**Analytical and clinical evaluation of a novel assay for anti-HBc IgG measurement in serum of subjects with overt and occult HBV infection**

Running title: anti-HBc IgG levels in overt and occult HBV

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**Conflict of Interest**

Gian Paolo Caviglia received research grants from Fujirebio Europe. All other authors have no conflict of interest to disclose.

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**Abbreviations**: ALT, alanine aminotransferase; 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; CH, chronic hepatitis; CHB, chronic hepatitis B; CI, chronic infection; CLEIA, chemiluminescent enzyme immunoassay; COI, cut-off index; CV, coefficient of variation; ddPCR, droplet digital polymerase chain reaction; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; LLoD, lower limit of detection; LLoQ, lower limit of quantitation; SD, standard deviation.

**ABSTRACT**

*Objectives:* We assessed the analytical and clinical performance of the Lumipulse® G HBcAb-N (Fujirebio, Japan) assay for IgG antibodies to hepatitis B core antigen (anti-HBc IgG) measurement in serum of subjects with overt and occult HBV infection (OBI).

*Materials/methods:* Serum anti-HBc IgG was assessed in 181 anti-HBc-positive subjects: 119 chronic hepatitis B (CHB) patients in different infection phases and 62 subjects (35 CHB and 27 OBI) with available liver specimens for HBV covalently-close-circular (ccc)DNA analysis.

*Results:* The anti-HBc IgG assay showed a linear dynamic range (R2=0.9967); lower limit of detection and quantitation were 0.5 IU/mL and 0.8 IU/mL. Reproducibility was 4.9% and accuracy 98.7%. Anti-HBc IgG levels varied according to HBV infection phase, linearly declined during antiviral treatment and resulted correlated to intrahepatic HBV cccDNA (*r*=0.752, *P*<0.001).

*Conclusions:* The quantitative anti-HBc IgG assay exhibited appropriate analytical performance and may represent a diagnostic complement in CHB patients and OBI subjects.

*Keywords:* Anti-HBc, ddPCR, HBcrAg, HBsAg, HBV cccDNA

**1. Introduction**

The proteins of hepatitis B virus (HBV) nucleocapsid are highly immunogenic and can elicit a strong B-cell immune response resulting in high levels of antibodies to the HB core antigen (anti-HBc) (Coffin et al. 2019).Anti-HBc antibodies are widely used for screening and diagnosis of HBV infection together with hepatitis B surface antigen (HBsAg) (Rodella et al. 2006). While the IgG type anti-HBc is predominant in chronic HBV infection (CHB) and in occult/resolved HBV infection, the anti-HBc of IgM type is raised to high titers in acute HBV infection and is diagnostic of this condition (Höner Zu Siederdissen et al. 2018; Trépo et al. 2014).

Recent studies showed that total anti-HBc levels vary among the different phases of CHB (Yuan et al. 2015; Song et al. 2015); they may help to predict hepatitis B e antigen (HBeAg) seroconversion in patients treated with peg-interferon or nucleos(t)ide analogues (NAs) (Yuan et al. 2013; Fan et al. 2016), and are associated with inflammatory activity and significant fibrosis in treatment naïve CHB patients (Li et al. 2016; Li et al. 2017).In HBsAg-negative subjects, the presence of anti-HBc antibodies is considered a surrogate of occult HBV infection (OBI) (Raimondo et al. 2008; Tandoi et al. 2014). Guidelines suggest that anti-HBc-positive patients undergoing pharmacological immunosuppression should receive pre-emptive therapy or prophylaxis according to the risk of HBV reactivation (Perrillo et al. 2015); the risk is determined not only by the immunosuppressive potential of therapy but also by the presence of higher levels of replication-competent HBV covalently-closed-circular (ccc)DNA in the liver (EASL 2017; Raimondo et al. 2019; Caviglia et al. 2018).

Commercial assays for anti-HBc antibodies measure predominantly the IgG component but also antibodies of other immunoglobulin classes; as a matter of fact, anti-HBc IgM can be detected in the serum of CHB patients reflecting disease remission and reactivation (Park et al. 2015). Recently, a qualitative chemiluminescence enzyme immunoassay (CLEIA) that detects anti-HBc IgG only, has been developed (Kobayashi et al. 2015). Our aim was to assess the analytical and clinical performance of anti-HBc IgG quantitation in serum of patients with overt and occult HBV infection.

**2. Materials and methods**

*2.1. Patients*

A total of 181 anti-HBc-positive subjects were included in the study. A cohort of 119 untreated CHB patients were retrospectively enrolled and classified into the different phases of CHB infection according to EASL clinical practice guidelines(EASL 2017) (13 HBeAg+ chronic hepatitis [CH], 51 HBeAg- CH, 55 HBeAg- chronic infection [CI]). The 51 patients with HBeAg- CH underwent antiviral treatment with NAs and serum samples were collected also at 6, 12, 36 and 60 months of therapy. All patients had no evidence of liver cirrhosis on the basis of liver biopsy, liver elastography (FibroScan®, Echosens, Paris, France) or liver echography. Data on HBV genotype was available in the majority of the patients (n = 105 [88.2%]); they were mostly infected with HBV genotype D (n = 96 [91.4%]). We further included in the study a cohort of 27 previously investigated anti-HBc-positive liver donors with detectable intrahepatic HBV cccDNA (OBI) (Caviglia et al. 2018), and a distinct group of 35 chronic HBsAg carriers (overt infection) with matched serum and liver tissue samples collected at routine liver biopsies or at surgical procedures. All patients gave their written informed consent and the study was approved by the Ethics Committee of the Città della Salute e della Scienza - University Hospital of Turin.

*2.2. Evaluation of the quantitative capacity of anti-HBc IgG assay*

The Lumipulse® G HBcAb-N assay (Fujirebio, Tokyo, Japan) was selected for our biomarker research study in consideration that is specific for the detection of IgG class of anti-HBc in serum. The assay is based on a fully automated two-step sandwich CLEIA technology working on LUMIPULSE® G system. Default anti-HBc IgG results are automatically reported as cut-off index (COI), calculated as a multiple of the cut-off value obtained from calibration data (COI = S/C x 0.09). The First International Standard for anti-HBc (NIBSC code 95/522, National Institute for Biological Standards and Control, Potters Bar, UK) was used for standard curve calibration. Quantitative anti-HBc IgG results were reported as IU/mL.

*2.3. HBV virological and serological assays*

Serum 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. Serum HBsAg and hepatitis B core-related antigen (HBcrAg) were determined by CLEIA on LUMIPULSE® G600 II analyzer (Fujirebio, Tokyo, Japan). HBcrAg values (Lumipulse® G HBcrAg) were expressed as Log U/ml and the LLoD was 2.0 Log U/mL (measurement range: 3.0 - 7.0 Log U/ml) (Caviglia et al. 2017). Serum HBsAg was measured with the HQ assay (Lumipulse® G HBsAg-Quant); the analytical sensitivity was 5 mIU/ml (Shinkai et al. 2013).

*2.4. Quantitation of intrahepatic HBV cccDNA*

Intrahepatic HBV cccDNA was quantitated using a droplet digital PCR (ddPCR)-based method as previously described (Caviglia et al. 2018). Before amplification, intrahepatic total DNA was treated overnight at 37°C with 10 U of plasmid-safe ATP dependent DNase (Epicentre, Madison, Wisconsin, USA) to digest single-strand DNA and linear double-strand DNA. Intrahepatic HBV cccDNA was quantified by QX200TM ddPCR System (Bio-Rad, Hercules, California, USA) and normalized to cell number by RPP30 copy number variation assay (Bio-Rad, Pleasanton, California, USA). The LLoQ and LLoD were 3.8 and 0.8 copies/105 cells, respectively.

*2.5. Statistical analysis*

Continuous variables were expressed as mean ± standard deviation (SD) or median and 95% confidence interval (CI) according to data normality. Normal distribution was checked by the D’Agostino-Pearson normality test. For the characterization of quantitative anti-HBc IgG method, the coefficient of determination (R2) was assessed by linear regression analysis, whereas 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. Mann-Whitney *U* test or Student’s *t*-test were used to analyze continuous variables when necessary. Fisher’s exact test was used to compare categorical variables. Correlation between continuous variables was tested by Pearson’s correlation coefficient (*r*) or Spearman’s statistics (*rs*). Repeated measure ANOVA was performed to evaluate biomarkers kinetics. A *P* value <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc® software, version 18.9. (MedCalc, Ostend, Belgium).

**3. Results**

*3.1. Analytical performance of the quantitative anti-HBc IgG assay*

Two-fold serial dilutions of the First International Standard for anti-HBc were used to calibrate the assay (Table S1). The standard curve showed a high accuracy (R2 = 0.9967) and no significant deviation from linearity (Cusum test for linearity, *P* = 0.360) (Figure 1A). An optimal concordance (*pc* = 0.9951) was observed between the concentration expressed in IU/mL and the anti-HBc concentration automatically calculated from the COI (Figure 1B), and no differences were observed between serial dilutions of the International Standard for anti-HBc and anti-HBc-positive samples (*P* = 0.159) (Figure 1C). The LLoD and LLoQ were 0.8 IU/mL and 0.5 IU/mL, respectively (Table S2) (Figure 1D). No reactivity for anti-HBc IgG was observed from testing 68 anti-HBc-negative serum samples (specificity 100%).

To evaluate intra-assay precision (repeatability), five replicates of two anti-HBc-positive samples were tested in the same experiment, while for inter-assay precision (reproducibility), individual experiments with two anti-HBc-positive samples were performed on five different days. The mean coefficient of variation (CV) was 3.7% and 4.9% for intra- and inter-run tests, respectively.

To assess the accuracy of recovery, different volumes of the First International Standard for anti-HBc were added in a serum sample negative for anti-HBc antibodies, in order to obtain different antibody concentration ranging from 1.6 IU/mL to 50 IU/mL (Table S3). The accuracy of quantitative anti-HBc IgG assay was calculated as the mean recovery percentage of all the measurements, corresponding to 98.7%.

*3.2. Measurement of anti-HBc IgG in the different phases of HBV infection and comparison with HBV serological markers*

The characteristics of the patients are reported in Table 1. Patients with overt HBV infection (n = 119, 3.72 ± 0.63 Log ng/mL) had significantly higher anti-HBc IgG values compared to subjects with OBI (n = 27, 1.21 ± 0.61 Log ng/mL) (*P* < 0.001). Among untreated patients with CHB, the mean anti-HBc IgG values varied significantly through the different phases of CHB infection, with higher values in CH phases (n = 64, 4.03 ± 0.45 Log ng/mL) and lower in HBeAg- CI phase (n = 55, 3.35 ± 0.61 Log ng/mL) (*P* < 0.001) (Figure 2). To note, HBeAg- CH patients on long term treatment with NAs (60 months), exhibited mean anti-HBc IgG values lower than those observed in patients with HBeAg- CI (2.90 ± 0.51 ng/mL *vs.* 3.35 ± 0.61 ng/mL, respectively; *P* < 0.001).

In patients with CHB, the measure of anti-HBc IgG showed a good correlation with that of HBV DNA (*r* = 0.560, 95%CI 0.422 - 0.672, *P* < 0.001) and ALT (*rs* = 0.571, 95%CI 0.436 - 0.682, *P* < 0.001), but a moderate correlation with the measure of HBsAg (*r* = 0.374, 95%CI 0.208 - 0.519, *P* < 0.001) and HBcrAg (*r* = 0.397, 95%CI 0.234 - 0.538, *P* < 0.001) (Figure S1).

*3.3. Kinetics of anti-HBc IgG in patients treated with NAs*

A total of 255 sequential serum samples collected from 51 patients with HBeAg- CH who underwent NAs treatment were analyzed for HBV DNA, HBsAg, HBcrAg and anti-HBc IgG levels. The patients were followed for a median of 9 (8 - 12) years; during the follow up, 3 out of 51 (5.9%) cleared HBsAg. The mean HBV DNA, HBsAg, HBcrAg and anti-HBc IgG values at the different time-points (baseline, 6, 12, 36 and 60 months on treatment) are reported in Table S4. We observed a significant decline of all biomarkers from baseline to 60 months of therapy (Figure 3); anti-HBc IgG measures progressively declined with a linear trend (linear fit, *r* = 0.958, *P* < 0.001) from baseline to 60M of therapy (repeated measures ANOVA, *P* < 0.001).

*3.4. Correlation of anti-HBc IgG and intrahepatic HBV cccDNA*

Table 1 summarizes the characteristics of the subjects with overt (n = 35) and occult HBV infection (n = 27) in whom intrahepatic HBV cccDNA was evaluated. In liver specimens, the mean HBV cccDNA levels were 3.11 ± 1.14 Log copies/105 cells in patients with overt HBV infection and 1.22 ± 0.70 Log copies/105 cells in subjects with OBI (*P* < 0.001). In serum samples, the corresponding anti-HBc IgG values were 3.68 ± 0.83 Log IU/mL and 1.21 ± 0.61 Log IU/mL, respectively (*P* < 0.001). In the whole cohort (n = 62), intrahepatic HBV cccDNA showed a positive correlation with anti-HBc IgG serum levels (*r* = 0.785, 95%CI 0.666 - 0.865) (Figure 4); in the subgroup of patients with overt infection, HBV cccDNA values were significantly correlated with HBsAg (*r* = 0.624, 95%CI 0.367 - 0.792, *P* < 0.001), HBcrAg (*r* = 0.733, 95%CI 0.589 - 0.857, *P* < 0.001) and anti-HBc IgG (*r* = 0.553, 95%CI 0.270 - 0.748, *P* < 0.001).

**4. Discussion**

In the present study, we characterized an assay for the measurement of anti-HBc IgG in serum of patients with overt and occult HBV infection using the CLEIA assay Lumipulse® G HBcAb-N, specifically developed for the detection of IgG type HBc antibody and we observed that anti-HBc IgG serum levels were related to the phase of HBV infection, to the treatment status and to the reservoir of intrahepatic HBV cccDNA.

The quantitative anti-HBc IgG assay was calibrated against the First WHO International Standard for anti-HBc and showed an excellent analytical performance; the calibration curve was linear within an analytical range set from 0.8 IU/mL to 50 IU/mL. The CVs for repeatability and reproducibility were 3.7% and 4.9%, far below the general accepted criterion of 10%. In terms of reliability, the intra- and inter-assay variation < 5% denote a high precision and consistency of the method.

In the clinical setting, we observed that anti-HBc IgG levels varied among the different phases of CHB infection, increasing significantly from patients with CI to those with CH; in addition, anti-HBc IgG levels were more than 100-fold higher in patients with overt HBV infection compared to subjects with OBI. In accordance with our findings, Song *et al.* reported that the levels of total anti-HBc increased stepwise from subjects with past HBV infection to subjects with OBI and further from patients with CI to patients with CH (Song et al. 2015). Results from previous reports support the concept that quantitative anti-HBc levels are closely associated to liver disease activity in patients with CHB; indeed, it has been reported that total anti-HBc levels were strongly correlated with ALT values and liver inflammation activity in both HBeAg+ and HBeAg- patients (Yuan et al. 2015; Song et al. 2015; Li et al. 2016). In agreement with these findings, we observed a positive correlation between the serum values of anti-HBc IgG and ALT (*rs* = 0.570). Likely, the release of core proteins during liver tissue necrosis may induce a strong B cell immune response resulting in high levels of antibodies production.

In patients with HBeAg- CH treated with NAs, anti-HBc IgG levels progressively declined with a linear fashion throughout 60 months of antiviral treatment reaching values even lower than those observed in serum samples of patients with HBeAg- CI; the HBV DNA and HBcrAg exhibited a marked decline in the first year of treatment and the HBsAg remained almost unvaried. Interestingly, it has been reported that baseline serum levels of total anti-HBc could predict the clearance of HBsAg and anti-HBs seroconversion in patients treated long-term with NAs and that they were associated with a reduced risk of clinical relapse after discontinuing antiviral therapy (Yuan et al. 2015; Chi et al. 2019). Unfortunately, we could not evaluate this issue as in our series only 3 of 51 treated patients experienced HBsAg clearance without anti-HBs seroconversion; long-term prospective studies should address this issue in larger cohort of patients.

Finally, in the overall cohort of subjects with overt and occult HBV infection we observed a strong correlation between anti-HBc IgG serum levels and HBV cccDNA values (*r* = 0.785). As a matter of fact, non-invasive circulating biomarkers able to accurately reflect the HBV cccDNA pool may play a crucial role for the definition of novel treatments endpoints in patients with CHB and for the management of subjects with OBI at risk of viral reactivation. So far, HBcrAg appears the most reliable surrogate for both HBV cccDNA pool and transcriptional activity in patients with CHB (Suzuki et al. 2009; Chen et al. 2017; Testoni et al. 2019; Chen et al. 2019). Consistently, in such patients we observed that HBcrAg achieved the best correlation with HBV cccDNA (*r* = 0.733), followed by HBsAg (*r* = 0.624) and anti-HBc IgG (*r* = 0.553). However, in subjects with resolved HBV infection, the clinical value of HBcrAg is limited (van Halewijin et al. 2019), and liver tissue samples for the detection of HBV cccDNA are often not available. Considering that anti-HBc is widely recognized as surrogate of OBI, antibody titer may reflect the residual production of HBV core antigens and consequently, intrahepatic replication-competent HBV cccDNA. This aspect may have relevant clinical implication particularly in OBI patients receiving cancer chemotherapy or other immunosuppressive agents. Indeed, it has been reported that higher total anti-HBc levels at baseline were able to predict HBV reactivation in patients with lymphoma and resolved HBV infection receiving rituximab-based chemotherapy (Yang et al. 2018); these findings suggest that anti-HBc quantitation may allow to further stratify the risk of viral reactivation and thus to tailor the prophylactic strategy.

In conclusion, we evaluated the quantitative capacity of the Lumipulse® G HBcAb-N assay that exhibited appropriate analytical performance and may represent a diagnostic complement for the management of both patients with CHB and subjects with OBI. In particular, the quantitation of serum anti-HBc IgG may provide additional information on both the immune response of the host and the HBV persistence reservoir.

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**Table 1**

Demographic, clinical and virological features of the 181 anti-HBc-positive subjects included in the study: (A) the cross-sectional cohort of CHB patients in different phases of HBV infection and (B) the cohort of subjects with overt and occult HBV infection in whom intrahepatic HBV cccDNA was evaluated.

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| --- | --- | --- | --- |
|  | **(A) Cross sectional cohort** |  | **(B)** **Intrahepatic HBV cccDNA evaluation** |
|  | **HBeAg+ CH** | **HBeAg- CH** | **HBeAg- CI** | ***P* value** |  | **Overt HBV**  | **OBI** | ***P* value** |
| Number | 13 | 51 | 55 |  |  | 35 | 27 |  |
| Age, years | 38 (28 - 45) | 49 (45 - 55) | 40 (38 - 44) | <0.001 b |  | 52 (42 - 56) | 63 (53 - 70) | 0.001 e |
| Gender, M/F | 10/3 | 41/10 | 27/28 | 0.005 c |  | 26/9 | 15/12 | 0.177 f |
| ALT, U/L | 106 (59 - 123) | 103 (57 - 133) | 22 (19 - 25) | <0.001 b |  | 3.11 ± 1.14 | NA | <0.001 g |
| HBV DNA, Log IU/mL | 7.75 ± 0.82 | 6.24 ± 1.36 | 2.74 ± 0.91 | <0.001 d |  | 5.48 ± 2.27 | NQ a |  |
| HBsAg, Log IU/mL | 4.47 ± 0.79 | 3.67 ± 0.51 | 2.62 ± 1.17 | <0.001 d |  | 3.13 ± 1.31 | UD |  |
| HBcrAg, Log U/mL | 6.9 ± 0.3 | 4.8 ± 1.5 | 2.4 ± 0.7 | <0.001 d |  | 3.8 ± 1.8 | UD |  |
| Anti-HBc IgG, Log IU/mL | 4.06 ± 0.69 | 4.02 ± 0.38 | 3.35 ± 0.61 | <0.001 d |  | 3.68 ± 0.83 | 1.21 ± 0.61 | <0.001 g |

Data are expressed as mean ± SD for normal continuous variables and as median (95% CI) for continuous non-normal variables.

a Five subjects had detectable serum HBV DNA (<20 IU/mL), while 22 subjects where serum HBV DNA negative. b *P* value calculated by Kruskal Wallis. c *P* value calculated by χ2-test. d *P* value calculated by one-way analysis of variance. e *P* value calculated by Mann-Whitney *U* test. f *P* value calculated by Fisher’s exact test. g *P* value calculated by Student’s *t*-test. Abbreviations: ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; cccDNA, covalently-closed-circular DNA; CH, chronic hepatitis; CI, chronic infection; F, female; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NA, not available; NQ, not quantifiable; M, male; UD, undetectable.

**Fig. 1.** Analytical performance of the quantitative anti-HBc IgG assay. (A) Calibration curve build on a two-fold dilution of the First International Standard for anti-HBc (50 IU/mL) against the raw fluorescence units (FU) of the assay. (B) Concordance analysis of anti-HBc IgG values expressed in IU/mL and the anti-HBc titer automatically calculated as cut-off index (COI). (C) To investigate dilution parallelism, three serum samples positive for anti-HBc (S#21, S#37 and S#139) were pre-diluted depending on their initial concentration to reach the upper limit of the analytical range (≈50 IU/mL). Then, five serial two-fold dilutions were applied to each sample and to the International Standard for anti-HBc. (D) Probit regression analysis was performed to determine LLoQ and LLoD; replicates of a serially diluted anti-HBc-positive serum were tested and we considered valid the calculated concentrations that differed no more than two fold from the expected concentrations. Abbreviations: Anti-HBc, antibody to hepatitis B core antigen; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; LLoD, lower limit of detection; LLoQ, lower limit of quantitation.

**Fig. 2.** Anti-HBc IgG levels according to different phases of HBV infection and biomarkers correlation.*P* values of comparison between groups have been calculated by paired or unpaired Student *t*-test. Abbreviations: Anti-HBc, antibody to hepatitis B core antigen; CH, chronic hepatitis; CI, chronic infection; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NAs, nucles(t)ide analogues; OBI, occult HBV infection.

**Fig. 3.** Kinetics of HBV biomarkers in patients with HBeAg- CH treated with nucleos(t)ide analogues. Abbreviations: Anti-HBc, antibody to hepatitis B core antigen; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; M, month.

**Fig. 4.** Correlation between intrahepatic HBV cccDNA and anti-HBc IgG serum levels. Correlation coefficient (*r*) was assessed by Pearson’s correlation test. Abbreviations: Anti-HBc, antibody to hepatitis B core antigen; HBV cccDNA, hepatitis B virus covalently-closed-circular DNA.