy transfer principle and real-time PCR, using the LightCycler and a pair of specific fluorescent hybridization probes. This LightCycler real-time PCR assay (LC-PCR) detected HBV DNA in a linear range from 10^1 to 10^8 copies per reaction ($250-2.5 \times 10^9$ copies ml^{-1}), with a rapid PCR cycling time of 35 min. The assay was validated with two EUROHEP HBV DNA standards (*ad* and *ay* subtypes) and exhibited low intra-assay ($< 6\%$) and inter-assay ($< 16\%$) variation for both subtypes over the complete range of 7 orders of magnitude. The assay was evaluated clinically using serum samples from 120 HBsAg⁺ individuals and 45 healthy controls who were negative for both HBsAg and anti-HBc. Levels of HBV DNA were measured in these samples using both the LC-PCR and Digene Hybrid Capture II HBV DNA (HCII) assays. The prevalence rates for HBV DNA in the HBsAg⁺ serum samples were respectively 95% (114/120) and 56% (67/120) by LC-PCR and HCII ($P < 0.01$). All 67 HCII-positive samples tested positive with LC-PCR, while the 47 discordant samples showed low levels of HBV DNA (down to 265 copies ml^{-1}), detectable only by the more sensitive LC-PCR assay. Levels of HBV DNA as measured by the two assays showed good correlation ($r = 0.902$; $P < 0.001$). The level of HBV DNA was significantly higher in HBeAg⁺ than anti-HBe⁺ samples (median 1.5×10^7 vs 4.6×10^4 copies ml^{-1} ; $P < 0.01$). It is concluded that this LC-PCR assay is clinically useful for the rapid, sensitive and accurate measurement of HBV DNA.

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INTRODUCTION

Hepatitis B virus (HBV) remains an important cause of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma in endemic areas. Although the incidence of new infections has decreased after the introduction of vaccination programmes, HBV infection remains an important global health problem, with the number of chronic HBV carriers exceeding 350 million worldwide (Lee, 1997).

There is increasing evidence that measuring the level of HBV DNA in serum is useful in monitoring the efficacy of antiviral therapy, detecting the occurrence of drug-resistant mutants and detecting relapse after discontinuing antiviral therapy (Chan *et al.*, 1998, 2002; Lai *et al.*, 1998; Omata, 1998). Assays to quantify HBV DNA that are currently available commercially are based on either direct nucleic acid hybridization (Hendricks *et al.*, 1995; Ho & Chan, 2000; Lai *et al.*, 1999) or competitive PCR (Kessler *et al.*, 1998; Noborg *et al.*, 1999). Amongst the former, we and others have reported the

superiority of the Hybrid Capture II HBV DNA test (HCII) (Digene Corp.) over other non-PCR-based assays in terms of accuracy and sensitivity (Ho *et al.*, 1999; Ho & Chan, 2000; Niesters *et al.*, 2000). To date, although PCR-based assays have increased sensitivity, the technical complexity, susceptibility to contamination and more variable reproducibility of results with these assays have hindered their application in clinical practice (Kaneko *et al.*, 1990; Quint *et al.*, 1995).

Recent advances in PCR instrumentation and the capacity for simultaneous fluorimetric detection of PCR amplification products have enabled real-time kinetic analysis of the latter (Higuchi *et al.*, 1992; Holland *et al.*, 1991). Since amplification, measurement and quantification of PCR product occur simultaneously in the same closed reaction vessel, the need for post-PCR manipulations is obviated and the risk of PCR product carry-over contamination is minimized. Real-time PCR technology has been used to measure HBV DNA in serum using the TaqMan probe in the ABI Prism 7700 sequence detector (Perkin Elmer Applied Biosystems) (Abe *et al.*, 1999; Loeb *et al.*, 2000; Pas *et al.*, 2000; Weinberger *et al.*, 2000). In the present study, we have developed a rapid, specific and highly sensitive real-time PCR

Abbreviations: CV, coefficient of variation; FRET, fluorescence resonance energy transfer; HBV, hepatitis B virus; HCII, Digene Hybrid Capture II; LC-PCR, LightCycler PCR.

assay for HBV DNA based on the fluorescence resonance energy transfer (FRET) principle, with a pair of fluorescent hybridization probes and the LightCycler system (LC-PCR) (Roche Diagnostics). The accuracy, intra-assay and inter-assay variability and the detection range for this novel assay were examined, together with validation using EUROHEP HBV DNA standards. Also, the clinical performance of the LC-PCR was compared with that of the commonly used HCII test.

METHODS

Blood samples and standards. Serum samples from 120 HBsAg⁺ patients and 45 HBsAg⁻ and anti-HBc⁻ healthy blood donors were assayed for HBV DNA by both the LC-PCR and HCII assays. The HBsAg⁺ patients were randomly included from chronic HBV carriers attending follow-up for serial monitoring of liver status. Blood samples were centrifuged within 4 h to obtain the serum fractions, which were then divided into aliquots and kept at -80 °C before testing. Serological tests for HBsAg were performed with a commercial microparticle enzyme immunoassay (AxSym; Abbott Laboratories). Two EUROHEP standards were included (kindly provided by Dr W. H. Gerlich, University of Giessen, Germany) with HBV DNA concentrations of 2.7×10^9 and 2.6×10^9 copies ml⁻¹ of serotypes *ad* and *ay*, respectively (Heermann *et al.*, 1999).

HCII assay. The HCII ('standard') assay (Digene Corp.) quantified HBV DNA by solution hybridization, immunocapture and chemiluminescent signal detection. The assay protocol followed the manufacturer's instructions. Briefly, 30 µl denaturing reagent was added to each microplate well containing 30 µl of test samples or HBV DNA standards (0–6000 pg ml⁻¹). The plate was incubated at 65 °C for 30 min to allow lysis of HBV and DNA denaturation. RNA–DNA hybridization was achieved by adding 30 µl RNA probe (specific for HBV *ad* and *ay* strains) to each well and incubating at 65 °C for 60 min. An aliquot (75 µl) of the hybrid-containing solution was then transferred into RNA–DNA capture wells and shaken (Thermolyne Maxi-Mix III) at 1100 r.p.m. at room temperature for 60 min. The solution in the wells was then removed by aspiration. An aliquot (75 µl) of alkaline phosphatase-conjugated antibodies to RNA–DNA hybrids was added to each well and incubated at room temperature for 30 min. After six washings, 75 µl chemiluminescent substrate was added and light emission after 15 min was measured with a chemiluminometer (DML 2000 Luminometer; Digene Corp.). Results were expressed in pg ml⁻¹ according to the plot of standards. The sensitivity according to the manufacturer was 0.5 pg or 1.42×10^5 copies ml⁻¹. We have previously reported an evaluation of this assay (Ho *et al.*, 1999).

LC-PCR assay. Nucleic acid was extracted from 200 µl serum and Digene Calibrator 5 using the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's protocol. DNA was eluted from the QIAamp spin column with 50 µl distilled water, which served as the template for the LC-PCR assay. This DNA preparation had a concentration factor of 4 and a yield of $83 \pm 3\%$ compared with the original amount of DNA in the sample. Calibrator 5 from the HCII assay, which contained 1.7×10^9 copies ml⁻¹ (6000 pg ml⁻¹) HBV plasmid DNA, was used to prepare the calibration curve for the LC-PCR assay, with a range from 10^1 to 10^8 HBV DNA copies per reaction. The Qiagen DNA extraction step was performed on this Calibrator 5 standard as on the other samples to ensure parity of nucleic acid yield. In addition, this step removed the sodium azide used as a preservative in the Calibrator 5 standard, which might affect the PCR.

PCR was performed using a Roche Diagnostics LightCycler and the fluorescent signal of the PCR products after each cycle was monitored

continuously by the FRET principle with a pair of fluorescent hybridization probes (Espy *et al.*, 2000; Ghosh *et al.*, 1994). We used a set of primers for the HBV core region (BcP1, 5'-ACCACC AAATGCCCCCTAT-3'; BcP2, 5'-TTCTGCGACGCGCGA-3'), according to published nested PCR protocols (Chung *et al.*, 1993; Ho *et al.*, 1999), which yielded a 130 bp product. The donor fluorescein probe (HBVcD, 5'-GAGTTCTTCTTCTAGGGGACCTGC-fluorescein-3') and the acceptor LightCycler-Red 640 (LCRed) probe (HBVcA, 5'-LCRed-TCGTCGTCTAACAACAGTAGTTCCG-phosphate-3') directed to the 130 bp product were designed according to the manufacturer's guidelines (Roche). Aliquots of 10 µl of the extracted nucleic acid template and 10 µl of a reaction mixture containing 2 µl $10 \times$ LightCycler FastStart DNA Master hybridization probes (Roche), 1 µM BcP1, 1 µM BcP2, 0.3 µM HBVcD donor probe, 0.3 µM HBVcA acceptor probe and 5 mM MgCl₂ were added to each capillary. All capillaries were sealed and then centrifuged briefly before amplification in the LightCycler. The PCR protocol consisted of an initial step at 95 °C for 10 min for activation of the FastStart *Taq* DNA polymerase, followed by 45 cycles of 0 s (hold time on reaching temperature) denaturation at 95 °C, 12 s annealing at 60 °C and 12 s extension at 72 °C. The temperature transition rate was 20 °C s⁻¹ for all steps. Fluorescence data were acquired once each cycle at the end of the annealing phase with detection channel setting at F2/F1.

For data analysis, baseline adjustment was carried out in the 'proportional' mode and fluorescence curve analyses were carried out in the 'fit points' mode of the LightCycler software. Positive results obtained in the LC-PCR assay using fluorescent hybridization probes were characterized by a sigmoid curve, showing an initial, rapid, exponential increase in fluorescence signal followed by a plateau. Negative reactions did not show any increase in fluorescence signal. A standard curve was created automatically with the LightCycler software in each run by plotting the threshold cycle number against the copy numbers of each standard and quantification of HBV DNA for unknown samples was inferred from the regression line.

Statistics. Determination of the inter- and intra-assay coefficients of variation (CV) of the LC-PCR in testing the EUROHEP standards at different dilutions and the *xy* scatter plot, Spearman's correlation (*r*) and logarithmic transformation of the readings for comparison of the HCII and LC-PCR assays were done by using the SPSS software (version 8.0, SPSS Inc.). Comparisons between HBsAg⁺ and anti-HBsAg⁺ subjects were by χ^2 and Mann–Whitney tests.

RESULTS

The time required to perform the LC-PCR assay was less than 2.5 h, including extraction of DNA from serum samples (completed in 1.5 h) and real-time PCR cycling and data analysis on the LightCycler (40 min). To determine the linear range and sensitivity of the LC-PCR assay, serial 10-fold dilutions of HBV DNA samples prepared with the Digene Calibrator 5 were tested in triplicate (10 copies per reaction) or duplicate (10^2 – 10^8 copies per reaction). All samples with concentrations ranging from 10^1 to 10^8 copies per reaction tested positive with LC-PCR, with typical sigmoid fluorescence curves (Fig. 1). The detection limit was 10 copies per reaction (250 copies ml⁻¹), while no amplification product was obtained with the negative control. There was a linear correlation between the cycle number (*y*) and the input concentration (copies per reaction) of HBV DNA template (*x*) [*r* = 1.0; *y* = $-3.533 \log_{10}(x) + 42.73$]. This standard curve over the 8 log₁₀ dynamic range was used in the quantification of HBV DNA in the test samples.

Fig. 1. Quantification of HBV DNA with fluorescent hybridization probes on the LightCycler. Serial 10-fold dilutions from 10^1 to 10^8 copies of plasmid HBV DNA per reaction in duplicate or triplicate were analysed with the LightCycler. Typical amplification curves are shown, which illustrate the relationship between fluorescence as measured in channel F2/F1 and the number of cycles. No amplification was noted in the 0 copies per reaction (negative control) capillaries.

Fig. 2. Testing of EUROHEP standards with the LC-PCR assay. Serial 10-fold dilutions were made from EUROHEP standards of subtype *ad* (◆) and *ay* (□). Each sample was analysed in triplicate in three runs. The expected (input) HBV DNA concentration was plotted against the mean concentration of triplicate runs as measured by LC-PCR. The dashed line represents the theoretical curve.

To determine the accuracy and CV of the LC-PCR assay at different HBV DNA concentrations, EUROHEP standards from a single extraction were tested in three separate runs, both undiluted and in sequential 10-fold dilutions. An almost linear results curve against the theoretical values was obtained over the complete range of 8 orders of magnitude for both *ad* and *ay* serotypes, with intra-assay CV of 6 % and inter-assay CV of 16 % (Fig. 2). All samples with *ad* subtype concentration ≥ 270 copies ml^{-1} or *ay* subtype concentration ≥ 260 copies ml^{-1} tested positive, confirming the sensitivity limit of 250 copies ml^{-1} indicated by the standard curve (Fig. 2).

To examine the clinical performance of the LC-PCR assay, HBV DNA concentrations were measured in serum samples from 120 HBsAg⁺ patients and 45 HBsAg⁻ healthy controls and the results were compared with those determined using the HCII assay. The seroprevalence for HBV DNA among the HBsAg⁺ patients was 114/120 (95 %) by LC-PCR and 67/120 (56 %) by HCII ($P < 0.01$) (Table 1). All 67 HCII-positive samples yielded positive results with the LC-PCR assay, and the log-transformed HBV DNA concentrations obtained with the two assays demonstrated a good correlation ($r = 0.902$; $P < 0.001$). The 47 discordant samples from HBsAg⁺ patients that tested negative by HCII (i.e. $< 1.42 \times 10^5$ copies ml^{-1}) but positive by LC-PCR contained HBV DNA at low concentrations, down to 265 copies ml^{-1} (Fig. 3). All 45 samples from healthy controls tested negative by both assays, thereby yielding specificity rates of 100 %.

Of the 120 HBsAg⁺ patients, 54 (45 %) were positive for HBeAg and 66 (55 %) were positive for anti-HBe, and the

Fig. 3. Log-log plot of HBV DNA concentrations in 114 samples from HBsAg⁺ patients as measured by LC-PCR or HCII assays. Good correlation was observed between results obtained with the two assays for the 67 concordant positive samples. Forty-seven samples tested negative by HCII but had detectable HBV DNA by LC-PCR.

prevalence of HBV DNA by LC-PCR was similar in the two groups (100 and 95 %, respectively; not significant). In contrast, the HCII assay failed to detect HBV DNA in a considerable number of anti-HBe⁺ patients (89 vs 29 %; $P < 0.001$) (Table 1). The HBV DNA concentration, as determined by LC-PCR, was significantly higher in HBeAg⁺

Table 1. Detection of HBV DNA by LC-PCR and HCII assays and the relationship to HBeAg/anti-HBe status

Serum samples from 120 HBsAg⁺ subjects were tested. Results are numbers (percentages) testing positive for HBV DNA.

Assay	All (n = 120)	HBeAg ⁺ (n = 54)	Anti-HBe ⁺ (n = 66)
LC-PCR	114 (95) ^{a*}	54 (100)	60 (95)
HCII	67 (56) ^a	48 (89) ^b	19 (29) ^b

*Values with the same superscript are significantly different at $P < 0.01$ (a) or $P < 0.001$ (b).

patients compared with those positive for anti-HBe (median 1.5×10^7 vs 4.6×10^4 copies ml⁻¹, range 1100 – 6.6×10^9 and < 250 – 1.8×10^9 ; $P < 0.05$) (Fig. 4).

DISCUSSION

Recent advances in PCR technology allow continuous measurement of the fluorescence emitted during amplicon production at each PCR cycle. The fluorescent reporters include the SYBR Green dye for sequence-non-specific detection of double-stranded DNA or TaqMan probe, hybridization probes and molecular beacons for target-sequence-specific detection. Although inexpensive and easy to use, the SYBR Green dye may produce false-positive signals after 30 cycles of real-time PCR and is therefore not applicable to the measurement of serum HBV DNA at $< 10^5$ copies ml⁻¹, except with the addition of a second-round nested reaction (Brechtbuehl *et al.*, 2001), which would be time-consuming and might increase the risk of PCR product carry-over contamination.

Fig. 4. HBV DNA concentration in serum samples from 120 HBsAg⁺ subjects in relation to HBeAg/anti-HBe status. The level of HBV DNA was significantly higher in HBeAg⁺ subjects compared with those positive for anti-HBe (median values are indicated by horizontal rules).

We have designed the sequences of the donor (HBVcD) and acceptor (HBVcA) hybridization probes on the basis of a set of primers (BcP1 and BcP2) that we have used for more than 8 years in our in-house HBV nested PCR assay (Chung *et al.*, 1993; Ho *et al.*, 1999). The upstream probe is labelled at the 3'-end with fluorescein and the downstream probe at its 5'-end with LCRed. As the PCR products are made, FRET from fluorescein (donor) to LCRed (acceptor) results in an increase in LCRed signal. The LCRed signal is determined by the amount of product at the end of each PCR cycle, and the first significant increase in signal during the exponential phase (i.e. the threshold cycle) correlates with the initial amount of target DNA template. Our probes have been designed to anneal one base apart on the target 130 bp amplicon in order to achieve maximum FRET signal production.

The use of the QIAamp DNA Blood kit for nucleic acid extraction minimizes contamination and ensures reproducibility of yield, both important properties of quantitative assays. The LightCycler FastStart DNA Master hybridization probe also contributes to the accuracy of the LC-PCR assay, since the constituent polymerase (a modified form of thermostable recombinant *Taq* DNA polymerase) in this ready-to-use 'hot-start' reaction mixture is only activated after heating at 95 °C for 10 min has removed the blocking groups.

The two EUROHEP reference standards have been used for standardization of HBV DNA test kits and in quality-control trials (Heermann *et al.*, 1999; Niesters *et al.*, 2000). Although it is theoretically better to use a biological standard, such as the HBV genome, rather than a plasmid calibrator, the limited supply of the EUROHEP references presents a major hindrance to their routine use in assays for clinical service. Therefore, we have used the readily available calibrator of the Digene assay as a standard in the LC-PCR assay and have validated the assay with the EUROHEP standards. Our results demonstrated linearity over the entire 8-log₁₀ detection range, with low CV for both EUROHEP *ad* and *ay* serotypes. Evaluation of clinical samples from HBsAg⁺ patients and HBsAg⁻ controls showed that the LC-PCR assay was highly specific. Its results correlated well with their HCII counterparts, thereby facilitating direct comparison of data obtained with different assays. Of particular significance is the more than 500-fold increase in sensitivity with the LC-PCR assay compared with the standard HCII test, with the ability to detect HBV DNA at levels as low as 250 copies ml⁻¹. This presents a distinct clinical advantage in patients receiving antiviral medications, which helps to ensure adequate suppression of virus replication, in order to detect relapse early or to monitor for the emergence of drug-resistant HBV mutants. In this context, there are recent reports that have demonstrated a relationship between virus load and the propensity to develop drug-resistant mutants (Puchhammer-Stockl *et al.*, 2000). Besides quantification, another application of fluorimetric real-time PCR assay with

the LightCycler has been the detection of emergence of drug-resistant HBV mutants (YMDD motifs) in patients during lamivudine therapy (Cane *et al.*, 1999; Whalley *et al.*, 2001).

We have shown that the level of HBV DNA is generally higher in HBeAg⁺ subjects compared with those who are positive for anti-HBe and that the improved sensitivity of LC-PCR offers a definite advantage in the detection of the relatively lower levels of viraemia in the latter group. Nevertheless, there is marked individual variation, and the virus load can be high in some anti-HBe⁺ patients. In this regard, five anti-HBe⁺ patients had HBV DNA levels higher than the median level in the HBeAg⁺ group. Two of the five patients were renal transplant recipients and the other three were non-immunosuppressed patients with persistently abnormal liver biochemistry. These results suggest that HBeAg/anti-HBe status is not a reliable surrogate marker for virus replication or disease activity (Chan *et al.*, 2002).

Our results demonstrate that the LC-PCR assay has a broad quantification range from 250 to 2.5×10^9 copies ml⁻¹ and low intra-assay and inter-assay CVs, similar to assays using the TaqMan probe and ABI PRISM (Abe *et al.*, 1999; Loeb *et al.*, 2000; Pas *et al.*, 2000; Weinberger *et al.*, 2000). Other LightCycler-based quantitative HBV DNA assays have used the standard from the Chiron HBV DNA Quantiplex assay and offer detection ranges from 10 to 10¹⁰ copies per reaction (Jardi *et al.*, 2001; Paraskevis *et al.*, 2002). Paraskevis *et al.* (2002) compared home-made HBV DNA plasmid and pooled patient sera with high HBV DNA levels as standards and concluded that the two yielded very similar results. The ABI PRISM 7700 detector has the advantage of simultaneous analysis of up to 96 samples, compared with 32 samples with the LightCycler. On the other hand, the turn-around time of the LC-PCR assay is less than 2.5 h, compared with 4 h required for other real-time PCR assays. The reagents of both real-time PCR methods are less expensive than those of the commercial HBV DNA kits. Hence, the presently reported LC-PCR assay represents another option amongst the choices for real-time PCR assays; users can therefore select according to their particular requirements.

In conclusion, this LC-PCR assay permits sensitive and accurate quantification of serum HBV DNA over a wide range within 2.5 h and has potential for both research and clinical use, in view of the increasing number of patients being monitored for changes in virus load.

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