RESEARCH ARTICLE

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Diagnostic Accuracy of Plate-Based Digital PCR Assays for Simultaneous Quantitation of Hepatitis B and D Viral Loads in Patients' Serum

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Abstract

Objective: This study aims to evaluate the diagnostic accuracy of plate-based digital PCR assays that will accurately and sensitively measure HBV and HDV viral loads in patients' serum simultaneously. **Methods:** First, we optimized the components and parameters for real-time PCR and RT-PCR assays to measure HBV and HDV levels. Next, we employed identical components and parameters in digital PCR and digital RT-PCR assays. Subsequently, we evaluated the accuracy and sensitivity of these assays compared to established techniques and assessed their efficacy by analyzing different serum samples. **Results:** The technical sensitivity of the optimized digital PCR procedures was 1-5 copies/reaction for both HBV and HDV. Compared to real-time PCR and real-time RT-PCR assays, HBV and HDV quantitative digital PCR assays demonstrated strong correlation coefficients (R^2_{HBV} = 0.944 and R^2_{HDV} = 0.900). Substantial levels of agreement were indicated by Lin's Concordance Coefficient (CCC_{HBV} = 0.963 and CCC_{HDV} = 0.933). In addition, 2% (2/98) of the clinical samples had low HBV concentrations that the digital PCR could identify but not the real-time PCR. The HDV prevalence found in the study was 3.1% (3/98) by real-time PCR but 5.1% (5/98) by digital PCR. **Conclusion:** In summary, the optimized digital PCR assays successfully measured hepatitis B and hepatitis D viruses in serum samples with low viral loads that real-time PCR assays could not detect.

Keywords: Digital PCR- hepatitis B virus- hepatitis D virus- microfluidic array plate- quantification- real-time PCR

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Introduction

Inflammation of the liver is known as hepatitis [1]. Hepatitis viruses most frequently bring on hepatitis, although other infections can also bring it on, toxic substances (such as alcohol and other medicines) and autoimmune illnesses [1, 2]. Most of the time, chronic viral hepatitis develops slowly. There are frequently no symptoms in the initial stages. However, if the condition had not been identified promptly, it might have progressed to fibrosis (scarring), cirrhosis, or liver cancer [3]. Five distinct hepatitis viruses have been discovered, denoted by the letters A, B, C, D, and E [3]. Ten to twenty years after infection, the disease load brought on by hepatitis B, C, and D becomes apparent. In light of this, public health officials must know how common these diseases are [1, 4].

With prevalence rates ranging from 10–20%, Vietnam has one of the highest rates of HBV infection among developing nations [5]. Eight million Vietnamese people (8.4% of the population) are chronic HBV carriers, including 3.22 million who are eligible for HBV treatment, yet only an estimated 43,230 (1.34%) of those with HBV infection obtain the necessary therapies [6]. Besides, Vietnamese HBsAg-positive patients revealed a high 15.4% HDV-genome prevalence, compared to 43.3% in acute hepatitis patients [7]. Northern and southern Vietnam had different HDV distributions and genotypes [8]. Patients with HBV infection who also have HDV coinfection or superinfection develop an acute infection, which causes more severe liver damage than HBV mono-infection [8, 9]. Therefore, it is important to detect both viruses simultaneously to prevent complications and provide appropriate treatment [10].

Different methods exist for detecting HBV and HDV infections, including serological and molecular tests. Serological tests can detect the presence of HBV surface antigen (HBsAg) and HDV antibody (anti-HDV) in the blood. Molecular tests can detect the presence of HBV DNA and HDV RNA in the blood or liver tissue [10, 11]. To quantitatively detect HBV [12] and HDV [13, 11],

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real-time PCR assays can measure both viruses' viral loads sensitively, precisely, and accurately; however, this may be costlier and time-consuming due to the need to run them separately. In this study, we optimized and assessed the abilities of a plate-based digital PCR to quantitate HBV DNA and HDV RNA copy numbers individually but in the same run. We compared the sensitivity of the developed digital (RT-) PCR to the real-time (RT-) PCR assays. We calculated the agreement between the two methods by correlation coefficient and Lin's concordance coefficient.

Materials and Methods

Patients and acid nucleic isolation

Between January 2023 and August 2023, 98 patients were enrolled in the study at the University Medical Center of Ho Chi Minh City-Branch 2 (Figure 1). The sample size was determined using a convenience sampling method. All eligible samples meeting the research facility's selection criteria were included during the study period. The sample size depended on the availability of suitable clinical samples within the timeframe and scope of the study. The ethical approval was obtained from the Ethics Committees of Hong Bang International University.

Based on clinical samples, the patients selected for this study were individuals infected with the Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV). Inclusion Criteria: Patients who tested positive for Hepatitis B surface antigen (HBsAg) exhibited signs of co-infection or superinfection with HDV, as determined by previous diagnostic results (e.g., anti-HDV antibody).

Sample Criteria

The study used serum samples from patients confirmed to have HBV/HDV infections, with intact and high-quality samples suitable for viral load quantification. Identification of Potential Patients: Patients were selected from those with a history of chronic Hepatitis B infection and previously diagnosed with HBV through clinical testing. Potential patients with confirmed co-infection or superinfection with HDV were identified through anti-HDV antibodies and HDV-RNA quantification tests. The study was conducted in medical facilities where diagnostic tests for HBV and HDV were routinely performed on patients with a history of hepatitis-related illnesses.

New samples were collected from the same patients if indeterminate results were obtained. The latest samples were re-extracted and re-tested, and the results were compared with those of the original samples in the same run to ensure consistency.

For samples with missing data, attempts were made to supplement the missing information. If the data could not be supplemented, the sample was excluded from the study, and a replacement sample was collected to maintain the integrity of the research.

Total nucleic acid (DNA and RNA) was isolated from the frozen serum using the AccuRive Viral sDNA/RNA Prep Kit (#EX-DRA03.1A, Khoa Thuong) and stored at -20°C before use. This study was designed as a retrospective study, with data collection performed after the index test (digital PCR) and the reference standard (real-time PCR) had been conducted. There was no significant time interval or clinical intervention between the performance of the index test (digital PCR) and the reference standard (real-time PCR). Both tests were conducted consecutively on the same set of serum samples to ensure consistency in comparison.

Real-Time PCR assays

The Real-Time PCR for HBV DNA quantification reactions were performed according to the manufacturer's instructions using the 7500 FAST Real-Time PCR System (ThermoFisher Scientific). The 20 µL qPCR reactions consisted of 5 µL TaqPath™ 1-Step Multiplex Master Mix (ThermoFisher Scientific), 500 nM BF/R, 250 nM BP, 200 nM ICBF/R, 100 nM ICBP, and 5 µL of the extracted DNA sample. The cycling conditions were as follows: an initial cycle of UNG activation for 2 min at 25°C and a denaturation cycle of 2 min at 95°C, followed by 40 cycles of denaturation for 5 sec at 95°C, and annealing for 30 sec at 60°C. No template control was included in all runs, and every sample was measured in triplicate. Ct values were generated, and the 7500 System SDS Software performed linear regression analyses of the calibration curves.

Real-Time RT-PCR for HDV RNA quantification reactions was also performed in the 7500 FAST Real-Time PCR System (ThermoFisher Scientific). The 20 µL qPCR reactions comprised 5 µL TaqPath™ 1-Step Multiplex Master Mix (ThermoFisher Scientific), 500 nM DF/R, 250 nM DP, 200 nM ICDF/R, 100 nM ICDP, and 5 µL extracted RNA sample. The cycling conditions were as follows: an initial cycle of UNG activation for 2 min at 25°C, a cycle of reverse transcription for 10 min at 53°C, a denaturation cycle of 2 min at 95°C, and followed by 40 cycles of denaturation for 5 sec at 95°C, annealing for 30 sec at 60°C.

The choice of real-time PCR as the reference standard was based on its well-established criteria for quantifying viral loads. Using known concentration standards run alongside clinical samples, the reaction criteria for quantitative real-time PCR were achieved, with a slope of -3.32, efficiency ranging from 95% to 105%, and an R^2 value of 1. The threshold cycle (Ct) value was determined based on these criteria, and sample results were interpreted accordingly using real-time PCR.

Plate-based digital PCR assays

The QuantStudio™ Absolute Q™ Digital PCR System (ThermoFisher Scientific, Pub. No. MAN0025621, 2021) was used to quantify the concentration of HBV copy number according to the manufacturer's instructions. The 10 µL digital PCR reaction comprised of 1 X QuantStudio™ Absolute Q™ DNA Digital PCR Master Mix (ThermoFisher Scientific), specific primers and probes (Table 1) for P and HBB genes similar to qPCR for HBV quantification, and 5 µL extracted DNA sample. The cycling conditions were as follows: an initial denaturation cycle of 10 min at 96°C, followed by 40 cycles of denaturation for 5 sec at 96°C, and annealing for 20 sec at 60°C. After amplification, the digital PCR data were analyzed using the QuantStudio Absolute Q Digital PCR software (ThermoFisher Scientific).

For HDV quantification, the extracted RNA was reversed by the SensiFast cDNA Synthesis Kit (Bioline), and 5 µL cDNA was used for the digital PCR reaction similar to the above HBV quantification reaction, except for primers and probes (Table 1) specific for HDV and RNase P. Similar cycling conditions were used.

The threshold for digital PCR was determined by running negative and positive controls with known concentrations alongside the clinical samples. The cut-off for a positive result was established based on the known negative and positive samples to ensure accurate viral quantification.

Statistical analysis

All statistical analyses were performed with SPSS Software Version 20 for Windows (IBM Corp., Armonk, NY, USA), except those explicitly stated. Pearson's correlation coefficient was used to measure the correlation between HBV copy numbers from real-time PCR and digital PCR, and the correlation is considered as strong as $r \geq 0.900$. Lin's concordance coefficient tests assessed the quantitative agreement between digital PCR and quantitative PCR measurements in patient samples. Agreement strength was categorized as poor (≤ 0.90), moderate (> 0.90-0.95), substantial (> 0.95 - 0.99), or perfect (> 0.99). Statistical significance was set at a two-sided p-value less than 0.05.

Results

Dynamic range and sensitivity of real-time PCR and digital PCR assays for HBV DNA quantification

The HBV amplicon sample (10^8 copies/µL) was diluted to 10^0 copies/µL to compare the sensitivity and accuracy between real-time PCR and digital PCR amplification systems. The real-time PCR system detected as little as 5 x 10^1 copies/reaction of HBV amplicon input (Figure 2) with $R^2 = 0.9936$ and E% = 99.97, displaying a high goodness of fit. The real-time PCR exhibited a wide range of detection and reliably measured $5x10^1$ - $5x10^8$ copies/reaction. The system detected as little as 5 copies/reaction of HBV amplicon for digital PCR, which is more sensitive to real-time PCR. However, the digital PCR reaction became entirely saturated when the input of HBV amplicon exceeded 10^6 copies/reaction (data not shown).

Detection and quantification of HBV DNA in patient samples

Compared to real-time PCR assays, digital PCR assays showed similar capabilities of quantifying HBV virus load in all patient samples with strong correlation values ($R^2 = 0.944$) (Figure 3). Lin's concordance coefficient presented substantial levels of agreement (CCC = 0.963, 95% CI = 0.946 – 0.975). In addition, 2% (2/98) of the clinical samples exhibited low HBV concentrations that the digital PCR could detect but not the real-time PCR. The percentage of HBV identified by real-time PCR and digital PCR were 71.4% (70/98) and 73.5% (72/98), respectively.

Dynamic range and sensitivity of real-time RT-PCR and digital RT-PCR assays for HDV RNA quantification

The HDV amplicon sample (10^8 copies/µL) was diluted to 10^0 copies/µL to assess the sensitivity and accuracy of real-time RT-PCR and digital RT-PCR amplification systems. The real-time RT-PCR technique recognized as few as 10^1 copies/µL ($5x10^1$ copies/reaction) of HDV amplicon input (Figure 4), and it had high goodness of fit ($R^2 = 0.996$ and E% = 99.85). The detection range of real-time RT-PCR was extensive and could reliably measure $5x10^1$ - $5x10^8$ copies/reaction. The digital RT-PCR was more sensitive than real-time RT-PCR, detecting just 5 copies of the HDV amplicon per reaction. However, when

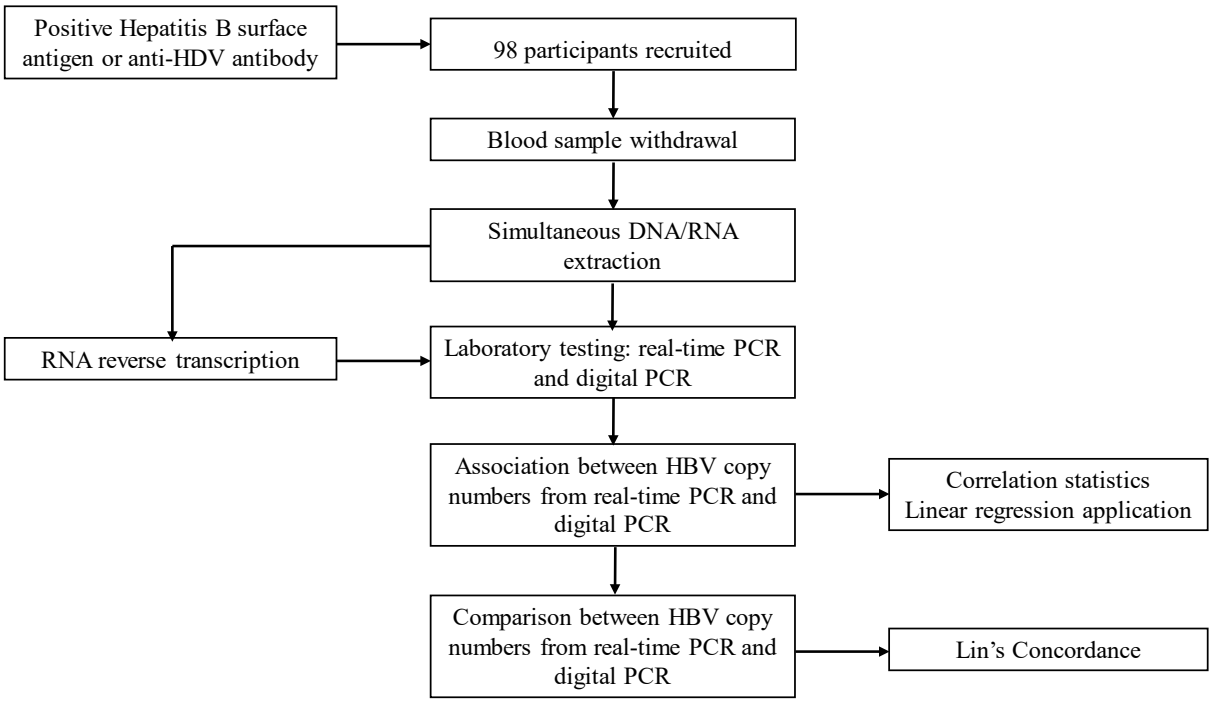

Figure 1. Workflow of the Study

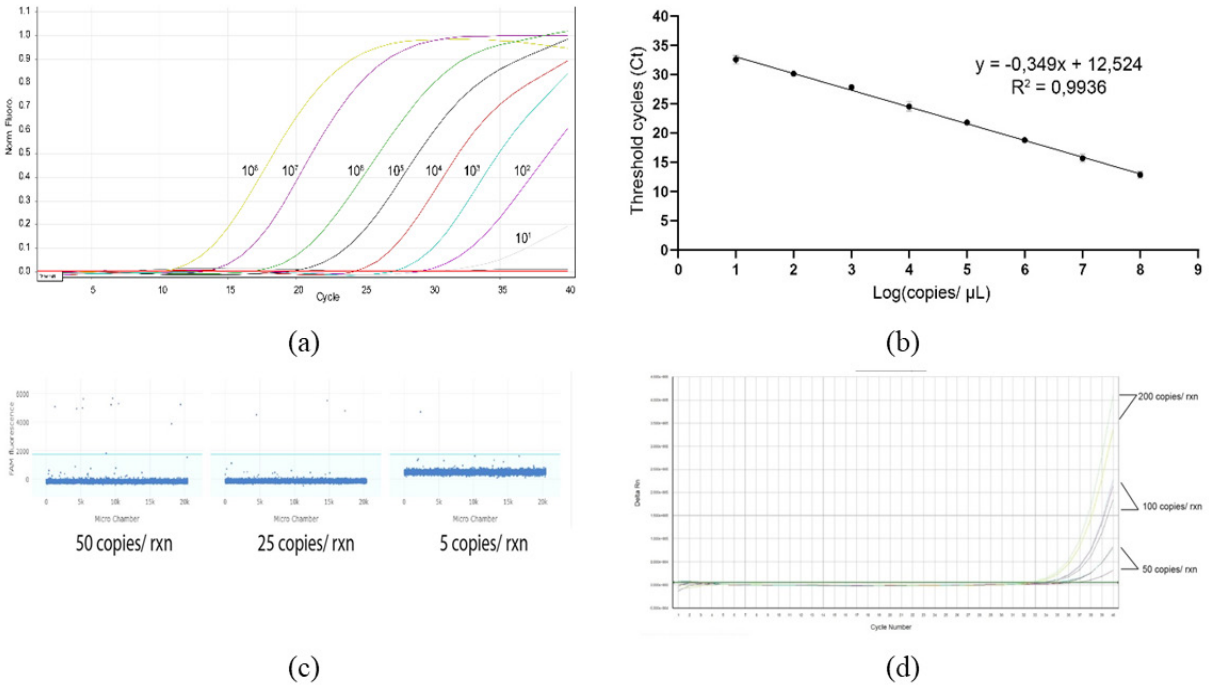

Figure 2. Quantification of Serially Diluted HBV Amplicon by Qualitative PCR and Digital PCR. The DNA amplicon was serially diluted from 10^8 copies/µL to 10^0 copies/µL. (a) qPCR amplification plot at 10-fold serial diluted HBV amplicon levels, (B) qPCR standard curve from a 10-fold dilution series of HBV amplicons, the slope of the fitted line is -0.349 and R^2 = 0.9936, (c) digital PCR analysis plot at low levels of HBV amplicons, (d) real-time PCR amplification plot at low levels of HBV amplicons.

the HDV amplicon input exceeded 10^6 copies/reaction, the digital RT-PCR reaction became completely saturated (data not shown).

Detection and quantification of HDV RNA in patient samples

Digital RT-PCR techniques demonstrated equivalent capability to real-time RT-PCR assays in estimating HDV virus load in all patient samples with high correlation values (R^2 = 0.900) (Figure 5). Lin's concordance coefficient (CCC = 0.933, 95% CI = 0.906 – 0.953) measured significant levels of moderate agreement between the two assays. Then again, only the digital RT-PCR could detect the low HDV quantities in 2% (2/98) of the clinical samples. Real-time RT-PCR and digital RT-PCR each revealed 3.1% (3/98) and 5.1% (5/98) of HDV, respectively.

No adverse events were reported or observed from performing either the index test (digital PCR) or the reference standard (real-time PCR), as both tests are non-invasive and involve standard laboratory procedures on serum samples.

Discussion

The epidemiology of hepatitis B has undergone

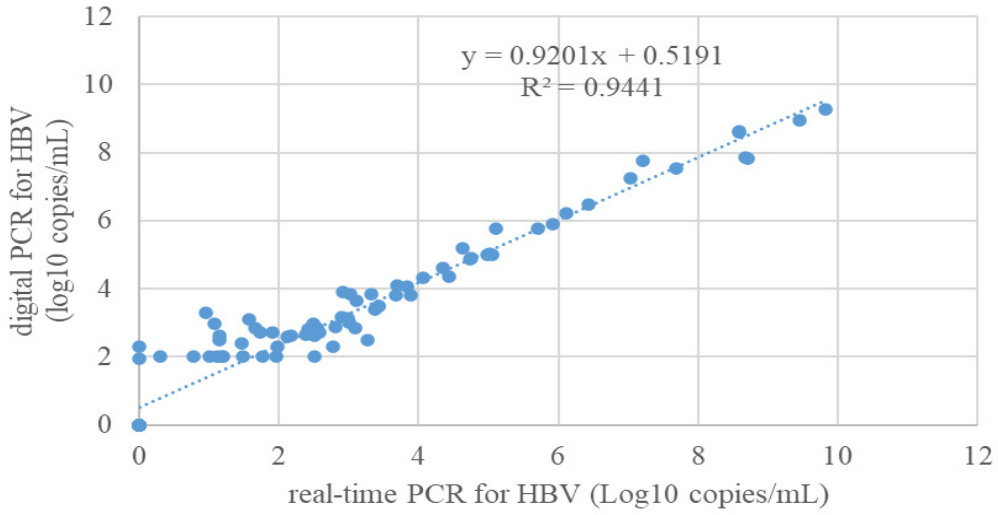

Figure 3. The Correlation Coefficient of HBV Virus Load Quantification by Real-Time PCR and Digital PCR

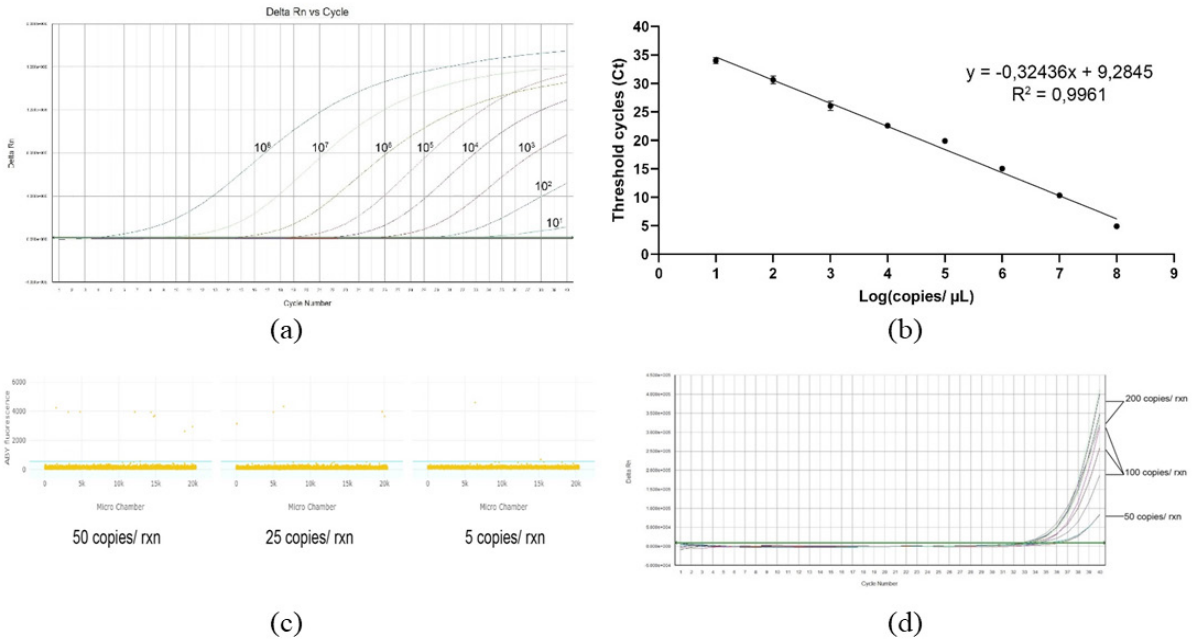

Figure 4. Quantification of Serially Diluted HDV Amplicons by Real-Time RT-PCR and Digital RT-PCR. The amplicon was serially diluted from 10^8 copies/µL to 10^0 copies/µL. (a) real-time RT-PCR amplification plot at 10-fold serial diluted HDV amplicon levels, (b) real-time RT-PCR standard curve from a 10-fold dilution series of HDV amplicon, the slope of the fitted line is -0.32436 and $R^2 = 0.9961$, (c) digital RT-PCR analysis plot at low levels of HDV amplicons, (d) digital RT-PCR amplification plot at low levels of HDV amplicons.

considerable changes in the past decade, primarily due to the introduction of vaccination initiatives against HBV, resulting in a notable decrease in the infection's prevalence among the global population [14, 15]. Typically, individuals who are infected with HBV frequently experience either HDV superinfection or co-infection, leading to hepatitis delta [9, 16]. Hepatitis delta continues to pose a significant medical challenge, owing to its unique and rapid progression towards liver cirrhosis and hepatocellular carcinoma, coupled with the limited treatment options presently available[16, 9]. The measurement of HDV viral load is crucial for evaluating the effectiveness of treatment and for providing a more precise clinical staging of chronic HDV infection[9, 16]. Indeed, HDV viremia has a considerable influence on the progression of the disease, demonstrating a correlation with poorer clinical outcomes and being associated with an elevated long-term risk of liver-related complications, including hepatic decompensation, hepatocellular carcinoma, or liver-related mortality/transplantation [17, 8]. In this research, we have utilized a novel digital PCR technique for the first time to develop and refine an assay aimed at quantitatively assessing HBV-DNA and HDV-RNA as a substitute for conventional RT-qPCR.

Digital PCR, in contrast to real-time PCR, utilizes the technique of dividing the PCR mix into numerous partitions in conjunction with Poisson statistics, which allows for accurate and definitive quantification of a molecular

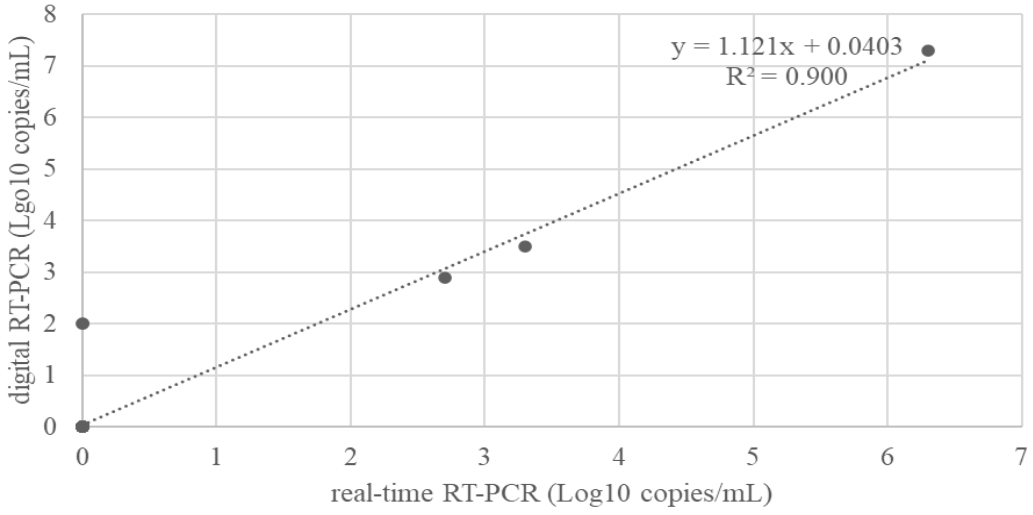

Figure 5. The Correlation Coefficient of HDV Virus Load Quantification by Real-Time RT-PCR and Digital RT-PCR

Table 1. Primer and Probe Sequences for Real-Time PCR and Digital PCR Assays

Name	Gene	Sequences (5' – 3')	Length (bp)	Products (bp)	Ref
BF	P	AGAATCCTCACAATACCGCAGAGT	24	74	21
BR		CACACGGTAGTTCCCCCTAGAA	22		
BP		FAM-AGACTCGTGGTGGACTTCTCTCAAT	25		
DF	Small delta antigen	GCATGGTCCCAGCCTCCT	18	213	22
DR		TCTTCGGGTCGGCATGG	17		
DP		ABY-ATGCCCAGGTCGGAC-MGB	15		
ICBF	HBB	5'-CAGGTACGGCTGTCATCACTTAGA-3'	24	185	23
ICBR		5'-CATGGTGTCTGTTTGAGGTTGCTA-3'	24		
ICBP		Cy5-GCCCTGACTTTTATGCCCAGCCCTG	25		
ICDF	RNase P	AGATTTGGACCTGCGAGCG	19	65	13
ICDR		GAGCGGCTGTCTCCACAAGT	20		
ICDP		Cy5-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	23		

target while reducing the impact of PCR inhibitors [18]. By eliminating the requirement for a standard curve to estimate target copy numbers as is done in real-time PCR, digital PCR achieves greater consistency and accuracy in quantification. Nevertheless, inconsistencies may arise in digital PCR when determining the threshold fluorescence that categorizes a well as either positive or negative. Fluctuations in micro-well fluorescence can result from factors such as primer/probe concentrations, baseline autofluorescence, and intermediate fluorescence, known as rain, which can hinder interpretation [17].

This study assessed the efficacy of digital PCR platforms in contrast to real-time PCR platforms to build a novel, reliable approach for the absolute quantification of HBV and HDV in clinical samples that would also provide power, accurate, and precise tools for general virology research. Good linearity was found using both approaches. However, digital PCR showed a relatively small dynamic range due to the saturation of positive digital chambers at higher target concentrations [19]. Even so, compared to the real-time PCR assays, the digital PCR assays demonstrated superior reproducibility, particularly for samples at lower concentrations [19, 20]. With digital PCR, direct measurements are made using the Poisson method, which yields an absolute quantification as opposed to qPCR data, which is based on a standard curve. Because end-point fluorescence signals are collected and binomial events are counted (positive or negative), digital PCR is less vulnerable to PCR inhibitors and efficiency fluctuation. In general, our study presented that the digital (RT-)PCR assays exhibited superior reliable and reproducible quantitative results over the real-time (RT-)PCR, especially in scenarios with few copy number samples.

In summary, we described using digital PCR systems as novel techniques for precisely and sensitively measuring HBV DNA and HDV RNA. The real-time PCR techniques could not identify the very low viral loads in serum samples of the hepatitis B and D viruses. At the same time, the optimized digital PCR assays were successful in measuring them. These assays were very successful and reproducible, making them valuable tools for monitoring hepatitis B / hepatitis D patients and determining the likelihood that the disease will advance and the efficacy of treatment.

The study faced limitations due to ethical restrictions, allowing only minimal patient demographic and clinical data collection. Convenience sampling was used, introducing potential selection bias, as samples were collected based on availability at a single medical facility within a set timeframe. This approach may limit the patient population's diversity and the findings' generalizability to other regions. Although digital PCR showed higher sensitivity than real-time PCR, the small sample size resulted in statistical uncertainty, with broad confidence intervals affecting the robustness of conclusions. Furthermore, the study did not differentiate between pre-specified and exploratory diagnostic accuracy or analyze disease severity, as comprehensive clinical details were unavailable. The analysis focused solely on viral load measurements. Additionally, alternative diagnoses for patients without HBV or HDV were not examined. Future research should address these limitations, explore diagnostic variability in different clinical settings, and incorporate a broader range of clinical and demographic data.

In conclusion, this study successfully optimized and validated plate-based digital PCR assays to simultaneously quantify Hepatitis B virus (HBV) DNA and Hepatitis D virus (HDV) RNA in patient serum samples. The digital PCR assays demonstrated superior sensitivity to conventional real-time PCR, particularly in detecting low viral loads missed by real-time PCR. This makes digital PCR a valuable tool for more accurate diagnosis and monitoring of patients with HBV and HDV, especially those with co-infections. The ability to detect lower viral quantities provides clinicians with better information for patient management, potentially improving outcomes by allowing for earlier intervention and tailored treatments. These findings highlight the potential of digital PCR as a reliable and reproducible method in clinical virology, particularly for infections where accurate viral load quantification is crucial for disease prognosis and therapy efficacy.

Author Contribution Statement

TTTH, ATH, TMH, ATN: Designed the study, obtained ethical approval for the research, conducted experiments using real-time RT-PCR, contributed to manuscript writing, and reviewed and approved the manuscript. TTTH, NTN, YTHV, DHN, ATN: Conducted digital RT-PCR experiments, contributed to manuscript writing, and reviewed and approved it. TTTH, ATH, ATN: Contributed to protocol development and manuscript writing and reviewed and approved the manuscript.

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Ethical Declaration

Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the Hong Bang, Ho Chi Minh City, Vietnam. The decision number of the permission from the Ethical committee: 01/PCT-HĐĐĐ-ĐVN, Hong Bang University, Ho Chi Minh City, Vietnam. All the samples used in this study were agreed upon by Hong Bang University and obtained from all participants in the current study. All patients signed informed consent before entering the study. No study drug or procedure was applied. This is an observational study.

Availability of data and materials

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors have no conflict of interest.

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