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High Stability of the HBV-DNA in Samples Stored at Different Temperatures or Stressed by Repeated Freeze-Thawing Treatment

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Abstract

Most of the patients chronically infected with hepatitis B are living in countries with limited resources. The diagnostic systems need to be robust enough to facilitate the assessment of samples under stress due to temperature and power failure. The stability of the HBV-DNA was assessed at different temperatures and under freeze-thawing conditions in three different kind of HBV containing samples.

HBV positive serum samples and their purified matched HBV-DNA samples were incubated both at 37°C and -20°C and subsequently quantified by HBV-specific qPCR. In addition, the performance in qPCR of the above-mentioned samples and also of an in-house HBV plasmid standard curve based on a plasmid comprising the genome of the hepatitis B virus as a part of a novel qPCR system were assayed under freeze-thawing conditions.

No significant differences were detected between groups of treatment in terms of HBV-DNA levels, both in the case of serum samples or their purified matched counterpart when stored to both 37°C and -20°C or when treated with the freeze-thawing protocol. Similar results were obtained with the HBV plasmid standards when they were treated with the freeze-thawing procedures.

In conclusion, both serum HBV-DNA samples as well as the purified matched HBV-DNA serum samples were stable at 37°C, or after repeated cycles of freeze-thawing like also was the standard curve from the novel qPCR, further demonstrating the robustness of this quantitative system. These results support the use of this novel quantitative system in areas with limitations in cold chain conditions during transportation.

Keywords

HBV-DNA stability;
HBV serum samples;
HBV-DNA samples;
HBV qPCR standards;
Novel HBV qPCR system.

INTRODUCTION

The most common cause of chronic viral hepatitis in human beings worldwide is Hepatitis B virus (HBV). It is estimated that 20% from the approximately 250-300 million HBV-infected chronic patients will develop complications such as liver cirrhosis

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and hepatocellular carcinoma. The mortality related to VHB infection has been estimated in 700 000 patients per year with a rising trend [1].

The quantitative determination of the HBV level in blood or the viral load (VL) represents one of the most important variables for the management of the disease and also for clinical trials follow-up during the development of new products against chronic hepatitis B (CHB) [2-4]. In order to assess the value of available methods used for HBV DNA quantification it is necessary to explore the effect of sample processing, storage, and transport to ensure the accuracy and reproducibility of results [5].

Current international systems to quantify HBV DNA are expensive and still far from widely available at developing countries, however a low-cost qPCR system is under development at the Center for Genetic Engineering and Biotechnology (CIGB, Cuba) [6].

Only a few published studies refer to HBV-DNA stability under different storage temperatures and or in front of stressing freeze-thawing conditions [5]. Taking into account these limitations, we studied the stability of the two more frequent types of HBV-DNA samples used for VL quantification: the serum of the CHB carriers and their purified matched HBV-DNA obtained from such samples. Serum samples are traditionally preserved at -20°C. The stability of both types of samples at temperature storage of 37°C, and under freeze-thawing condition was evaluated. It was also studied the stability of the standard curve used by the qPCR system following a stressing freeze-thaw procedure. The samples from the standard curve, based on a plasmid comprising the complete genome of the HBV and covering a wide range of concentrations were studied, in order to assess the effect of these conditions when the samples contains decreasing concentrations of HBV DNA.

MATERIALS AND METHODS

Subjects

Serum HBV-DNA from 16 CHB patients (patients 1-16) was used for the stability assays. All patients were recruited at "Camilo Cienfuegos" Hospital in Sancti-Spiritus, Cuban central region. As a positive qPCR control (C+), the HBV-DNA positive serum from one CHB patient with a known concentration of $1x10^6$ copies/ mL was used.

HBV DNA quantification

HBV DNA purification: The HBV DNA of the 16 CHB patients was purified from 200 µL of serum with the "QIAamp DNA Mini kit" (Qiagen, Germany), according to the manufacturer's instructions.

Quantitative PCR reaction: qPCR reaction was prepared as described previously by Aguiar et al 2014 [6] using the unspecific commercial SYBR Green reaction mix from the "Quantitect SYBR Green PCR kit" (Qiagen, Germany).

Primers: The oligonucleotide pair used at a concentration of 0.67 µM was previously published by other authors [7,8]. The sequences of both primers are: sense 5′-GTGTCTGCGGCGTTTTATCA-3′ and antisense 5′- ACAAACGGGCAACATACCTT- 3′.

Thermal cycling: Thermal cycling was performed in a Rotor Gene 3000 Real-time PCR (Corbett Research, Australia). Reaction conditions were at 95°C for 15 minutes followed by 40 cycles at of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds.

Standard curve: The pST012012 plasmid that carries the complete 3.2 kb genome of the HBV was used as the quantification standard curve in the qPCR, as previously described by Aguiar et al., 2014 [6]. For the qPCR performance, we used in this research only a four - point standard curve: $9×10^5$ copies/reaction (c/r), $9×10^3$ c/r, 90 c/r and 9 c/r.

qPCR positive controls (C+): Each qPCR test run included a positive control (C+) sample ($1x10^6$ copies/ mL) and a negative control sample (human serum from Sigma).

Description of the stability experiments to different kinds of samples that contain HBV-DNA

Stability of the samples at the temperature of 37°C: Sixteen serum samples positive to HBV-DNA by qPCR and their sixteen purified matched HBV-DNA samples were incubated at 37°C or at -20°C during ten consecutive days. The VL was quantitated by qPCR and the results of the determinations of the samples stored at 37°C were contrasted with the VL

results of the samples stored at -20°C during the same period of time.

Freeze-thawing procedure: The same sixteen serum samples positive to HBV-DNA by qPCR and their sixteen purified matched HBV-DNA, as well five in-house standards prepared using the pST012012 plasmid [6], were thawed each day (ted) during one hour (1h) at room temperature (RT) and frozen again at -20°C for a period of ten days. All samples were compared to the matched samples stored at -20°C for the same period of time.

Statistical analysis

The VL estimated concentration using qPCR experiments was the variable followed to study the stability of the HBV-DNA containing samples. A novel low cost and robust qPCR assay, that was validated [6,9] and cross-validated [10] was implemented. GraphPad Prism Version 5.03 was used to obtain all the descriptive statistics parameters generated in the present study: For the comparison among the VL levels from the eight groups containing HBV DNA it was used an Ordinary one-way ANOVA, and in the specific case of the two treated groups composed by the five standards curve points, a Spearman Correlation analysis was also employed.

In general, the primary qPCR results (VL levels) were expressed in copies/ reaction, and after were converted to copies/ mL (c/ mL) for statistical analyses. Also, the VL values expressed in c/ mL were previously transformed by the log.

RESULTS

The robustness of a novel qPCR technique [6], was confirmed through the stability experiments under stressing conditions. The purified HBV-DNA samples as well as the serum samples from matched CHB carriers, stored at 37 and -20°C, and also the samples treated as part of a freeze-thawing protocol, demonstrated stability after storage under stressing conditions compared to the traditional temperature of storage (-20°C). No significant differences in qPCR results (p=0.9985) were detected among the serum samples of the CHB patients and their already purified matched HBV-DNA when both types of samples were exposed to temperatures of thermal stress (37°C) during 10 days (Figure 1). The implementation of the freeze-thawing protocol did not lead to significant changes in the qPCR results (p=0.9985) when both types of samples were studied (Figure 1). Variation of VL levels among all positive controls (C+) from the several qPCR performed were in the expected range (±0.5 log $1x10^6$ c/ mL), and the negative controls (C-) render undetectable results in all cases (data not shown).

Two types of CHB patients in relation with its VL levels to conform the cohort of 16 samples in each group were detected (Figure 1), some with relatively high VL levels, and others with low VL levels, in each case the VL levels were constant, suggesting that the effect of the DNA concentration was not a relevant factor under the studied conditions (Figure 2).

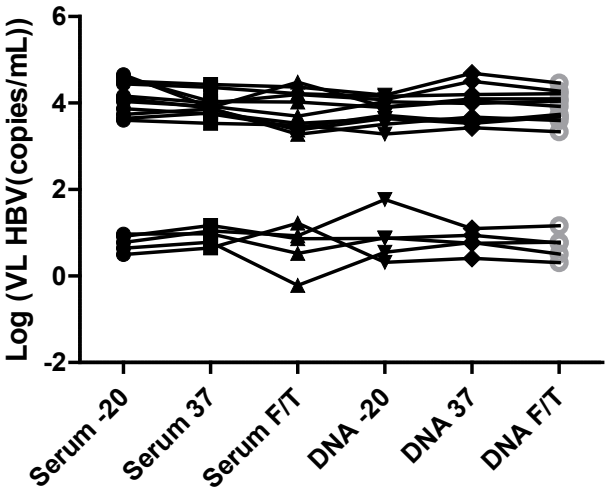

Figure 1. The graph represents the comparison among the values of the VL levels (converted firstly from copies/reaction to copies/mL) and transformed by the log, from the six groups containing HBV DNA: three groups with HBV DNA inside the serum of the CHB carriers treated at -20°C or 37°C, or by the freeze-thaw procedures (Serum -20, Serum 37, Serum F/T), and three more groups composed by pure HBV DNA obtained previously from the matched CHB serum carriers and treated (DNA -20, DNA 37, DNA F/T). There is no statistical significance when data were processed by one-way ANOVA (p=0.9985).

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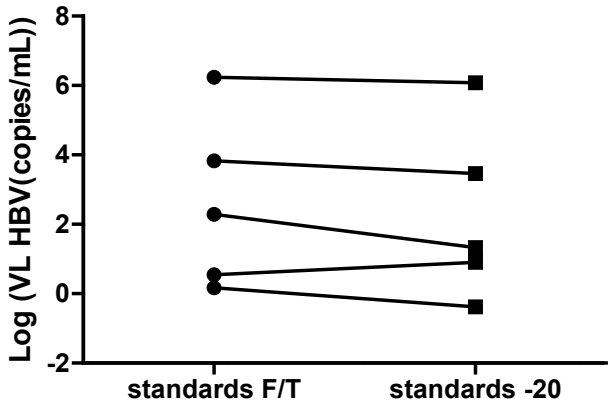

Figure 2. Comparison between the VL levels values (converted firstly from copies/reaction to copies/mL) obtained from the two treated groups composed by the five standards curve points described in the qPCR system for HBV quantification reported by Aguiar et al, 2014[6], based on the pST012012 plasmid that carries the complete HBV genome. These VL values were obtained after the Stability Experiments where the work concentration of these five standard curve points received two different treatments: at -20°C all the time (10 days) or freeze-thaw each day for one hour at room temperature. There are no statistical significance when data were processed by one-way ANOVA (p = 0.2014).

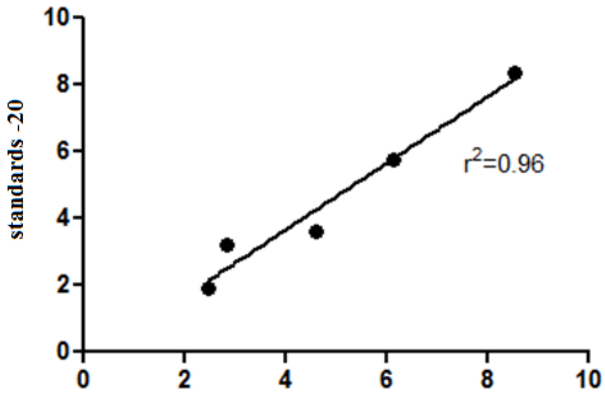

Figure 3. Correlation analysis between the VL levels (converted firstly from copies/reaction to copies/mL) from the two treated groups composed by the five standards curve points described in the qPCR system for HBV quantification reported by Aguiar et al, 2014[6], based on the pST012012 plasmid that carries the complete HBV genome. These VL values were obtained after the Stability Experiments where the work concentration of these five standard curve points received two different treatments: at -20°C for ten consecutive days or treated by freeze-thaw procedures each day for one hour at room temperature during the same time. Correlation was significant with a high Spearman Correlation Coefficient (r = 1.000) also a high Square Regression Coefficient (r^2=0.96), which strongly indicated that the freeze-thaw treatments applied to the standards curve evaluated has no significance in its performance.

The freeze-thaw procedure was applied to the five in-house standards at their working concentration. Subsequently the samples were evaluated by qPCR to study the stability of HBV DNA used as standard under stress condition. Statistical analysis showed no significant diffreneces of the estimated HBV-DNA concentration among the standards stored at -20°C or treated under freeze-thaw conditions (p=0.2014). The correlation analysis between the VL levels from the groups comprising the five standards stored at -20°C or treated by freeze-thaw procedures evidenced a statistically significant correlation, with a Spearman Correlation Coefficient (r=1.000) and also a high Square Regression Coefficient (r^2=0.96) (Figure 3).

DISCUSSION

Although additional studies to increase the sample size of the study may be conducted, the presented results demonstrate the robustness of the quantitative PCR system used to determine the concentration of HBV DNA. The two different types of samples used to preserve the HBV-DNA for quantification experiments were stable, suggesting that laboratories using the qPCR kits may store their samples both as serum samples and also as purified DNA. In addition, a wide range of DNA levels performs similarly although more specific studies may be conducted with this specific objective.

The high stability of the HBV-DNA at 37°C and the freeze-thaw procedures also support the robustness of the HBV-DNA quantification method to quantify

the virus in serum samples or in purified DNA, especially in cases where samples need long hours of transportation as well as in areas with instability in power supply where freeze chain could be affected temporally. This is especially important because the prevalence of HBV chronic infection is higher in resource limited countries.

Currently, there are few published studies on HBV-DNA stability under high storage temperatures [5]. Krajden et al., 1998 [11] demonstrated that he Quantiplex HBV-DNA assay (Chiron Corporation), based on bDNA technology, that the HBV-DNA contained in frozen serum, or in serum that was never frozen, and exposed under 45°C, 37°C, 23°C, 4°C and -70°C, were only stable at 4°C and -70°C for at least 5 days. In addition, José et al., 2005 [12], using the real time PCR quantitative technique to verify the HBV-DNA stability, reported that HBV-DNA stored at 5°C and 25°C was stable for at least 28 days, regardless of the initial titer. Furthermore, Lee et al., 2002 [13] demonstrated by qPCR technique that HBV DNA in plasma does not need a stabilizing solution for up to 14 days at 37°C or at temperatures lower than 37°C, because HBV DNA is more stable than HCV and HIV RNA. Recently, by qPCR technique, Almeida et al., 2015 [5], demonstrated that HBV-DNA contained in plasma is stable for at least 7 days at 42°C (Table 1).

Few published studies focused on the stability of the serum or plasma HBV-DNA samples under several cycles of freeze-thaw. All these researches agree that samples of plasma HBV-DNA are stable between eight and ten cycles of freeze-thaw [14-17].

Our research is in agreement with the abovementioned publications in the sense that serum HBV-DNA at higher temperature conditions than the traditional one at -20°C, is stable for several days. Also, the stability of the serum HBV-DNA treated with ten cycles of freeze-thawing is similar to the commented results regarding the temperature storage. In general, no statistical differences were found among groups (Figure 1). Differences were in a range of +/- 0.5 log which is an acceptable variability in this type of assays: VL less than 0.5 log (10) RNA/DNA copies number/ mL [5,18,19].

Serum samples as well as purified DNA are the most frequently samples used at the DNA quantification laboratories. The same results in the evaluation of these two types of samples open the possibility of accepting both types of samples for the DNA assessment, although the method need to be conveniently validated for both scenaries.

Other type of HBV-DNA containing sample, like the HBV standards of the qPCR system for HBV-DNA quantification reported by Aguiar et al., 2014 [6], based on a plasmid that carried the complete genome of the HBV, were previously demonstrated to be very stable at 37°C, 25°C and 4°C during one month [9].

Likewise, standard curves are very important tools in qPCR, for example to analyze precisely the concentration of pathogens like the viruses. Handling of DNA standards often implies multiple cycles of freezing and thawing that might affect DNA stability and integrity. This in turn might

Table 1: Summary of the eight groups treated in the Stability Experiment.

Type of Samples	Treatments for a period of ten days		Group-Names
	Temperatures (°C)	Freeze-Thaw conditions	
CHB serum sample	-20°C		Serum -20
CHB serum sample	37°C		Serum 37
CHB serum sample		ted 1h at RT and after -20°C	Serum F/T
HBV-DNA sample	-20°C		DNA -20
HBV-DNA sample	37°C		DNA 37
HBV-DNA sample		ted 1h at RT and after -20°C	DNA F/T
Plasmid Standards	-20°C		Standards -20
Plasmid Standards		ted 1h at RT and after -20°C	Standards F/T

influence the accuracy, reliability and reproducibility of quantitative measurements in qPCR experiments. In this work, the five standard curve points from the qPCR system [6,9,10], that are normally stored at -20°C, resulted very stable during the freeze-thawing procedure, evidencing the stability in broad range of HBV DNA levels of plasmid used as standards. Finally, currently available methods for DNA quantification are still expensive, the presented results may contribute to the generalization of a qPCR system to be used as part of the long-term follow-up of patients in resource limited settings.

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