Digital Droplet PCR (ddPCR) for Detection and Quantitation of Hepatitis Delt Virus

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Research Article

Keywords: Anti-HDV, Reverse-transcriptase polymerase chain reaction, ELISA, HBV-related liver disease, Liver failure

DOI: https://doi.org/10.21203/rs.3.rs-791508/v1

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Abstract

Background & Aims

The prevalence of hepatitis delt virus (HDV) far exceeds our expected level, there remains a lack of reliable quantitative assays for HDV RNA detection. We sought to develop a new method based on digital droplet PCR (ddPCR) for HDV RNA quantitative detection.

Methods

With plasmid (pMD19T) containing HDV full-genome, we determined the method for ddPCR-based HDV RNA quantification. To compare various assays for HDV detection, 30 cases diagnosed hepatitis D and 14 controls were examined by ELISA, RT-PCR and ddPCR. 728 HBV-related patients including 182 chronic hepatitis B (CHB), 182 liver cirrhosis (LC), 182 hepatocellular carcinoma (HCC) and 182 liver failure (LF) were screened for HDV infection.

Results

The limit of detection of ddPCR for HDV is significantly low, which lower limit of detection (LLoD) and lower limit of quantitation (LLoQ) to be 5.51 copies/reaction (95% CI: 1.15–6.4*105) and 0.18 copies/reaction (95% CI: 0.0012151- 0.76436), respectively. Among the 44 samples, ELISA detected 30 cases positive for anti-HDV, ddPCR reported 24 samples and RT-PCR reported 10 samples positive for HDV RNA. Moreover, the positive rates of anti-HDV IgG were 1.1%, 3.3%, 2.7% and 7.1% in patients with CHB, LC, HCC, and LF; the detection rates of RT-PCR in HDV RNA were 0%, 16.67%, 15.4% and 20%, however, the detection rates of ddPCR were 0%, 33.33%, 30.77% and 60%.

Conclusion

We establish a high sensitivity and high specificity quantitative HDV RNA detection method based on ddPCR compared to RT-PCR. HBV-related end-stage liver disease, especially liver failure, are associated with a remarkably high rate of HDV infection.

Introduction

Hepatitis delta virus (HDV) was found in the nucleus of hepatocytes in 1977 by Italian scholars and is the smallest known human virus. HDV is a defective RNA virus, and its proliferation and propagation are dependent on the assistance of hepatitis B virus (HBV) to provide the viral envelope (1). Full-length genomic nucleotide sequencing and phylogenetic analyses have identified eight genotypes of HDV, with separation among genotypes up to 40% over the full-length sequence. HDV-1, which comprises 4 subgenotypes, is the most prevalent genotype worldwide, and the geographic distributions of genotypes show obvious differences (2). Compared with HBV monoinfection, patients with HDV and HBV co-infection have the most severe form of viral hepatitis. HDV infection significantly accelerates disease progression of chronic hepatitis B, which progresses to cirrhosis within 5 years and to hepatocellular carcinoma within 10 years on average (3, 4).

The latest meta-analysis showed that 0.16% of the general population is estimated to be positive for anti-HDV antibodies, with approximately 10.6% of patients infected with HBV being coinfected with HDV worldwide (5). Another recent study estimated that 48–60 million individuals are infected globally (6). Additionally, the prevalence of HDV differs significantly among geographic regions. One study conducted in 2011 to 2016 in the United States showed that 42% of adult HBsAg carriers have antibodies against HDV (7). In China, a recent meta-analysis showed an anti-HDV antibody prevalence rate of 2.1% among HBsAg carriers and 0.4% among the general population (5). However, the number of hepatitis D cases reported from 2016 to 2020 was 441, 481, 356, 352 and 187 for these years, revealing a large discrepancy from the number based on the prevalence of the national viral hepatitis seroepidemiological survey (8). Approximately 10% of HBsAg-positive individuals in China are estimated to be infected with HDV, and approximately 90 million individuals in China have HBV infection (9, 10). Due to the limited ability to effectively detect HDV, infection with this virus is likely to be a major obstacle for achieving the WHO's goal of eliminating viral hepatitis, including chronic hepatitis B, C, and D, worldwide by 2030.
At present, the limitations of HDV laboratory examinations are one of the important reasons why HDV infection is underestimated. All immunocompetent patients infected with HDV can produce anti-HDV antibodies, including IgM and IgG. Positivity for anti-HDV IgM indicates HDV replication, whereas IgG suggests a previous HDV infection and persists for many years. Serum HD antigen is detectable only transiently in blood specimens collected early at the onset of HDV infection, before the rise of antibody titers. In clinical laboratories, ELISA to detect anti-HDV IgM or IgG is the most common measure to screen for HDV infection. On the one hand, there is a lack of uniform quality standards for kits produced by different manufacturers, and the results of different laboratories are not comparable; on the other hand, test results are inaccurate if the virus infection occurs within the window period and is closely related to the patient's own immune status (11). Although the quantitative microarray antibody capture assay, which has high specificity and sensitivity, is likely an ideal tool for population screening (12), it is not widely used. However, HDV RNA detection is considered the "gold standard" for diagnosing hepatitis D infection. Recently, RT-PCR (real-time reverse-transcriptase polymerase chain reaction) assays have been employed for relatively quantitative detection of HDV, and the sensitivity and accuracy have improved significantly (13). The detection limit of RT-PCR for HDV is approximately 1000 copies/µl, but it is not useful for all the various genotypes (1). Moreover, due to the complexity of primers and probe design, this assay is not well standardized, and results from different laboratories are difficult to compare (13).

The droplet digital PCR (ddPCR) system is based on sample dropletization. After PCR amplification, the concentration of the target molecule can be quantified to 1 copy/µl. In this study, we developed and present a new assay for HDV measurement based on ddPCR that is characterized by improved sensitivity and accuracy compared with RT-PCR. Here, we compare ELISA, RT-PCR and ddPCR for HDV RNA detection and explore the prevalence and quantity of HDV in patients with HBV-related liver disease.

Patients And Methods

Clinical data

From 2015 to 2020, a total of 772 unique serum samples from HBV-infected patients were collected at Beijing Youan Hospital, Capital Medical University. Among them, the 44 subjects enrolled in the study included 30 cases of HBV and HDV coinfection; 14 cases of HBV infection alone were used as negative controls. In addition, 728 HBsAg+ serum samples were employed for HDV screening, including 182 patients diagnosed with chronic hepatitis B (CHB), 182 diagnosed with HBV-related liver cirrhosis (LC), 182 diagnosed with HBV-related liver failure (LF) and 182 diagnosed with HBV-related hepatocellular carcinoma (HCC). The HBV viral load and HBV serum markers of all enrolled samples were analyzed by the laboratory department of Beijing You'an Hospital. Patient information, including age and sex, was obtained from medical records. A serum sample from each patient was collected and stored at -80°C until processing. For all samples collected, details were anonymized during subsequent laboratory tests.

The protocol of the current investigation was conforming to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Beijing You'an Hospital's human research committee. Informed consent was obtained from all patients. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki established in 1975 and revised in 1983.

Enzyme-linked immunosorbent assay (ELISA)

Serum anti-HDV IgM and anti-HDV IgG were determined with enzyme-linked immunosorbent assays (ELISAs) using commercial kits (Abbexa Ltd, Cambridge, UK) according to the manufacturer's instructions. Briefly, serum samples were diluted at 1:11 with diluent; the appropriate positive and negative controls were established. Sample and blank wells on the precoated plate were added, and the plate was covered and incubate at 37°C for 30 min. Then, the plate was washed 5 times with wash buffer, the detection reagent was added, and the plate was incubated and washed as above. Finally, 50 µl of TMB substrates A and B were added, followed by incubation at 37°C for 10 min; 50 µl of stop solution was added to each well, and optical density was measured at 450 nm.

RNA extraction and reverse transcription

Total RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) from 150 µl serum samples according to the manufacturer's instructions. Finally, 50 µl nucleic acid extract was obtained and used for reverse transcription.

RNA was reverse transcribed using PrimeScriptTM 1st Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) with random primers and oligo(dT) primers. The reaction program was as follows: 30°C, 10min; 42°C, 45min; 95°C, 5min, 70°C, 15min, and 4°C forever. The cDNA product was used for subsequent RT-PCR and ddPCR tests simultaneously.
Results

Sensitivity and dynamic range of the ddPCR assay to detect HDV

The sensitivity of the ddPCR method for HDV detection was assessed using the plasmid pMD19T containing the HDV full genome, as shown in the supplemental materials. A 10-fold serial dilution of the plasmid, ranging from $10^6$ to $10^0$, was prepared to test the linearity of the ddPCR method using primer and probe sets targeting the HDV common sequence (Fig. 1A). Linear regression analysis showed that this method has excellent linear correlation between the detected value and expected value, with $R^2 = 0.9985$ (Fig. 1B). In addition, RT-PCR was used to detect the above serially diluted plasmid, with a reported range of $10^3$ to $10^6$ copies/reaction. When the target concentration was higher than $10^3$ copies/reaction, RT-PCR displayed good linear correlation, with $R^2 = 0.9995$ (Fig. 1C). These results demonstrate that compared with RT-PCR, ddPCR has a wider detection range, with a lower limit of detection, even reaching 1 copy/reaction.

Determining the LLoD and LLoQ of the ddPCR assay for HDV detection

Next, we performed probit analysis with a sigmoid curve to determine the lower limit of detection (LLoD) and lower limit of quantitation (LLoQ) of both ddPCR and RT-PCR. First, HDV RNA was extracted from a positive serum sample and then reverse transcribed to cDNA and tested using ddPCR. The sample was serially diluted 10-fold to concentrations from $10^{-2}$ to $10^3$ copies/reaction. Each concentration was tested in 7 replicates, and the LLoQ and LLoD were determined by probit regression analysis with MedCalc software 19.0.4, and the lowest concentrations of 95% and 50% positive samples were detected.

Continuous variables are presented as mean ± standard deviations (SDs). A p value < 0.05 was considered statistically significant.

Statistical analysis

For ddPCR assay characterization, the data were analyzed with specific software to calculate the concentration of the target. The coefficient of determination was assessed by linear regression analysis using GraphPad Prism 8.00. In addition, LLoD and LLoQ were calculated by probit regression analysis with MedCalc software 19.0.4, and the lowest concentrations of 95% and 50% positive samples were detected.
384.62 (95%CI: 116.10–1047.0) and 4328.89 (95%CI: 1431.00–167353.0), respectively (Fig. 2B). Obviously, both ddPCR and RT-PCR were reliable when testing high-concentration samples, but ddPCR performed more precisely when detecting samples with concentrations lower than $10^3$ copies/reaction.

**Specificity of the ddPCR assay for detecting HDV**

To assess the specificity of ddPCR for HDV detection, we used various kinds of plasmids, including plasmids containing the full-length HBV genome, HCV plasmids, HIV plasmids and HDV plasmids, to perform three independent tests. According to the results, amplification of plasmids other than the HDV plasmid led to no positive signal, which confirms the high specificity of the ddPCR method with the primers/probe set targeting HDV (Fig. 3A).

In addition, we evaluated the specificity and effectiveness of ddPCR for HDV detection. We extracted virus nucleic acids from the serum of patients who were clinically confirmed, including cases of monoinfection with HBV, coinfection with HBV and HCV, coinfection with HBV and HIV, and coinfection with HBV and HDV. The above samples were tested using the ddPCR method in parallel, and positive events were only found for patients with coinfection of HBV and HDV (Fig. 3B). In summary, ddPCR for HDV detection is a highly specific method that significantly reduces false positive results.

**Detection efficiencies of ddPCR, RT-PCR and ELISA for HDV**

In this study, 44 samples were collected from clinical patients, and the clinical information is summarized in Table 1 and supplement Table 1. Thirty samples were from patients clinically diagnosed with hepatitis D, and 14 samples were determined to be HBV infection alone. First, we performed ELISA to detect anti-HDV IgG, and 30 samples were positive, consistent with the clinical diagnosis. Afterwards, viral nucleic acids were extracted from 44 samples simultaneously, and cDNA products were obtained after reverse transcription. These products were used for ddPCR, RT-PCR at the same time.
| Variables | HBV related markers | HDV related markers |
|-----------|---------------------|---------------------|
|           | HBV viral load (IU/mL) | HBsAg | Anti-HBs | HBeAg | Anti-HBe | Anti-HBc | RT-PCR (copies/reaction) | ddPCR (copies/reaction) |
| Patient 1 | 200 | + | + | - | - | + | + | N.D. | 2229.00 |
| Patient 2 | 1006 | + | + | - | - | - | + | 17051.94 | 41597.30 |
| Patient 3 | 3*10^5 | + | + | - | + | - | + | 16992.01 | 21315.50 |
| Patient 4 | 4616 | + | - | - | + | - | + | 8019.70 | 19976.60 |
| Patient 5 | 208 | + | + | - | + | - | + | 6307.53 | 4461.50 |
| Patient 6 | 2323 | + | + | - | + | - | + | N.D. | 965.10 |
| Patient 7 | 1.6*10^4 | + | + | - | + | - | + | N.D. | 283.90 |
| Patient 8 | 2.7*10^5 | + | - | + | - | + | + | 184320.00 | 299876.00 |
| Patient 9 | 3008 | + | + | + | + | - | + | 7009.88 | 8992.20 |
| Patient 10 | 7.9*10^4 | + | + | + | + | - | + | N.D. | 600.24 |
| Patient 11 | 6009 | + | + | - | + | + | + | N.D. | 329.01 |
| Patient 12 | 3760 | + | + | - | + | + | + | N.D. | 230.02 |
| Patient 13 | 600 | + | + | - | + | - | + | 60088.21 | 88230.00 |
| Patient 14 | 2989 | + | + | - | + | - | + | 25632.02 | 605.10 |
| Patient 15 | 2.1*10^4 | + | - | - | + | + | + | N.D. | 300.00 |
| Patient 16 | 6.7*10^6 | + | - | + | - | + | + | N.D. | 221.0 |
| Patient 17 | 5.2*10^4 | + | - | + | - | + | + | N.D. | 772.0 |
| Patient 18 | 3021 | + | + | + | - | + | + | 6000.21 | 5580.00 |
| Patient 19 | 6701 | + | + | - | + | - | + | N.D. | N.D. |
| Patient 20 | 880 | + | + | - | + | - | + | N.D. | 390.00 |
| Patient 21 | 1554 | + | + | - | - | - | + | N.D. | N.D. |
| Patient 22 | 990 | + | - | - | + | - | + | 7442.10 | 8810.00 |
| Patient 23 | 1228 | + | + | - | + | - | + | N.D. | 280800 |

N.D.: Not detected.
| Variables | HBV related markers | HDV related markers |
|-----------|---------------------|---------------------|
|           | HBV viral load (IU/mL) | HBsAg | Anti-HBs | HBeAg | Anti-HBe | Anti-HBc | Anti-HDV | RT-PCR (copies/reaction) | ddPCR (copies/reaction) |
| Patient 24 | 2.2*10^4 | +     | +       | +      | -       | -       | +       | N.D. | 190800 |
| Patient 25 | 394     | +     | +       | -      | +       | -       | +       | N.D. | N.D.   |
| Patient 26 | 5.3*10^5 | +     | -       | +      | -       | +       | +       | N.D. | N.D.   |
| Patient 27 | 7217    | +     | +       | -      | +       | -       | +       | N.D. | 190.20 |
| Patient 28 | 209     | +     | +       | -      | +       | -       | +       | N.D. | N.D.   |
| Patient 29 | 1020    | +     | +       | -      | +       | -       | +       | N.D. | 330800 |
| Patient 30 | 330     | +     | +       | -      | -       | -       | -       | +   | N.D.   |
| Patient 31 | 3.3*10^4 | +     | +       | +      | +       | +       | -       | N.D. | N.D.   |
| Patient 32 | 8.1*10^6 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 33 | 7.3*10^7 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 34 | 2001    | +     | +       | -      | +       | -       | -       | N.D. | N.D.   |
| Patient 35 | 9.2*10^5 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 36 | 1.0*10^6 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 37 | 8.2*10^4 | +     | +       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 38 | 5.6*10^4 | +     | +       | +      | +       | +       | -       | N.D. | N.D.   |
| Patient 39 | 6009    | +     | +       | +      | +       | -       | -       | N.D. | N.D.   |
| Patient 40 | 2.9*10^4 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 41 | 5.1*10^6 | +     | -       | +      | -       | +       | -       | N.D. | N.D.   |
| Patient 42 | 3.2*10^4 | +     | +       | -      | +       | -       | -       | N.D. | N.D.   |
| Patient 43 | 377     | +     | +       | -      | +       | -       | -       | N.D. | N.D.   |
| Patient 44 | 6.2*10^5 | +     | +       | -      | +       | -       | -       | N.D. | N.D.   |

N.D.: Not detected.
The results of nucleic acid analysis showed that RT-PCR was only able to detect 10 positive samples; ddPCR detected 24 positive samples, 14 more than RT-PCR (Fig. 4A, B). Based on our results, positivity for anti-HDV IgG indicates that the patient had a previous infection but that perhaps no virus remained due to the application of antiviral drugs. For the presence of low levels of HDV in patients, the detection efficiency of ddPCR was significantly higher than that of RT-PCR.

Detection of HDV in patients with HBV-related diseases by ELISA, RT-PCR and ddPCR

A total of 728 samples were examined in this study, including 182 from patients with chronic hepatitis B, 182 from patients with HBV-related liver cirrhosis, 182 from patients with HBV-related liver failure and 182 from HBV-related hepatocellular carcinoma (Supplement Fig. 1), which basic information were summarized in supplement Table 2. First, anti-HDV IgG and anti-HDV IgM in these samples were detected using ELISA methods. As shown in Table 2, the results were as follows: 1.1% of patients with chronic hepatitis B were anti-HDV IgG positive; 3.3% of patients with liver cirrhosis were anti-HDV IgG positive, and 1.1% were anti-HDV IgM positive; 2.7% of patients with hepatocellular carcinoma were anti-HDV IgG positive, and 1.6% were anti-HDV IgM positive; and 7.1% of patients with liver failure were anti-HDV IgG positive, and 2.2% were anti-HDV IgM positive. Next, total RNA was extracted from the 26 samples that anti-HDV positive mentioned above. RT-PCR results showed that no HDV RNA was detected in patients with chronic hepatitis B; 0.55% of liver cirrhosis and hepatocellular carcinoma cases were positive for HDV RNA and only 0.1% of patients with liver failure. The ddPCR results were as follows: no HDV RNA positivity in patients with chronic hepatitis B; 1.1% of patients with liver cirrhosis; 1.6% of patients with hepatocellular carcinoma; and 2.2% of patients with liver failure. These results indicate that the ddPCR we established is a high-sensitivity and high-accuracy detection method for HDV, especially for HDV RNA less than $10^2$ copies/µl, which is significantly superior to RT-PCR.

| Patient cohorts | Anti-HDV IgG | Anti-HDV IgM | RT-PCR | ddPCR |
|-----------------|--------------|--------------|--------|-------|
|                 | Number       | Positive rate| Number | Positive rate| Number | HDV positive rate in total | RNA positive rate in HDV infection | Number | HDV positive rate in total | RNA positive rate in HDV infection |
| Chronic hepatitis B (n = 182) | 2            | 1.1%         | 0      | -          | 0      | -                         | 0      | -                         | 0      | -                         |
| Liver cirrhosis (n = 182)   | 6            | 3.3%         | 2      | 1.1%       | 1      | 0.55%                     | 16.67% | 2                         | 1.1%   | 33.33%                    |
| Liver failure (n = 182)     | 13           | 7.1%         | 4      | 2.2%       | 2      | 1.1%                     | 15.4%  | 4                         | 2.2%   | 30.77%                    |
| Hepatocellular carcinoma (n = 182) | 5            | 2.7%         | 3      | 1.6%       | 1      | 0.55%                     | 20%    | 3                         | 1.6%   | 60%                       |

In addition, we found that HDV prevalence differs among patients with chronic hepatitis B, HBV-related liver cirrhosis, HBV-related liver failure and HBV-related hepatocellular carcinoma. Patients with confirmed chronic hepatitis B showed a relatively low HDV infection rate of 1.1% according to anti-HDV IgG compared with those with liver cirrhosis, hepatocellular carcinoma, and liver failure. In patients with liver cirrhosis and hepatocellular carcinoma, HDV infection rates were 3.3% and 2.7% according to anti-HDV IgG, respectively. In patients with liver failure, anti-HDV IgG positivity was detected in 7.1%, which indicated that coinfection of HDV and HBV significantly accelerates the progression of liver disease.

Discussion
It is important to develop an accurate HDV RNA quantitative test, which is critical for HDV diagnosis and guiding treatment response. Several meta-analyses in recent years have reported an infection rate of HDV of approximately 0.8% of the general population and 13.02% of the HBsAg-positive population (6), even though the anti-HDV positive rate increases by 3–4 times among those with liver diseases (5). According to estimates, there are approximately 12 million people infected with HDV globally. Nevertheless, detection of HDV is often neglected clinically. There may be two reasons: on the one hand, many clinicians lack awareness of the serious disease consequences caused by HDV infection; on the other hand, there are no standard and accurate methods for detecting HDV infection (14, 15). There have been many efforts for HDV detection, including HDV antibody screening by ELISA (16–18) and quantitative microarray antibody capture assays (12). However, there is a lack of progress in testing HDV RNA quantitatively, and RT-PCR, which is insufficient regarding sensitivity and accuracy, remains the method for confirming the diagnosis and management of patients (19–22). Therefore, a method of HDV RNA quantitation with high sensitivity and specificity for the confirmation of HDV infection and treatment monitoring is urgently needed.

In this study, we developed a new method of detecting HDV RNA based on ddPCR, which enables absolute quantification of the serum virus with excellent sensitivity and high specificity. ddPCR is a very sensitive and reproducible technique that has been used for testing in various fields. Studies have indicated that ddPCR can be used for the detection and quantitation of HBV cccDNA in the liver of individuals with occult HBV infection (23), for the detection of TP53 deletions and point mutations in chronic lymphocytic leukemia (24), and as a more accurate tool for SARS-CoV-2 detection in low-viral load specimens (25). The more accurate HDV RNA detection method established in this study significantly improves the diagnostic ability of HDV infection, including confirmation of HDV infection, especially in patients with low viral loads (cases that are undetermined by RT-PCR), monitoring the treatment effect of various antiviral drugs and evaluation of disease progression.

ddPCR methods have obvious superiority in HDV RNA detection compared with other available methods. Compared with RT-PCR, our results showed that the sensitivity threshold of these assays was approximately 10^3 copies/µl, which is significantly lower than the sensitivity of ddPCR at 1 copy/µl. Moreover, the results obtained from different laboratories are often not comparable due to the use of different primer sets and the nonuniformity of the amplified RNA region (26–28). More importantly, commercial and in-house RT-PCR assays in 55% of laboratories often underestimate or fail to quantify HDV viremia, according to the French national quality control study (29). Therefore, considering the limitation of RT-PCR assays for quantitative HDV RNA, the development of more accurate methods for nucleic acids is particularly necessary at the present time. In addition, detecting HDV-specific IgM or IgG with ELISA is an indispensable approach, especially for the screening of a large range of HBsAg-positive populations. Nonetheless, the widow period for detection is relatively short (30). Anti-HDV IgM typically appears in serum at 2 to 3 weeks after the onset of symptoms and disappears by 2 months after acute HDV infection. Therefore, anti-HDV IgG, as a serologic scar, is commonly used for HDV screening because it can persist in serum after the resolution of acute HDV infection and in chronic HDV infection. Furthermore, anti-HDV detection is usually a false negative in immunodeficiency patients, such as those with AIDS. However, confirmation of HDV infection still relies on detectable HDV RNA as a “gold standard” for HDV diagnosis and management.

In addition, we screened HDV infection in different patient cohorts, including cases of chronic hepatitis B, HBV-related cirrhosis, HBV-related HCC, and HBV-related liver failure. Most striking is the finding that the HDV infection rate was as high as 7.4% in patients with HBV-related liver failure and that it was relatively low in patients with cirrhosis or HCC and lowest in patients with chronic hepatitis B. Moreover, we developed RT-PCR and ddPCR for all anti-HDV-positive samples; expectedly, the detection rate of HDV RNA with ddPCR was significantly higher than that of RT-PCR. Such RNA-undetectable but anti-HDV IgM-positive samples might be due to RNA degradation during storage or cases with no active viral replication. Nonetheless, the results strongly indicate that we should conduct early screening for HDV infection in HBsAg-positive individuals, as early detection of HDV infection and timely antiviral intervention can effectively slow the progression of severe liver disease. According to a recent study, there are approximately 120 million people who are positive for HBsAg in China. Most liver failure patients develop chronic hepatitis B; based on our results, 7.1% of them have HDV infection, which is overlooked. Our results have far-reaching implications for public health and institutions related to the development and implementation of appropriate response measures.

In conclusion, we developed an assay based on ddPCR for HDV detection with high sensitivity and specificity. The assay can directly quantitatively measure the HDV viral load in patients and is of great significance for guiding the clinical treatment of HDV and monitoring drug efficacy.

**Abbreviations**
HDV, hepatitis delt virus; HBV, hepatitis B virus; RT-PCR, real-time reverse-transcriptase polymerase chain reaction; ddPCR, droplet digital PCR; LLoD, lower limit of detection; LLoQ, lower limit of quantitation; ELISA, Enzyme-linked immunosorbent assay; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; LF, liver failure.

Declarations

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

Plant Reproducibility

Not applicable.

Clinical Trials Registration

Not applicable.

Authors' contributions: F.R. and Z.D. designed the experiments. L.X., and X.Z. performed the experiments and wrote the manuscript. Y.C., Z.F. and Y.T. prepared the samples and collected the data. H.Z. performed statistical analyses. All authors have read and approved the submission of the manuscript.

Conflicts of interest: The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

Funding: This study was supported by grants from the National Natural Science Foundation of China (81770611, 82002243); key projects of Beijing Municipal Education Commission's Science and Technology Plan (KZ202010025035); Capital Health Development Scientific Research Project (2020-1-1151, 2021-1G-2181); Demonstrating application and research of clinical diagnosis and treatment technology in Beijing (Z191100006619096, Z191100006619097); Beijing Talents foundation (201800021469G289); Beijing Hospitals Authority Youth Programme (QML20201702).

References

1. Roulot D, Brichler S, Layese R, et al. Origin, HDV genotype and persistent viremia determine outcome and treatment response in patients with chronic hepatitis delta. J Hepatol. 2020;73:1046–62.
2. Rizzetto M. Hepatitis D, Virus. Introduction and Epidemiology. Cold Spring Harb Perspect Med. 2015;5:a021576.
3. Koh C, Da BL, Glenn JS. HBV/HDV Coinfection: A Challenge for Therapeutics. Clin Liver Dis. 2019;23:557–72.
4. Dias J, Hengst J, Parrot T, et al. Chronic hepatitis delta virus infection leads to functional impairment and severe loss of MAIT cells. J Hepatol. 2019;71:301–12.
5. Stockdale AJ, Kreuels B, Henrion MYR, et al. The global prevalence of hepatitis D virus infection: Systematic review and meta-analysis. J Hepatol. 2020;73:523–32.
6. Miao Z, Zhang S, Ou X, et al. Estimating the Global Prevalence, Disease Progression, and Clinical Outcome of Hepatitis Delta Virus Infection. J Infect Dis. 2020;221:1677–87.
7. Patel EU, Thio CL, Boon D, Thomas DL, Tobian AAR. Prevalence of Hepatitis B and Hepatitis D Virus Infections in the United States, 2011–2016. Clin Infect Dis. 2019;69:709–12.
8. Chen HY, Shen DT, Ji DZ, et al. Prevalence and burden of hepatitis D virus infection in the global population: a systematic review and meta-analysis. Gut. 2019;68:512–21.
9. Goyal A, Murray JM. Recognizing the impact of endemic hepatitis D virus on hepatitis B virus eradication. Theor Popul Biol. 2016;112:60–9.
10. Goyal A, Murray JM. The impact of vaccination and antiviral therapy on hepatitis B and hepatitis D epidemiology. PLoS One. 2014;9:e110143.
11. Wu S, Zhang Y, Tang Y, et al. Molecular epidemiology and clinical characteristics of hepatitis delta virus (HDV) infected patients with elevated transaminases in Shanghai, China. BMC Infect Dis. 2020;20:565.
12. Chen X, Oidovsambuu O, Liu P, et al. A novel quantitative microarray antibody capture assay identifies an extremely high hepatitis delta virus prevalence among hepatitis B virus-infected mongolians. Hepatology. 2017;66:1739–49.
13. Le Gal F, Brichler S, Sahli R, Chevret S, Gordien E. First international external quality assessment for hepatitis delta virus RNA quantification in plasma. Hepatology. 2016;64:1483–94.
14. Wedemeyer H, Negro F. Devil hepatitis D: an orphan disease or largely underdiagnosed? Gut. 2019;68:381–2.
15. Brichler S, Le Gal F, Butt A, Chevret S, Gordien E. Commercial real-time reverse transcriptase PCR assays can underestimate or fail to quantify hepatitis delta virus viremia. Clin Gastroenterol Hepatol. 2013;11:734–40.
16. Lu XX, Yi Y, Su QD, Bi SL. Expression and Purification of Recombinant Hepatitis Delta Virus (HDV) Antigen for Use in a Diagnostic ELISA for HDV Infection Using the High-Density Fermentation Strategy in Escherichia coli. Biomed Environ Sci. 2016;29:417–23.
17. Shen L, Gu Y, Sun L, et al. Development of a hepatitis delta virus antibody assay for study of the prevalence of HDV among individuals infected with hepatitis B virus in China. J Med Virol. 2012;84:445–9.
18. Lin GY, Wu YL, Wang CS, et al. Performance of commercially available anti-HDV enzyme-linked immunosorbent assays in Taiwan. Virol J. 2020;17:76.
19. Smedile A, Niro MG, Rizzetto M. Detection of serum HDV RNA by RT-PCR. Methods Mol Med. 2004;95:85–93.
20. Theamboonlers A, Hansurabhanon T, Verachai V, Chongsrisawat V, Poovorawan Y. Hepatitis D virus infection in Thailand: HDV genotyping by RT-PCR, RFLP and direct sequencing. Infection. 2002;30:140–4.
21. Hetzel U, Szirovicza L, Smura T, et al. Identification of a Novel Deltavirus in Boa Constrictors. mBio 2019;10.
22. Wang C, Shen X, Lu J, Zhang L. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system for rapid detection of HDV genotype 1. Lett Appl Microbiol. 2013;56:229–35.
23. Caviglia GP, Abate ML, Tandoi F, et al. Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection. J Hepatol. 2018;69:301–7.
24. Frazzi R, Bizzarri V, Albertazzi L, et al. Droplet digital PCR is a sensitive tool for the detection of TP53 deletions and point mutations in chronic lymphocytic leukaemia. Br J Haematol. 2020;189:e49–52.
25. Suo T, Liu X, Feng J, et al. ddPCR: a more accurate tool for SARS-CoV-2 detection in low viral load specimens. Emerg Microbes Infect. 2020;9:1259–68.
26. Koh C, Heller T, Glenn JS. Pathogenesis of and New Therapies for Hepatitis D. Gastroenterology. 2019;156:461–76 e461.
27. Le Gal F, Dziri S, Gerber A, et al. Performance Characteristics of a New Consensus Commercial Kit for Hepatitis D Virus RNA Viral Load Quantification. J Clin Microbiol. 2017;55:431–41.
28. Olivero A, Smedile A. Hepatitis delta virus diagnosis. Semin Liver Dis. 2012;32:220–7.
29. Brichler S, Le Gal F, Neri-Pinto F, et al. Serological and molecular diagnosis of hepatitis delta virus infection: results of a French national quality control study. J Clin Microbiol. 2014;52:1694–7.
30. Shah PA, Choudhry S, Reyes KJC, Lau DTY. An update on the management of chronic hepatitis D. Gastroenterol Rep (Oxf). 2019;7:396–402.

Figures
Figure 1

Sensitivity and dynamic range of the ddPCR assay to detect HDV. Data are representative of at least three independent experiments. (A) The 10-fold serial dilution of pMD19T plasmid is analyzed by ddPCR, the blue points represent positive signal. (B) Correlation analysis to determine the dynamic range of ddPCR. The expected values (converted to log10) of pMD19T were plotted on Y axis versus detected values (converted to log10) on X axis to perform linear analysis. (C) Correlation analysis to determine the dynamic range of RT-PCR. The Ct values reported by RT-PCR were plotted on Y axis versus expected values (converted to log10) of pMD19T on X axis to perform linear analysis.
Figure 2

Determining the LLoD and LLoQ of ddPCR assay for HDV detection. (A) The probit analysis sigmoid curve was used to determine the lower limit of detection (LLoD) and lower limit of quantitation (LLoQ) of ddPCR. The HDV positive sample with the determined concentration was diluted in a 10-fold series, each concentration repeated 7 times for ddPCR HDV detection. The concentration corresponding 95% probability on curve represent LLoQ and 50% represent LLoD. (B) The probit analysis sigmoid curve was used to determine the lower limit of detection (LLoD) and lower limit of quantitation (LLoQ) of RT-PCR. The analytical method conducted as the same as (A).
Figure 3

Specificity of the ddPCR assay to detect HDV (A) Various plasmid including HBV, HCV, HIV and HDV was used to determine the specificity of ddPCR for HDV detection. The details of plasmids were provided on supplementary materials. (B) Samples from patients of HBV mono-infection, coinfection of HBV and HCV, coinfection of HBV and HIV, coinfection of HBV and HDV, to determined the specificity of ddPCR for HDV detection.
Figure 4

Comparison the detection efficiency of ddPCR, RT-PCR and ELISA for HDV. (A) The Venn diagram shows the detection number of ddPCR, RT-PCR and ELISA for HDV in 44 clinical confirmed patients including 30 HDV infection subjects. The purple area shows the number of HDV antibodies positive detected by ELISA; The red area shows the number of HDV RNA detected by ddPCR; The blue area shows the number of HDV RNA detected by RT-PCR. (B) Comparison the detection values between ddPCR and RT-PCR. The measured values of RT-PCR were plotted on X axis, the vertical dashed line denote the LLOQ of RT-PCR; The measured values of ddPCR were plotted on Y axis, the parallel dashed line denote the LLOQ of ddPCR.

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