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SHORT COMMUNICATION

Distribution of different HBV DNA forms in plasma and peripheral blood mononuclear cells (PBMCs) of chronically infected patients with low or undetectable HBV plasma viremia

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SUMMARY

Few studies have documented hepatitis B virus (HBV) DNA in peripheral blood mononuclear cells (PBMCs). We developed real-time PCR methods for differential amplification of covalently closed circular (cccDNA) and total HBV DNA (tDNA). The different distribution of cccDNA and tDNA in plasma and PBMCs was evaluated in 37 patients with low or undetectable viremia. Plasma tDNA measured by the Abbott reference system and the in-house assay correlated well (Spearman rho = 0.804; P<0.0001). tDNA was detected in four PBMC samples, all from patients with detectable plasma viremia (range 633-6,406 IU/ml), cccDNA was not detected in any sample. The reasons for apparently discrepant results need further investigation but possibly include the high diversification of HBV status and plasma viremia levels.

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Liver disease associated with persistent hepatitis B virus (HBV) infection remains an important public health problem with significant morbidity and mortality. In spite of the existence of an effective vaccine, worldwide approximately 260 million people are chronic HBV (CHB) surface antigen (HBsAg) carriers and current treatment with interferon and/or nucleoside analogues (NA) is not able to achieve a complete cure (EASL, 2017; Schweitzer *et al.,* 2015). The key obstacle to HBV eradication is the persistence of HBV DNA in the nuclei of infected hepatocytes, either integrated into the host genome or as a covalently closed circular DNA (cccDNA) episomal form (Allweiss and Dandri, 2017; Summers and Mason, 2004). While HBV integration is rare and its clinical implications still require investigation (Tu *et al.,* 2017), cccDNA plays an essential role in the long-term persistence of HBV infection and can often be detected even following NA therapy and HBsAg seroconversion (Boyd *et al.,* 2016; EASL, 2017; Lai *et al.,* 2017; Singh *et al.,* 2004). Since quantification of cccDNA in infected hepatocytes (Lenci *et al.,* 2010; Luo *et al.,* 2016; Werle–Lapostolle *et al.,* 2004) requires invasive liver biopsy, more accessible tissues, such as serum or peripheral blood mononuclear cells (PBMCs), have been investigated in different patient populations although using non-standardized techniques (Chen *et al.,* 2004; Li *et al.,* 2017). Some reports have shown that cccDNA in patients' sera is a marker of off-treatment virological relapse (Chen *et al.,* 2004; Singla *et al.,* 2014; Takkenberg *et al.,* 2009), whereas others have not found cccDNA in the serum of CHB patients (Jun-Bin *et al.,* 2003; Köck *et al.,* 1996; Werle–Lapostolle *et al.,* 2004). Some investigators speculate that PBMCs support HBV replication only partially, with linear and circular relaxed HBV DNA but not cccDNA formed in these cells (Köck *et al.,* 1996; Laskus *et al.,* 1999; Murakami *et al.,* 2004; Umeda *et al.,* 2005), while others have detected cccDNA in PBMCs and/or plasma (Cabrerizo *et al.,* 2000; Coffin *et al.,* 2015, 2014; Mazet-Wagner *et al.,* 2006; Pasquinelli *et al.,* 1990; Torii *et al.,* 2003) in a variable proportion of patients with chronic and occult HBV infection. Discrepancies in HBV distribution and compartmentalization were observed according to the serologic profile or the coinfection status of patients analysed (Coffin *et al.,* 2015; Loustaud-Ratti *et al.,* 2013). While HCV coinfection seems not to be associated with a different distribution of tDNA and cccDNA in serum and/or PBMCs of HBV positive patients, independently of their serological profile, HIV-1 seropositivity seems to influence the distribution of tDNA, which is prevalently detected in serum with respect to PBMCs in HBcAg positive and HBsAg negative patients. This profile suggests that HIV-induced immune dysfunction results in the maintenance of a low HBV viral load (Wagner *et al.,* 2004).

We developed molecular methods to evaluate the distribution of total HBV DNA (tDNA) and cccDNA in plasma and PBMCs of patients with low or undetectable HBV viremia. Written informed consent was obtained from each patient

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Table 1 - *Primers and probe sequences used for tDNA and cccDNA differential amplification in qPCR system. Coordinates are referred to X02763 HBV genotype A used as reference sequence (https://hbvdb.ibcp.fr/HBVdb/HBVdbNomenclature).*

Primer	*Sequence (5' – 3')*	*Coordinates*	*Labelling*	*Direction*	*Target*
P783	GTCTGTGCCTTCTCATCTGC	1551-1570	NO	Forward	cccDNA only
P784	AGTAACTCCACAGTAGCTCCAAATT	1923-1947	NO	Reverse	cccDNA and tDNA, universal primer
P785	GCAACTTTTTCACCTCTGCCTA	1816-1837	NO	Forward	tDNA
P787	TTCAAGCCTCCAAGCTGTGCCTTGGGTGGC	1863-1892	Fam/TAMRA TaqMan Probe	Forward	cccDNA and tDNA, universal probe

included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013) as reflected in a priori approval by the institutional human ethics committee. Plasma tDNA was quantified using the certified Abbott Real-time HBV Viral Load Assay (Abbott Diagnostics Inc.). HBsAg, HBeAg, anti-HBs, anti-HBe were detected by using the Elecsys system (Roche Diagnostics, Italy).

PBMCs and plasma were isolated from 8 ml of blood using Ficoll gradient centrifugation (Pharmacia) and stored frozen until used. Before collection, PBMC pellets were washed twice with phosphate-buffered saline (PBS) to remove viral particles possibly adsorbed on the cell membrane. Viral DNA was extracted from 200 µl of plasma or all the stored PBMCs preparation using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) methods for differential amplification of cccDNA and tDNA in plasma and PBMCs samples were developed. Briefly, cccDNA was preferentially amplified with specific primers flanking the gap region and the incomplete strand of HBV DNA while tDNA primers detected all forms of HBV DNA, including relaxed circular forms and cccDNA (*Table 1*). The beta-globin gene was simultaneously targeted in the tDNA qPCR performed on PBMCs to estimate the number of cells in each PCR reaction. The HBV qPCR standard curve was generated using serial 10-fold dilutions of a plasmid carrying a 1,000-bp fragment of HBV genome and normalized on the calibration curve of the International WHO linear HBV DNA Standard (NIBSC code 10/264). Similarly, the beta-globin standard curve was generated using human DNA derived from Sup-T1 cells (AIDS Reagent Program catalog number 100). The tDNA qPCR reaction mixture included 750 ng of spectrophotometrically measured PBMCs DNA or 5 µl of plasma DNA, 10 µl Premix Ex Taq (Takara Bio), 7.5 pmol of each of tDNA and beta-globin primers and 2.5 pmol of each of the tDNA universal probe and beta-globin probe in a final volume of 20 µl. Negative controls for qPCR and DNA extraction were included in each run, and each sample was amplified in duplicate. To improve the specificity of cccDNA detection, 2 µg of PBMCs DNA and plasma viral DNA were digested with 10 Units of Plasmid Safe DNase (Epicentre) for 1 hour at 37°C, as indicated by the manufacturer. This step was performed to destroy the single stranded DNA isolated from PBMCs and plasma prior to cccDNA detection. The cccDNA qPCR reaction mixture included 5 µl of digested DNA, 10 µl Premix Ex Taq (Takara Bio), 2% of DMSO, 7.5 pmol each of cccDNA primers and 2.5 pmol of tDNA universal probe in a final volume of 20 µl. All the reactions were run in a Light Cycler 96 system (Roche) for 50 cycles each including 15 seconds at 95°C and 1 minute at 57°C after the first denaturation step (5 minutes at 95°C). Data acquisition and handling were carried out using the Light Cycler 96 Software version 1.1.0.1320.

The association between tDNA values obtained with the Abbott Reference System and the in-house qPCR in plasma samples was evaluated by Spearman correlation analysis. The correlation between qPCR tDNA values and HbeAg status or HBV genotype were analysed by the Mann-Whitney test.

The limit of detection of qPCR was 10 IU/ml of plasma and 60 copies/10^6 PBMCs for tDNA and 5 IU/ml of plasma and 30 copies/10^6 PBMCs for cccDNA. The coefficient

Table 2 - *Baseline characteristics of the 37 patients included in the study.*

Population characteristics	*Values expressed as n (%), except* median (Inter Quartile Range)*
Patient Data	
Age, years	48.4* (37.8-60.5)
Gender, males	26 (70)
Caucasian ethnicity	27 (73)
Time from diagnosis of infection	7.3 (4.9-13.8)
HIV coinfection	2 (5.4)
Inactive carrier	16 (43)
Serological Markers	
HBsAg+	36 (97)
HBsAb+	1 (3)
HbeAg+	4 (11)
HBeAb+	33 (89)
Drug Therapy	
Entecavir	13 (35)
Tenofovir	7 (19)
Virological Markers	
Genotype	D 19 (68) A 6 (21), E 2 (7), B 1 (4)
HBV DNA Undetectable	15 (40.5)
HBV DNA <10 IU/ml	5 (13.5)
HBV DNA ≥10 IU/ml	17 (46)

of variability (CV) of the Ct values ranged from 5.55% to 1.37% for tDNA qPCR and 2.04% to 1.00% for cccDNA qPCR when measuring 5 to 50,000 plasmid target copies, with a linear dynamic range covering the 5 $\log_{10}$. The efficacy and safety of the Plasmid Safe DNase protocol was confirmed by a complete loss of PCR signal following treatment of 5,000-50,000 IU of the International linear HBV DNA Standard and no measurable loss of PCR signal following treatment of 5,000-50,000 IU of the HBV plasmid, respectively.

The baseline characteristics of the 37 patients recruited for the study are listed in *Table 2*. Plasma tDNA quantified with the Abbott reference system was undetectable in 15 samples, detectable below 10 IU/ml in five samples and above 10 IU/ml in 17 (median tDNA levels 336 IU/ml, IQR 69-793). Plasma tDNA levels measured by the Abbott reference system and the in-house qPCR correlated well (Spearman rho = 0.80; P<0.0001), with mean±SD difference 0.20±0.62 $\log_{10}$ IU/ml (*Figure 1*). Discordant results (bias of tDNA quantification larger than one log) were obtained only in 6 (16.2%) patients. Plasma tDNA levels showed no apparent association with HBeAg status (P=0.588) or HBV genotype (P=0.371). In the two HIV-1 coinfected patients, tDNA was below the limit of detection in plasma and PBMC samples. tDNA was detected in four PBMC samples, with values of 1,963, 2,017, 3,609, and 26,868 copies per 10^6 PBMCs, all from patients with detectable plasma viremia (range 633-6,406 IU/ml). Of note, a control cccDNA qPCR performed before digestion with Plasmid Safe DNase scored positive in 13.5% of samples (2 plasma and 3 PBMCs). However after enzymatic treatment, cccDNA was not confirmed in any sample, suggesting that the digestion step must be included in cccDNA detection protocols.

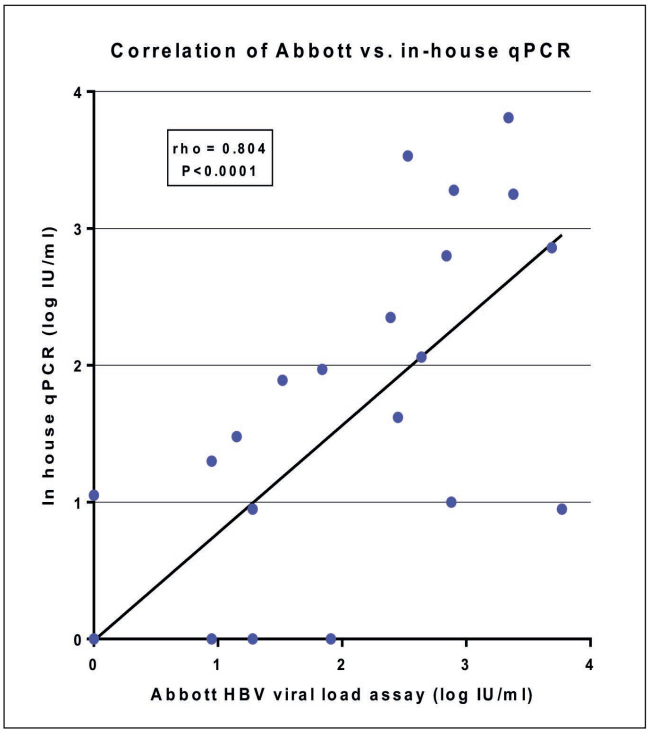

Figure 1 - *Correlation between in-house qPCR and Abbott HBV viral load assay (Roche Diagnostics) in the plasma of 37 patients with low or undetectable HBV viremia.*

Quantitative analysis of residual HBV DNA replication can be useful when assessing the risk of reactivation in patients with occult infection or "inactive" HBsAg carriers, or in order to determine the potential for HBV transmission in donor individuals and the efficacy of treatment regimens on HBV reservoirs. While cccDNA can be detected in liver biopsies as the key HBV replicative form, less invasive blood markers have been proposed including quantitative HBsAg (Wong *et al.,* 2017) and the Hepatitis B core-related antigen (HBcrAg) (Park *et al.,* 2012). However, these markers do not appear to be completely representative of cccDNA levels reflecting only partially the amount of HBV DNA and cccDNA in hepatocytes (Honda et al., 2016). Quantitation of cccDNA in blood is an attractive option but it remains controversial whether extrahepatic tissues contain cccDNA and whether cccDNA is released into the serum. This hypothesis is supported by HBV re-activation after orthotopic liver transplantation (Takaki *et al.,* 2015).

In the small patient group with low to undetectable plasma tDNA levels analysed in our study, we detected no cccDNA in plasma and PBMCs while tDNA was detected only in a few cases in the PBMC compartment, all associated with measurable plasma HBV DNA. Several (Cabrerizo *et al.,* 2000; Coffin *et al.,* 2014; Loustaud-Ratti *et al.,* 2013; Stoll-Becker *et al.,* 1997; Torii *et al.,* 2003), but not all (Köck *et al.,* 1996; Mazet-Wagner *et al.,* 2006; Umeda *et al.,* 2005), studies previously documented tDNA and occasionally cccDNA in a proportion of patients with chronic and occult HBV infection. Our case file included low viremic patients and thus complements previous studies, supporting the concept that PBMC tDNA and cccDNA are mostly associated with the OBI profile as recently reviewed (Joshi and Coffin, 2018). As suggested by our own control experiments, the lack of PBMCs/plasma pre-treatment with a DNase to digest the relaxed circular form of the HBV genome could explain the overestimation of cccDNA in some studies (Cabrerizo *et al.,* 2000; Takkenberg *et al.,* 2009; Torii *et al.,* 2003). Indeed, it must be emphasized that methods for cccDNA detection are far from standard. Other reasons for the discrepancy with previous literature may be related to the high diversification of patient population analysed in terms of infection status (e.g. inactive/active carriers or occult infection), plasma viremia levels and the proportion of treated patients. The prevalence and role of blood cccDNA remains elusive, suggesting analysis of a large and comprehensive HBV population with a standardized method.

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Competing interests

Conflict of Interest: None. The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Compliance with Ethical Requirements

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013) as reflected in a priori approval by the institutional South East Ethical Committee (study approved on October 2015, identification code OBI-2015). Informed consent was ob-

tained from all individual participants included in the study. This article does not contain any studies with animals performed by any of the authors.

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