Journal of
Genetics and Genome Research

Short Communication: Open Access

Application of Synthetic Standard Curves for Absolute Quantification of Hepatitis A and E by Real-Time PCR

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Short Communication

Hepatitis A, which presents a major health problem globally, is caused by hepatitis A virus (HAV). This virus, whose primary site of replication is the liver, is the most common agent causing acute liver disease worldwide [1]. Hepatitis E virus (HEV) is the most recently discovered of the hepatotropic viruses, its genome having been identified in 1991. Despite the similarity with hepatitis A virus in their pathogenesis and transmission form, HEV has a zoonotic potential [2].

Two major challenges of virus analysis include a lack of adequate information in infectivity and the difficulty to cultivate these viruses *in vitro*. Conservatively, immunological, nucleic acid-based methods, and cell culture have been applied as molecular techniques for virus analysis [3,4]. The disadvantages of these methods have incited for new detection approaches that are rapid, sensitive and specific as the real-time PCR technique. Real-time viral detection provides sufficient information regarding multiple steps in infection process at molecular level, which is valuable for the prevention and control of viral infections [5].

Conventionally, in absolute quantitative real-time PCR detection, the viral load is measured as copy number per cell or per total RNA/DNA following a transformation of the data using a standard curve. A standard curve is generated by performing a real-time PCR using a dilution series of template DNA made from plasmid DNA or, more recently, DNA oligonocleotides. The current literature suggests that there are advantages and disadvantages of using different templates for the construction of a standard curve in the absolute quantification [6]. As the results of our laboratory cost-benefits studies, we could observe that, despite displaying reliable results, using plasmid DNA as template is more expensive and takes longer to synthetize than DNA oligonocleotides. The advantage of using DNA oligonucleotides is that it can be custom synthesized, and only requires the nucleotide sequence information.

Over the past decade, synthetic biology has taken advantage of a comprehensive inventory of biomolecular parts [7] and it is providing alternative diagnostic tools for viral detection and monitoring. This report highlights the application of synthetics standard curves for quantification of hepatitis A and E genomes by real-time PCR. The aim of this study was to examine the potential of two DNA oligos for hepatitis A (HAV) and hepatitis E virus (HEV) genomes quantification that could be adopted readily into established testing protocols.

The HPLC-pure oligonucleotides specifying the 89bp HAV (5' non-coding region) and 74bp HEV (ORF3 region) amplicons were custom synthesized by IDT® (CoralVille, USA) (Table 1), corresponding to the same genome fragments used for plasmid DNA standard curve construction. Following manufacturer's specifications, the DNA oligo was dissolved in DNase/ RNase-free distilled water to 100pmol/uL which is equivalent, approximately, to 10^{13} DNA molecules/uL according to the Avogadro number calculation – ([DNAg/uL]/ Plasmid size (bp) x 660) x (6,022x10^{23}). The standard curves were generated by performing a TaqMan real time PCR, previously described by De Paula et al. and by Jothikumar et al. for HEV [8,9]. In this study, the plasmid standard curve was replaced to ultramer template ten-fold dilution series (10^{-1}-10^{-20}). The specific primers and probes were used according to the literature above. After determining the dilution range for each synthetic curve, they were

Table 1: Hepatitis A and E synthetic standard curve sequences

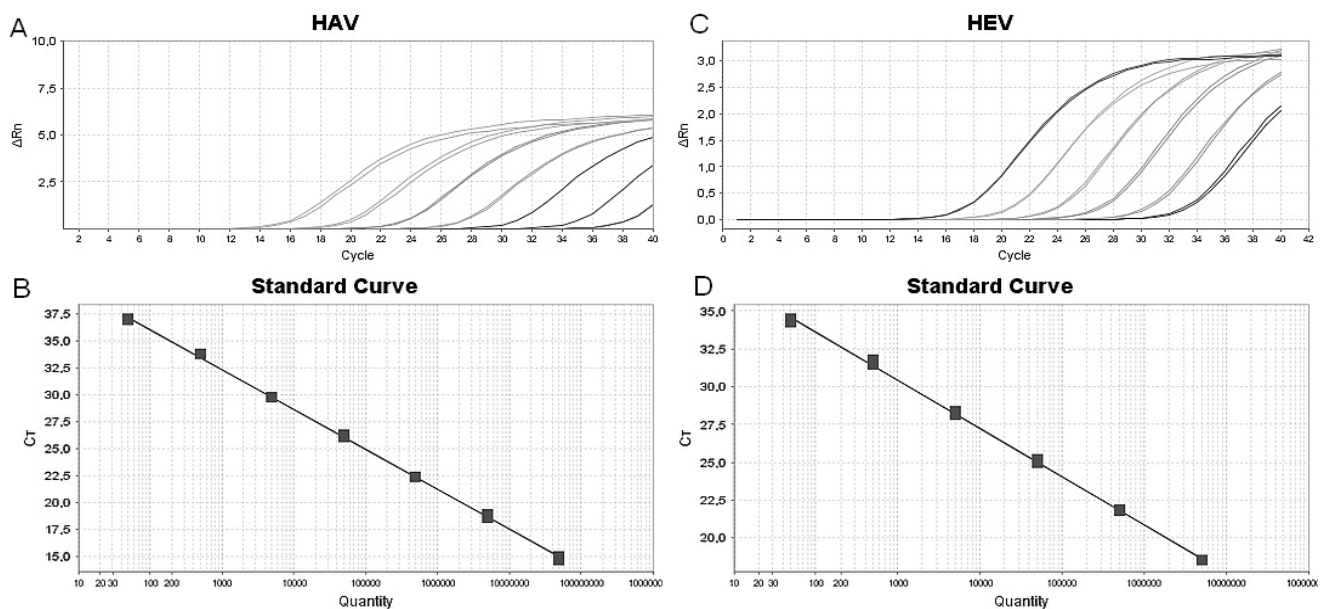| Virus	Ultramer sequence	Size (bp)	Amount of oligo
HAV	5'-TTC GTG AGA GCC CTG GAA GAA AGA AGA CGT ATC AGA AAG CGT GAA AAA TGA GTA TGC GTG ATT TAA GAA CCC TGA ACC TGC AGC TGA TA-3'	89	4nmol
HEV	5'-TTC GTA GGG GTT GGT TGG ATG AAC GTA GCG AAG GGC TGA GAA TCA ATG CGT GTC ACC CCA GAA ACC ACC TTC GT-3'	74	5.1nmol

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Figure 1: Synthetic oligo DNA standard curves for absolute quantification of hepatitis A and E virus by real-time PCR. Amplification plot and linear regression of HAV synthetic standard curve, respectively, slope -3,499, R^2 0,99 (A) and (B). Amplification plot and linear regression of HEV synthetic standard curve, respectively, slope -3,301, R^2 0,999 (A) and (B).

assayed along with plasmid DNA as standard curve to compare their quantification in copies/uL. Therewith, the absolute quantification using DNA oligo as standard curve could be performed using a panel of 14 HAV and HEV available samples with known quantification.

Firstly, in our experiments, we could observe that the range dilution for HAV synthetic curve was 10^6-10^0 molecules/uL and for HEV 10^6-10^1 molecules/uL (Figure 1). The difference between the dilution ranges of both oligo DNAs could be explained by HEV genome quantification results. It was detected non-specific amplification at high dilutions or at a low copy number. The Ct value of the HEV oligo DNA standard curve was leveled off at high Ct values due to low input DNA concentrations. Bowers and Dhar also reported it; however, this effect was apparent when the plasmid DNA and 500 bases *in vitro* transcribed RNA template were used as templates. The data from their study suggested that the size of the template used to generate standard curve should be very similar to the size of the amplicon which will reduce or eliminate the nonspecific amplification. In this report, we use this strategy but we could not observe similar results. Nevertheless, later data demonstrated that this effect did not have a directly influence on HEV genome absolute quantification due to the exclusion of non-specific amplification templates.

Following, it was assayed both plasmid and synthetic DNA curves with the panel samples. As the plasmid DNA curve was optimized for both viral genomes (range $2x10^{-2}$-10^{-8}ng/ul), they were used as "gold standard" curve to compare sample quantification for HAV and HEV. Samples quantification using each type of curve as standard was compared each other and the difference between their results were analyzed. HAV results demonstrated that quantification ratio between the two types of curves ranged from 0.83 to 2.14 times. The same comparison was performed for HEV samples and quantification ratio ranged from 1.87 to 2.23 times. Difference in absolute quantification between both types of curves, for HAV and HEV genomes, did not exceed one log. Furthermore, there was not discordance between qualitative results of tested samples.

Amplification efficiency values were similar for both types of curves, as indicated by the slopes of separate standard curves. The results of HAV absolute quantification demonstrated a slope average for plasmid DNA of -3,345, and oligo DNA of -3,499. For HEV, the slope average for plasmid DNA was -3,27 and for oligo DNA -3,301 (R^2>0,99 for all assays).

The absolute quantification of viral genomes by real time PCR has been become routine. Conventionally, a standard curve is generated using a dilution series of cloned genomic segment of the target virus as template, which is time consuming, and costly [6].

Recently, oligonucleotide representing the target gene has been used as template to generate a standard curve, as reported in this study and ISO/TS 15216-1:2013 that describe a method for quantification of levels of HAV from test samples of foodstuffs or food surfaces.

Our RT-PCR quantification methods were not compared with ISO/TS 15216-1:2013. However, the real-time PCR to hepatitis A, described here, have been used since 2007 to detect and quantify HAV from water, food, saliva, cell culture, serum, liver and stool samples with plasmid standard curve [10-15]. The results showed that the standard curve can be replaced for a synthetic curve.

The synthetics biology can overcome the biological limitations of plasmid curve. We could demonstrate that oligo DNA could be used as template of standard curve due to its similar results with the plasmid DNA curves (gold standard). Oligo DNAs standard curves could be a helpful alternative or laboratories that do not have enough space and financial conditions for molecular cloning. Due to these characteristics, they could be a very useful tool on viral diagnosis.

Financial Support

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