Journal of Virological Methods 193 (2013) 683–686

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Application of quality control planning methods for the improvement of a quantitative molecular assay

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Article history:
Received 18 December 2012
Received in revised form 15 July 2013
Accepted 20 July 2013
Available online 6 August 2013

Keywords:
Hepatitis B virus
Quantitative real-time PCR
Viral load
Operational process specification

A B S T R A C T

Hepatitis B virus (HBV) DNA measurement has an important role in the diagnosis and management of patients with chronic HBV infection. In cases of chronic hepatitis B, clinical decision is based on either the absolute amount of HBV DNA level, or else the relative change in HBV DNA level. To produce high quality and comparable results, assay performance characteristics must be verified and statistical quality control methods must be planned. In this study, systematic and random error values in an assay of plasma HBV DNA were determined. Performance of the method was examined by employing a normalized operational process specifications (OPSpecs) chart. The systematic error at low and high control levels were 0.33 and 0.22 log(IU/mL) respectively. At both levels, the standard deviations (SD) of the assay were 0.17 log(IU/mL). In addition, a single rule of $1_{2.5}$SD with 2 control measurements was selected as a candidate quality control method. The assay performed well and was acceptable for clinical use. Further improvement may be attained by switching to automated purification methods. In this study, the well-established discipline of statistical quality control was applied to a real-time quantitative PCR. It was concluded that by employing statistical quality control (QC) methods, which utilize long-term controls, critical changes in the measurement system could be detected.

1. Introduction

Infection with chronic hepatitis B virus (HBV) poses a significant health risk to patients. An HBV DNA level in such patients has been shown to correlate with the grade of hepatocellular necroinflammation, increased risks of developing hepatocellular carcinoma and mortality. Therefore, measurement of HBV DNA has an important role in patient diagnosis and management (Gish and Locarnini, 2006).

The medical decision points for HBV DNA level are 20,000 IU/mL, and 2000 IU/mL for HBeAg positive and HBeAg negative patients, respectively (Chu and Lok, 2002; Gish and Locarnini, 2006). Optimal management of chronic hepatitis B requires a baseline HBV DNA level to be established through quantitative PCR (qPCR), which may then be used for monitoring the response and viral rebound associated with viral resistance (Lai and Yuen, 2007). HBV DNA measurements are usually performed over a period of several months to years and so a stable analytical system is essential for producing reproducible and comparable measurements. Although achieving this goal is more straightforward with approved kits compared to in-house methods, Clinical and Laboratory Standards Institute (CLSI) requires analytical quality control of the whole system with at least one long-term independent control (Espy et al., 2006; Wayne, 2006), even for approved assays. In recent decades, diagnostic laboratories have investigated different aspects of quality control (QC) methods, such as QC method selection, probability of error detection and false alert rate (Linnet and Boyd, 2006; Westgard, 1992a). In a QC system, the probability of error detection and false alert rate depend on the selection of QC material, target value, frequency of measurement and QC rules (Westgard, 1992a,b).

In contrast to routine biochemistry methods, statistical QC is yet to be comprehensively investigated in qPCR (Shahsiah et al., 2010). The aim of this study was to determine the performance characteristics for HBV DNA measurement, and further, to plan a statistical QC system of a real-time qPCR assay.

2. Methods

2.1. Study design

The clinical minimum threshold for treatment of HBV infected patients is defined to be 1 log(IU/mL) change in HBV DNA level (Gish

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http://dx.doi.org/10.1016/j.jviromet.2013.07.056

and Locarnini, 2006). In other words, the efficacy of antiviral treatment is defined as the ability of an antiviral drug to cause at least 1 log(IU/mL) reduction in HBV DNA from the baseline (Gish and Locarnini, 2006). Therefore, a total error of less than 1 log(IU/mL) does not affect the clinical decision-making. Because other parameters, including minor changes in serum, may also affect the HBV DNA level, the arbitrary value of 0.5 log(IU/mL) was considered as the maximum total allowable error (TAE) of the assay in this study. Based on this assumption, the values of systematic and random error were measured at 4.2 log(IU/mL) and 3.2 log(IU/mL), which are near clinical decision points.

For statistical calculations, logarithmic transformations of the plasma HBV DNA values were used. This is valid operation, since it may be assumed that the transformed values are distributed normally (Wayne, 2006; Yen-Lieberman et al., 1996). The operating Point was determined on a normalized Operational Process Specifications (OPSpecs) chart (Westgard, 1992b). Finally, the performance of the analytical method was assessed and candidate QC procedures were selected.

2.2. DNA purification

Nucleic acid was extracted from plasma using a Roche High Pure Nucleic acid purification kit (Roche Diagnostics, Indianapolis, IN) as per the manufacturer's instructions. The process is explained briefly: 200 μL plasma was incubated with 200 μL binding buffer, 5 μL polyA, 5 μL internal control and 50 μL proteinase K for 10 min at 72 °C. After adding a further 100 μL binding buffer, the mixture was brought to column. Following three rounds of washing with inhibitor removal and wash buffers, viral DNA was eluted into a final volume of 50 μL and stored at 4 °C for up at most two days before the qPCR was performed.

2.3. Polymerase chain reaction

The polymerase chain reaction was performed using a QIAGEN Artus HBV RG PCR kit (Hilden, Germany) with a procedure adapted from the manufacturer's instructions. In short, the process was as follows: 15 μL master-mix was blended with 10 μL of one of the following to prepare 25 μL reaction mixture: a purified sample, a negative control, or a standard. The purified samples contained HBV DNA and an internal control. Three to five standards and a negative control were included in each run. The reactions were performed in a Rotor-Gene 3000 real-time machine (Mortlake, Australia). The process was as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles of three steps, comprising: (1) 95 °C for 15 s, (2) 55 °C for 30 s, and (3) 72 °C for 15 s. The signal was acquired on FAM and JOE channels at the end of annealing step. The target amounts were quantified by a standard curve method, by means of Rotor-Gene 6, software version 6.1. Sample material in the plasma was quantified as IU/mL by the formula: Result (IU/μL) × Elusion Volume (μL)/Sample Volume (mL). In all reactions, the efficiency was more than 0.9 and the coefficient of regression was greater than 0.99.

2.4. Systematic error measurement

A lyophilized secondary WHO standard was purchased from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom) at a level of 10^6 IU/mL. The standard was reconstituted with 0.5 mL distilled water, as per the manufacturer's instructions, by means of a class A pipette. Using a class A pipette, dilutions were prepared by adding 100 μL of the reconstituted standard to 5 mL HBsAg-negative plasma, in order to achieve an HBV DNA concentration of 19,608 IU/mL. Subsequently, 500 μL of the diluted standard was added to 5 mL HBsAg-negative plasma, to achieve an HBV DNA concentration of 1783 IU/mL. Finally, 200 μL aliquots were prepared at each level and were frozen at −20 °C.

For each day over a five day period, duplicates were extracted of the two diluted standards separately, and quantified by a QIAGEN Artus HBV RG PCR assay.

The difference of the mean of the values of each level from the target value was calculated. The logarithmic transformation of this value was considered as an estimation of systematic error.

2.5. Random error measurement

Two plasma samples with HBV-DNA levels of about 2 log(IU/mL) and 4 log(IU/mL) were prepared by the pooling of HBV DNA positive and negative human plasma. Two-hundred microliters aliquots were prepared at each level and were frozen at −20 °C. Over a period of 15 months, a total of 20 samples, of each level were extracted separately, as described above, and quantified by a QIAGEN Artus HBV RG PCR assay. The total standard deviation (SD_t) of the values, which were transformed logarithmically at each level, was considered as an estimation of total random error.

To determine the standard deviation of the PCR phase, fourteen purified samples of each level were mixed, divided in aliquots and measured in five different PCR runs. Because the variation of purification was negated by pooling, the measurement would only reflect the variation of the PCR phase.

2.6. Statistical calculations

To confirm the normal distribution of HBV DNA levels following logarithmic transformation (log HBV), the one sample Kolmogorov–Smirnov test was applied. The mean, standard deviation (SD) and coefficient of variation (CV) of log HBV DNA were obtained at each control level after the exclusion of outliers, i.e. the results that deviated by more than 3.5 SDs from the mean. Ninety-five percent confidence intervals of the mean, SD and CV were calculated using a bootstrapping method. The total imprecision of the method is due mainly to the DNA purification and qPCR procedures. Therefore, the imprecision of the purification phase was calculated by: $SD_e^2 = SD_t^2 - SD_p^2$, where SD_t is the total standard deviation, and SD_e and SD_p are the standard deviations of the purification phase and of the PCR phase, respectively. An F test was employed to compare the imprecision of the purification phase in the upper and lower levels of HBV DNA. Consideration of the sample sizes used in this study and the calculated type I error of 0.05, F values of greater than 2.46 were considered significant. SPSS version 18.0 (2009) was used for statistical calculations, with p-values of less than 0.05 considered significant.

2.7. Normalized Operational Process Specification Chart

A Normalized Operational Process Specification (OPSpec) chart was constructed as described by Westgard (1992b). A brief explanation of the process is given: the percentage of observed systematic error and the coefficient of variation (CV) were calculated at each level, and divided by TAE of 0.5 log(IU/mL) to normalize. The operating point was then plotted on the OPSpecs chart (Fig. 1), for each level.

3. Results

The results of inter-run random error measurement were obtained from 12th January 2011 to 12th April 2012 (Fig. 2). The results of the systematic error measurement were obtained in April and May 2012.

The HBV-DNA values, which were transformed logarithmically, showed normal distribution (Kolmogorov–Smirnov test, $p > 0.05$).