Evaluation of digital PCR for absolute and accurate quantification of Hepatitis A virus

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Abstract: Hepatitis A virus (HAV) infection is the leading worldwide cause of acute viral hepatitis, and outbreaks caused by this virus often occur in contaminated water and food. Foodborne enteric viruses are conventionally processed by quantitative RT-PCR (RT-qPCR), which gives sensitive and relative quantitative detection results based on the standard curve. While RT-qPCR has limitations and may lead to incomparability of results from different laboratories. Here, we developed a reverse transcription digitial PCR (RT-dPCR) for absolute quantification of HAV genomic RNA. Our data showed that RT-dPCR assay achieved highest precision (RSD<6%), when optimum range was between 284 and 2029 copies per panel, especially the lowest RSD was 1.6%, when the number of copies per panel was 1008. In addition, RT-dPCR assays had a lower limit of detection of 2.6 copies/μL HAV genomic RNA, compared with that of approximately 10 copies/μL by RT-qPCR assay. This study demonstrates that RT-dPCR is capable of absolution and accurate quantification of low copy RNA targets of HAV in food products, water samples and vaccine products.

1. Introduction

Hepatitis A virus (HAV) is a small non-enveloped virus belonging to the *Picornaviridae* family, and has a 7.5 kb linear, positive-sense, single-stranded RNA genome[1-4]. Hepatitis A virus (HAV) is one of the common causative agents for acute hepatitis worldwide[5], about 1.5 million new HAV infections occur each year[6-7]. Sanitation and socioeconomic status are two key factors defining the geographical distribution of HAV[8], so in Africa, Asia, Latin America, and the Middle East, the positive rate of immunoglobulin G (IgG) antibody to HAV in adults is close to 90%, and many children as young as 10 years of age are infected with HAV[9].The largest outbreak of HAV infection occurred in Shanghai, China, in 1988, in which almost 310,000 cases were caused by consumption of clams harvested from a sewage-polluted area.

The population profile of HAV infection has transitioned from that of high to intermediate endemicity in several Asian countries including China in the last 20-30 years, due to socio-economic growth and sanitary improvement leading to lower prevalence among children. This has resulted in an increased average age of infection and consequent increased morbidity. In China, several outbreaks of HAV infection have been reported recently (Ministry of Health of People's Republic of China, 2012).

HAV is mainly transmitted via the fecal–oral route either by person-to person contact or by ingestion of contaminated water and food: particularly shellfish, soft fruits and vegetables[10-12].

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HAV is more resistant in the environment than other enteric viruses and can survive in fresh water, seawater, sewage, and seafood (such as clams) for a few days or even several months, it can cause illness even at a low concentration. Therefore, it is both important and necessary to develop sensitive, accurate and quantitative methods for detecting HAV.

To date, real-time quantitative RT-PCR (RT-qPCR) is the most popular approach used to quantify HAV due to its sensitivity, specificity and speed[13]. However, an external standard or a calibrant is needed to quantify the environmental sample when using RT-qPCR, which requires careful calibration and consistent source material. In the absence of a certified reference material, one usually develops one's own standard and quantifies the standard nucleic acid concentration by measuring absorbance at 260 nm by use of a UV spectrophotometer[1, 14]. Therefore, due to differences in standard curve construction and potential analysis subjectivity, this approach based on relative quantification has limitations and may lead to incomparability of results from different research groups[15]. There is a need for an accurate and absolute method that can be used for quantification of HAV and characterization of HAV certified reference material, for effective comparison of quantitative measurements, quality control in laboratory routine analysis. Digital PCR (dPCR) as a novel promising nucleic acid absolute quantification method, is more accurate and precise than qPCR, and most importantly, it can independently quantify nucleic acid without calibration or internal control[16-17]. dPCR transforms the exponential, analogue signal of classic PCR into a linear, digital signal, retaining the single-molecule sensitivity of PCR. Single molecules are isolated by dilution and individually amplified by PCR; each product is then analyzed separately. This is achieved by partitioning a sample before PCR amplification. The distribution of target DNA molecules among the partitions follows Poisson statistics, and at the so-called limiting dilution most reactions contain either one or zero target nucleic acid molecules[18]. After a PCR amplification step, we can accurately determine the copy number of the original nucleic acid samples[17], by analyzing the number of positive partitions[19].

In this study, reverse transcription digital PCR (RT-dPCR) was developed to quantify HAV genomic RNA. The dynamic range and factors involved in RT-dPCR measurement accuracy and bias were investigated. Additionally, RT-dPCR was compared to RT-qPCR for quantification of HAV genomic RNA, the results reveal RT-dPCR gives absolute and accuracy quantification of Hepatitis A virus.

2. Materials and methods

2.1 Hepatitis A virus (HAV)

Hepatitis A (live) vaccine (attenuated HAV H2 strain) was purchased from the Institute of Medical Biology, Chinese Academy of Medical Sciences, China, and stored at 4 °C until use.

2.2 Nucleic acid extraction and construction of HAV standard plasmid

Genomic RNA of Hepatitis A (live) vaccine (attenuated HAV H2 strain) was extracted from 250 μL of viral stock using the MagMAX™ Viral RNA Isolation Kit (Life technologies) according the manufacturer's directions. The RNA was eluted from the beads in a final volume of 30μL with RNase-free water and stored at -80 °C until use. Reverse transcription (RT) of virus RNA were performed according to the instructions supplied with the mouse reverse transcription kit (MLV, Promega), respectively[7]. HAV viral RNA was amplified using primers derived from the most constant region, the 5' non-coding region (5'NCR). The primers used were, forward primer HAV-1 (5'-TTTCCGGAGCCCCTCTTG-3'), reverse primers HAV-2 (5'-AAA GGGAAATTTAGCCTATAG CC-3') and HAV-3 (5'-AAAGGGAAAATTTAGCCTATA GCC-3'), representing the 5'NCR region was constructed according to Costa-Mattioli et al[20]. HAV gene fragments were amplified by polymerase chain reaction (PCR) using a Phusion High-Fidelity PCR kit (New England BioLabs). The positive plasmid pGEM-5'NCR was constructed and considered the standard plasmid. The concentration and quality of the plasmid was measured with a Nano-Drop 2000 (Thermo Fisher

Scientific, Inc.) and the result was converted into the viral copy number. The plasmid used for the standard curve was packed and stored at -20 °C.

2.3 RT-qPCR

The quantitative detection of HAV was performed by RT-qPCR. For RT-qPCR assay, the pGEM-5'NCR plasmid was serially diluted 10-fold into RNase-free water, and solutions of 10^0 copies/μL,10^1 copies/μL, 10^2 copies/μL, 10^3 copies/μL, 10^4 copies/μL, 10^5 copies/μL, 10^6 copies/μL, 10^7 copies/μL and 10^8 copies/μL were used to draw a standard curve, with three replicates per dilution. One-step RT-qPCR system with AgPath-ID™ One-Step RT-PCR Kit (Life technologies) was utilized. 25 μL experiments were performed with 5 μL of template, 12.5 μL of 2× RT-PCR Buffer, 1 μL of 25× RT-PCR Enzyme Mix. The primers used were the same as reverse transcription (RT) described previously, and the probe used was HAV-Probe (5'-FAM-ACTTGATACCTCACCGCCGTTTGCCT -TAMRA-3')[20]. Final concentration of 900 nM forward primer HAV-1, 900 nM reverse primer HAV-2, 900 nM of reverse primer HAV-3, and 250 nM of HAV-Probe were used. HAV genomic RNA extract was tested in five replicates and a negative control containing all the reagents except the RNA template were included in each run.

The one-step RT-qPCR reactions were run on the LightCycler 480 (Roche) with the following cycling conditions: 42 °C for 30 min (1 cycle); 95 °C for 10 min (1 cycle); 95 °C for 15 s and 60 °C for 1 min (45 cycles); and 50 °C for 30 s (1 cycle). A single fluorescence read was taken at the end of each 60 °C step, and a sample was considered positive if the Cq value was less than 40 cycles(Stefan et al. 2016). Negative samples gave no Ct value. A standard curve for pGEM-5'NCR plasmid was generated from 10-fold dilutions in RNase-free water of the titrated clarified suspension stocks, and the concentration of genomic viral RNA extract was calculated based on the standard curve. The slopes (*S*) of the regression lines were used to calculate the amplification efficiency (*E*) of the RT-qPCR reactions, according to the equation (1)(Brod et al. 2013) to determine the performance of RT-qPCR assays.

$$E = 10^{(-1/s)} - 1 \tag{1}$$

Where *E* is PCR efficiencies, *S* is the slope of the calibration curves.

2.4 RT-dPCR

RT-dPCR targets the same region of target 5' non-coding region (5'NCR) of HAV as RT-qPCR. The primers and probe used were the same as RT-qPCR described previously. RT-dPCR quantifications were performed on the BioMark System (Fluidigm, South San Francisco) using the 12.765 digital arrays (Fluidigm). The digital array comprises twelve panels and each panel contains 765 individual partitions of approximately 6 nL volume each with a total volume per panel of approximately 4.59 μL (6 nL×765)[21]. The instrument software generates PCR amplification curves and real-time cycle threshold (Ct) values for each of the 9,180 chambers (765×12). By counting the number of positive reactions, the number of target molecules in each sample can be accurately estimated according to Poisson distribution.

One-step RT-dPCR system with AgPath-ID™ One-Step RT-PCR Kit (Life technologies) was utilized. Reactions were performed in a 10 μL reaction mixture containing 5 μL of 2× RT-PCR Buffer, 0.4 μL of 25× RT-PCR Enzyme Mix, 0.5 μL of 20× GE Sample Loading Reagent (BioMark), 900 nM of forward primer HAV-1, 900 nM of reverse primer HAV-2, 900 nM of reverse primer HAV-3, 250 nM of HAV-Probe and 1 μL of template RNA. No template control was prepared by adding same amount of RNase-free water in place of RNA. All components excluding RNA were pre-mixed, and then the final reaction mix was prepared gravimetrically by combining the RNA and the pre-mixed solution, to minimize the uncertainty from pipetting. Then the reaction mixture (10 μL) was dispensed into each sample inlet, and approximately 4.59 μL of this reaction mix was distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm)[22].The one-step RT-dPCR program involved a 30 min reverse transcription of RNA at 42 °C, followed by a 10 min denaturation step at 95 °C, and lastly 50 cycles of 15 s at 95 °C, 1 min at

60 °C.

To evaluate linearity and precision over the dynamic range of RT-dPCR, gravimetric serial dilution of the HAV genomic RNA extract in RNase-free water were prepared to provide a final nominal RNA concentration approximately ranging from 3 to 4000 copies per panel (from dilution 1 to dilution 10, labeled S1–S10). Concentration of HAV genomic RNA extract was ascertained using RT-qPCR. Each solution was analyzed in five replicates, thus, a total of 50 reactions plus two NTCs were prepared to evaluate the dynamic range.

The copy number of HAV viral RNA per panel determined by RT-dPCR was calculated by used of equation (2)[22], the copy number of HAV viral RNA per microliter was calculated by used of equation (3)[22].

$$M = \frac{\log(1-\frac{H}{C})}{\log(1-\frac{1}{C})} \tag{2}$$

Where M is the RNA copy number per panel, H is the number of positive partitions, N is the total number of partitions analyzed.

$$T = \frac{D \times M}{V_p} \tag{3}$$

Where T is the RNA copy numbers per microliter, Vp is the partition volume, D is the dilution factor combining both the factor used to dilute RNA during PCR preparation and the factor used to further dilute the RNA with the PCR mixture.

3. Results

3.1 HAV quantitative detection by RT-qPCR

The standard curve of RT-qPCR using serial dilution of pGEM-5'NCR plasmid ranging from 1×10^1 copies/μL to 1×10^8 copies/μL showed strong correlation coefficients (R^2) of 0.9995 (Fig. 1). The PCR efficiencies (E) of PCR were calculated to be between 95 and 105 % using the equation (1). The limit of detection (LOD) was calculated to be approximately 10 copies/μL, and no unspecific amplification was found in 1×10^0 copies/μL and negative controls.

HAV genomic virus quantification was firstly assessed on serial dilutions of pGEM-5'NCR plasmid which were titrated by using OD (Nano-Drop 2000) and used as a standard for HAV quantification by RT-qPCR. As our RT-qPCR results showed, Cq values of HAV genomic viral RNA extract in five replicates were 20.28, 20.27, 20.19, 20.07 and 20.18, respectively, the concentration of genomic viral RNA extract calculated based on the standard curve was (1816726 ± 107641) copies/μL. These results demonstrated the validity of the plasmid production and RT-qPCR assay, which was important for the following RT-dPCR analysis.

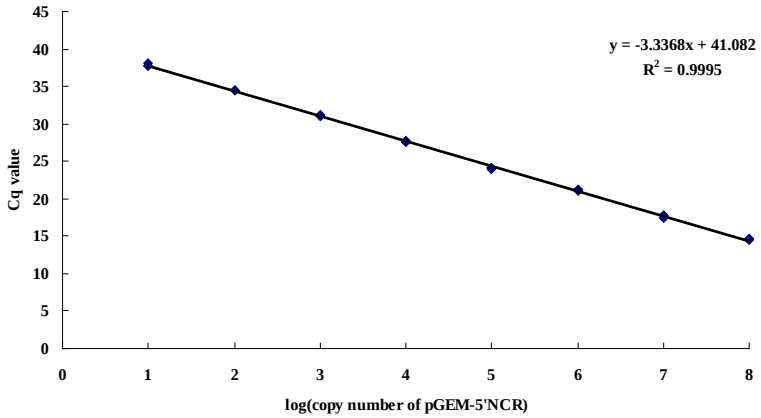

Fig. 1 RT-qPCR standard curve for pGEM-5'NCR

3.2 Linearity and precision of RT-dPCR

For the dynamic range of RT-dPCR, HAV genomic RNA was diluted to 4286, 2186, 1077, 538, 268, 135, 65, 32, 16, or 3 copies per panel (4.59 μL). The RT-dPCR response was linear over the dynamic range of 3-4286 copies per panel, as shown in Table 1. There was a linear relationship (R^2=0.999, Fig. 2) between the nominal concentration and the measured concentration by RT-dPCR. It is interesting to note that approximately 0 to 2 partitions were assigned in five replicate panels, when the nominal concentration was 3 copies per panel (corresponding to 7 copies/μL before diluted with RT-PCR mixture), indicating that the limit of detection (LOD) of HAV genomic RNA was 1.2 copies per panel (calculated to be 2.6 copies/μL) when using RT-dPCR.

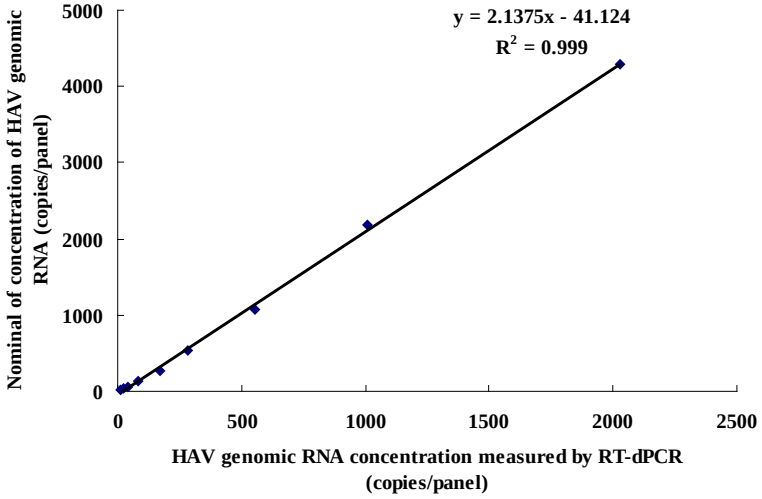

Fig. 2 Linearity of RT-dPCR response

Precision is usually expressed numerically in measures of imprecision, such as relative standard deviation (RSD). As the number of copies per panel increased, the RSD of the analyzed results decreased. The RT-dPCR achieved highest precision (RSD<6%), when the number of copies per panel was between 284 and 2029, and the lowest RSD was 1.6%, when the number of copies per panel was 1008 (Fig. 3). When the RNA copy numbers were lower than 40, the RSDs for these were larger than 10%. The number of copies per panel of dilution S10 in RT-dPCR replicates is the most variable, because the copy number concentration of dilution S10 is the lowest (1.2 copies per panel). When the number of copies per panel was outside of the range of 284 to 2029, the RSDs increased due to either the stochastic effect or to near-saturation of the dPCR chip. Thus, to achieve higher accuracy and better precision in RT-dPCR measurement, the RNA concentration should be within the optimum range.

Table 1. Linearity of hepatitis A virus (HAV) RNA quantification by reverse transcriptase digital PCR (RT-dPCR).

Nominal concentration of HAV genomic RNA (copies/panel)	HAV genomic RNA quantification by RT-dPCR in five replicates (copies/panel)						
	1	2	3	4	5	Mean	SD
4286	2149	2049	1846	2007	2092	2029	114.8
2186	1009	1017	1028	984	1004	1008	16.4
1077	556	539	561	570	547	555	12.1
538	277	268	288	283	303	284	13.1
268	151	188	180	163	175	171	14.6
135	71	81	87	73	88	80	7.8
65	28	53	42	33	44	40	9.8
32	12	29	21	18	27	21	6.9
16	8	12	4	15	9	10	4.2
3	1	2	1	2	0	1.2	0.8

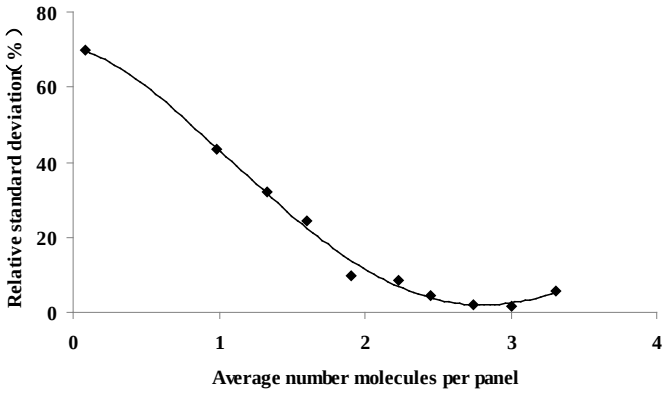

Fig. 3 Relative standard deviation of RT-dPCR response

3.3 Comparison of HAV RNA Quantification between RT-dPCR and RT-qPCR

To compare HAV RNA quantification by both quantitative detection methods (RT-qPCR vs. RT-dPCR), four dilutions of HAV genomic RNA 2186, 268, 32, 3 copies per panel (corresponding to the HAV genomic RNA concentration of theoretical expected in RNA extract, 4763, 584, 70, 7 copies/μL) were tested by both methods. The concentration of HAV genomic RNA measured in five replicates with the qPCR and ddPCR techniques is shown in Table 2. Overall, the data generated by the two detection method fell within the same order of magnitude.

Table 2. Quantification of HAV genomic RNA in series dilutions by RT-qPCR and RT-dPCR (*n*=5)

Nominal concentration of HAV genomic RNA (copies/μL)	HAV quantification by RT-qPCR		HAV quantification by RT-dPCR	
	HAV genomic RNA (copies/μL)	RSD (%)	HAV genomic RNA (copies/μL)	RSD (%)
4763	4771 ± 351	7.35	2197 ± 32	1.45
584	585 ± 43	7.34	373 ± 18	7.60
70	73 ± 7	8.96	47 ± 13	28.74
7	8 ± 1	12.71	3 ± 2	62.36

The mean of the five replicates was used for each reproducibility experiment. Results are expressed as mean ± SD (standard deviation of the mean).

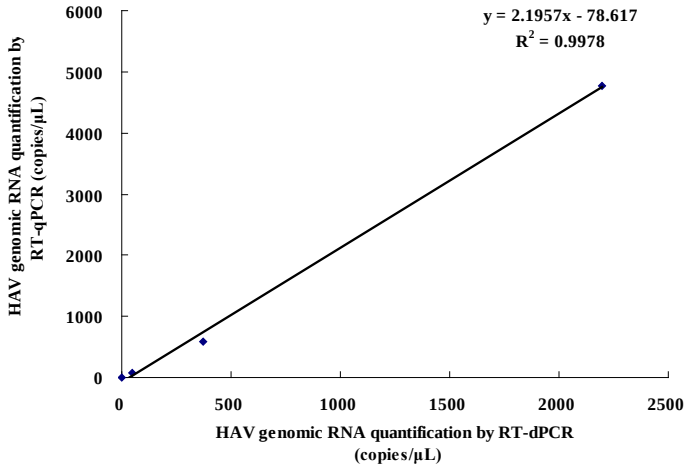

Fig. 4 Correlations between HAV genomic RNA quantification by RT-qPCR and RT-dPCR.

The variability of the HAV genomic RNA measured with the RT-qPCR and RT-dPCR techniques was shown in the quantitative correlations of RT-qPCR and RT-dPCR measurements (Figure 4), where the slopes were 2.1957 with an R^2 value of 0.9978. The numbers of target copies measured by the two methods correlated well, however, absolute quantification by RT-dPCR was approximately 2.20-fold lower than quantification by RT-qPCR using standards quantified by OD (Nano-Drop 2000) (Figure 4). This is in agreement with previous report in the quantitative correlation of qPCR and dPCR in *Cylindrospermopsis*, *Microcystis*[23], HEV[24], Hepatitis A virus and Norovirus[1, 14] and other viruses[14].

4. Discussion

Hepatitis A virus (HAV) infection is the leading worldwide cause of acute viral hepatitis, and outbreaks caused by this virus often occur in fecal polluted waters and food[25-26]. Approximately 1.5 million clinical cases of hepatitis A occur worldwide annually but the rate of infection is probably as much as ten times higher[6].

Currently, RT-qPCR is the most widely used molecular method for the detection and identification of viruses in biological and environmental sources in water and food[27-29], this approach based on relative quantification has limitations and may lead to incomparability of results from different research groups. Recently, important applications of digital PCR have been described for the clinical virology and oncology fields for the detection of rare mutants[1]. Digital PCR is a promising novel technology for viral load quantification. One of the advantages of using digital PCR technology is the ability to quantify from single RNA molecules and detect low copy numbers of template. This genomic RNA quantification approach does not require calibrated standards leading to a potential simplification of quantitative.

In this study, reverse transcription digital PCR (RT-dPCR) was evaluated to quantify HAV genomic RNA. The RT-dPCR assays had a lower limit of detection of 2.6 copies/µL HAV genomic RNA, compared with that of approximately 10 copies/µL by RT-qPCR. It achieved highest precision (RSD<6%), when optimum range was between 284 and 2029 copies per panel, especially the lowest RSD was 1.6%, when the number of copies per panel was 1008. Full understanding of the optimum range of RT-dPCR assay may help users render the measurement results more accurate. In addition, RT-dPCR was compared to RT-qPCR for quantification of HAV genomic RNA, the results revealed that the numbers of target copies measured by the two methods correlated well, however, the number of RNA copy number determined by RT-dPCR was lower than the number of copies expected using spectrophotometry[14]. The main cause of discrepancy between relative and absolute quantification could be errors introduced by spectrophotometric determination of the nucleic acid concentration, leading to an overestimation of the copy genome number[30]. This could explain why samples from

viral stocks and stools potentially containing cellular genomes (non-target RNA) and degraded (non-amplified) targets were particularly affected by quantification discrepancies. Therefore, RT-dPCR may provide more accurate measurements than RT-qPCR, as it is not dependent on amplification efficiency. We believe that RT-dPCR would be a promising tool for absolution and accurate quantification of HAV in food products, water samples and vaccine products.

Acknowledgments

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