Original Article

Quality Control Planning of a Hepatitis B Viral Load Laboratory-Developed Assay

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ABSTRACT

Background and Objectives: **HBV DNA monitoring is important in management of chronic viral hepatitis B infection. HBV DNA measurements are carried out over period of months to years. So the analytical system must be stable and reproducible. The aim of this study was to determine the performance characteristics and to plan a statistical quality control system of a laboratory-developed real-time quantitative PCR assay for HBV DNA quantification.**

Methods: **Values of systematic and random error at two clinical decision points;4.2 Log IU/mL (20000 IU/mL) and 3.2 Log IU/mL (2000 IU/mL) were determined. Candidate quality control procedures were selected and performance of the method by application of normalized operational process specification (OPSpecs) charts was determined.**

Results: **The performance of the assay at level of 4.2 Log IU/mL and 3.2 Log IU/mL were excellent and good respectively. Moreover, $a1_{3.5S}$ rule with two measurements offered 90% probability of error detection at level of 4.2 Log IU/mL, while no rule offered 90% probability of error detection at level of 3.2 Log IU/mL.**

Conclusion: **Minimizing the formation of primer-dimer and nonspecific products and concentrating the target DNA during the purification process are proposed for accurate quantitative PCR particularly when CT values are high.**

Keywords: **Hepatitis B, Quality Control, Real-Time Polymerase Chain Reaction, Virus Load**

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Introduction

Nowadays several HBV DNA assays are available. In real-time quantitative PCR (QPCR) assays, amplification and detection occur simultaneously in the same tube and the samples are quantified by deriving the value from a standard curve generated with standards (1, 2). HBV DNA measurement is important in initial diagnosis and monitoring of patients with chronic viral hepatitis B. Determination of HBV DNA Level is associated with the degree of hepatocellular necroinflamation, risk of developing drug resistance, mortality of liver disease, the risk of developing hepatocellular carcinoma, infectivity through the vertical transmission from mother to child (3, 4). Since HBV DNA measurements are carried out over period of months to years, the analytical system must be stable and under control for the results to be reproducible and comparable. Achieving this goal is easier with approved assay kits, especially for laboratories with less experience in molecular methods. However, according to clinical and laboratory standards institute (CLSI) documents, even for approved assays, quality control (QC) of the analytical system with at least one long-term control material is required (5). Probability of error detection, false alert rate and the best QC rule in a quality control system depend on the values of imprecision and inaccuracy in the analytical assay(6).

The aim of this study was to evaluate the performance characteristics and application of a statistical quality control planning for a laboratory-developed real-time QPCR assay for HBV DNA measurement.

Materials and Methods

Study Design

In the treatment of patients with chronic hepatitis B, when the reduction in HBV DNA from the baseline is at least 1 Log IU/mL, antiviral treatment is considered efficient (3, 7). For this reason an analytical total error less than 1 Log IU/mL is not likely affect the clinical decision. Therefore, in the current study maximum amount of allowable total error of the assay was considered 1 Log IU/mL. Threshold points for treatment of patients with HBe Ag positive and negative patients are defined 4.2 Log IU/mL (20000 IU/mL) and 3.2 Log IU/mL (2000 IU/mL) respectively (3, 7). According to this assumption, values of inaccuracy and imprecision at these two levels, were measured. Subsequently, operating point was determined on a normalized Operational Process Specifications (OPSpecs) chart (8). Finally, performance of method was assessed and the best QC rules were selected.

Specimens

Patients are regularly admitted to our molecular diagnostics Laboratory for HBV viral-load quantification which is performed by means of Artus HBV ASR. Routinely 2 mL venous blood is collected from each patient and anticoagulated in EDTA. The plasma is then separated within 6 hours by 1000 g centrifugation for 20 minutes. Plasma is then stored in two separate aliquots up to 4 weeks at minus 20ºC.

Extraction

Using Roche High Pure Neucleic acid purification kit (Roche Diagnostics, Indianapolis, IN) nucleic acid was purified from plasma as ordered by the manufacturer. Briefly, 200 µL plasma, 200 µL Binding buffer and 50 µL proteinase K were mixed and incubated in 55ºC for 10 minutes. 100 µL binding buffer was added to the mixture. Then it brought to the column. Three cycles of washing by inhibitor removal and wash buffers were done and finally, viral DNA was eluted into the volume of 50 µL. Purified nucleic was stored in 4ºC up to 3 days.

Laboratory-Developed HBV Viral Load Assay

The HBV viral load was measured by quantitative

real-time polymerase chain reaction (Q-PCR) using primers designed to amplify a 79 nucleotide region of precore/core protein gene, primers and probe sequences are shown in Table 1. The same gene is used by COBAS AMPLICOR (Roche Diagnostics, Indianapolis, IN) for viral-load quantification. Each reaction volume was 20 µL containing 10 µL of 2X QuantiFast Probe PCR Kit (QIAGEN, Hamburg, Germany), 500 nM of each primer, 200 nM probe and 9 µL template. The reactions were performed in a RotorGene 3000 real-time (Corbett Research, Mortlake, Australia) as follows: initial denaturation at 95 ºC for 3 minutes, 40 cycles of 95 ºC for 5 seconds, 61 ºC for 30 seconds. Pooled plasma of HBV positive patients was prepared and extracted as described above. Five 1:10 dilutions of extracted HBV DNA were quantified by Artus HBV RG PCR kit (QIAGEN Hamburg, Germany) and used as Standards.

Table 1- Primer and probe sequences used in this study

Forward	TCAIGCCTCCAAGCTGTGC
Reverse	CTCCACAGTAGCTCCAAATTC
Probe	FAM-TGTCCATGCCCCAAAGCCACCCAAG-BHQ

Systematic Error Measurement

Nineteen plasma samples with HBV-DNA above 500 IU/mL, in different ranges of concentration were selected. Viral DNA was extracted as described above and then quantified by QIAGEN Artus HBV RG PCR assay. Same extracted DNA of samples was also measured by laboratory-developed assay. Afterwards, a regression line was obtained using logarithmically transformed values of each measurement considering Qiagen as reference method and plotting its values on horizontal axis.

Random Error Measurement

By pooling of HBV-DNA positive samples with about 2000 and 20000 IU/mL viral load, two plasma samples as QC materials were prepared. Each sample was extracted 6 times separately as described above and measured by laboratory-developed assay. Standard deviation of logarithmically transformed values at each level was considered as the amount of random error.

Statistical calculation

One sample Kolmogorof Smirnow test was used to evaluate the Gaussian normal distribution of logarithmically transformed HBV DNA level. The results greater than 3.5 SD from the mean were considered as the outliers and excluded. The values of systematic and random errors of log HBV DNA at each control level were obtained as described above. After dividing them by the amount of TEa, Operating points on a normalized Operational Process Specifications (OPSpecs) chart with 90% quality assurance and two control levels were determined. Performance of method was assessed and the best QC rules were selected. For statistical calculations, SPSS version 18.0 (2009) was used and a *P* value less than 0.05 was considered significant.

Results

The results of HBV DNA measured by Artus HBV RG kit, which was considered reference method, ranged from 3.11 to 8.86 Log IU/mL (1,277 IU/mL to 719,920,285 IU/mL). The results are shown in Table 2. The equation of the regression line for the logarithmic data was $y = 1.0334x - 0.5662$. For a particular value of X the difference between value of X and Y equals systematic error (9), which at 3.2 and 4.2 Log IU/mL were 0.46 and 0.42 Log IU/mL respectively (Fig. 1). Standard deviation of replicates was calculated

at two different levels of 3.2 and 4.2 Log IU/mL were 0.18 and 0.08 Log IU/mL respectively. The operating points on OPspecs chart were determined at level of 3.2 and 4.2 Log IU/mL (Fig. 2). The performance of the assay at level of 4.2 Log IU/mL was excellent but at level of 3.2 Log IU/mL was good to marginal. Moreover, an $1_{3.5S}$ rule with two measurements offered 90% probability of error detection at level of 4.2 Log IU/mL, while no rule offered 90% probability of error detection at level of 3.2 Log IU/mL (Fig. 2).

Table 2- Results of the replicates in two levels, mean and standard deviation of each group

	1st Replicates			2nd Replicates		
No	**Ct**	**Log IU/mL**	**IU/mL**	**Ct**	**Log IU/mL**	**IU/mL**
1	27.73	4.092942	12,386	29.27	3.700858	5,022
2	27.13	4.245702	17,608	32.21	2.952334	896
3	26.98	4.283892	19,226	30.81	3.308774	2,036
4	26.99	4.281346	19,114	30.54	3.377516	2,385
5	27.82	4.070028	11,750	32.09	2.982886	961
6	27.09	4.255886	18,025	30.91	3.283314	1,920
M		4.227371	17,145		3.180965	1,640
SD		0.080017			0.177172	

M: mean; SD: standard deviation

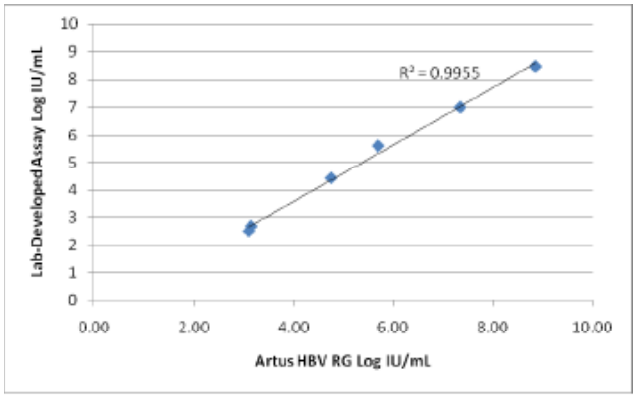

Fig. 1: The regression line between Artus HBV method on X-axis and Laboratory-developed HBV assay on Y-axis.

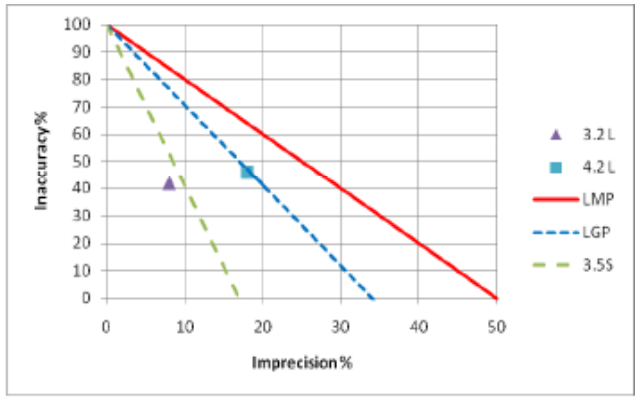

Fig. 2: Normalized OPSpecs Chart with 90% Analytical Quality Assurance, 3.2 L: 3.2 Log IU/mL, 4.2: 4.2 Log IU/mL, LMP: Limit of Marginal Performance, LGP: Limit of Good Performance, 3.5S: 13.5S rule with 2 times Repeat

Discussion

HBV DNA monitoring is important in initial diagnosis and management of patients with chronic viral hepatitis B. As HBV DNA measurements by QPCR are obtained in a period of months or years, the analytical system must be stable and reliable. So application of a long time quality control procedure for detection of critical errors in the measurement system is necessary. Compared with other laboratory practices, application of traditional quality control procedures for molecular diagnostic tests has not yet come to end (10). Some of the reasons in this regard are as follows: lack of adequate quality control materials and lack of quantitative test results (10).

Nowadays QC procedures in most of PCR assays, either quantitative or qualitative, is limited to use

of positive, negative and internal controls. But in quantitative assays, use of external controls with concentrations near the clinical decision making points in each run for detection of errors is required (6).

Ling-liang *et al.* applied traditional QC procedures in microsatellite-based bone marrow transplantation engraftment assays. Their study contains two steps. The initial phase established QC parameters over 20 runs. In the second step, they plotted Levey -Jenning charts by using the initial data. Westgard multi-rules were used for detection of errors (11).

In this study, patient samples at two levels of clinical decision making, were used as QC materials. As HBV DNA is a circular double stranded DNA (12), it is stable over the times and can be used as a suitable QC material.

As each laboratory should establish its own empiric rules (6), OPSpecs chart was employed in this work to determine the assay performance and to select the best quality control rule for HBV-DNA viral load assay. In this study the performance of the assay at level of 4.2 Log IU/mL was excellent but at level of 3.2 Log IU/mL was good to marginal. An $1_{3.5S}$ rule with two measurements offered 90% probability of error detection at level of 4.2 Log IU/mL. While no rule offered 90% probability of error detection at level of 3.2 Log IU/mL. When the sample contains only few target molecules, primer–dimer and nonspecific products compete with specific products and decrease the efficiency (13,14). This may explain lower assay performance, with larger SDs, at lower target molecule levels in this study. Minimizing the formation of primer-dimer and nonspecific products is important for minimizing SD in quantitative PCR particularly when CT values are high (14). Therefore better performance of the assay can be achieved by optimizing the primer design, concentration and PCR conditions. Another strategy for gaining a better performance is to push the PCR reaction to lower CT values by concentrating the target DNA during the purification process.

Yen-Lieberman *et al*. for comparing two values based on sample size formula:$(Z_{1-\alpha/2}+Z_{1-\beta})= \mathrm{Log}(X_1/X_2)/(1.4\times SD)$ calculated the minimum standard deviation for HIV-RNA copy number measurement (15). According to this formula, for detection of 1 log difference in HBV DNA between two samples at a type 1 error (α) of 0.05 and type 2 error (β) of 0.01, the minimum standard deviation is 0.22 Log(IU/mL). In other words reporting values at levels with a SD larger than 0.22 Log (IU/mL) may cause clinical decision-making problems. Meanwhile, the lesser the SD the more the probability of error detection will be even with acceptable SD in statistical quality control procedures.

This study aims to provide a quality control planning for HBV DNA real time QPCR, which to our knowledge, in such a way has not been examined so far. However according to documents, the sample size for method comparison and random error measurement, must be at least 40 and 20 measurements, respectively (6), which was the limitation of our study.

In this study, a laboratory developed HBV DNA QPCR assay was evaluated. For routine laboratory measurements by approved assay kits, a similar discipline of statistical quality control for assessment of the analytical system can be designed and applied.

Conclusion

By using patient samples as QC materials and obtaining the best QC rules, long time QC procedures can be applied for detection of critical errors in the measurement system. In addition, minimizing the formation of primer-dimer and nonspecific products and concentrating the target DNA during the purification process are proposed for accurate quantitative PCR particularly when CT values are high.

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