Comparison of Droplet Digital PCR and Real Time PCR Method for HBV DNA Quantification

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Background: *HBV infection causes a potential serious public health problem. The ability to detect HBV DNA concentration is an important issue that had been continuously improved. When using quantitative polymerase chain reaction (qPCR), several factors are of concern, for example, sources of material, standard curve calibration, and PCR efficiency. Digital PCR (dPCR) is an alternative PCR-based technique for absolute quantification using Poisson's statistics without requiring a standard curve.*

Objective: *Compare the data set of HBV DNA generated between dPCR and qPCR methods.*

Material and Methods: *Fifty-four samples were quantified by Abbot's real time PCR and with 2-6 log10 HBV DNA were selected for comparison with dPCR.*

Results: *Of these 54 samples, there were two outlier samples defined as negative by dPCR, whereas 52 samples were positive by both of these assays. The difference between two assays was less than 0.25 log IU/mL in 24/52 samples (46%) of paired samples; less than 0.5 log IU/mL in 46/52 samples (88%) and less than 1 log in 50/52 samples (96%). The correlation coefficient (r) was 0.788 (p-value <0.0001). Comparison with qPCR method, data generated by dPCR tend to be an overestimation in the sample with the low level of HBV DNA concentration and underestimated in the sample with high viral load. The variation of DNA by dPCR measurement might be due to the pre-amplification procedure and PCR template.*

Conclusion: *Measurement of HBV DNA by using dPCR, the results of the HBV DNA copy number tended to be deviated by over- or under-estimated when comparison to real time PCR method. In addition, a large quantity of DNA was used when compared to qPCR. However, the optimum processes of this assay have to be further investigated.*

Keywords: *Hepatitis B virus, Real time PCR, Digital PCR, DNA quantification*

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Hepatitis B virus (HBV) is a high impact on the public health problem. In worldwide, it is estimated that over 350 million people being chronically infected[1]. For HBV infection diagnosis, hepatitis B surface antigen (HBsAg) is a useful marker of infection. However, there is increasing evidence that detection and quantification of hepatitis B virus DNA (HBV DNA) in serum play a key role in monitoring the viral replication, disease progression, and assessment of the response to therapy. In addition, it is relevant to detect the occurrence of drug-resistant mutants and to detect relapse after discontinuing antiviral therapy[2-4]. By these reasons, quantitation of viral nucleic acids (HBV DNA was assessed by using quantification PCR (qPCR), which is the method of choice. In order to standardize the HBV DNA Level, World Health Organization (WHO) has established an international standard for universal standardization of HBV DNA quantification units and defined in IU/ml. In general, an IU is equivalent to approximately 5 to 6 copies, which are depending on the method of measurement.

The principle of quantification polymerase chain reaction (qPCR) or real-time PCR is based on the detection and quantification of a fluorescent reporter. During the PCR reaction, the dynamic range of amplification product is increased and measured. Quantification is depended on the quantification cycle (Cq)[5] and standard curve generated simultaneously with known standards[6]. Software is used to calculate the amount of viral genomes in the initial sample by comparison with a panel of quantified standards[7]. Both PCR efficiency and Cq value, had influence on the accuracy and precision of qPCR[8]. By these reasons,

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several qPCR methods for detecting HBV DNA with high accuracy and precision have been developed[9-12].

Unlike qPCR, digital PCR (dPCR) (Bio-Rad, CA, USA) achieves the sensitive and accurate quantitation of DNA in the sample without the requirement of standard calibration[13]. This novel system is based on limiting dilution of samples into the small compartments or droplets, each containing a single molecule of interest. The droplet's fluorescence is examined. If the fluorescence reaches a cutoff, the droplet is then defined as positive, if not it is defined as negative. The concentration within the sample as a whole is subsequently calculated using the proportion of positive and negative droplets and Poisson's statistics (http://definetherain.org.uk/). Although both methods are sensitive, however, no need of the standard calibration is the potential advantage of dPCR over conventional qPCR[14,15]. Therefore, the aim of this study was to evaluate the characteristics and accuracy of dPCR method for quantification of HBV-DNA by comparison to commercial real time PCR.

Material and Method

Clinical samples

Fifty-four plasma samples were taken from HBV infected patients who visited Maha-Chakri Sirindhorn Medical Center hospital between 2011 and 2012. The level of HBV viral load was initially quantitated by using Abbott Real-Time HBV assay (Abbott GmbH & Co. KG, Wiesbaden, Germany). The concentration of HBV DNA samples was calculated from the calibration curve, which was reported in copies/ml. Samples of HBV-DNA, ranging from 1.5 to 6 log copies/ml, were selected and stored at "80°C for further investigation. The amount of HBV DNA was determined by using BioRad QX100 system and the copy numbers were quantified by QuantaSoft Software version 1.2.10.0 (Bio-Rad, Hercules, CA, USA).

DNA extraction

The nucleic acid in infectious specimens was extracted from 200 µl of selected samples with Nuclisens extraction kit (Biomeriux, France) according to the manufacture's protocol. The extracted products were stored in elution buffer.

Primers and probe

The primers and probe sequences were used as follow:

HBVTAQ1 5'-GTG TCT GCG GCG TTT TAT CA-3' Location 379-398…..

HBVTAQ2 5'-GAC AAA CGG GCA ACA TAC CTT-3' Location 456-476…..

Probe HBS(16) 5'-CCT CTT CAT CCT GCT GCT ATG CCT CAT C-3' Location 403-430…..

All primers were aliquot and stored at "40°C.

PCR amplification

To determine the amount of HBV-DNA by dPCR, DNA samples were amplified separately in a GeneAmpPCR System 9700 thermal cycler (Applied Biosystems, CA, USA). Each reaction consisted of a 20 µL solution containing 10 µL Express qPCR MIX UNI, 900 nM primers, 250 nM probe, and 5 µl of DNA template with the following cycling conditions, 10 min at 95°C, 12 cycles each consisting of a 15 sec denaturation at 95°C followed by a 62°C extension for 30 sec, and then 40 cycles each consisting of a 10 sec denaturation at 95°C followed by a 56°C extension for 60 sec, and a final 10 min at 98°C. After cycling, droplets were evaluated immediately or stored at 4°C overnight.

Data analysis

The Cq values and amplification data were analyzed using the Bio-Rad iQ5 optical system software, version 2.0 (Bio-Rad, Hercules, CA, USA). Data were collected at the extension step (72°C) of every cycle, and the quantification cycle values were determined with "PCR Base Line Subtracted Curve Fit".

Statistical analysis

The DNA concentration was transformed into log (10) copies/ml. Correlation of HBV DNA of two methods was assessed by Pearson's correlation and non-linear regression. The difference between two data sets was analyzed by Bland-Altman tests. The *p*-value of <0.05 was considered significant. All statistical analyses were performed by using GraphPad Prism version 4.02 (GraphPad Software, San Diego, California, USA).

Bioethical approval

The Bioethical Committee of Srinakharinwirot University approved the conduct of the study (reference No. SWUEC-065/56E).

Results

Quantification of HBV-DNA copy number by dPCR

The concentrations of HBV-DNA in all 54 samples were measured by commercial real time PCR as the reference assay. The copy numbers of HBV-DNA

were transformed into a log. For dPCR assay, the final output was log10 HBV-DNA copy numbers per input unit (5 µl of the input cDNA), and the data set was compared to those from commercial real time PCR. Although the quantitated HBV-DNA showed slightly high correlation between these two methods, (r = 0.788), there were two outlier samples. Of these samples were defined as negative by dPCR, whereas 52 samples were positive by both methods. The difference of paired samples between the two assays was less than 0.25 log IU/mL in 24/54 (44.4%); less than 0.5 log IU/mL in 46/54 (85%), and less than 1 log IU/mL in 50/54 (93%). Of these 52 samples, the determination of DNA copy was 1.5 to 6 log copies/ml by both two assays. False-negative were detected in the samples which contained HBV-DNA concentration ranging from 2 to 4 log copies/ml. Data were shown in Fig. 1.

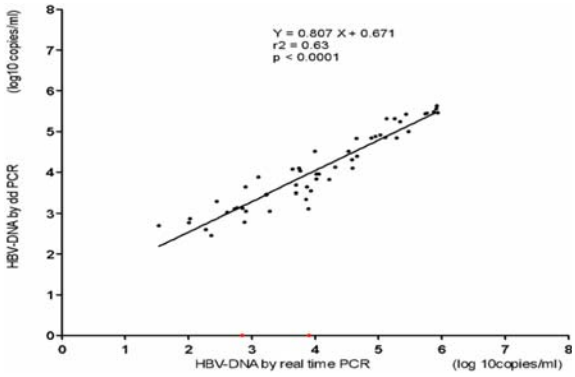

Fig. 1 Correlation between log10 HBV-DNA copy number by qPCR and dPCR. The black line indicated the best fit non-linear regression line. Only positive samples were plotted and calculated for their correlation.

Correlation and regression of HBV DNA Data set between two quantification assays

In order to assess the correlation of HBV-DNA level from 54 samples between two methods, two-tails Pearson's test correlation coefficient (r) was 0.788 (*p*-value <0.0001). For non-linear regression analysis, the linearity (R2) was 0.63, and the equation of the straight line (Y) was 0.8076 X +0.807 as shown in Fig. 1.

The comparison of commercial qPCR and dPCR techniques, these two data sets were analyzed by Bland-Altman's test. The mean difference (bias+SD) between HBV-DNA copy numbers generated by qPCR and dPCR was 0.103+0.774 log10 and 95% limits of agreement was ranged from -1.414 to 1.622 log10. Overall, the qPCR showed mean positive bias relative to dPCR. An overestimation of HBV DNA levels by the dPCR system was detected relative to the qPCR when the concentration of HBV DNA was low. In high level of viral load, HBV DNA detection by dPCR method tended to be lower than those quantified by qPCR (Fig. 2).

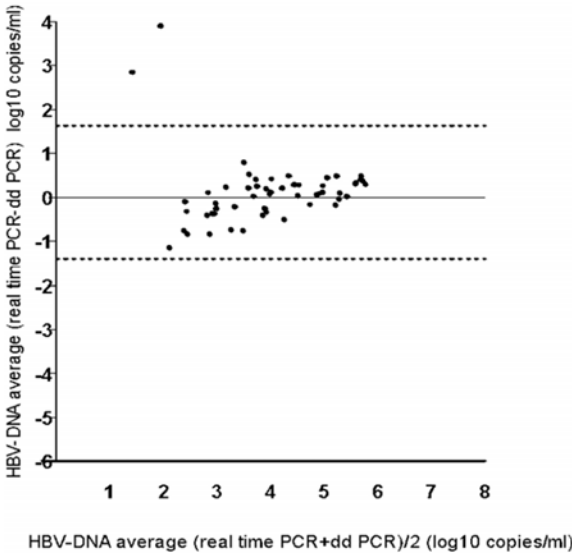

Fig. 2 Quantifiable viral load by qPCR and dPCR assessed by Bland-Altman Bias plot. The mean difference between the qPCR and dPCR was 0.104 log copies/ml. The dot lines represent the 95% limits of agreement, which was ranged from -1.414 to 1.622 log 10.

Discussion

The quantification of HBV DNA level is very useful for monitoring the progression of the disease and the efficacy of treatment in chronic HBV infection[16,17]. At present, most of the HBV DNA quantification assays are usually measured by qPCR, which widely used as a gold standard method[18]. For qPCR assay, quantification is based on a standard curve, which requires calibration, consistent source material, and subjectivity of the quantification cycle[5,19]. These factors might lead to the inconsistency of measurement of HBV DNA in the same samples. To eliminate these problems, dPCR was introduced as a method of nucleic acid quantification, which is detected the amount of HBV DNA without using standard curve[14]. Although dPCR is used to measure an absolute quantification of target DNA, the available data were inadequate for the consistency data in this assay. An application in quantification of HBV DNA for clinical use is still under

developed.

In this study, the data set of HBV DNA quantification by the qPCR and dPCR were performed. By using Bland-Altman Bias plot, it was clearly demonstrated that the differences in accuracy and the precision of these two assays. The correlation of measurements was slightly high for both assays (r = 0.788). However, two false-negative samples were found by dPCR. Base on the accuracy in defining the negative and positive droplets for threshold setting, the inaccurate setting leads to the considerable diversity. The discordant results were influenced on the final determination of zero copy with unknown reason. Overall, the absolute numbers of HBV DNA copy numbers quantified by dPCR were slightly lower than the corresponding HBV DNA copy numbers assessed by real time PCR. According to the previous study, both dPCR and qPCR had similar sensitivity, but dPCR enumerated 10 to 40% fewer DNA copies compared with qPCR[19]. Most of the underestimate results by the dPCR assay emphasized in high level of HBV DNA viral load samples. In contrast, data generated by dPCR was tended to be overestimated in the sample with low concentration of HBV DNA. In this study, the discrepant results (differences of HBV DNA $\geq$0.5 log10 IU/ml) were infrequent (15%). In comparison between two commercial PCR assays in HCV RNA quantification, it is recommended that HBV DNA load variations should be less than 3-fold (i.e., 0.5 log10), whereas variations of more than 3-fold (i.e., 0.5 log10) can reliably be considered[6]. The variation in DNA by dPCR measurement might be due to a lot of reading steps, and time-consuming than qPCR. All of these factors might affect the concentration of measurement[5]. Although little or no pre-amplification bias had been observed for low level of DNA by qPCR, this application was sensitive for the dPCR assay. The pre-amplification step requires careful for method validation because the potential bias can be introduced[20]. Another promising explanation for the inconsistencies in dPCR quantification is that linear template was existed in both double stranded (ds) and denatured single stranded (ss) of DNA. It is therefore, possible for one dsDNA molecule to populate two individual dPCR reactions[20].

Conclusion

Information in the measurement of HIV-DNA by dPCR was already known in 2012, and Pearson's linearity correlated between real time PCR and dPCR was R^2 = 0.64. At present, comparison between these two methods for HBV-DNA had never been reported. In this study, we proposed that the copy number of HBV- DNA showed some deviations from the reference value. Nevertheless, the linearity of HBV-DNA was R^2 = 0.63. A minor drawback of dPCR is a high concentration of DNA had to be used when compare to the qPCR. However, this assay should be further investigated.

What is already known on this topic ?

Information in the measurement of HIV-DNA by dPCR was reported in 2012, and Pearson's linearity correlated between real time PCR and dPCR was R^2 = 0.64.

What this study adds ?

Comparison between real time PCR and dPCR for HBV-DNA found that the Pearson's linearity was R^2 = 0.62. The copy number of HBV DNA showed some deviations from the reference value. A minor disadvantage of dPCR is a high concentration of DNA used when compare to the qPCR.

Acknowledgement

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Potential conflicts of interest

None.

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การวัดปริมาณของ HBV DNA โดยวิธี Droplet Digital PCR เปรียบเทียบกับวิธี Real Time PCR

ชัชวาลย์ วงศ์จิตรัตน์, นาวิน ห่อทองคำ, รวงผึ้ง สุทเธนทร์, สุรางค์รัตน์ ศรีสุรภานนท์

ภูมิหลัง: *การติดเชื้อไวรัสตับอักเสบ บี เป็นปัญหาสำคัญทางด้านสาธารณสุขการตรวจหา HBV DNA จึงมีความสำคัญและมีการพัฒนาอย่างต่อเนื่อง การตรวจหาโดยวิธี quantitative polymerase chain reaction (qPCR) มีปัจจัยด้านมาตรฐานการตรวจวัด เช่น สารที่ใช้ในการทา standard curve ประสิทธิภาพของการทา PCR ไม่มีความคงที่การใช้ Digital PCR (dPCR) เป็นทางเลือกใหม่ในการหาปริมาณโดยอาศัยหลักการทางสถิติ ทำให้ไม่ต้องมีการใช้ standard curve*

วัตถุประสงค์: *การศึกษานี้ต้องการเปรียบเทียบค่าของ HBV DNA ที่ได้จากการตรวจวัดโดยวิธี qPCR กับ dPCR*

วัสดุและวิธีการ: *ตัวอย่างตรวจที่ได้รับการตรวจโดย by Abbott's real time PCR และมีค่า HBV DNA อยู่ระหว่าง 2-6 log10 HBV DNA ถูกคัดเลือกและทำการตรวจด้วย dPCR*

ผลการศึกษา: *ผลการตรวจด้วย dPCR พบว่าจากตัวอย่างตรวจ 54 ตัวอย่างมี 2 ตัวอย่างที่ให้ค่าเป็นลบ ส่วนที่เหลือ 52 ตัวอย่าง มีจานวน 24 ตัวอย่างที่ให้ผลการตรวจต่างกันไม่เกิน 0.25 log 10 จำนวน 46 ตัวอย่างที่ให้ผลการตรวจต่างกันไม่เกิน 0.5 log10 และมีจำนวน 50 ตัวอย่างตรวจที่ให้ผลการตรวจต่างกันไม่เกิน 1 log10 โดยมีค่าสัมประสิทธิ์ของความสัมพันธ์ r = 0.788 และ p-value <0.0001.*

สรุป: *ในการเปรียบเทียบพบว่า ค่าที่ได้จากการตรวจโดยวิธี dPCR มีแนวโน้มที่จะสูงกว่าหรือต่ำกว่าการตรวจโดย qPCR ถ้าตัวอย่างตรวจที่มีปริมาณ HBV DNA น้อย หรือ HBV DNA ที่มีปริมาณมากตามลำดับ การที่วัด HBV DNA ได้ต่างกันน่าจะเนื่องมาจากขั้นตอน pre-amplication และคุณลักษณะของ template นอกจากนี้ข้อเสียของ dPCR คือต้องใช้ DNA จำนวนมากเปรียบเทียบกับ qPCR จากการที่ dPCR เป็นเทคนิคใหม่ ข้อจำกัดต่างๆ เหล่านี้จึงควรมีการพัฒนา*