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Short communication

Droplet digital PCR as a useful tool for the quantitative detection of Enterovirus 71

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A B S T R A C T

Hand, foot and mouth disease (HFMD) is a contagious viral disease that frequently affects infants and children and present with blisters and flu-like symptoms. This disease is caused by a group of enteroviruses such as enterovirus 71 (EV71) and coxsackievirus A16 (CA16). However, unlike other HFMD causing enteroviruses, EV71 have also been shown to be associated with more severe clinical manifestation such as aseptic meningitis, brainstem and cerebellar encephalitis which may lead to cardiopulmonary failure and death. Clinically, HFMD caused by EV71 is indistinguishable from other HFMD causing enteroviruses such as CA16. Molecular diagnosis methods such as the use of real-time PCR has been used commonly for the identification of EV71. In this study, two platforms namely the real-time PCR and the droplet digital PCR were compared for the detection quantitation of known EV71 viral copy number. The results reveal accurate and consistent results between the two platforms. In summary, the droplet digital PCR was demonstrated to be a promising technology for the identification and quantitation of EV71 viral copy number.

1. Introduction

Enterovirus 71 (EV71) is a positive sense, single stranded, non-enveloped RNA virus of the Picornaviridae family (Nasri et al., 2007a,b). EV71 is one of the main etiological agents for hand foot and mouth disease (HFMD) that commonly affects infants and children (McMinn, 2002, 2012; Patel and Bergelson, 2009; Ooi et al., 2010; Solomon et al., 2010; Lui et al., 2013b). This disease is caused by a group of enteroviruses which is usually self-limiting, characterised by various symptoms such as fever, rashes, poor appetite and multiple ulcers in mouth (Wong et al., 2010; Ho et al., 2011; Huang et al., 2012). However, unlike other HFMD causing enteroviruses, EV71 been commonly associated with severe clinical diseases, including neurological diseases such as aseptic meningitis, brainstem and cerebellar encephalitis leading to cardiopulmonary failure and death (Chu et al., 2001; Lee and Chang, 2010; Lee et al., 2011, 2012; McMinn, 2012). Clinically, HFMD caused by EV71 is indistinguishable from other HFMD causing enteroviruses such as coxsackievirus A16 (CA16) (McMinn, 2002, 2012; Patel and Bergelson, 2009; Ooi et al., 2010; Solomon et al., 2010; Lui et al., 2013b, 2014a,b).

Currently, the gold standard for identification of enteroviruses involves propagation by cell culture followed by neutralisation with specific antisera to confirm the serotype (Tan et al., 2006, 2008b). However, this procedure is limited by its long turnaround time which may take a few weeks. Furthermore, some enteroviruses may proliferate sub-optimally in cell cultures, require several passages or may not be typeable (Tan et al., 2006, 2008a,b). Recent technological advances have resulted in the development of molecular methods e.g. such as PCR and real-time PCR, which are more sensitive and have shorter turnaround time (Mackay et al., 2002). Quantitative real-time PCR (qPCR) has been widely implemented and use to determine viral load in both clinical and research laboratories (Mackay et al., 2002).

Over the past two decade, fluorescence based qPCR chemistry have revolutionised nucleic acid diagnostics and become the gold standard for quantitation of viral load (Henrich et al., 2012; Hayden

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et al., 2013). Quantitative determination of viral load has been used to monitor the efficiency of antiviral therapy and to determine changes in that therapy (Hindson et al., 2013; McDermott et al., 2013). Rising viral burden have been used as a trigger for pre-emptive treatment to prevent symptomatic infection (Henrich et al., 2012; Hayden et al., 2013). Although qPCR have made major impact in the advance of disease diagnostics, this technology has notable limitation (Palmer, 2013; Sanders et al., 2013).

Quantitative determination of the viral load in the real-time PCR cycler is dependent on a series of known standards across a linear range to an unknown sample to determine the concentration to that of the calibration curve (Henrich et al., 2012; Hayden et al., 2013; Sanders et al., 2013; Sedlak and Jerome, 2013). However, the variation in performance and material in the assay lead to disagreement between different laboratories (Henrich et al., 2012; Hayden et al., 2013; Sanders et al., 2013; Sedlak and Jerome, 2013). The use of international standards made by the World Health Organisation (WHO) mitigate the issue however these standards are only available for a few common target analysts (Hayden et al., 2013). As such, droplet digital PCR (ddPCR), an emerging nucleic acid detection methodology that provides absolute quantitation of targeted sequence would be a useful tool to resolve this issue. This method relinquishes the need for calibration/standard curve and the reliance of rate-based measurement (Ct values) (Hayden et al., 2013). Since the establishment of this technology, this platform has been used to determine the presence of microorganism such as HIV, cytomegaloviruses and other viral pathogens in patients to monitor antiviral therapeutics and the quality of water etc (Henrich et al., 2012; Hayden et al., 2013; Palmer, 2013; Strain et al., 2013; Racki et al., 2014). The objective of this study is to evaluate if ddPCR is a comparable platform for the detection and quantitation of EV71.

2. Material and methods

2.1. Quantitation of EV71

The standard use in this study was generated and used previously in the laboratory (Tan et al., 2006). The EV71 strain used in this study was isolated from a fatal case of HFMD during October 2000 outbreak in Singapore, Enterovirus 5865/sin/000009 strain from subgenogroup B4 (accession number 316321; hereby designated as Strain 41). Briefly the standard was generated by conventional PCR to amplify a 204 bp VP1 fragment using forward primer 5′-GAGAGTTCTATAGGGGACAGT-3′, and the reverse primer 5′-AGCTGTGCTATGTGAATTAGGAA-3′ from within the VP1 region of EV71 (nt2466 to nt2669). The PCR product (204 bp) was then cloned into the pGEM-T plasmid vector and subsequently transformed into *Escherichia coli* JM109 competent cells (Promega, USA). RNA was synthesised in vitro from the recombinant plasmids and the concentration was measured spectrophotometrically at 260 nm. The number of viral copies was then calculated. A series of ten-fold dilutions were then prepared, yielding a series of viral copies ranging from 2.5×10^0 copies to 2.5×10^7 copies.

2.2. Quantitative real time polymerase chain reaction

The quantitative real time polymerase chain reaction (qRT-PCR) was performed using the iQ™ Multiplex Powermix (Bio-Rad Laboratories, CA, USA) on the BioRad CFX96™ Real-Time PCR system (Bio-Rad Laboratories, CA, USA). The EV71 specific primers targeting the conserve VP1 regions were 5′-GAGAGTTCTATAGGGGACAGT-3′, Taqman probe 5′-HEX-GATGACTGCTCACCTGTGTGTTTTGACC-BHQ-1 and the reverse primer 5′-AGCTGTGCTATGTGAATTAGGAA-3′ (Tan et al., 2006). Briefly, standard of viral copies ranging of from 2.5×10^0 copies to 2.5×10^6 copies were added with primers and probe to iQ™ Multiplex Powermix (Lui et al., 2013a,b; Tan et al., 2008a,b). The reaction mix was then subjected to thermal profile of denaturation at 95 °C for 10 m, followed by amplification and quantification in 40 cycles at 95 °C for 10 s, 60 °C for 30 s followed by 50 °C for 30 s. At the end of amplification cycles, melting temperature analysis was performed by the BioRad CFX96™ Real-Time PCR system (Bio-Rad Laboratories, CA, USA).

2.3. Droplet digital PCR

The droplet digital polymerase chain reaction (ddPCR) was performed using the ddPCR mastermix (Bio-Rad Laboratories, CA, USA) on the BioRad QX100 droplet digital PCR system (Bio-Rad Laboratories, CA, USA). Briefly, standard of viral copies ranging of from 2.5×10^0 copies to 2.5×10^3 copies were added with primers and probe to the ddPCR in a final volume of 20 μl in accordance to the manufacturer's instructions (Bio-Rad Laboratories, CA, USA). The reaction mixture was then processed with 70 μl of droplet generation oil (Bio-Rad Laboratories, CA, USA) using the droplet generator (Bio-Rad Laboratories, CA, USA). The droplets generated were then transferred into a 96 well plate (Eppendorf, Germany) and PCR amplification was performed with a thermal profile of denaturation at 95 °C for 10 m, followed by 40 cycles at 94 °C for 30 s, 60 °C for 60 s followed by 98 °C for 10 m on a T100 thermal cycler (Bio-Rad Laboratories, CA, USA). Finally, the plate was loaded onto the droplet reader (Bio-Rad Laboratories, CA, USA) and the data was generated and analysed using the QuantaSoft analysis software (Bio-Rad Laboratories, CA, USA).

2.4. Data analysis

GraphPad Prism Version 6.0c (GraphPad Software, USA) was used to generate standard curve from real-time PCR and ddPCR data. All statistical analysis was performed on GraphPad Prism Version 6.0c (GraphPad Software, USA).

3. Results

Viral load testing procedures has been used extensively in research laboratories and are now integrated to a diverse setting ranging from drug companies to clinical diagnostics laboratories. Quantitative values have been used to monitor the efficacy of antiviral treatment and/or to determine changes in the therapy (Kiselinova et al., 2014; Sedlak and Jerome, 2013). Despite the advances and the use of a highly established platform like the real-time PCR, challenges in viral load testing remains due to the intrinsic limitation of the method which central on the issue of precision, accuracy and standardisation (Hayden et al., 2013; Hindson et al., 2013; Sanders et al., 2013; Sedlak and Jerome, 2013; Strain et al., 2013; Kiselinova et al., 2014). ddPCR, a direct measurement of target molecules which relinquishes the need for calibration/standard curve and obviate the need to developed costly international standards is an attractive tool for viral load testing (Henrich et al., 2012; Sanders et al., 2013; Sedlak and Jerome, 2013).

To investigate if ddPCR is a comparable platform for the detection and quantitation of EV71, real-time PCR and ddPCR were performed using known standards of EV71 (Tan et al., 2006, 2008b). Known standards of EV71 viral copies were used for the determination of linear regression correlation coefficients (R^2) for the log transformed copy number obtain from both real-time PCR and ddPCR data. The expected copy numbers measured