Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

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

Comparison of reverse-transcriptase qPCR and droplet digital PCR for the quantification of dengue virus nucleic acid

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ARTICLE INFO

Keywords:
Droplet digital PCR
Dengue virus
RT-qPCR
RT-ddPCR
Viral quantification

ABSTRACT

Polymerase chain reaction (PCR) is an important molecular biology technique for *in vitro* amplification of nucleic acids. Reverse transcriptase quantitative PCR (RT-qPCR) and more recently reverse transcriptase digital droplet PCR (RT-ddPCR) have been developed for the quantification of nucleic acids. We developed an RT-ddPCR assay for the quantification of attenuated dengue virus serotype 2 nucleic acid and compared it with a routine RT-qPCR assay. While the routine RT-qPCR assay targets the NS5 gene, the E gene was selected for the optimization of the RT-ddPCR assay conditions. The specificity of the assay was demonstrated using the attenuated dengue virus serotype 2 alone and in the presence of the other three dengue serotypes. The results from both assays for 25 samples of the attenuated dengue virus serotype 2 were found to be comparable, with an R^2 from the linear regression analysis of > 0.98. A major advantage of the RT-ddPCR assay is that it allows quantification of nucleic acid, without the need of a standard curve. RT-ddPCR can be implemented for the absolute quantification of dengue vaccine virus nucleic acid during the vaccine manufacturing process.

1. Introduction

Dengue is a mosquito-borne disease causing an estimated 390 million infections annually. It is a major public health problem throughout the tropical world [1]. The causative dengue viruses (DENVs) are members of the genus Flavivirus, which are lipid-enveloped, positive-sense, single-stranded RNA viruses, about 55 nm in diameter. The DENV genome is translated into a polyprotein that is co- and post-translationally cleaved to yield three structural proteins and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Fig. 1). The capsid or core (C) protein forms a nucleocapsid complex with the virion RNA located inside the lipid envelope. The membrane (M) and envelope (E) proteins are embedded in the lipid envelope via carboxy-terminal transmembrane domains and are displayed on the surface of virions.

There are four serotypes (DENV-1 to DENV-4) that are now present in Asia, Africa and the Americas [2]. A tetravalent dengue vaccine, Dengvaxia®, developed by Sanofi Pasteur, has been shown to reduce the incidence of virologically-confirmed dengue in children aged 2–16 years in two large, phase III clinical trials in Asia and Latin America [3,4]. The vaccine has been licensed in several countries in Asia and Latin America, making it the first vaccine to be licensed for the prevention of disease caused by four dengue virus serotypes.

During the vaccine manufacturing process, as for any viral vector, the European Medicines Agency (EMA) recommends that all relevant information including virus concentrations should be provided for characterization purposes [5]. One measure that is recommended by the World Health Organization (WHO) and the EMA is the ratio of particles to infectious units. This can be determined by assessing the number of genome equivalents (Geq) and the infectivity as measured using plaque assays or 50% cell culture infectious dose ($CCID_{50}$) assays. As recommended in the EMA guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines, the vaccine viruses need to be quantified at the pre-master seed lot, the master seed lot and the working seed lot stages, as well as at various stages during the vaccine production [6]. Currently we determine the genome equivalents are using an RT-qPCR assay that requires purification and concentration of the viral RNA before it is retro-transcribed to cDNA and amplified by PCR [7]. Although the development of quantitative polymerase chain reaction (qPCR) techniques was a major advancement, the technique requires comparison of an unknown sample with a 'standard' to obtain quantitative measurements. During the process, the target nucleic acid sequence is copied exponentially with sequence-specific primers, until a signal produced by a fluorescent probe is

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https://doi.org/10.1016/j.biologicals.2018.01.001
Received 22 May 2017; Received in revised form 13 October 2017; Accepted 19 January 2018

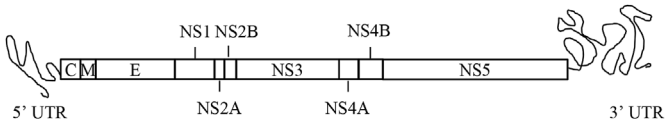

Fig. 1. Schematic representation of the dengue virus genome.

detected. The number of cycles needed to produce a detectable signal is related to the amount of template nucleic acid. A standard curve, produced with a 'reference' sample is used to correlate the cycle number with a known amount of nucleic acid. The preparation and distribution of the standard dilutions are possible sources of error during the process. Also, since there is no universal standard, when the reference standard stock is depleted and has to be replaced, the equivalence of the new reference standard stock with the previous one has to be checked, making its management labor-intense for control laboratories.

Digital PCR (dPCR), which can detect a single DNA molecule, was first reported in the 1990s and has been used increasingly for the quantification of various DNA targets [8–11]. More recently a droplet digital PCR (ddPCR) has been developed [12]. This technology uses a combination of microfluidics and proprietary surfactant chemistries to divide randomly nucleic acid into water-in-oil droplets uniform in size and volume [13]. Each droplet contains the reagents required for the PCR amplification of the template molecules and they are subject to workflows similar to those used for most standard TaqMan probe-based assays which use the 5′ nuclease activity of Taq DNA polymerase to cleave a fluorescently-labeled probe (FAM-labeled minor grove binder). Following PCR, the fluorescence emitted by each droplet is detected to determine the number of droplets in which the amplified sequence is detected (considered as positive) and the number of droplets in which there is no amplification (considered as negative). These data are used to give an estimated absolute quantification which is derived from the count of the proportion of positive droplets relative to the total number of droplets and their known volume, using Poisson statistics [14].

RT-ddPCR, unlike the earlier digital PCR techniques, offers scalable and automated technologies for implementation. Serial dilutions, which are labor-intense and a possible source of error, are not necessary since the sample can be partitioned into droplets in one step. This partitioning results in superior precision and linearity because it measures individual molecules (target copy number) rather than an ensemble concentration.

Here we report results from the development of an RT-ddPCR assay for the quantification of nucleic acid from an attenuated serotype 2 dengue virus and its comparison with our routine RT-qPCR assay.

2. Materials and methods

2.1. Dengue virus samples

The RT-ddPCR assay was developed using an attenuated serotype 2 dengue virus sample at a concentration of 10.6 $\log_{10}$ Geq/mL, estimated by qPCR. Since the RT-ddPCR assay has to be serotype-specific because the vaccine contains the four dengue serotypes, we assessed the specificity of the RT-ddPCR assay by mixing the attenuated serotype 2 dengue virus with the other three serotypes (1, 3 and 4) to obtain a tetravalent sample.

2.2. RT-qPCR

RNA was extracted with the QIAsymphony Certal Vaccine Kit (QIAGEN®) for the routine RT-qPCR assay, following the manufacturer's instructions. The primers are located in the well-conserved dengue NS5 region which codes for the viral polymerase (Fig. 1). The target RNA was detected in a single reaction after amplification in a mix of RNA and DNA polymerases in the presence of a specific TaqMan fluorescent probe. The intensity of the fluorescence was measured at the start of the PCR exponential phase when the nucleic acid copy number is represented by a linear equation. The viral RNA concentration was estimated using a standard curve established using known concentrations of dengue NS5 RNA, expressed as $\log_{10}$ genome equivalent per mL ($\log_{10}$ Geq/mL).

2.3. RT-ddPCR primers and probes

The in-house primers and probe that are currently used in the qPCR assay that target the NS5 gene were assessed during the RT-ddPCR assay development (Fig. 1; Table A1). In addition, three primers/probes that targeted the E and NS3 genes and the 3′ UTR region, described in the literature, were adapted and assessed (Fig. 1; Table A1) [15–17].

2.4. Reverse-transcriptase-ddPCR method

The RT-ddPCR was developed using a one-step RT-ddPCR kit for probes, 2X buffer control kit, droplet reader oil and droplet generator DG8TM oil (Bio-Rad®). The equipment used included Droplet Generator Cartridges, Droplet Generator DG8TM Gaskets, and Pierceable Foil Heat Seal (Bio-Rad®) and Twin-tec PCR 96-well plate (Eppendorf®).

RNA was extracted from the attenuated serotype 2 dengue virus using QIAcube and QIAamp Viral RNA Mini Kit (QIAGEN) during the RT-ddPCR assay development phase. For the RT-ddPCR assay, 5 µL of extracted RNA was combined with 20 µL of the master mix, primers and probe and placed in a well in the 8-well droplet generator cartridge with 70 µL of droplet generator oil. The cartridge was placed in the droplet generator machine that can generate 1000 droplets/well/second. The droplets were transferred to a PCR plate which was placed in a PCR thermal cycler apparatus where the reverse transcriptase reaction occurred followed by 45 PCR cycles. The droplets were then sucked into the reader at 1500 droplets/second and individually read by the fluorescence reader that can read 1,400,000 droplets per 96-well PCR plate. Droplets were considered positive when the specific fluorophore (FAM: 6-carboxyfluorescein) was detected. The result for each droplet was plotted on a graph of fluorescence intensity versus the droplet number. Droplets with fluorescence above threshold intensity were scored as positive and assigned a value of 1. All droplets with fluorescence below the threshold were scored as negative and assigned a value of 0 (zero). The fraction of positive droplets was fitted to a Poisson distribution, which corrected the results by taking into consideration the probability that the droplet may have contained > 1 copy, when only one was expected. These results were analyzed, taking into consideration the total number of droplets and their known volume, to determine the concentration of sample, expressed as the $\log_{10}$ genome equivalent per mL ($\log_{10}$ Geq/mL). These results were presented as scatter plots with the fluorescence amplitude on the y-axis and the cumulative number of droplets analyzed on the x-axis.

We varied the concentrations, time and temperature parameters for the PCR reaction to determine which gave optimal results, i.e., the best separation between the droplet populations in the positive and negative clusters. The manufacturer recommends performing annealing and elongation of the primers at 60 °C. Using the Thermocycler T100® (Bio-Rad), we assessed the impact of temperature, ranging from 55.0 °C to 68.0 °C, on the separation between the positive and negative clusters and the virus quantification. The manufacturer recommends that primer concentrations should be between 500 nM and 900 nM and the probe at 250 nM. We tested concentrations of 300 nM, 500 nM, 700 nM and 900 nM for the primers with the probe at 250 nM and also the probe at 125 nM, 250 nM, 350 nM and 500 nM with the primer at 500 nM.

2.5. Comparison of RT-qPCR and RT-ddPCR

The comparison of RT-ddPCR and RT-qPCR was performed using 25 samples of attenuated serotype 2 dengue virus from different stages of