Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection

Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: relationship with HBV serum markers

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Abbreviations: anti-HBc, antibodies to hepatitis B core antigen; anti-HBe, antibodies to hepatitis B e antigen; anti-HBs, antibodies to hepatitis B surface antigen; cccDNA, covalently closed circular DNA; CI, confidence interval; CLEIA, chemiluminescent enzyme immunoassay; COI, cut-off index; ddPCR, droplet digital polymerase chain reaction; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; LLoD, lower limit of detection; LLoQ, lower limit of quantitation; OR, odd ratio; ROC, Receiver operating characteristic; RT-PCR, real time polymerase chain reaction.

Abstract

Background & Aims: The accurate diagnosis of occult HBV infection (OBI) requires the demonstration of HBV DNA in liver biopsies of HBsAg-negative subjects. However, in clinical practice a latent OBI is deduced by the finding of the antibody to the HB-core antigen (anti-HBc). We investigated the true prevalence of OBI and the molecular features of intrahepatic HBV in anti-HBc-positive subjects.

Methods: The livers of 100 transplant donors (median age 68.2 years; 64 males, 36 females) positive for anti-HBc at standard serologic testing, were examined for total HBV DNA by nested-PCR and for the HBV covalently closed circular DNA (HBV cccDNA) with an in-house droplet digital PCR assay (ddPCR) (Linearity: $R^2 = 0.9998$; lower limit of quantitation and detection of 2.4 and 0.8 copies/10^5 cells, respectively).

Results: A true OBI status was found in 52% (52/100) of the subjects and cccDNA was found in 52% (27/52) of the OBI-positive, with a median 13 copies/10^5 cells (95% confidence interval 5-25). Using an assay specific for anti-HBc of IgG class, the median antibody level was significantly higher in HBV cccDNA-positive than negative donors (5.7 [3.6-9.7] vs. 17.0 [7.0-39.2] COI, p = 0.007). By multivariate analysis, an anti-HBc IgG value above a 4.4 cut-off index (COI) was associated with the finding of intrahepatic HBV cccDNA (OR = 8.516, p = 0.009); a lower value ruled out its presence with a negative predictive value of 94.6%.

Conclusions: With a new in-house ddPCR-based method, intrahepatic HBV cccDNA was detectable in quantifiable levels in about half of the OBI cases examined. The titer of anti-HBc IgG may be a useful surrogate to predict the risk of OBI reactivation in immunosuppressed patients.

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Lay summary: The covalently closed circular DNA (cccDNA) form of the Hepatitis B virus (HBV) sustains the persistence of the virus even after decades of resolution of the florid infection (Occult HBV infection=OBI). In the present study we developed an highly sensitive method based on droplet digital PCR technology for the detection and quantitation of HBV cccDNA in the liver of subjects with OBI .We observed that the amount of HBV cccDNA may be inferred from the titer in serum of the IgG class antibody to the hepatitis B core antigen (anti-HBc IgG). The quantitation of anti-HBc IgG may represent a surrogate to discriminate the patients at the highest risk of HBV reactivation following immunosuppressive therapies.

Introduction

Occult hepatitis B virus (HBV) infection (OBI) refers to the presence of intrahepatic HBV DNA in the absence of detectable hepatitis B surface antigen (HBsAg) [1]. OBI is secondary to overt HBV infections; it guarantees the persistence of the virus in a cryptic form protected from the immune response of the host. The virological key is the covalently closed circular DNA (cccDNA), an HBV DNA form generated as a plasmid-like episome from the protein-linked relaxed circular DNA genome; it resides in the nucleus of infected cells and gives rise to progeny viral sequences acting as a transcription template for all viral RNAs [2].

OBI has clinical significance; it can reactivate hepatitis B when the immune response of the host is compromised, as in liver transplants or under chemotherapy, may accelerate the progression of hepatic fibrosis in patients with chronic hepatitis C and has been considered a risk factor for hepatocellular carcinoma [3].

An accurate diagnosis of OBI would require a liver biopsy to measure the intrahepatic HBV DNA, yet obtaining liver specimens is difficult. In practice, recognition of OBI is based on the finding of the antibody to the HB core antigen (anti-HBc), on the premise that this reactivity represents a serological scar to a clinically resolved exposure to HBV and may therefore be an indirect marker of a latent HBV infection [4]. Intrahepatic HBV cccDNA is resistant to antivirals and cannot be eradicated [5], but it is possible to prevent OBI reactivation by prophylaxis with HBV antivirals [6]. This strategy is recommended for anti-HBc-positive patients undergoing pharmacological immunosuppression; in default of virologic data, the individual indication to prophylaxis is not determined by parameters of HBV infectivity but by the immunosuppressive potential of therapy.

We developed and present in this study a new assay for HBV cccDNA measurement based on droplet digital PCR (ddPCR), an emerging technique which exhibits improved sensitivity and accuracy to detect low DNA concentrations compared with conventional quantitative real-time PCR (qPCR)[7]. We used this assay to determine the prevalence and quantity of HBV cccDNA in

subjects with OBI recruited among anti-HBc-positive liver donors and to assess the relationship between the viral findings in the liver and the markers of HBV infection in serum.

Patients and Methods

Patients

From November 2010 to December 2016, 112 consecutive HBsAg-negative/anti-HBc-positive deceased heart-beating liver donors were recruited at the Liver Transplant Center of the University of Turin. Eighteen liver biopsies from liver donors without any HBV marker were collected as negative controls. No organ came from executed prisoners or other institutionalized persons. A serum sample and a liver needle biopsy were obtained from each donor; the latter was collected in RNA-later solution and stored at -80°C until processing.

Due to the study design, no specific approval was sought from the Local Institutional Ethics Committee. By the Italian law, Regional Transplant Centers are the custodians of donor biomedical data also for research purposes. All study procedures complied with the ethical standards of the 2000 Declaration of Helsinki and the Declaration of Istanbul 2008.

Detection of circulating and intrahepatic total HBV DNA

Circulating HBV DNA was detected and quantified by a fully automated qPCR system (COBAS Amplicor-COBAS TaqMan, Roche, Switzerland). Results were expressed in IU/mL; the lower limit of detection (LLoD) and quantitation (LLoQ) were 9 IU/mL and 20 IU/mL, respectively.

Frozen liver biopsies were disrupted in 500µL of lysing buffer using a Tissue Lyzer homogenizer and incubated overnight with proteinase K (20 mg/mL) at 37°C. Intrahepatic total DNA was isolated by phenol/chloroform method; concentration and quality were assessed by NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, Delaware, USA). OBI was investigated as previously described [8]. Briefly, extracted liver DNA samples were analyzed for the presence of HBV genomes by four parallel nested-PCRs to detect HBV surface, core, polymerase and X sequences.

PCR primers were complementary to highly conserved nucleotide sequences of HBV genome. Two rounds of amplification, 35 cycles each, were performed using HotStartTaq Polymerase (Qiagen, Hilden, Germany). Appropriate negative and positive controls were included in each PCR experiment. To check for false negatives a parallel PCR for the β-globin gene was performed. Samples positive for at least two HBV targets were scored as OBI-positive according to Taormina expert meeting statements [1].

Quantification of intrahepatic HBV cccDNA by ddPCR

Plasmid AM-12 containing the whole HBV genome [9] and DNA extracts from HBsAg-positive liver recipients and HBV-seronegative liver donors were used for the characterization of the ddPCR assay. To assess the specificity of our ddPCR-based HBV cccDNA assay, restriction endonuclease analysis was performed using EcoRI (Promega, Madison, Wisconsin, USA), which has only one restriction site in plasmid AM-12, or HindIII (Promega, Madison, Wisconsin, USA), which has no restriction sites in either HBV cccDNA or in plasmid AM-12, according to manufacturers' instructions.

For HBV cccDNA quantification in liver donors DNA extracts, 2 µg of intrahepatic total DNA were treated overnight at 37°C with 10 U of plasmid-safe ATP dependent DNase (PSAD) (Epicentre, Madison, Wisconsin, USA) to digest single-strand DNA and linear double-strand DNA. PSAD digestion was carried out using a 50 µL reaction volume containing 5 µL of 10x PSAD buffer, 2 µL of ATP 25 mM, 1 µL of PSAD, 37 µL of de-ionized water and 5 µL (400 ng/µL) of intrahepatic total DNA. Digested samples were further purified by phenol/chloroform/ethanol precipitation and resuspended in 20 µL of sterile water. The comparison between PSAD and S1 Nuclease digestion is reported in Supplementary Method Document 1.

Specific pan-genotypic primers targeting the HBV DNA gap region and a fluorescence hybridization probe were used to detect the viral cccDNA. Forward and reverse primers were 5'-CGTCTGTGCCTTCTCATCTGC-3' (nt 1550-1570) and 5'-GCACAGCTTGGAGGCTTGAA-3'

(nt 1882-1863) respectively [10], and the probe 5'-FAM-CTGTAGGCATAAATTGGTCTGCGAA-FQ-3'. A 20 µL ddPCR reaction mix comprised 10 µL of 2x ddPCR Supermix for probes (no dUTP) (Bio-Rad, Pleasanton, California, USA), 1 µL of 20x primers/probe mix (900 nM and 250 nM) and 9 µL of digested/purified DNA sample. Reaction droplets were generated according to manufacturer's protocol by QX200™ Droplet Generator (Bio-Rad, Hercules, California, USA). Intrahepatic HBV cccDNA was amplified using T100™ Thermal Cycler (Bio-Rad, Hercules, California, USA) with the following amplification profile: an initial denaturation cycle of 10 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 62°C (ramp rate 2.5°C/s) and a final incubation of 10 min at 98°C. Appropriate negative and positive controls were included in each ddPCR experiment. After amplification, positive and negative droplets were quantified by a QX100™ Droplet Reader (Bio-Rad, Hercules, California, USA) using QuantaSoft™ analysis software version 1.7.4 (Bio-Rad, Hercules, California, USA). All intrahepatic HBV cccDNA values were normalized to cell number assessed by RPP30 CNV assay (Bio-Rad, Pleasanton, California, USA) and reported as HBV cccDNA copies/10^5 cells.

Quantification of intrahepatic HBV cccDNA by qPCR

The qPCR reactions were performed using CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). A 20 µL qPCR reaction mix comprised 10 µL of 2x Sso Advanced Universal Probes Supermix (Bio-Rad, Hercules, California, USA), 1 µL of 20x primers/probe mix (900 nM and 250 nM) used for ddPCR and 9 µL of digested/purified DNA sample. The conditions of amplification were an initial denaturation cycle of 3 min at 95°C, followed by 40 cycles of denaturation for 30 s at 95°C and a combined annealing/extension for 60s at 60°C. The obtained Cq values were analyzed by CFX™ Manager software version 3.1 (Bio-Rad, Hercules, California, USA). Serial dilutions of plasmid AM-12 served as quantification standard and data were normalized for cellular DNA content using RPP30 CNV assay.

Serological assays

Standard total anti-HBc assays were originally used for the work-up of the donors. Serum HBsAg, hepatitis B core-related antigen (HBcrAg) and anti-HBc IgG were determined with sensitive chemiluminescent enzyme immunoassays (CLEIA) on the fully automated system Lumipulse® G600 II analyzer (Fujirebio, Tokyo, Japan). The HBsAg was measured with the HQ assay (Lumipulse® G HBsAg-Quant) which detects linearized HBsAg using 2 monoclonal antibodies against determinant "a" external and internal epitopes with an analytical sensitivity of 5 mIU/mL [11]. HBcrAg values (Lumipulse® G HBcrAg) were expressed as Log U/mL and the analytic measurement range was 2.0 - 7.0 Log U/mL [12]. Anti-HBc IgG levels (Lumipulse® G HBcAb-N) were reported as cut-off index (COI), automatically calculated as multiple of the cut-off value obtained from calibration data (COI = S/C x 0.09). Appropriate negative and positive controls were included in all tests.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) or median (95% confidence interval [CI]) according to data normality. Normal distribution was checked by the D'Agostino-Pearson normality test.

For ddPCR method characterization, coefficient of determination (R^2) was assessed by linear regression analysis, whereas lower LLoQ and LLoD were determined by probit regression analysis. LLoQ and LLoD were defined as the lowest concentration at which 95% and 50% of positive samples were detected, respectively. A Bland-Altman plot was constructed to analyze the agreement between the quantitative results obtained with ddPCR and qPCR, and the Cohen's kappa coefficient (k) was calculated to evaluate inter-rater agreement between the rates of HBV cccDNA positivity by the two methods.

Mann-Whitney non-parametric test was used to analyze continuous variables. Fisher's exact test or chi-square (χ^2) test for trend were performed to compare categorical variables where appropriate. Correlation between continuous variables was tested by Pearson's correlation coefficient (r). Receiver operating characteristic (ROC) curve analysis was performed to assess the area under the curve (AUC) and the cut-off value maximizing sensitivity and specificity. The odd ratios (OR) of variables associated with HBV cccDNA presence were estimated using a multiple stepwise logistic regression analysis.

A *p* value <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc® software, version 16.0. (MedCalc, Ostend, Belgium).

Further details about the materials used, are reported in the supplementary CTAT table.

Results

Liver donors features

One hundred of the 112 anti-HBc-positive donors (median age 68.2 [65.5-71.5] years, 64 males and 36 females) were included. Reasons for exclusion were a positive test for antibodies to the hepatitis C virus (n = 4), the finding of an unknown gastrointestinal stromal tumor (n = 1), donor age <18 years (n = 1) and serum/plasma samples unavailable (n = 5). A donor was dismissed for the subsequent finding of HBsAg-HQ positivity (57 mIU/mL). Six donors had HBV DNA detectable in blood (all <20 IU/mL), 75 donors were anti-HBs-positive and 32 were antibody to hepatitis B e antigen (anti-HBe)-positive (25 were positive for both antibodies).

The characteristics of the donors with HBV DNA in blood are reported in Supplementary Table 1 and 2.

Prevalence of occult HBV infection (OBI)

OBI prevalence was 52% (i.e. 52 of the 100 the donors had a positive nested-PCR for at least two different HBV genomic regions). In detail, 35 liver biopsies were 4/4 targets positive, 9 were 3/4, 8 were 2/4, 13 were 1/4 while 35 were completely negative. Among the 18 anti-HBc-negative liver donors no positivity was ever observed for the four HBV genomic regions.

Sensitivity and accuracy of the ddPCR–based HBV cccDNA assay

The performance of ddPCR method for HBV cccDNA quantitation was assessed using the plasmid AM-12 containing the full length HBV genome. Serial 10-fold dilutions, ranging from 10^6 to 10^0 copies/reaction was used to test linearity (Fig. 1A). The method showed an excellent linear correlation between expected and observed HBV cccDNA copy number (10^5 - 10^0 copies/reaction, $R^2 = 0.9998$, $p < 0.0001$) (Fig. 1B). To determine LLoQ and LLoD, 10 replicates of a serially diluted HBV cccDNA-positive liver extract were tested after digestion with PSAD (obtained from an HBsAg-positive liver transplant recipient); LLoQ was 3.8 copies/10^5 cells whereas LLoD was 0.8 copies/10^5 cells (Fig. 1C). Reproducibility was assessed by intra- and inter-run tests using different HBV cccDNA-positive DNA extracts. For intra-run test, 8 replicates of 2 HBV cccDNA-positive samples (673 ± 55 and 4283 ± 353 copies/10^5 cells) were tested in the same ddPCR experiment, whereas for inter-run test, 7 individual ddPCR experiments with 2 replicates of an HBV cccDNA-positive sample (4164 ± 387 copies/10^5 cells) were performed. The coefficient of variation (CV) was calculated as the SD of HBV cccDNA copies/reaction divided by replicates mean. Mean CV was 8.21% and 9.29% for intra-run and inter-run test, respectively. To evaluate the efficiency of the extraction and purification method, 10 replicates of a DNA extract from HBV-seronegative livers spiked with a known number of plasmid AM-12 copies ,followed by PSAD digestion and phenol/chloroform/ethanol precipitation, were quantified by ddPCR. The mean recovery rate, calculated as the ratio between observed and expected HBV cccDNA copy number, was 79% (expected: 300 copies/reaction, observed: 236 ± 46 copies/reaction).

Specificity of the ddPCR–based HBV cccDNA assay

To assess the specificity of the HBV cccDNA assay, plasmid AM-12 was digested with PSAD only or with PSAD following plasmid linearization by EcoRI. As shown in Fig. 2A, amplification of plasmid AM-12 with EcoRI pre-treatment showed no positive events in comparison to plasmid AM-12 digested with PSAD only, confirming the specificity of PSAD digestion of linear DNA molecules.

In addition, to evaluate the specificity and efficiency of our method for selective HBV cccDNA quantitation in a complex background consisting of genomic DNA, integrated HBV DNA and different replicative HBV DNA intermediates, we compared the number of copies detected in a HBsAg-positive sample with or without PSAD digestion; we observed a reduction in the number of copies following PSAD digestion. Conversely, no further reduction in copies number was observed following HindIII pre-treatment (Fig. 2B), suggesting that the combined use of PSAD digestion and HBV cccDNA-selective primers may effectively eliminate potentially integrated HBV DNA sequences without the need of genomic DNA fragmentation.

No signal was detected in any of the 18 liver biopsies from donors without HBV markers in serum (Fig. 2C).

Intrahepatic HBV cccDNA quantitation by ddPCR

HBV cccDNA was detected in 27 out of the 100 liver biopsies with a median of 13 (5-25) copies/10^5 cells or 1.1 (0.7-1.4) Log copies/10^5 cells (Fig. 3A). Among OBI-positive liver donors, the prevalence of HBV cccDNA positivity was 52% (27 out of 52), whereas none of the OBI-negative liver donors resulted HBV cccDNA-positive (p <0.001) (Fig. 3B). Intrahepatic HBV cccDNA levels significantly correlated to the positivity of the four nested-PCRs for OBI detection (r = 0.545, 95%CI 0.391-0.670; p <0.001) (Fig. 3C). The results of nested-PCR assay for total HBV DNA *versus* ddPCR assay for HBV cccDNA are reported in Supplementary Table 3.

Comparison of HBV cccDNA quantitation with the ddPCR and the qPCR assays

To compare the reproducibility of the ddPCR and qPCR assays, all the 100 liver DNA extracts were further tested by qPCR. No HBV cccDNA-negative sample at ddPCR was positive at qPCR, while only 11 of the 27 samples HBV cccDNA-positive at ddPCR were positive with HBV cccDNA quantifiable by qPCR (k = 0.501, 95%CI 0.307-0.695). The samples positive both at ddPCR and qPCR had significant higher HBV cccDNA concentration compared to those negative at qPCR (40.0 [5.7-331.6] *vs.* 7.5 [2.3-15.5] copies/10^5 cells, $p = 0.012$, respectively). Same as for the characterization of ddPCR, to determine LLoQ and LLoD of qPCR assay, 10 replicates of a serially diluted HBV cccDNA-positive liver extract were tested after digestion with PSAD; LLoQ was 71.1 copies/10^5 cells whereas LLoD was 19.1 copies/10^5 cells. According to the Bland-Altman plot (Fig. S1A) and linear regression analysis (Fig. S1B), there was only a moderate agreement between ddPCR and qPCR assays ($R^2 = 0.6037$, $p = 0.005$).

Relationship between HBV serum markers and intrahepatic HBV cccDNA

The demographic and virologic features of the donors stratified according to HBV cccDNA positivity are reported in Table 1. No differences were observed in HBcrAg levels between HBV cccDNA-positive and -negative liver donors ($p = 0.483$); in almost all cases test results were below LLoQ (<2 Log U/mL). Five donors showed HBcrAg values ≥2 Log U/mL: 2 HBV cccDNA-positive (2 and 2.5 Log U/mL) and 3 HBV cccDNA-negative (2.3, 2.5 and 2.6 Log U/mL). No differences were found both in anti-HBs positivity rates and in anti-HBs levels between HBV cccDNA-positive and -negative liver donors ($p = 0.599$ and $p = 0.481$, respectively), whereas the rates of anti-HBe positivity and anti-HBc IgG levels were significantly different ($p = 0.003$ and $p = 0.007$, respectively).

The median anti-HBc IgG levels increased significantly (5.1 [3.0-8.7] *vs.* 13.7 [7.0-22.0] COI, $p = 0.004$) between the OBI-negative and the OBI-positive group and it increased moderately without statistical significance between the OBI-positive with and without HBV cccDNA (Fig. 4). ROC

curve analysis showed that 4.4 COI was the optimal cut-off that maximized sensitivity (92.6%) and specificity (48.0%) for discrimination between HBV cccDNA-positive and -negative liver specimens (AUC = 0.680, 95% CI 0.577-0.771, $p = 0.002$); 25 donors (52%) in OBI-negative group, 10 (40%) in OBI-positive/HBV cccDNA-negative and 2 (7%) in HBV cccDNA-positive group had anti-HBc IgG levels below 4.4 COI (χ^2 test for trend, $p < 0.001$). In a multivariate logistic regression model including age, gender, anti-HBe positivity and anti-HBc IgG > 4.4 COI, only the latter was significantly and independently associated with measurable intrahepatic HBV cccDNA (OR = 8.516, 95% CI 1.709-42.425, $p = 0.009$); lower antibody titers predicted the lack of measurable intrahepatic HBV cccDNA with a negative predictive value of 94.6%.

Discussion

The hallmark of OBI is the presence of intrahepatic HBV DNA, therefore an accurate diagnosis of this condition would require a liver biopsy. OBI is an asymptomatic condition of viral latency and it would be unethical to submit healthy persons to an invasive procedure; in clinical practice the finding of anti-HBc in serum with standard immunoassays is considered sufficient evidence to diagnose OBI, with the corollarium that all HBsAg-negative persons displaying this marker are candidate to HBV prophylaxis should they be treated with potent immunosuppressive drugs. However, the association between the expression and level of anti-HBc and the presence of intrahepatic HBV DNA remains unknown. To address this issue, we examined liver specimens collected at grafting from anti-HBc-positive liver donors, first to determine the prevalence of intrahepatic total HBV DNA (i.e. OBI), then to determine in the OBI-positive subjects the prevalence of HBV cccDNA. We used a newly developed assay based on ddPCR technology, designed to quantify intrahepatic HBV cccDNA with good reproducibility at a LLoD of 0.8 copies/10^5 cells, without reliance on a calibration curve; the extraction and the purification procedures with phenol/chloroform/ethanol only slightly reduced the DNA recovery. In comparison with other qPCR-based methods with a LLoD ranging from 0.005 to 0.0003 copies/cell for

intrahepatic HBV cccDNA quantitation [13-16], our method was 10 to 100-fold more sensitive and thus particularly suitable for the detection of very low concentrations of target DNA; only 11 of the 27 donors positive at ddPCR, were also positive at qPCR. Previously, Tang *et al.* compared ddPCR to qPCR for total HBV DNA quantification in plasma and Mu *et al.* described a ddPCR method for HBV cccDNA quantitation studying HepG2.2.15 DNA samples, which they applied to detect intra-hepatic HBV cccDNA in liver biopsies from chronic hepatitis B patients [17-19]. However, so far no data have been reported on the performance of ddPCR in the quantitation of HBV cccDNA in subjects with markers of previous HBV exposure but no history of liver disease.

The prevalence of OBI in the whole anti-HBc-positive cohort was 52%, a figure consistent with the prevalence of 62.5% in anti-HBc-positive subjects reported by Raimondo *et al.* [20], in the only study in which intrahepatic HBV DNA was directly assessed in liver biopsies of individuals without hepatic disease. In the present study, the cccDNA species was detectable in about half the HBV DNA-positive livers with a median of 13 copies/10^5 cells; though it cannot be ruled out that the HBV cccDNA negative livers contained viral sequences in amounts lower than the analytical sensitivity of our ddPCR assay, this assay appears at present the most accurate test to directly determine HBV sequences in liver specimens.

We correlated the finding of HBV cccDNA in liver with the presence in serum of HBV biomarkers. No distinctive virologic or serologic features were observed in the 6 donors with HBV DNA detectable in serum; the borderline viremia is likely to be an occasional incidental event, as previously reported by Cemin *et al*. [21] Almost all sera were negative for the HBcrAg; this is at variance with the data of Suzuki *et al.*, who found that 6 of 13 HBsAg-negative/HBV cccDNA-positive patients had detectable HBcrAg in serum (3.23 ± 0.27 Log U/mL), concluding for a clinical utility of this marker in identifying OBI [22]. However, our donors are different from the patients of Suzuki *et al.*; in our cases, anti-HBc was an anamnestic response to an uneventful distant viral exposure while the patients of Suzuki *et al.* had a chronic HBsAg-positive hepatitis which subsequently became HBsAg-negative.

Conventional assays for anti-HBc antibodies measure predominantly the IgG component but also antibodies of other immunoglobulin classes. To optimize the measure of this marker, we used the fully automated CLEIA assay Lumipulse® G HBcAb-N, specifically developed for the detection of IgG type HBc antibody. Compared to other chemiluminescent immunoassays for anti-HBc, this assay showed the largest linear dynamic range as well as the highest sensitivity and specificity [23]. The median titer of the IgG anti-HBc was significantly higher in the OBI-positive than the OBI-negative group. Therefore, the titer of the antibody may be by itself broadly predictive of the risk of OBI reactivation in patients undergoing immunosuppressive therapy; interestingly, only a quarter of the anti-HBc-positive livers exhibited the replication competent cccDNA intermediate, a finding in agreement with the clinical experience that HBV recurs in a minority of the anti-HBc-positive patients undergoing immunosuppression. Though no significant difference in the median antibody titer was observed between the OBI-positive subjects with and without HBV cccDNA, in the former the minimal anti-HBc IgG values were distinctly higher than the minimal values in the latter, allowing to extrapolate in a multivariate analysis corrected for age and gender a cut off value of >4.4 COI independently associated with the detection of intrahepatic HBV cccDNA; this threshold ruled out the presence of measurable HBV cccDNA with a negative predictive value of 94.6%. Thus, the anti-HBc IgG antibody may not only identify by a low serological level the patients at no or a minor risk of OBI reactivation, it may also provide by its level above a cut-off of 4.4 COI an unbiased numerical surrogate to discriminate the patients at the highest risk of OBI reactivation, helping clinicians to define individualized schemes of OBI prophylaxis.

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Table 1. Demographic and virological features of the donors divided according to a negative or positive HBV covalently closed circular DNA test.

Variables	HBV cccDNA- (n = 73)	HBV cccDNA+ (n = 27)	*p* value
Age, years*	70.8 (66.1-72.9)	63.0 (53.3-70.3)	0.049
Gender, M/F	49/24	15/12	0.350
Circulating HBV DNA positivity, n (%)	0	6 (22.2%)	<0.001
OBI positivity, n (%)	25 (34.2%)	27 (100%)	<0.001
Positive HBV regions by nested PCR, n (%)			<0.001**
• 0	25 (47.9%)	0	
• 1	13 (17.8%)	0	
• 2	7 (9.6%)	1 (3.7%)	
• 3	6 (8.2%)	3 (11.1%)	
• 4	12 (16.4%)	23 (85.2%)	
HBcrAg, Log U/mL*	<2.0 (<2.0-<2.0)	<2.0 (<2.0-<2.0)	0.483
Anti-HBs positivity, n (%)	54 (74.0%)	21 (77.8%)	0.599
Anti-HBs, mIU/mL*	56.0 (27.3-97.7)	58.0 (25.8-184.3)	0.481
Anti-HBc IgG, COI*	5.7 (3.6-9.7)	17.0 (7.0-39.2)	0.007
Anti-HBe positivity, n (%)	17 (23.3%)	15 (55.6%)	0.003

*Data are expressed as (95% confidence interval). **p value has been calculated by χ^2 test for trend. Unless otherwise specified, continuous variables were compared by Mann-Whitney test whereas categorical variables were analyzed by Fisher's exact test.

Fig. 1. Sensitivity and accuracy of the ddPCR–based HBV covalently closed circular DNA assay. (A) Amplification of a 10-fold dilution of plasmid AM-12 by ddPCR. (B) Correlation between ddPCR expected and observed HBV covalently closed circular DNA copy numbers. (C) Probit analysis sigmoid curve reporting the lower limit of quantitation (LLoQ) and the lower limit of detection (LLoD).

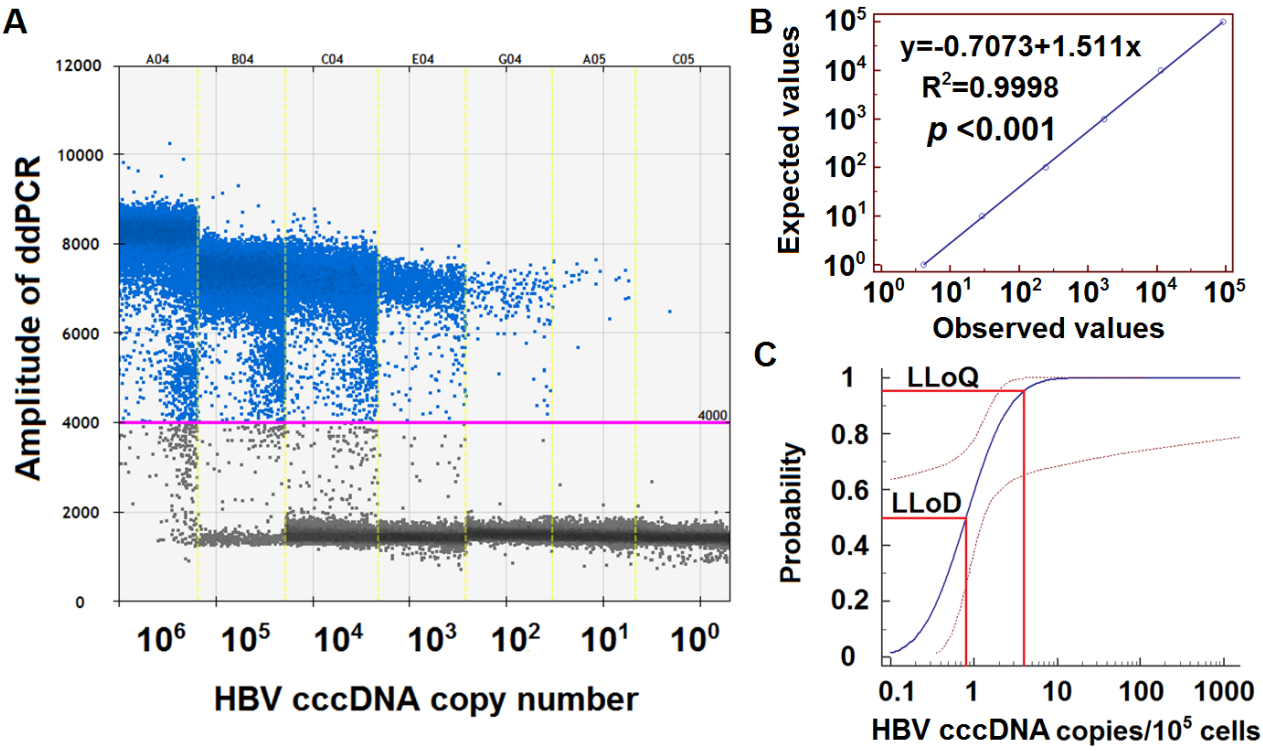

Fig. 2. Evaluation of the specificity of the ddPCR-based assay for HBV covalently closed circular DNA quantitation. (A) Amplification by ddPCR of plasmid AM-12 digested with plasmid-safe ATP dependent DNase (PSAD) only or with PSAD following plasmid linearization by EcoRI. (B) Effect of restriction endonuclease and PSAD digestion on liver DNA extract from hepatitis B surface antigen-positive sample. (C) Amplification of HBV cccDNA by ddPCR in liver biopsy specimens from 18 HBV-seronegative liver donors.

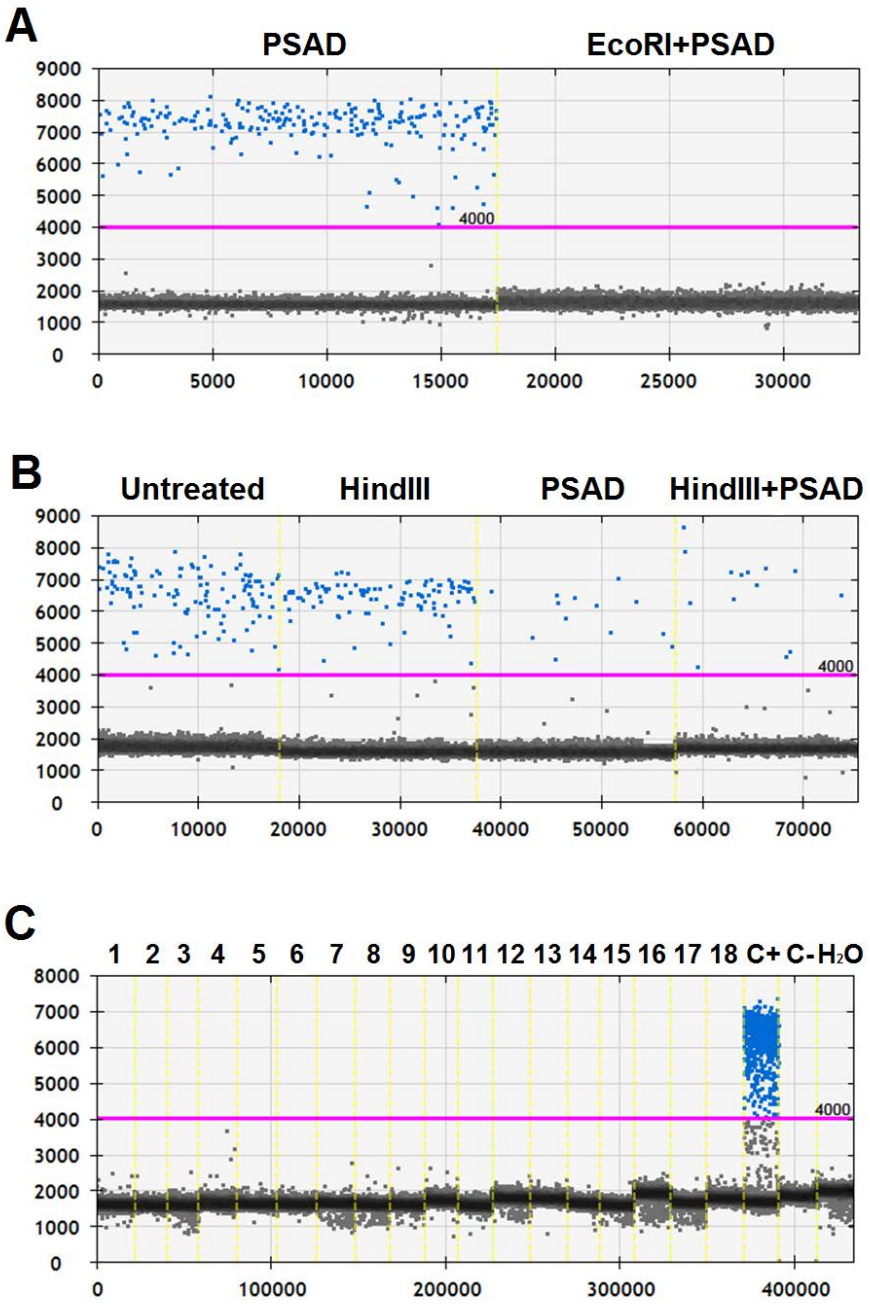

Fig. 3. Intrahepatic HBV cccDNA levels and OBI positivity. Intrahepatic HBV cccDNA levels in cccDNA-positive liver donors (A), distribution of HBV cccDNA positivity according to the number of positive nested PCRs for total HBV DNA detection (B) and correlation between intrahepatic HBV cccDNA copies and number of positive nested PCRs (C).

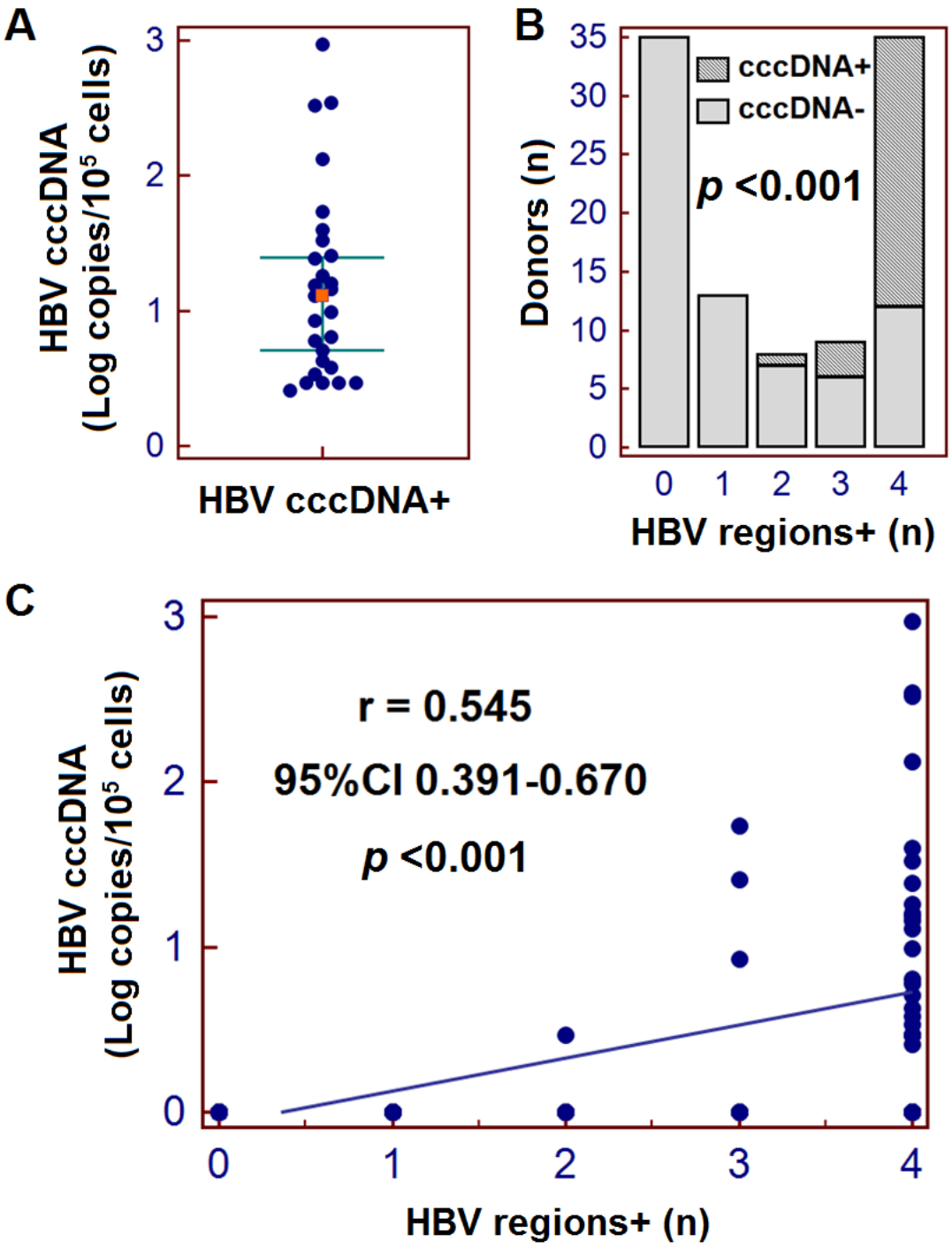

Fig. 4. Anti-HBc IgG serum levels in OBI-negative, OBI-positive/HBV cccDNA-negative and OBI-positive/HBV cccDNA-positive liver donors. Boxes identify the median and the 25th and 75th percentiles, while whiskers stretch to the 5th and 95th percentiles. The median titer of anti-HBc IgG were 5.1 (3.0-8.7) COI in OBI-/cccDNA-, 10.4 (3.6-31.3) COI in OBI+/cccDNA- and 17.0 (7.0-39.2) COI in OBI+/cccDNA+. Comparison between groups was performed by Mann-Whitney test.