Journal of King Saud University – Science xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

qRT-PCR for enterovirus detection: Conversion to ultrafast protocols

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ARTICLE INFO

Article history:
Received 22 July 2016
Accepted 8 April 2017
Available online xxxx
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Keywords:
Enterovirus
qRT-PCR
Ultra-Fast protocol

ABSTRACT

Enterovirus group (EV) still causes significant morbidity with economic impact worldwide. Quantitative RT-PCR (qRT-PCR) technology offers many advantages over to conventional RT-PCR in terms of rapidity and specificity. The TaqMan hydrolysis probe technique and Syber Green I intercalating dye strategy are by then largely used. Several published protocols are applied routinely for EV detection in food and environmental analysis advanced in chemical strategies and thermal profiles, in order to reduce the response times. In this study an Ultra-Fast protocol to detect and quantify EV RNA genome was tested and the application prospective of the protocol was described and discussed. The assay effectiveness was evaluated comparing two different set of primers/probe, targeting a 5′UTR region of EV genome. Three different oligonucleotides concentration were tested: 200 nM, 250 nM and 300 nM for TaqMan technique whereas 200 nM, 300 nM and 400 nM were employed for Syber Green I chemistry. The results demonstrated the validity of this Ultra-Fast approach, compared to the Traditional and Fast protocols. The best performance was obtained using 200 nM of proper oligonucleotides, in both of the chemical strategies tested. The response time of analysis was reduced at 50′ (probe) and 57′ (intercalating dye) per run, against the other longer protocols. The oligonucleotides features can affect the assay performance and should satisfy specific characteristics.

1. Introduction

Enterovirus group (EV) includes divers human enteric viruses, such as poliovirus, coxackievirus group A, coxackievirus group B, echovirus, etc, that still causes significant morbidity with economic impact in both developed and not developed countries (Dubot-Pérès et al., 2014; Hindiyeh et al., 2014; Zhang et al., 2014). EV persists in the environment for long time especially in the water bodies and may be transfers in food, keeping the chain of infection. In environmental matrices the EV load is variable, very low in the waters bodies compared to the food and/or the wastewater matrices. For this reason, a lot of time is consumed for the pretreatment and the concentration steps needful prior to evaluate the presence of viral particles.

Active environmental surveillance protocols require sensitive and specific tests which may reduce times and manipulation of samples at least during the detection assays respect to the time consuming culture isolation method. Molecular tests as the latest quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) represent an excellent and rapid analytical tool giving accuracy, efficiency and reproducibility of the assays (Bustin, 2005). Among fluorogenic chemical strategies the TaqMan hydrolysis probe and the Syber Green I intercalating dye techniques are commonly used in qRT-PCR and several published protocols have entered into the routine for EV detection in food and environmental matrices (Nijhuis et al., 2002; Donia et al., 2005, 2010; Dubot-Pérès et al., 2014; Jebri et al., 2014). The amplification of the highly conserved 5′-untraslated region (5′UTR) of EV genome is still largely used in environmental surveillance and clinical analyses (Kurdziel et al., 2001; Donaldson et al., 2002; Donia et al., 2010; Gervasi et al., 2012; Harwood et al., 2013; Zhang et al., 2014; Jebri et al., 2014; Kaas et al., 2016) because can detect all members of the EV group (Yates, 2014).

A qRT-PCR protocol for EV detection has been optimized by Donia et al. (2005) and applied in environmental assays (Donia et al., 2010; Masciopinto et al., 2011). Advancing in biochemical strategies and thermal profiles have been gradually enhanced the published protocols, reducing analysis response times. For this purpose the industries have improved the instruments and the performances of the reagent mixes suggesting Ultra-Fast run formulations, to ensure reliable and reproducible data in shorter

Peer review under responsibility of King Saud University.

ELSEVIER Production and hosting by Elsevier

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http://dx.doi.org/10.1016/j.jksus.2017.04.003
1018-3647/

times by the qRT-PCR application. Indeed, during the years, the qRT-PCR run times were reduced, on average, from 220 min to 120 min per test consequent the technology enhancements. The latest generation instruments, updated by new spectra optical systems and analysis software, promise superior precision data in 50 min per test using Ultra-Fast chemistry approaches. By the protocols conversion it is expected energy and cost savings, over the time, without to affect data analysis. Based on the current knowledge there are no data in the literature about the application of Ultra-Fast protocols for enteric virus's detection.

In this paper the results of this application were exposed and preliminary results were discussed in order to consider this procedure in environmental viruses monitoring. For this purpose two qRT-PCR protocols were compared: the first one optimized by Donia et al. (2005) and successfully employed by researchers in environmental (Le Guyader et al., 2008; Vilariño et al., 2009) and clinical assays (Le Guyader et al., 2008), the second suggested by Donaldson et al. (2002) and widely cited in several articles and used to date by researchers in the environmental EV surveillance and food safety (Tuladhar et al., 2012; Zhang and Wang, 2014; Kaas et al., 2016).

2. Materials and method

2.1. Experimental design

A comparison between two qRT-PCR protocols in use for EV detection and quantification was developed converting the thermal profile in Ultra-Fast run.

The protocols conversion was evaluated applying the chemical strategies commonly used TaqMan hydrolysis probe and Syber Green I intercalating dye, testing different oligonucleotides concentration.

2.2. Stool sample processing and positive controls preparation

To assess the applicability of the protocol a stool sample positive for EV genome presence was processed as unknown specimen. Stool sample was acquired from hospitalized children, diagnosed with acute gastroenteritis and screened for the panel of enteric viruses by RT-PCR end point. Feces were diluted with phosphate-buffered saline (PBS) (Merck) or 0.89% NaCl to 10% suspensions. After vigorous mixing the fecal specimen was clarified by centrifugation at 2,500*g* for 20 min at 4 °C, and then ultracentrifuged to remove any molecular test inhibitors in a bench Beckman ultracentrifuge Optima TL (Milan, Italy) equipped with a TLA-100 rotor at 50,000*g* for 1 h at 4 °C.

Three enteroviruses were chosen to simulate positive specimens and as positive EV controls: Poliovirus 1 Sabin type (P1S), Coxsackievirus B4 (CB4) and Echovirus 9 (Ecv9). Viruses were cultured as previously described (Donia et al., 2005) and titrated in laboratory by qRT-PCR.

Sample and controls were tested in two replicates each one. Extraction and amplification effectiveness was evaluated using the synthetic RNA standard cloned, tested as the reaction control at several known concentrations, 1×10^4, 1×10^2, 1×10^1 gce/μL.

RNA was extracted by the QIAmp viral RNA kit (Qiagen, Milan, Italy) following the manufacturer's instructions and dissolved in RNase -free water containing 0, 5 U/μL of RNase inhibitor (Promega, Milan, Italy).

2.3. Standard cloning

A 263 bp fragment of the 5′UTR region of the complete sequence of P1S strain (Accession number AY184219, position 428–690 nucleotides) was cloned in pCR 4 TOPO vector (Invitrogen- Milan, Italy), in accordance with the manufacturer's recommendations. Synthetic RNA was transcribed in vitro (T7 RNA polymerase – Promega, Milan, Italy) and after digested with Dnase and Rnase-free RQ1 (Promega, Milan, Italy). Synthetic RNA was purified with the QIAmp viral RNA kit (Qiagen, Milan, Italy) following the manufacturer's instructions and dissolved in RNase -free water containing 0, 5 U/μL of RNase inhibitor (Promega, Milan, Italy). The synthetic RNA yield was determined spectrophotometrically (NanoDrop- Euroclone - Milan, Italy) and converted in gce/μL (1×10^8 gce/μL, stock solution).

Table 1
Data expressed by the protocols compared in the study.

Ts/Tas/Tp (Donia et al., 2005)	Primers/probe concentration	Slope values (means)	CC (R^2) (means)	EFF (means)
TaqMan 220 min.#	200 nM	−3.3	0.991	99.5%
	250 nM	−3.0	0.886	109%
	300 nM	−4.0	0.966	76%
TaqMan 120 min.*	200 nM	−3.4	0.999	98%
	250 nM	−3.0	0.994	106%
	300 nM	−2.9	0.975	114%
TaqMan 50 min.§	200 nM	−3.2	0.998	102%
	250 nM	−2.2	0.960	134%
	300 nM	−2.0	0.890	140%
SybGr I 240 min.#	200 nM	−3.9	0.995	78%
	300 nM	−3.7	0.996	85%
	400 nM	−3.3	0.997	100%
SybGr I 150 min.*	200 nM	−3.2	0.998	99%
	300 nM	−3.0	0.995	97%
	400 nM	−2.8	0.988	106%
SybGr I 57 min.§	200 nM	−3.2	0.999	102%
	300 nM	−2.9	0.988	106%
	400 nM	−2.5	0.899	141%
Up/Dw/Pr				
Donaldson et al. (2002)				
TaqMan 220 min.#	600/250 nM	−3.4	0.999	98%
	400 nM	−2.4	0.898	150%
TaqMan 120 min.*	600/250 nM	NA	NA	NA
	200 nM	NA	NA	NA
TaqMan 50 min.§	600/250 nM	NA	NA	NA
	200 nM	NA	NA	NA

#Traditional qRT-PCR protocol; *Fast qRT-PCR protocol; §Ultra-Fast qRT-PCR protocol; NA = No Amplification.

Please cite this article in press as: Donia, D.T.. Journal of King Saud University – Science (2017), http://dx.doi.org/10.1016/j.jksus.2017.04.003