Methods xxx (xxxx) xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Droplet digital PCR assay as an innovative and promising highly sensitive assay to unveil residual and cryptic HBV replication in peripheral compartment

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ARTICLE INFO

Keywords:
Droplet digital PCR
HBV
Serum HBV-DNA
Highly-sensitive assay
Occult HBV infection
Chronic HBV infection

ABSTRACT

Droplet digital PCR is an innovative and promising approach for highly sensitive quantification of nucleic acids that is being increasingly used in the field of clinical virology, including the setting of hepatitis B virus (HBV). Here, we comprehensively report a robust and reproducible ddPCR assay for the highly sensitive quantification of serum HBV-DNA. The assay showed a limit of detection of 4 copies/ml (<1IU/ml) by Probit analysis, showed a good linearity ($R^2 = 0.94$) and a high intra- and inter-run reproducibility with differences between the values obtained in the same run or in two independent runs never exceeding 0.14logcopies/mL and 0.21logcopies/mL, respectively. By analysing serum samples from chronically HBV infected patients (mostly under antiviral treatment), ddPCR successfully quantified serum HBV-DNA in 89.8% of patients with detectable serum HBV-DNA < 20 IU/mL [equivalent to <112copies/ml] by classical Real-Time PCR assay, with a median (IQR) of 8 (5–14)IU/mL [45(28–78)copies/ml], and in 66.7% of patients with undetectable serum HBV-DNA, with a median (IQR) of 5(4–9)IU/mL [28(20–50)copies/ml]. Similarly, by analysing serum samples from patients with a serological profile compatible with occult HBV infection (anti-HBc+/HBsAg-), ddPCR successfully quantified serum HBV-DNA in 40% of patients with a median (IQR) value of 1(1–2)IU/mL [5(5–11)copies/ml], in line with the extremely limited viral replication typically observed in occult HBV infection.

Overall, the availability of assays for the highly sensitive quantification of serum HBV-DNA can provide an added value in optimizing the diagnosis of occult hepatitis B infection, improving the therapeutic management of chronically HBV infected patients, also in the light of innovative drugs (upcoming in clinical practise) aimed at achieving HBV functional cure.

1. Introduction

Droplet digital PCR (ddPCR) is an innovative and promising approach for highly sensitive quantification of nucleic acids. The principle of this method is based on water–oil emulsion droplet technology in which the sample is partitioned into 20,000 droplets, each representing a micro-reactor for individual PCR reaction in which the presence of the target is revealed by the emission of fluorescence. This results in the separation of positive droplets, containing at least one copy of the target and exhibiting increased fluorescence and negative droplets, both individually counted on a dedicated droplet fluorescence reader. Lastly, the fraction of positive droplets is fitted to a Poisson distribution to determine the absolute starting copy number in the sample.

This approach ensures high sensitivity with a very accurate quantification independently by PCR reaction efficiency and without the need of a standard curve, representing a reliable method for the quantification of very low levels of nucleic acids. For this reason, in recent years, ddPCR is being increasingly used in the field of clinical virology for the

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https://doi.org/10.1016/j.ymeth.2021.05.011
Received 8 February 2021; Received in revised form 29 April 2021; Accepted 12 May 2021
Available online 14 May 2021

quantification of few copies of viral genomes in different biological samples [1–7].

In the scenario of hepatitis B virus (HBV), the availability of highly sensitive ddPCR-based assays provides an added value in revealing residual viral replication during antiviral treatment that can expose patients to a persistent risk of developing long-term HBV-related complications, such as liver cancer. The detection of residual viremia is also particularly relevant considering the upcoming introduction in clinical practise of novel drugs aimed at achieving HBV functional cure, defined as off-treatment HBsAg loss, together with undetectable HBV-DNA [8]. Furthermore, such assays can play an important role in the setting of occult HBV infection, usually characterized by very low concentrations of serum HBV-DNA, rarely detected by current commercially available Real-Time PCR assays [9]. Occult HBV infection is clinical relevant since it can be transmitted during liver transplantation and in cases of blood transfusion, maintains the pro-oncogenic properties of overt HBV infection and can expose patients to a risk of viral reactivation under immunosuppression [9]. In this light, the availability of highly sensitive assays for the quantification of serum HBV-DNA is crucial in optimizing the diagnosis of this challenging form of HBV infection.

Based on these assumptions, we developed a robust and reproducible ddPCR assay for the highly sensitive quantification of serum HBV-DNA. A comprehensive methodological characterization of the assay, along with its diagnostic accuracy and applications in clinical practise, is reported.

2. Material and methods

2.1. Primers and probe design

The assay is designed to amplify a 97 nucleotide region encoding HBV surface antigen (HBsAg), spanning the nucleotides 354 to 451 of full-length HBV genome (based on the Genbank accession number X65259.1). The sequences of forward and reverse primers and of the probe are reported in Table 1. The primers and probe were designed on the basis of highly conserved regions of HBV genome (N-terminus HBsAg encoding region), in order to set-up an assay covering all HBV genotypes and not affected by HBV variability. In particular, these regions were selected since they showed a high degree of HBV genetic conservation (ranging from 94% to 99%) by analysing > 1500 different HBV sequences from HBV-infected patients, covering all HBV genotypes (A, B, C, D, E, F and G) collected for routine clinical practice.

2.2. Workflow of the ddPCR procedure

HBV-DNA quantification by ddPCR workflow is composed by 5 steps: *1)* DNA extraction *2)* master-mix preparation and sample inoculum, *3)* droplets generation, *4)* amplification and *5)* droplets reading and data analysis (Fig. 1A-C).

2.2.1. DNA extraction

HBV-DNA is extracted from 1 mL of serum through the Nucleic Acid Extraction System eMAG (bioMérieux, Boxtel, The Netherlands) according to the manufacturer's instructions (Fig. 1A). The extracted HBV-DNA, eluted in a volume of 50 μL, is dried by using Savant DNA SpeedVac™ Concentrators at low temperature for 1 h (Thermo Fisher Scientific, U.S.A.), and then resuspended in a final volume of 10 μL Nuclease-free water. This final volume of extracted HBV-DNA allows to run two ddPCR reactions, each containing 5 μL of extracted DNA according to the Manufacturer's instructions.

The step of DNA concentration has been validated in ad hoc experiments in order to exclude potential contamination. In particular, extracted DNA from HBV-negative serum samples (N = 6) were concomitantly concentrated with extracted DNA from HBV-positive serum samples (N = 24) by using the vacuum concentrator and then quantified by ddPCR. No positive results were obtained for HBV-negative samples, suggesting the absence of contamination during this step.

2.2.1.1. Suggestions. In case of serum samples derived from patients with a serological profile compatible with occult HBV infection, characterized by minimal amount of serum HBV-DNA, we suggest to start from 2 mL of serum in order to maximize the DNA extraction and then to follow the procedure as above-mentioned in agreement with Taormina Statements [9].

If 1 h is not sufficient to dry HBV-DNA, we recommend to extend the time until the complete drying of HBV-DNA is achieved.

2.2.2. Master-mix preparation and sample inoculum

The second step is represented by the preparation of the so-called Master-mix for the subsequent ddPCR reactions (Fig. 1B). The total amount of Master-mix to be prepared depends on the total number of ddPCR reactions that should be run. For each ddPCR reaction well, a volume of 15 μL is required.

For each reaction well, it is necessary to mix:

1. 10 μL of 2X ddPCR Supermix for Probes (No dUTP) provided by the Manufacturer (Bio-Rad, USA);
2. 1 μL of Primers/Probe mix (final concentration of 900 nM and 250 nM, respectively);
3. 4 μL of Nuclease free water.

Once the total amount of Master-mix is prepared, it is necessary to aliquot in each ddPCR reaction well:

- 15 μL of Master-mix;
- 5 μL of DNA.

This allows to obtain a final volume of 20 μL of ddPCR reaction mix.

Suggestions

During the Master-mix preparation, we suggest to increase by 10% the volume of each reagent in order to guarantee a 15 μL of Master-mix for each reaction well.

2.2.3. Droplets generation

The third step is the generation of the droplets, which represent the PCR micro-reactors for HBV-DNA amplification (Fig. 1B). This step requires the use of DG8™ Cartridges for QX200™/QX100™ Droplet Generator (Bio-Rad, USA), fixed within an *ad-hoc* DG8 Cartridge Holder (Bio-Rad, USA). Each cartridge is composed by three 8-wells rows. Firstly, 20 μL of each reaction mix are inoculated in the middle row of wells, and then 70 μL of Droplet Generation Oil for Probes (Bio-Rad, USA) in the bottom wells of the cartridge. Then, each cartridge is covered with an ad-hoc gasket provided by the Manufacturer (Bio-Rad, USA), hooking the gasket in the place of secure. Finally, the assembled cartridge is placed in the QX200™ Droplet Generator instrument (Bio-Rad, USA), capable to automatically generate the reaction-mix containing droplets in the top row of wells in 2 min.

Suggestions

We suggest to pipet 20 μL of the complete reaction mixes in the

Table 1
Primers and probe designed for the highly sensitive HBV-DNA quantification.

	Sequence	Position[a]	Length
Forward	5′-TTATCGCTGGATGTGTCT-3′	354–373	18
Reverse	5′-CAAGAAGATGAGGCATAGC-3′	432–451	19
Probe	5′-AGAGGAAGATGATAAAACGC-3′	390–412	20

[a] Positions are defined according to the HBV full genome sequence GenBank accession number X65259.1

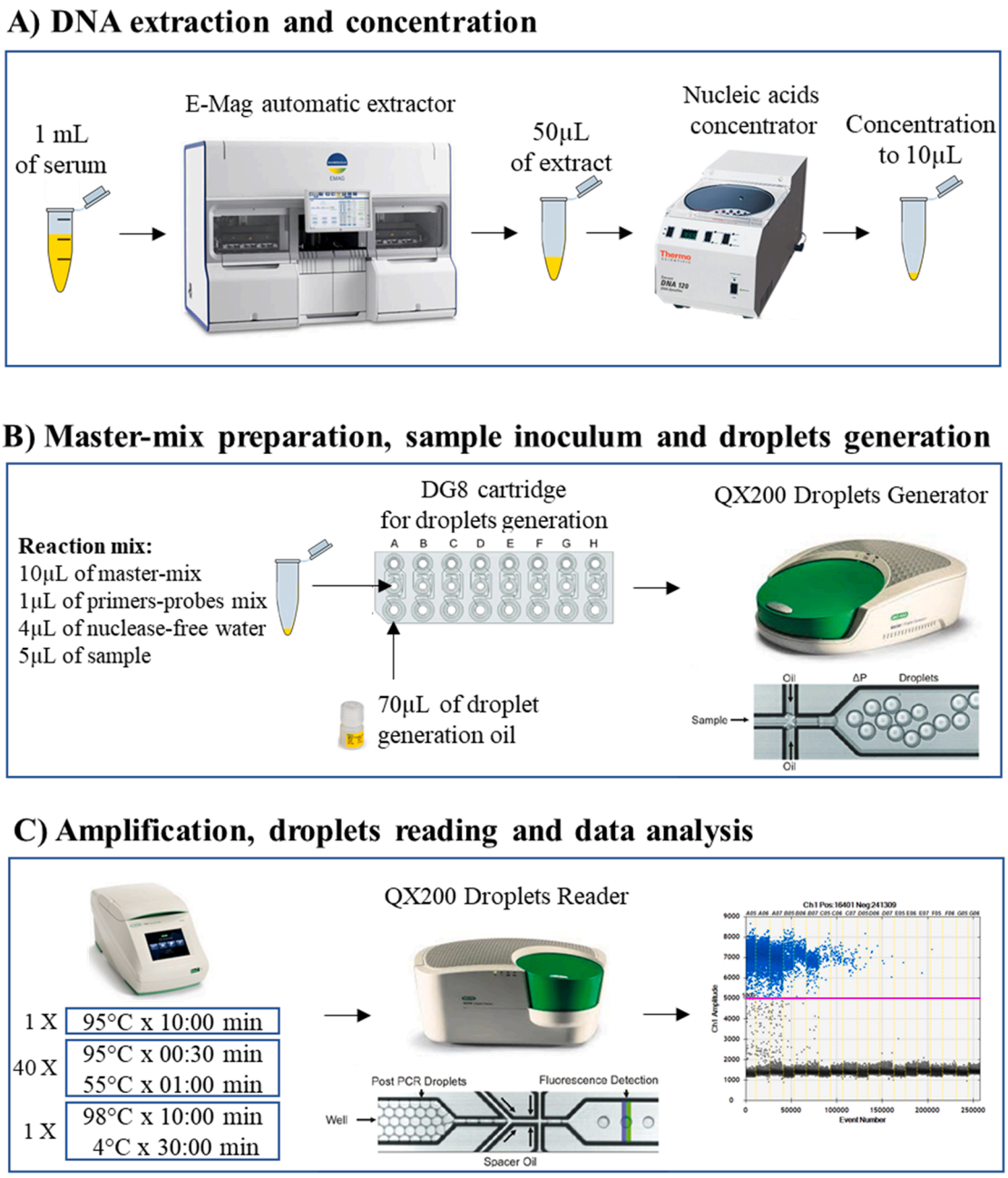

Fig. 1. Schematic representation of ddPCR workflow. The figure represents a schematic overview of the ddPCR workflow. Panel A shows the step of DNA preparation, from extraction to concentration of target DNA. Panel B shows the master-mix preparation, sample inoculum and droplets generation. Panel C shows the amplification phase, the droplets read and the results analysis.

dedicated wells of the cartridge, taking care to avoid transferring air-bubbles.

It is mandatory to fill all the 8 wells of the middle and bottom rows. If the number of samples is < 8, the remaining wells of the middle row should be filled with ddPCR™ Buffer Control for Probes while the remaining wells of the bottom row with the oil.

2.2.4. *Amplification*

After their generation, the reaction-mix containing droplets are transferred from the cartridges to a 96-wells plate (Fig. 1C). When all the droplets have been transferred, the plate should be sealed using Aluminum Pierceable Foil Heat Seals and the BioRad PX1 Plate Sealer, and placed in the T100 thermocycler (Bio-Rad, USA).

The thermic profile of ddPCR amplification is: 10 min at 95 °C, 40 cycles of 30 sec at 95 °C and 1 min at 55 °C. In the end, 10 min at 98 °C and 30 min at 4 °C, with a ramping rate of 2 °C/sec

Suggestions

To avoid shearing the droplets, we suggest to transfer them slowly and carefully.

After the amplification, the droplets can be stored at 4 °C for 24 h, however the immediate reading is advisable.

2.2.5. *Droplets reading and data analysis*

For the results acquisition, the plate is placed into the QX200 Droplet Reader (cat.1864003, Bio-Rad, USA) (Fig. 1C). The QuantaSoft software (Bio-Rad, USA) is used to set up the reading parameters, according to the type of probes and master-mix used. Once the raw results are obtained, it is crucial:

1. to check the total number of droplets for each well. If the droplet count per well is < 10,000, the result is not reliable and the quantification should be repeated.
2. to set a threshold line to discriminate positive and negative droplets. For this scope, the inclusion of ≥ 2 negative and 1 positive control is mandatory for each run of quantification in order to simplify the definition of the positivity threshold. In particular, 2 negative controls were added for every 24 samples analysed. The positivity threshold is usually set above the clouds generated by negative controls. In particular, the threshold was manually set above the highest signal observed in negative controls by excluding the droplets positioned in the rain zone closest to the negative cloud, in order to minimize the inclusion of false positive droplets with an artificial increased fluorescence. Furthermore, in line with Bio-Rad recommendations, all samples, considered positive in this study, were characterized by percentage of positive droplets > 0.02%.

After the definition of positivity threshold, the software generates a result expressed as copies/μL of reaction (cp/μL$_{Rx}$). As previously-mentioned, for a single sample two ddPCR reactions are run, each containing 5 μL of resuspended DNA. Thus, to obtain the copies of serum HBV-DNA in the volume of 5 μL, the following formula is applied:

$cp/5\mu L = (cp/\mu L_{Rx} \times 20\ \mu L)$ where 20 μL represent the total volume of ddPCR reaction mix.

Then, the results of each ddPCR reaction are summed in order to obtain the number of HBV-DNA copies present in the 10 μL of resuspended DNA derived from 1 mL of serum. To convert the copies/mL to International Unit/mL (IU/mL), it is necessary to multiply copies/mL for the conversion factor 5.6.

Suggestions

According to Witte et al., and Rowlands et al. [10,11], in order to obtain more compact droplet clouds and to substantially reduce the droplet rain, it is possible to reduce the ramping rate to 1 °C/sec in every PCR step, to increase the annealing/extension time to 2 min (up from 1 min), and to increase the number of cycles. In this protocol, 3 ramping rates were tested: 1 °C, 2 °C and 3 °C per second. Among them, 2 °C per second was the best ramping rate capable to minimize the rain and to maximize the fluorescent signal (Fig. S1).

2.2.6. Assessment of the assay accuracy

The linearity of the assay was tested by using serial dilutions of full-length HBV-DNA extracted from HepAD38 cell line, known to contain a copy of full-length HBV genome. In particular, HepAD38 cells are a stably transfected cell line containing a cDNA copy of the HBV pgRNA (3.5 kb) under the control of a tetracycline-responsive promoter, thus supporting HBV replication under the control of the antibiotic [12].

The ten-fold serial dilutions from 10^4 to 1 copy of HBV-DNA were tested, each in 8 different replicates. The dilution corresponding to 1 copy was extracted and eluted in 100uL and then concentrated to 20uL. Following Bio-Rad recommendation, all these 20uL were tested in 4 ddPCR reactions in order to maximize the probability of single copy detection. The number of copies, obtained in each reaction, was summed in order to provide the final concentration. This procedure was applied to 8 samples, corresponding to the dilution of 1 copy, and 7 were successfully quantified.

Furthermore, the intra-run reproducibility was assessed by quantifying serum HBV-DNA from 6 serum samples in duplicate in each run, while inter-run reproducibility was assessed in 6 additional serum samples, in three independent runs, each in duplicate.

The specificity of the assay was assessed by applying ddPCR assay in 50 serum samples from HBV-negative patients (HBsAg-negative and anti-HBc-negative). The specificity (expressed as %) was calculated by applying the following formula: (Number of samples with a negative result / Number of true-negative samples) * 100.

The lower limits of blank (LoB) and detection (LoD) were determined by applying the approach described in Armbruster and Pry [13]. In particular, LoB is defined as the highest apparent analyte concentration expected to be found when replicates of a negative sample (blank) are tested. LoB was calculated by testing 50 replicates of negative samples (DNA extracted from serum of HBV-negative patients), according to the following formula: LoB = mean of blank + 1.645*(Standard Deviation of blank).

LoD is determined by utilising both the measured LoB and by testing replicates of a sample at low concentration (HBV-DNA: 1 copy/reaction), according to the following formula: LoD = LoB + 1.645 (Standard deviation of the low concentration sample). As recommended by Armbruster and Pry [13] the LoD was confirmed by testing additional 19 samples.

Furthermore, the LOD, defined as the lowest concentration at which 95% of positive samples were detected, was also determined by probit regression analysis through IBM SPSS Statistics 19 (IBM Corp., USA)

2.2.7. Quantification of serum HBV-DNA in a set of HBV-infected patients from clinical practise.

Serum HBV-DNA was quantified by using the above-described ddPCR assay in a population of 112 HBV infected patients (82 with chronic HBV infection and 30 with a serological profile compatible with occult HBV infection), collected for clinical practice at the University Hospital of Rome "Tor Vergata". For these patients, serum HBV-DNA was available for clinical purposes, obtained on fresh samples by using the fully automated Real-Time PCR system COBAS® AmpliPrep/ COBAS® TaqMan® HBV Test, v2.0 (lower limit of quantitation [LLoQ] of 20 IU/mL, Roche, Basel, Switzerland) allowing the sequential DNA extraction (based on magnetic beads) immediately followed by real-time PCR. For ddPCR procedure, an aliquot of serum samples from these patients were frozen at −80 °C between the collection and nucleic acid extraction.

In the set of patients with serum HBV-DNA > 20 IU/ml by Real-Time PCR, the Bland-Altman test was used to compare the results for HBV-DNA quantification by a commercially available validated real-Time PCR respect to those obtained by ddPCR. The Bland-Altman method is a well-consolidated and validated approach to assess the degree of agreement between two different analytical methods. The results of Bland-Altman approach are expressed graphically and the related plot displays a scatter diagram of the differences plotted against the averages of the two measurements, resulted from the application of the two different assay. Horizontal lines represent the mean difference, and at the limits of agreement, defined as the mean difference ± 1.95*Standard Deviation of differences. If these limits do not exceed the maximum allowed difference between the two assays, the two assays are considered to be in agreement and interchangeably.

3. Results

3.1. Linearity assay

The linearity of the assay was tested by quantifying serial dilutions of a known amount of full-length HBV-DNA derived from HepAD38 cell line (ten-fold serial dilutions from 10^4 to 1 copy/reaction). A linear relationship (R^2 is 0.996) was observed between the expected values and those generated by the ddPCR assay, suggesting a good accuracy of the assay (Fig. 2A). In particular, the differences between the observed and expected values ranged from 0.05 to 0.2 log copies of HBV-DNA.

3.2. Intra-run and inter-run reproducibility

To assess the intra-run and inter-run reproducibility, serum HBV-DNA derived from 12 HBV infected patients collected from clinical practice, was quantified by ddPCR.

The analysis of intra-run reproducibility showed a high reliability of the method, with differences between the values obtained in the same run never exceeding 0.14 log copies/mL. The mean coefficient of

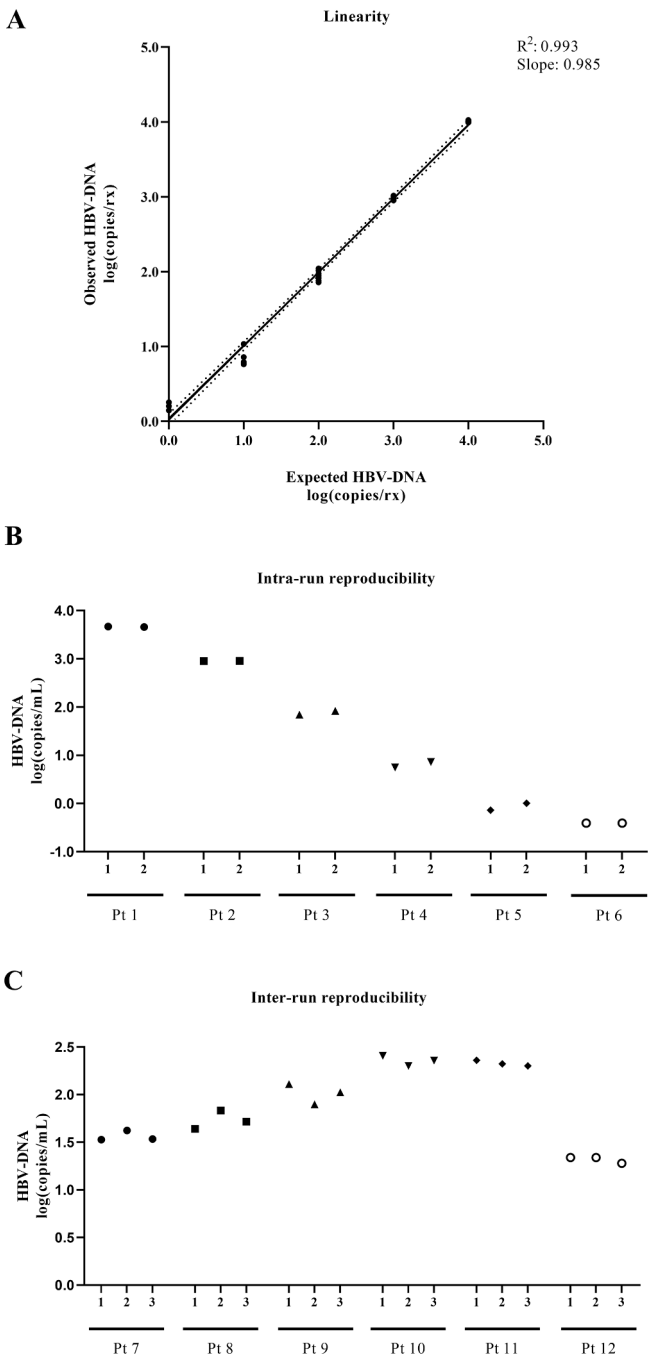

Fig. 2. Linearity, intra- and inter- run reproducibility of the assay. Panel A reports the linearity of the assay, estimated by quantifying ten-fold serial dilutions from 10^4 to 1 copy of HepAD38-derived HBV-DNA per reaction. The X axis reports expected values by ddPCR while the Y axis the observed values from serial dilutions. Dotted lines represent the 95% confidence intervals. Panel B and Panel C report the intra- and inter- run reproducibility, respectively. Values of HBV-DNA are reported in log (copies/mL). The intra-run reproducibility was assessed by quantifying serum HBV-DNA from 6 serum samples in duplicate in each run, while inter-run reproducibility was assessed in 6 additional serum samples, in three independent runs, each in duplicate. The experiments aimed at assessing inter-run reproducibility cover the entire ddPCR procedure with the exception of DNA extraction step.

variation (CV) was 2.7% (corresponding to 9.4% for absolute values) (Fig. 2B).

The analysis of inter-run reproducibility confirmed the above-mentioned results. Indeed, differences between the values obtained in three independent runs never exceeded 0.21 log copies/mL with a mean CV of 3.4% (corresponding to 14.3% for absolute values) (Fig. 2C).

3.3. Specificity and sensitivity

The specificity was assessed by applying ddPCR assay in a set of 50 serum samples from HBV-negative individuals (negative to HBsAg and anti-HBc). A weak positivity was observed only for two samples, corresponding to the specificity of 96%.

According to the approach by Armbruster and Pry [13], the limits of blank (LoB) and of detection (LoD) were 0.4 copies/reaction and 2 copies/reaction, corresponding to 0.8 copies/mL and 4 copies/mL of serum, respectively. Converting in IU/ml, the LoD corresponds to 0.8 IU/mL, supporting the capability of the assay to detect even 1 IU/ml. This LoD was confirmed by applying Probit analysis.

3.4. Application of the assay in a population of HBV-infected patients

3.4.1. Characteristics of HBV infected patients

Serum HBV-DNA was quantified by ddPCR in a set of 112 HBV-infected patients: 82 with chronic HBV infection (anti-HBc+/HBsAg +) and 30 with a serological profile compatible with occult HBV infection (anti-HBc+/HBsAg-). Most patients were male (61.6%) with a median (IQR) age of 50 (41–60) years (Table 2).

Focusing on the 82 patients with chronic HBV infection, 75 (91.5%) were treated with nucleoside analogues (NUC) (Table 2). For each patient, the quantification of serum HBV-DNA by the classical Real-Time PCR assay used in clinical practise was available. According to Real-Time PCR assay, 18 patients had an undetectable serum HBV-DNA, 49 had a detectable serum HBV-DNA $<$ 20 IU/mL, and the remaining 15 had a serum HBV-DNA $\geq$ 20 IU/mL with a median (IQR) value of 160 (30–660) IU/mL (Table 2).

HBV genotype was available for 41 out of 82 chronically HBV-infected patients. 68.3% (28/41) of patients was infected with HBV genotype D, followed by genotype A (17.1%, 7/41), E (4.5%, 2/41), B (4.5%, 2/41), C (4.5%, 2/41).

Focusing on the 30 patients with a serological profile compatible with occult HBV infection, all patients had an undetectable serum HBV-DNA by the classical Real-Time PCR assay (Table 2).

3.4.2. Quantification of serum HBV-DNA by ddPCR in chronically HBV infected patients

ddPCR successfully quantified serum HBV-DNA in all patients with serum HBV-DNA $>$ 20 IU/ml by the classical Real-Time PCR assay (15/15, 100%) (Fig. 3). By ddPCR, median serum HBV-DNA was 136 [30–414] IU/mL). The values of serum HBV-DNA by ddPCR showed a

Table 2
Patients' characteristics.

Patients' characteristics	**N = 112**
Male, N (%)	69 (61.6)
Median (IQR) age, years	50 (41–60)
Patients with chronic HBV infection	**N = 82**
Treated with anti-HBV drugs, N (%)	75 (91.5)
Serum HBV-DNA by Real-Time PCR assay, N (%)	
Undetectable	18 (22.0)
Detectable < 20 IU/mL	49 (59.7)
Detectable > 20 IU/mL	15 (18.3)
HBV genotype, N (%) [a]	
D	28 (68.3)
A	7 (17.1)
B	2 (4.5)
C	2 (4.5)
E	2 (4.5)
Patients with occult HBV infection	**N = 30**
Serum HBV-DNA by Real-Time assay, N (%)	
Undetectable	30 (100.0)

[a] data available for 41 patients.

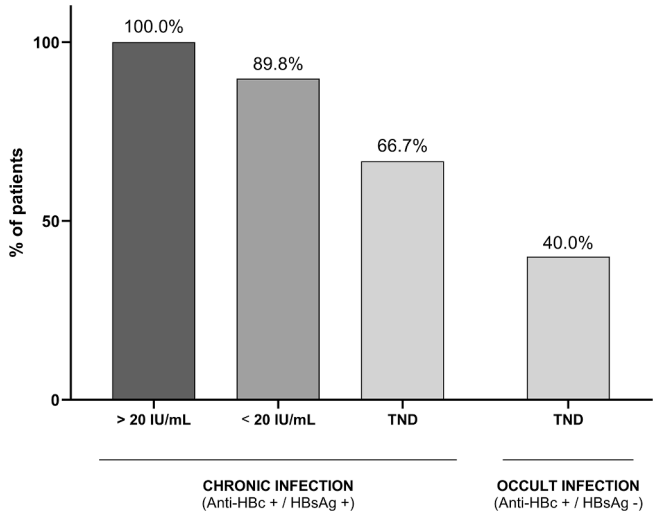

Fig. 3. Proportion of serum successfully quantified by ddPCR. The graph reports the percentage of serum samples successfully quantified by ddPCR stratified according to the type of infection and to serum HBV-DNA by Real-Time PCR assay used in clinical practice.

high concordance with values obtained by the classical Real-Time PCR assay with a R^2 of 0.94 and confirmed by Bland Altman test (Fig. 4A,B). In particular, the Bland-Altman plot showed that all the differences between the results for HBV-DNA obtained by ddPCR and by Real-Time PCR falled within the limits of agreement, supporting the agreement between the two assays (Fig. 4B).

Furthermore, ddPCR provided the quantification of serum HBV-DNA in 44 out of 49 (89.8%) patients with detectable serum HBV-DNA < 20 IU/ by classical Real-Time PCR assay (including those infected with non-D genotype), revealing a median (IQR) of 8 (5–14) IU/mL (Fig. 3).

Finally, ddPCR quantified serum HBV-DNA in 12 out of 18 patients (66.7%) with undetectable serum HBV-DNA by classical Real-Time assay, with a median (IQR) of 5 (4–9) IU/mL, highlighting the presence of a residual viremia in these patients (Fig. 3).

3.4.3. Quantification of serum HBV-DNA by ddPCR in anti-HBc-positive/HBsAg-negative patients

ddPCR successfully quantified serum HBV-DNA in 12 out of 30 (40%) patients with a median value of 1 (1–2) IU/mL (Fig. 3). In line with the status of occult infection (characterized by extremely limited viral replication), this value is significantly lower than that observed in the above-mentioned group of chronically HBV infected patients with undetectable serum HBV-DNA by classical Real-Time assay (1 [1–2] IU/mL vs 5 [4–9] IU/mL, P = 0.003).

4. Discussion

This study comprehensively describes a ddPCR-based assay for the highly-sensitive quantification of serum HBV-DNA. This methodology is characterized by a good accuracy, linearity and sensitivity showing a lower limit of detection of 4 copies/mL by Probit analysis. This supports the robustness and reliability of this assay that can represent a useful tool to detect the burden of residual/cryptic HBV replication in the setting of both chronic and occult infection.

Regarding the sensitivity and specificity of the assay, it should be considered that the choice of an accurate cut-off for defining positive and negative results remains a crucial and still controversial issue. To date, several methods have been proposed to ameliorate the discrimination between positive and negative droplets respect to the automatic method based on QuantaSoft software (Bio-Rad).

In particular, Jones et al. [14] developed a novel open source software, named 'definetherain', aimed to provide improved accuracy for

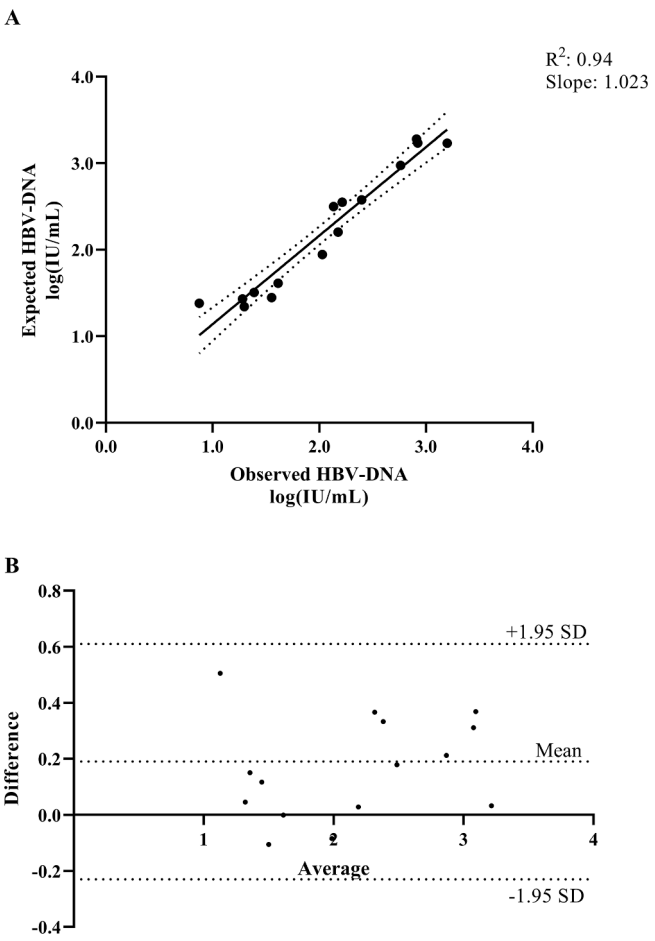

Fig. 4. Comparison of HBV-DNA levels between ddPCR and Real-Time PCR. The graph in panel A reports the comparison of serum HBV-DNA values obtained from ddPCR (X axis) and by Real-Time PCR assay (Y axis) in the subset of 15 patients with serum HBV-DNA > 20 IU/mL. Both values are reported as log (IU/mL). Dotted lines represent the 95% confidence interval of the best fit-line. Panel B shows the Bland-Altman comparison method. Upper dotted line represent the + 1.95 SD, the middle the mean of values and the lowest the −1.95 SD.

the assessment of low template copy numbers. Indeed, this novel method determines the threshold by using the mean and standard deviations of negative and positive control sample's amplitude data and removing ambiguous rain droplets.

Similarly, Trypsteen et al. (2015) [15] suggested an alternative methodology to define the threshold by running negative template controls for each assay and by modelling the extreme values in the negative droplet population using the extreme value theory.

However, despite these methods can increase the ddPCR assay specificity, they have the limits to be dependent on the use of controls that could be not necessarily representative of each experiment and could be associated with a potential underestimation of the true sample concentration if the rain is the consequence of suboptimal PCR reactions in droplets containing the target template. Based on these assumptions, in this study, the threshold was manually set above the highest signal observed in negative controls by excluding the droplets positioned in the rain zone closest to the negative cloud, in order to minimize the inclusion of false positive droplets with an artificial increased fluorescence.

Overall, further efforts are necessary to set up more accurate and standardized approaches for improving the definition of positivity threshold in ddPCR assays.

By analysing clinical samples, ddPCR provided a quantification of serum HBV-DNA in the vast majority of chronically HBV-infected

patients (almost 90%) with detectable serum HBV-DNA below the LLOQ by the classical Real-Time PCR assays currently used in clinical practise. The lack of detection of serum HBV-DNA by ddPCR assay in the remaining 10% of patients (N = 5) could be potentially explained by the fact that Real-Time PCR was directly run by using the DNA extracted from fresh serum, while ddPCR was run by using the DNA extracted form frozen serum. This cycle of freezing/thawing could have contributed to the lack of amplification in low-viremic patients.

An in depth analysis of clinical and virological characteristics of these 5 patients did not reveal any specific factor capable to potentially hamper HBV-DNA detection (i.e. non-D HBV genotype and/or presence of specific mutations in primers' annealing regions). Furthermore, in 3 out of these 5 patients, serum HBV-DNA was detected by real-time PCR in a single time point and was not confirmed in the closest follow-up samples.

Similarly, the ddPCR assay quantified serum HBV-DNA in a conspicuous proportion of patients (almost 70%) with undetectable serum HBV-DNA by classical Real-Time PCR assays. This allows to appreciate any viral load fluctuations (reflecting an enhanced intrahepatic reservoir activity) particularly during antiviral treatment, thus improving the monitoring of chronically HBV infected patients under treatment. This is in keeping with a recent study showing a residual viremia (composed by fully infectious viral particles) in the vast majority of chronically HBV-infected patients even after several years of antiviral treatment [16]. This residual viremia can contribute to replenish intrahepatic HBV reservoir, thus explaining the persistent risk to develop liver cancer despite prolonged antiviral treatment. Furthermore, this low-level viral replication can pose the basis for viral rebound upon discontinuation of antiviral treatment. At this regard, a previous study has shown that patients with a residual serum HBV-DNA > 2.5 copies/mL are at higher risk of viral relapse after suspension of nucleoside analogues inhibitors [17]. In this light, the quantification of residual viremia by ddPCR can guide the identification of patients candidate to the suspension of antiviral treatment, recently proposed as a novel approach to minimize drug exposure of patients with chronic HBV infection [18–24].

So far, several drugs, directly targeting either HBV replication cycle or enhancing anti-HBV immune response, are under development with the ultimate goal to achieve HBV functional cure [8,25–27]. In this light, highly sensitive assays for the quantification of serum HBV-DNA can provide an added value in order to monitor the full efficacy of these new drugs.

Finally, a clinical setting in which the highly sensitive quantification of serum HBV-DNA can provide an advantage is represented by patients with a serological profile compatible with occult HBV infection, defined as the presence of replication-competent HBV DNA in the liver and/or HBV DNA in anti-HBc-positive and HBsAg-negative patients [9].

In our group of patients with a serological profile compatible with occult HBV infection (anti-HBc-positive/HBsAg-negative), ddPCR successfully quantified serum HBV-DNA in 12 out of 30 (40%) patients with a median value of 1 (1–2) IU/mL. This result is in line with the extremely limited viral replication typically observed in this set of patients.

The detection of cryptic viremia in anti-HBc-positive/HBsAg-negative is crucial since it can contribute to HBV transmission during liver transplantation or blood transfusion [9] and can represent a persistent stimulus for HBV pro-oncogenic properties. Furthermore, this cryptic replication can promote immune-suppression driven HBV reactivation [28] that can lead, if not properly managed, to severe forms of hepatitis including fulminant hepatitis [28]. At this regard, recent studies have shown that the positivity to HBV-DNA (detected below LLOQ) is a factor predicting HBV-reactivation [29,30]. In this light, the use of highly sensitive assays for the quantification of serum HBV-DNA can help to finely monitor fluctuations of HBV replication under immunosuppression, thus optimizing the use of antiviral prophylaxis in this fragile population.

5. Conclusions

In this study, we showed that ddPCR-based assays for the quantification of serum HBV-DNA have a good accuracy, reproducibility and high sensitivity, as demonstrated by testing a different range of HBV-DNA levels. All these results support the efficiency and the reliability of ddPCR platform. This assay can provide an added value in optimizing the diagnosis of occult hepatitis B infection, improving the therapeutic management of chronically HBV infected patients, also in the light of innovative drugs (upcoming in clinical practise) aimed at achieving HBV functional cure.

Funding

This work was supported by Aviralia and Vironet C Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Lorenzo Piermatteo: Data curation, Formal analysis, Methodology, Writing - original draft, Investigation. **Rossana Scutari:** Data curation, Formal analysis, Methodology, Writing - original draft, Investigation. **Riccardo Chirichiello:** Data curation, Methodology. **Mohammad Alkhatib:** Visualization. **Vincenzo Malagnino:** Visualization, Resources. **Ada Bertoli:** Visualization, Resources. **Nerio Iapadre:** Visualization, Resources. **Marco Ciotti:** Visualization, Resources. **Loredana Sarmati:** Visualization, Supervision, Resources. **Massimo Andreoni:** Visualization, Supervision, Resources. **Francesca Ceccherini-Silberstein:** Funding acquisition, Writing - review & editing, Supervision. **Romina Salpini:** Conceptualization, Writing - original draft, Writing - review & editing, Investigation. **Valentina Svicher:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We kindly thank Bio-Rad Italia for providing technical support and reagents, particularly Dr. Letizia Ciccone and Dr. Stefano De Scandi. We also thank Katia Yu La Rosa for providing technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.05.011.

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