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COBAS AmpliPrep-COBAS TaqMan Hepatitis B Virus (HBV) Test: a Novel Automated Real-Time PCR Assay for Quantification of HBV DNA in Plasma▽

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Success in antiviral therapy for chronic hepatitis B is supported by highly sensitive PCR-based assays for hepatitis B virus (HBV) DNA. Nucleic acid extraction from biologic specimens is technically demanding, and reliable PCR results depend on it. The performances of the fully automatic system COBAS AmpliPrep-COBAS TaqMan 48 (CAP-CTM; Roche, Branchburg, NJ) for HBV DNA extraction and real-time PCR quantification were assessed and compared to the endpoint PCR COBAS AMPLICOR HBV monitor (CAHBM; Roche). Analytical evaluation with a proficiency panel showed that CAP-CTM quantitated HBV DNA levels in one single run over a wide dynamic range (7 logs) with a close correlation between expected and observed values ($r = 0.976$, interassay variability below 5%). Clinical evaluation, as tested with samples from 92 HBsAg-positive patients, demonstrated excellent correlation with CAHBM ($r = 0.966$, mean difference in quantitation = 0.36 $\log_{10}$ IU/ml). CAP-CTM detected 10% more viremic patients and longer periods of residual viremia in those on therapy. In lamivudine (LAM)-resistant patients, the reduction of HBV DNA after 12 months of Adefovir (ADF) was higher in the combination (LAM+ADF) schedule than in ADF monotherapy (5.1 logs versus 3.5 logs), suggesting a benefit in continuing LAM. CAP-CTM detected HBV DNA in liver biopsy samples from 15% of HBsAg-negative, anti-HBcAg-positive graft donors with no HBV DNA in plasma. The amount of intrahepatic HBV DNA was significantly lower in occult HBV infection than in overt disease. CAP-CTM can improve the management of HBV infection and the assessment of antiviral therapy and drug resistance, supporting further insights in the emerging area of occult HBV infection.

Hepatitis B virus (HBV) infection is a major cause of chronic liver disease. It is estimated that nearly 2 billion people are infected worldwide by HBV and that more than 350 million have persistent and chronic infection (38). HBV carriers have a high risk of developing long-term sequelae of hepatitis B, including cirrhosis and hepatocellular carcinoma that, in countries such as Italy with a moderate prevalence of HBV infection, account for nearly 25% of the indications for liver transplantation in reference centers (29). Recent advances in antiviral therapy, based on the development of new and more powerful nucleos(t)ide analogues, have dramatically improved chronic hepatitis B management, including the prevention of allograft reinfection in those patients undergoing liver transplantation for HBV-related disease.

The success of antiviral therapy has been supported by the introduction of highly sensitive tests for monitoring HBV DNA. Molecular tests help to determine the activity of HBV infection, the selection of patients for treatment, and the efficacy of antiviral therapy, identifying the development of HBV drug-resistant strains (16, 36, 38).

Several assays based on PCR are currently commercially available for HBV DNA. The recently introduced real-time PCR technique represents the method of choice compared to previous, conventional endpoint PCR due to a very sensitive quantification of the viral load over a wide dynamic range (7, 12, 15, 18, 20, 21, 24, 31, 32, 35, 41). At present, sample preparation is a major weakness in molecular tests, and improvements are constantly introduced to decrease the variability of the techniques and the risk of contamination, such as ready-to-use reagents and automation of the extraction procedure.

A fully automated system, the COBAS AmpliPrep-COBAS TaqMan HBV test (CAP-CTM; Roche Molecular Systems, Inc., Branchburg, NJ) consisting of two integrated platforms—the COBAS AmpliPrep for automated nucleic acid extraction from plasma specimens and the COBAS TaqMan 48, a real-time PCR assay based on TaqMan technology—has recently been developed (13, 35). Important improvements compared to other real-time PCR assays for HBV DNA are the incorporation of an internal quantitation standard to monitor the efficiency of the entire process and the introduction of a system to prevent carryover contamination. CAP-CTM is suitable for large routine series and has been demonstrated to equally quantitate HBV genotypes A through G (13, 35), but at present there are only few clinical data (13, 28).

In the present study, the performance of the CAP-CTM was evaluated and compared to the endpoint PCR assay COBAS AMPLICOR HBV monitor (CAHBM; Roche Molecular Systems). Analytical sensitivity and precision were assessed with an HBV proficiency panel, while correlation and differences in

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DNA quantification were determined in specimens from patients with chronic HBV hepatitis, focusing on HBV DNA kinetics in patients on antiviral therapy.

The specific aim of the study was to compare the real-time and endpoint PCR systems in the evaluation of viral load changes in HBsAg-positive patients with chronic hepatitis. An additional group of HBsAg-negative liver donors with antibodies to hepatitis B virus core antigens (HBcAg) (the majority of whom were also reactive for the antibody to HBsAg) was studied due to evidence suggesting that, with a highly sensitive molecular technology, HBV DNA can be detected in the blood or tissue samples of HBsAg-negative individuals anti-HBcAg-positive with or without antibody to hepatitis B surface antigen (anti-HBs), defined as occult-HBV carriers (1, 2, 4, 38). A specific aim with this group of subjects was to study the feasibility of using CAP-CTM for HBV DNA detection and quantification in plasma and liver tissue since occult-HBV carriers harbor the potential of HBV transmission if such grafts are transplanted into HBsAg-negative recipients (3, 10, 23–27, 30).

MATERIALS AND METHODS

Analytical evaluation. The evaluation of the assay was carried out analyzing a 12 member standard HBV DNA panel with titers ranging from 2 to 10^9 IU/ml (AcroMetrix, Benicia, CA). Each standard was tested in three subsequent days and interassay coefficient of variation (CV%) was evaluated.

Clinical evaluation. (i) Samples from HBsAg-positive patients. Plasma specimens ($n = 81$) from 81 HBsAg-positive, HBeAg-negative, anti-HBe-positive patients with HBV-related chronic disease were submitted to the microbiology laboratory of Molinette Hospital, Turin, Italy, for routine quantitative HBV DNA testing by the CAHBM. The samples were randomly selected for HBV DNA testing with CAP-CTM. Parallel experiments were run on specimens stored at −20°C for up to 3 days before testing with CAP-CTM and CAHBM.

Testing and assay comparison were performed in a second group of plasma specimens from 11 lamivudine-resistant patients (10 anti-HBe positive, 1 HBeAg positive) undergoing antiviral therapy with lamivudine (LAM) in combination with Adefovir (ADF; five patients) or ADF alone (six patients). Patients were monitored for a mean of 542 days (range, 377 to 974 days) after the introduction of ADF, and HBV DNA was evaluated in plasma every 3 months. A total of 71 samples were studied.

(ii) Samples from HBsAg-negative patients. A group of 26 HBsAg-negative, antiHBcAg-positive liver donors were included in the study. Antibody to HBsAg was available for 19 of them, and 16 showed an anti-HBs titer of >10 mIU/ml; in three donors anti-HBs was absent (titers of <10 mIU/ml). At the time of organ donation, plasma ($n = 26$) and liver biopsy samples ($n = 26$) were collected and stored at −20°C and at −80°C, respectively. Plasma and intrahepatic HBV DNA detection was performed with CAP-CTM, and the results were compared to those from two highly sensitive independent PCRs for the core and X genes of HBV (10). Occult-HBV infection was defined by the presence of both sequences from the core and the X gene in an HBsAg-negative, anti-HBcAg-positive donor (2, 4, 10, 38). CAP-CTM quantification of intrahepatic HBV DNA in liver biopsy samples from six HBsAg-positive recipients undergoing liver transplantation for HBV-related disease was assessed to compare the amounts of intrahepatic HBV DNA in occult-HBV carriers with those in HBsAg-positive patients with overt HBV disease.

HBV DNA quantification. (i) CAP-CTM. CAP-CTM is an automated real-time PCR test based on a dual-labeled hybridization probe targeting the precore and core regions associated with an HBV DNA automated extraction based on the affinity of DNA for silica gel-covered magnetic beads. The procedure processes 1,050 μl of plasma and consists of subsequent steps of lysis with chaotropic agents and proteinase K, DNA capture by use of glass particles, and purification. After DNA elution at high temperature (80°C), a robotic arm loads nucleic acids in microvials containing the PCR master mix prepared for each sample by the same robotic arm. An internal quantitation standard (QS) is added to each sample during the processing step. After HBV DNA extraction with the COBAS AmpliPrep instrument, a real-time PCR test is performed by the COBAS TaqMan 48 analyzer with a multiplex TaqMan assay. Two targets are amplified: HBV DNA and the internal QS. The QS is a noninfectious construct containing fragments of HBV sequences with primer binding regions identical to those of the HBV target sequence but with a detection probe different from that for HBV. The results are expressed as international units per milliliter with a 5.82 copies per IU conversion factor. Prevention of carryover contamination and sample integrity is provided by the use of the Amperase system based on uracil-*N*-glycosylase and dUTP incorporation (22). The sensitivity of CAP-CTM is 12 IU/ml, with a dynamic range from about 54 to 1.1×10^8 IU/ml and is designed for the extraction of 24 plasma specimens in about 2 h (13, 28).

(ii) CAHBM. The CAHBM is an endpoint PCR test for HBV DNA quantification. The system involves a manual DNA extraction from 100 μl of plasma sample and HBV DNA quantification by coamplifying a region of the precore or core HBV gene with an internal QS added at known concentrations in the extraction step. The sensitivity of the system is 200 copies/ml (~74 IU/ml) with a linear range of up to 200,000 copies/ml. Samples above the upper limit of the dynamic range must be retested at a 1/100 dilution. A conversion factor of 2.7 copies/IU has been reported for this assay.

HBV polymerase gene mutants. HBV LAM-resistant strains were detected with a reverse hybridization line probe assay (INNO-LIPA; Innogenetics, Ghent, Belgium) after amplification of domain B and C of the viral polymerase gene with specific primers.

Plasma and liver tissue analysis for HBV DNA in HBsAg-negative, anti-HBcAg-positive liver donors. (i) DNA extraction. Manual nucleic acid extraction was performed with 200 μl of plasma and 15-mm liver needle biopsy samples from 26 HBsAg-negative, anti-HBcAg-positive liver donors. Tissue specimens were frozen at −80°C at the time of organ donation. DNA was extracted with a procedure based on QIAgen silica gel membranes adsorbed onto spin columns (QIAGEN, Milan, Italy).

(ii) Intrahepatic HBV DNA detection. Two independent nested-PCRs for the core and X HBV genes were set in a qualitative format. PCR for the core gene consisted of two sets of primers derived from nucleotide positions 2230 to 2660 of the core gene, allowing for a final 221-bp amplified product. A second independent nested PCR with a set of primers for the X gene (nucleotide positions 1220 to 1818, for a final amplified product of 478 bp) was used for HBV DNA detection (10, 33). A portion (1 μg) of extracted DNA from liver samples was amplified in two commercially available PCR master mixtures, one containing specific primers for the core region (Nanogen, Turin, Italy) and one containing 2.5 U of *Taq* polymerase, 200 mM deoxynucleoside triphosphates, 1 mM $MgCl_2$ (QIAGEN, Milan, Italy), and 20 pmol of each primer for the X gene. Amplified products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The sensitivity of both assays was arbitrarily established at 10 copies of DNA/μg by 10 serial dilutions of a plasmid containing the whole HBV genome, as previously published (10).

In order to check the reliability of the DNA extraction performed from liver biopsy samples and plasma, the ribonuclease P gene was amplified in each sample as a control for DNA competence by a separate real-time PCR with a preset master mix (Applied Biosystems, Foster City, CA).

CAP-CTM for HBV DNA quantification in tissue samples. CAP-CTM provides an absolute quantification of the amount of HBV DNA by an internal quantitation standard that is added to plasma specimens from the extraction step. In order to maintain the required condition of biologic matrix and quantification for HBV DNA quantification in liver tissue, 1 μg of genomic DNA previously extracted from liver biopsy samples with the silica gel procedure was diluted in 1,000 μl of negative plasma control and processed with the CAP-CTM HBV test without further protocol modifications. HBV DNA levels were reported as $\log_{10}$ copies of DNA/1 μg.

Serologic markers of HBV infection. HBV serology was performed by using the ChLIA method (Abbott Laboratories, Abbott Park, IL).

Statistical analysis. Means, standard deviations, and coefficients of variation were calculated by using conventional statistical tests. Correlation between CAP-CTM and CAHBM was determined by linear regression analysis, and mean differences in quantification were determined by a Bland-Altman plot. HBV DNA results were expressed as $\log_{10}$ IU/ml. Differences between the amount of viral load as quantified for liver biopsy samples in different series of patients with CAP-CTM were considered statistically significant at P values of <0.05 (Student t test).

RESULTS

CAP-CTM performance for HBV DNA quantification has been assessed on a HBV standard panel, as well as on specimens from 92 HBsAg-positive patients with chronic hepatitis B and 26 HBsAg-negative, antiHBcAg-positive liver donors. A

TABLE 1. Clinical performance of the CAP-CTM assay compared to CAHBM in a group of 81 samples from HBsAg-positive patients submitted for routine HBV DNA testing by CAHBM

CAP-CTM	CAHBM (no. of samples)	
	Positive	Negative
Positive	34	8
Negative	0	39
Total	34	47

total of 178 plasma samples were analyzed for HBV DNA (152 specimens from HBsAg-positive patients and 26 from HBsAg-negative patients and anti-HBcAg-positive liver donors).

Analytical evaluation. A 12-member HBV DNA standard panel from AcroMetrix was tested, with expected values ranging from 2 to 10^9 IU/ml. A single sample of each standard was analyzed on three sequential days by CAP-CTM, and the mean interassay variation was determined. The CAP-CTM observed results were very close to the ones expected for each standard, with an excellent correlation (r = 0.976; 95% confidence interval [CI] = 0.915 to 0.993) and linearity (R^2 = 0.953). Standards with less than 12 IU/ml (n = 3) tested negative in all of the experiments performed. The interassay coefficients of variation for the standards 38, 95, 1.9×10^2, 1.9×10^3, 1.9×10^4, 1.9×10^5, 1.9×10^6, 1.9×10^7, and 1.9×10^8 IU were 3.3, 11, 2.7, 0, 2.2, 1, 0.9, 0.7, and 0%, respectively.

Clinical evaluation and systems comparison. (i) Samples from HBsAg-positive patients. Samples from 81 patients with chronic HBV hepatitis submitted for routine HBV DNA testing were studied. At the time samples were selected for CAP-CTM test, 34 were HBV DNA positive and 47 were HBV DNA negative as determined by CAHBM. Concordant results were determined in 90% of samples (34 positive and 39 negative concordant samples). CAP-CTM detected HBV DNA in eight CAHBM-negative samples (HBV DNA levels from 0.3 to 2.5 log IU/ml as determined by CAP-CTM), as shown in Table 1. The correlation between the two tests in HBV DNA quantification was excellent (r = 0.966, 95% CI = 0.9487 to 0.9786). Bland-Altman plotting for averaged logs demonstrated that the mean difference between the two tests (i.e., the $\log_{10}$ IU/ml [CAHBM] − the $\log_{10}$ IU/ml [CAP-CTM]) was 0.36 ± 0.43 $\log_{10}$ IU/ml (Fig. 1). When evaluated within the CAHBM dynamic range (from 200 to 200,000 copies/ml), differences were smaller (0.1 ± 0.4 $\log_{10}$ IU/ml).

In the second group of specimens from 11 HBsAg-positive LAM-resistant patients, the HBV viral load was measured with CAHBM and CAP-CTM from the time ADF was started (baseline), in combination with LAM (five patients) or alone (six patients) and then at months 6, 9, and 12. In the combination schedule, the mean reduction of HBV DNA level from baseline was 3.5 $\log_{10}$ IU/ml at month 6, 3.7 $\log_{10}$ IU/ml at month 9, and 5.1 $\log_{10}$ IU/ml at month 12. In the ADF monotherapy group, the mean reduction of HBV DNA from baseline was 3.0 $\log_{10}$ at month 6, 3.6 $\log_{10}$ at month 9, and 3.1 $\log_{10}$ at month 12. At month 12, 8 of 11 patients (10 of 11 with CAHBM) were below the lower detection limit of the CAP-CTM assay, whereas 3 patients (one with CAHBM), 2 on LAM+ADF and 1 on ADF monotherapy, were still HBV DNA positive (HBV DNA values of 2.5, 2.3, and 3.9 $\log_{10}$ IU/ml, respectively). Therefore, in evaluating HBV DNA levels while on therapy, CAP-CTM detected residual HBV DNA for longer periods than did CAHBM. The reduction of HBV DNA levels after 12 months of ADF administration was much more significant on the combination schedule (5.1-log decline) than on ADF monotherapy (3.5-log decline). These data suggest a much higher efficacy of the combination schedule LAM+ADF on HBV replication, even if it does not reach a statistically significant difference (P = 0.08) due to the small number of patients studied.

In LAM-resistant patients, comparison between CAP-CTM and CAHBM in HBV DNA quantification showed an excellent correlation (r = 0.958, 95% CI = 0.932 to 0.973), a finding similar to that found in the first group of HBsAg-positive

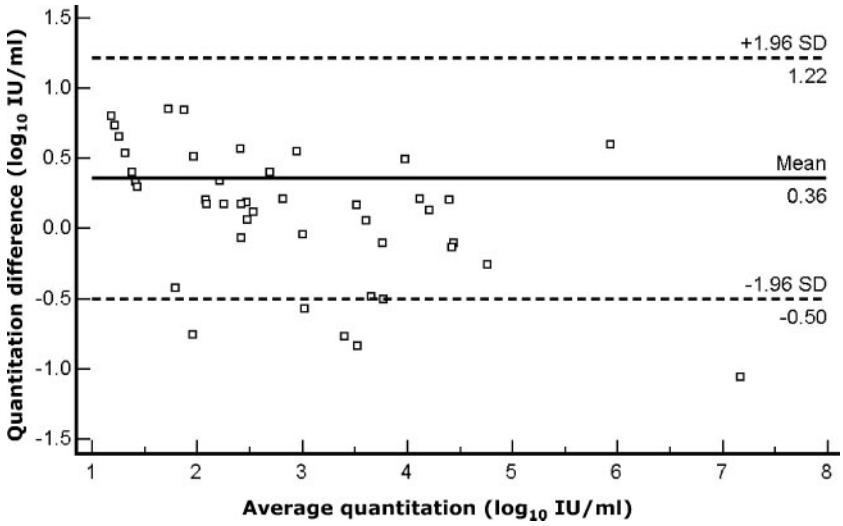

FIG. 1. Bland-Altman analysis of the differences in HBV DNA quantification between CAHBM and CAP-CTM tests as assessed on 81 routine clinical samples.

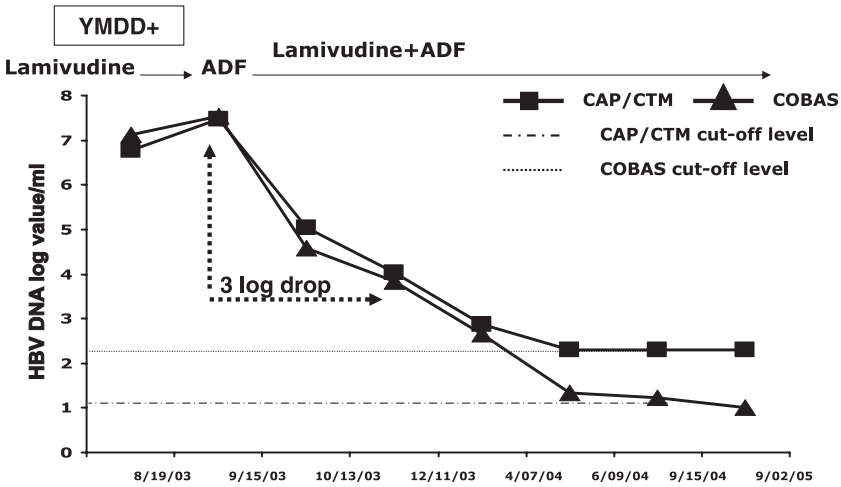

FIG. 2. Time course of HBV DNA in a patient with chronic hepatitis B treated with LAM+ADF after the emergence of LAM-resistant mutants.

patients. The differences in quantification were very small: 0.14 ± 0.71 $\log_{10}$ IU/ml. Figure 2 shows the dynamic of HBV DNA as quantitated by both CAP-CTM and CAHBM in one of the five HBsAg-positive LAM-resistant patients who received ADF in association with LAM. In this representative case, a 3-log drop of HBV DNA could be observed with both of the PCR techniques after 3 months of ADF administration. In this patient, HBV DNA was negative with CAHBM 9 months after the addition of ADF, but CAP-CTM detected decreasing levels of HBV DNA for 3 more months until it was undetectable by CAP-CTM at month 12.

(ii) Samples from HBsAg-negative patients and occult-HBV infection. Two independent qualitative PCRs for the core and X HBV genes and CAP-CTM were used to assess HBV DNA in plasma and liver biopsy samples from 26 HBsAg-negative, anti-HBcAg-positive liver donors (16 of 19 were also reactive for antibody to HBsAg). The two in-house PCRs detected HBV DNA with a higher sensitivity (10 copies of DNA/μg) than CAHBM, as demonstrated by previously published studies (5, 10). Plasma from all donors was negative for HBV DNA with CAP-CTM at the time of organ donation and with the two nested PCRs at a retrospective analysis. Nested PCRs for the both the core and the X viral genes concordantly detected HBV DNA in 8 of 26 (31%) liver biopsy samples from HBsAg-negative, anti-HBcAg-positive donors. These donors were defined as occult-HBV carriers. Anti-HBs was available for seven of them and tested positive in six, with a titer ranging from 14 to >1,000 mIU/ml. CAP-CTM detected HBV DNA in 4 of 26 donor liver biopsy samples (15%) that were HBV DNA positive by the two in-house PCRs for HBV genes. Six liver biopsy samples from six HBsAg-positive patients undergoing liver transplantation for HBV-related cirrhosis were selected as the control group for HBV DNA intrahepatic levels. The median intrahepatic viral level found by CAP-CTM was significantly lower in occult-HBV carriers than in HBsAg-positive patients with overt disease: 2.0 $\log_{10}$ copies of DNA/μg compared to 4.6 $\log_{10}$ copies of DNA/μg ($P = 0.003$) (Table 2).

DISCUSSION

Quantification of HBV DNA has become the most direct and reliable method for the accurate management of chronic hepatitis B as antiviral therapy has dramatically improved the prognosis of HBV disease in active carriers. Moreover, HBV DNA level is an important prognostic factor for HBV-related hepatocellular carcinoma (6, 16, 26, 30, 38). Highly sensitive and reproducible molecular tests are required to monitor viral load in plasma and assess the efficacy of the treatment and the development of HBV drug-resistant strains.

Endpoint PCR-based techniques for HBV DNA have shown a limited dynamic range, usually within 4 logs, affecting the quantification of moderate to high levels of viremia (17, 24).

TABLE 2. HBV DNA in liver biopsy samples from HBsAg-negative, anti-HBcAg-positive liver donors[a]

Patient group (no. of patients)	Total no. of biopsy samples	No. (%) of HBV DNA-positive liver biopsy samples as determined by:		Median HBV DNA level ($\log_{10}$ copies of DNA/μg)[c]
		Nested PCR (core and X genes)[b]	CAP-CTM	
HBsAg negative, anti-HBcAg positive (26)	26	8 (31)	4 (15)	2.0*
HBsAg positive, LT[d] (6)	6	6 (100)	6 (100)	4.6*

[a] Detection of intrahepatic HBV DNA by independent nested PCRs for the core and X HBV gene and quantification by CAP-CTM. HBV DNA was undetectable in plasma by either PCRs or CAP-CTM.
[b] Two independent nested PCRs were performed for the core and X genes. See the text for details.
[c] As determined by CAP-CTM. *, $P = 0.003$.
[d] LT, liver transplantation.

Real-time PCR is designed to quantify nucleic acids in the exponential phase of the reaction, allowing for a more reliable and precise quantification over a much wider dynamic range. Reliable PCR results also depend on sample preparation. Nucleic acid extraction from biologic specimens is technically demanding and a potential source of run-to-run variability and sample contamination (12, 19, 35). As a result, there is a growing need for automated sample processing systems to provide accurate quantification of nucleic acid for clinical purposes.

Real-time PCR assays for HBV DNA based on the TaqMan technology, as well as on other real-time platforms, have been designed on manual, semiautomatic, or automatic nucleic acid extraction procedures suitable for small series. Thus far, these formats have shown an excellent correlation with conventional endpoint PCR systems, including CAHBM, which is the most commonly used assay in comparative evaluations (7, 15, 18, 21, 24, 31, 32).

The real-time PCR system COBAS TaqMan 48 for HBV DNA has been evaluated with both automatic and manual extraction platforms, i.e., the MagNA Pure technology for small routine series and the generic High-Pure system viral nucleic acid extraction kit respectively, and has proved to be very reproducible and sensitive (9, 12, 18, 35). The integration of the real-time PCR analyzer with a fully automated DNA extraction platform on the COBAS AmpliPrep instrument (CAP-CTM) for large routine series has been recently introduced. CAP-CTM has been demonstrated to equally quantitate HBV genotypes A through G (13, 35), but few clinical data are available at present (13, 28).

The present report describes the evaluation of the integrated platform CAP-CTM in patients with chronic hepatitis B compared to the endpoint PCR assay CAHBM, focusing on differences in quantification and viral kinetics in patients on antiviral treatment. Moreover, the important issue of HBV DNA quantification in specimens other than plasma, such as liver biopsy sample, is addressed, as well as the potential role of the integrated system in detecting and quantifying HBV DNA in occult-HBV carriers, who are a major concern in the setting of blood and organ donation.

The analytical evaluation of CAP-CTM with a HBV proficiency panel (from 2 to 10^9 IU/ml) showed that CAP-CTM accurately quantitated HBV DNA levels in a single run over a wide dynamic range (7 logs) with a close correlation between expected and observed values and a very low interassay variability (coefficient of variation of $<5\%$). The upper detection limit of CAP-CTM versus CAHBM potentially extends beyond 10^8 IU/ml the possibility of a reliable quantification of high HBV DNA levels. This is an important achievement since untreated HBV-infected patients can be characterized by high levels of viremia requiring sample dilution by conventional endpoint PCR tests. The reported sensitivity of 12 IU/ml was confirmed since all of the standards above this value were positive, with a significant improvement compared to the endpoint PCR assay (74 IU/ml) (13, 35).

The evaluation of quantitation differences in clinical samples demonstrated that CAP-CTM has an excellent correlation with the endpoint PCR system CAHBM, a finding in agreement with other studies (12, 13, 19, 35). Differences for averaged $\log_{10}$ values between CAP-CTM and CAHBM were below ± 0.5 IU/ml and even less when only samples within the endpoint PCR dynamic range are considered. A difference below ± 0.5 $\log_{10}$ IU/ml among quantitative methods is clinically acceptable since changes in HBV DNA levels to either predict the early virologic response or the development of drug-resistant strains are generally based on viral load differences within 1 log (34). A high correlation of quantitative results with conventional PCR techniques is essential when a laboratory is considering the replacement of one test with a new one.

In patients with chronic HBV hepatitis, CAP-CTM was more sensitive than CAHBM, detecting 10% more viremic patients. The sensitivity of the method is important in the management of HBV-infected patients because of the increasingly recognized clinical significance of low viremia levels, their value in predicting the response to antiviral therapy, and the emergence of drug-resistant strains (6, 11, 16, 37, 38).

In the subset of LAM-resistant patients, significant decreases in HBV viral load under ADF therapy, either alone or in combination with LAM, were observed with CAP-CTM after 6 months of treatment (mean 3-log reduction) and, at month 12, 72% of the patients were below the lower detection limit of CAP-CTM (90% below the detection limit of CAHBM). Therefore, longer periods of residual viremia were detected with CAP-CTM than with CAHBM. The possibility of appreciating residual HBV DNA during treatment could allow for improvements in the prediction of antiviral response and the development of HBV drug-resistant strains, with a better definition of virologic endpoints (11, 36, 37). The mean reduction of HBV DNA level from baseline to month 12 was higher in patients on the combination (LAM+ADF) schedule than in those on ADF monotherapy (5.1 versus 3.5 $\log_{10}$ IU/ml). These data suggest a potential benefit, due to a much higher genetic barrier offered by the combination schedule than by monotherapy, in continuing LAM treatment in patients who are switched to ADF because of LAM resistance, but further observations on a larger population of patients are required to address this issue.

We demonstrated here that CAP-CTM can be applied to specimens other than plasma, allowing the detection of cell-associated HBV DNA. This is of particular value in the context of occult-HBV infection in HBsAg-negative individuals with or without serologic evidence of prior HBV infection, even if the role of occult infection in the context of HBV disease is not very well known (1, 4, 14, 39). In HBsAg-negative, anti-HBcAg-positive patients and anti-HBs-positive patients recovering from HBV infection or in patients with chronic infection and in healthy carriers exhibiting the presence of anti-HBcAg alone, HBV DNA can persist for years in serum or in the liver. Apart from situations associated with serologic evidence of HBV infection, occult-HBV infection has also been observed in healthy individuals without serologic evidence of prior HBV infection and normal liver function, such as in blood donors (1, 4, 39). Grafts or blood from occult-HBV carriers carry the risk of HBV transmission; this risk is particularly high in the setting of liver transplantation, where it varies from 33 to 100% (mean, 75%) in the absence of specific prophylaxis (25). The allocation of liver from occult-HBV carriers due to graft shortage has important implications in the donor-recipient matching, as well as for the prophylaxis of HBV reactivation and hepatitis after transplantation (23, 25, 27).

According to previously published studies, we demonstrated that in occult-HBV carriers HBV DNA is undetectable in plasma, even with the most sensitive methods, such as CAP-CTM, leaving open the question of how to detect occult-HBV carriers in important settings such as blood donations, apart from the presence of anti-HBcAg as a surrogate marker (1, 4, 8, 10, 25). In contrast, HBV DNA can be detected in a significant percentage of livers from these individuals by very sensitive PCRs and CAP-CTM. As suggested by the previously reported definition of occult-HBV infection, the presence of intrahepatic HBV in the HBsAg-negative, anti-HBcAg-positive donors in our series was confirmed by a positive amplification of sequences from at least two different HBV genes in independent PCRs (2, 4, 25, 38). In fact, the in-house nested PCRs for the core and X HBV genes and CAP-CTM detected intrahepatic HBV DNA in 31 and 15% of the HBsAg-negative, anti-HBcAg-positive donors, respectively. These subjects were defined as occult-HBV carriers. The majority of these individuals also carried anti-HBs, as shown by published observations of a high prevalence of occult-HBV carriers in this particular subset of patients, in spite of high anti-HBs titers (1).

Even if CAP-CTM could detect and quantify HBV DNA in only half of the nested-PCR-positive liver biopsy samples, it was possible to demonstrate that the amount of the intrahepatic HBV DNA in occult carriers was significantly lower (about 2 logs) than in liver from HBsAg-positive patients with overt HBV disease. This is in agreement with studies suggesting a complete or partial suppression of viral replication as the major distinctive feature in occult-HBV infection (2, 3, 5, 10, 40).

In conclusion, the present study shows that CAP-CTM is a versatile, automated, and labor-saving platform for HBV DNA in plasma, allowing for a rapid and accurate quantification of HBV DNA levels over a wide dynamic range with excellent sensitivity. The system may further improve the management of acute and chronic HBV infection, the assessment of antiviral therapy, the emergence of drug resistance. It is of note that CAP-CTM quantifies cell-associated HBV DNA, supporting further insights into the emerging area of occult-HBV infection. Therefore, the new assay can be considered an important step for the clinical application of PCR data in HBV disease.

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