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Short communication

Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices

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SUMMARY

A new nanofluidic digital RT-PCR method was developed for sapovirus (SaV) using control material obtained according to standards for enteric viruses. Primers employed amplify a fragment of 112 bp of the polymerase capsid junction, allowing the detection of human genogroups I, II and IV. Analytical validation was performed in clinical, shellfish and environmental water samples. This novel protocol rendered great effectiveness and repetitiveness, as well as higher sensitivity than real time RT-PCR assay, with differences in quantification ranging from 0.1 to 2.6 log-units. The method described here can constitute a promising tool for standardizing SaV quantification.

1. Introduction

Real-time RT-PCR (RT-qPCR) has been used worldwide over the last years to study the prevalence of enteric viruses, including human sapovirus (SaV). At present, RT-qPCR is established for a great variety of matrices: clinical, food and water, and environmental samples. It enables a sensitive, reliable and quantitative amplification of target sequences down to a few copies, which is especially challenging in some of those matrices, due to their low viral load and the presence of inhibitory substances (Greening and Cannon, 2006). RT-qPCR strengths are based on high sensitivity, specificity, reproducibility, speed, and minimal risk of carryover contamination (Fraisse et al., 2017). This technique has been established in the ISO 15216 as the method for detection and quantification of norovirus and hepatitis A virus in foodstuffs (ISO/TS, 2013; ISO, 2017). Nevertheless, RT-qPCR presents several limitations that must be taken into account. For instance, standard curves are needed for the quantification, which is calculated relatively and not absolutely, and reference materials are usually not easy to acquire. On the other hand, it has been demonstrated that inhibitory molecules typical from some matrices (especially food and environmental samples matrices) interfere with the polymerase activity during the PCR (Bustin and Nolan, 2004; Girones et al., 2010; Polo et al., 2016). The inclusion in the ISO protocol of two types of controls (process and external amplification) to palliate some of these limitations increases the economic and time costs of the procedure.

Digital PCR (dPCR) may solve some of these limitations partitioning each reaction mix on nanofluidic chips or micro-droplets across thousands of individual PCR reactions containing zero, one or more copies of the target sequence (Fraisse et al., 2017; Rački et al., 2014). Target molecule quantification is calculated after endpoint using binomial Poisson statistics by the ratio of positive to total partitions (Dube et al., 2008; Pinheiro et al., 2012). As dPCR is an absolute approach, there is no need for reference materials or a standard curve. It therefore avoids the amplification efficiency bias, observed with qPCR (Bhat et al., 2009). Partitioning on dPCR may be especially advantageous for the complex detection of viruses in food and environmental samples, decreasing the impact of inhibitory matrix-type compounds. Also, it presents higher sensitivity for detecting low viral loads (Rački et al., 2014; Hindson et al., 2011).

Human SaV is one of the most important agents implicated in foodborne outbreaks of acute gastroenteritis (Todd and Greig, 2015). It has been detected worldwide in different sample matrices as feces (Page et al., 2016), foods (Varela et al., 2016b) and environmental waters (Sano et al., 2011). This enteric virus is non-enveloped, with positive sense, single-stranded RNA genome and member of the family *Caliciviridae*. It is mainly transmitted via faecal-oral route and highly stable in the environment (Oka et al., 2015). The main purpose of the study was the development of a nanofluidic digital PCR methodology for the detection and quantification of human SaV.

2. Material and methods

Primers and probes employed for nanofluidic RT-dPCR and RT-qPCR were the same, targeting the polymerase capsid junction,

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Table 1
RT-dPCR conditions assayed in the optimization experiments.

Reverse Transcription	Inactivation		45 cycles		Final elongation
			Denaturation	Annealing & elongation	
10 min 45 °C[×] 20 min 45 °C	15 min 95 ºC	One-Step[×] Two-Steps	15 s 95 °C	1 min 50 °C 2 min 50 °C[×]	3 min 50 °C

·Conditions selected as the best from all tested.

conserved for genogroups I, II and IV (Oka et al., 2006). Final concentrations were 400 nM for primers and 200 nM for Taqman probe. The reaction volume was 15 µL consisting in: 12 µL mix (1 X RT-PCR buffer, 0.99 µL of enhancer and 0.6 µL RT-PCR enzyme) from AgPath-ID™ One-Step RT-PCR (Applied Biosystems, Life Technologies, Bleiswijk, Netherlands), and 3 µL RNA sample. In order to optimize the methodology, the protocol was designed and ran with dilutions of a standard SaV material obtained by cloning the target fragment into a plasmid, transforming competent cells, then purified and quantified as previously described (Varela et al., 2016a). Dilutions of the SaV positive control plasmid were tested in duplicates and also by RT-qPCR to compare the results obtained with the two procedures.

Different conditions were tested using QuantSudio™ 3D Digital PCR System (Applied Biosystems, Life Technologies, Bleiswijk, Netherlands). Retro-transcriptase (RT) was carried out both separately and in one-step, and also trying 10 and 20 min at 45 °C. The 45 cycles of amplification started with 15 s denaturation at 95 °C, and then two options were tested for annealing and elongation: 1 and 2 min at 50 °C; and a final elongation step for 3 min at 50 °C (Table 1). After running, chip information was read into QuantStudio™ 3D Digital PCR Instrument and data analysed on QuantStudio™ 3D Analysis Suite Cloud Software.

Clinical, shellfish and environmental water samples were considered to assess the analytical performance of the method in these matrices. A total of 25 of samples were analysed, being distributed as follows: 6 clinical stool samples from Spanish outpatients with acute gastroenteritis (C1 to C6), classified into different human SaV genogroups I, II and IV (unpublished results); 7 shellfish samples from different species: *Meretrix lyrata*, *Callista chione*, *Ensis macha*, *Donax* sp. and *Argopecten purpuratus* (S1 to S7); and 12 environmental water samples from six sewage treatment plants (STPs) in Portugal (W1 to W6), one sample from the influent of the plant and another one from the effluent in each STP. Viral recovery was performed as previously described (Varela et al., 2016b) following basically the principles of the ISO standard protocol (ISO/TS, 2013). Samples were analysed in duplicate to test the repetitiveness of the method using the final optimized conditions. Experiments were carried out also by RT-qPCR as previously described (Varela et al., 2016b). All RT-dPCR and RT-qPCR assay included the correspondent positive (plasmid) and negative (molecular grade water) controls.

Statistical analysis was conducted with R Statistical Software version 3.4.3 (R-Project, Vienna, Austria) in order to compare quantification by RT-qPCR and RT-qPCR. Analytical experimental results of the three matrices were tested through a one-way analysis of variance (ANOVA) (R Core Team, 2013). Data were converted into a logarithmic format, samples below the limit of quantification were converted into that limit value, and negative samples were settled for the limit of detection. Linear regression analyses were conducted using Microsoft Excel 2007.

3. Results

The reaction partition in this nanofluidic dPCR equipment breaks the mixture in a chip among approximately 20,000 wells, which gives a theoretical maximum quantification range of five magnitude orders. Concentrations above that were not analyzable, and dilutions were used on those expected to present high loads, based on the RT-qPCR analysis. Results showed no difference between the two options of retro-transcriptase or its duration, therefore a 10 min one-step RT was selected as it presents benefits both in time and cost of the procedure. Better results were achieved with the 2 min annealing and elongation time (data not shown).

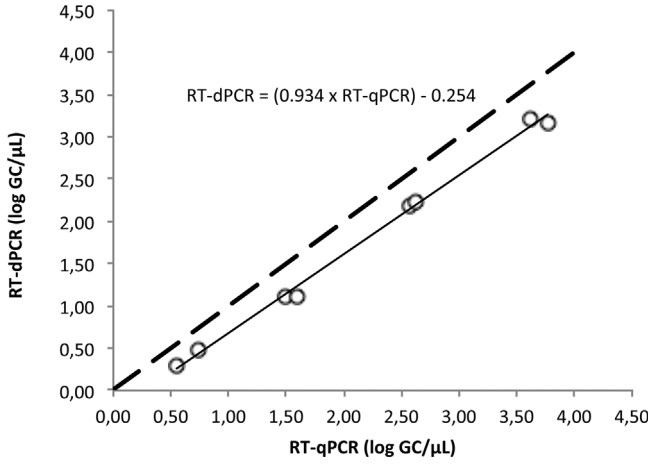

Fig. 1. Linear regression analysis of human SaV concentration determined by RT-dPCR and RT-qPCR using dilutions of standard SaV controls. Black line represents regression, dashed line identity of quantification, and dots log quantification measures from both procedures for each dilution.

Dilutions of the SaV positive control plasmid used corresponded to 10^0–10^3 genome copies per µL (GC/µL). Comparison along dilutions between RT-dPCR and RT-qPCR showed that RT-qPCR gives 0.2–0.5 log higher values of concentration. Linear regression between the two procedures established log RT-dPCR as (0.934 × log RT-qPCR) − 0.254 (Fig. 1).

Analytical experiments ran correctly with the final conditions regardless the matrix analysed, including molluscs which are considered to be the most difficult samples to amplify based on the high concentration of inhibitors in them. In general, RT-dPCR quantification ranges varied from a minimum of 3.7×10^{-1} GC/µL of extracted RNA observed in the effluent of a sewage treatment plant, to a maximum of 3.6×10^{6} GC/µL of extracted RNA detected in one of the clinical samples (Table 2). Samples with very low SaV concentrations (below 1.0×10^{0} GC/µL of extracted RNA) were repeated for dPCR to avoid possible false negative results. In all cases results were confirmed (data not shown).

The two techniques were compared calculating the difference between the logarithm of RT-qPCR quantification and the RT-dPCR equivalent. For some samples it was not possible to make these estimations, as some of them were below the quantification limit for RT-qPCR, which was calculated in 6.7×10^{-1} GC/µL. Results showed a general log difference average of 0.75 between both methodologies with these samples, meaning higher viral load quantifications by the RT-qPCR. Stool matrix collection presented the highest difference of viral load, estimated in almost 1 log. In two samples, quantification was higher using RT-dPCR (shellfish and sewage samples). Four samples were positive for human SaV only when using RT-dPCR (1 shellfish and 3 sewages), and one was only detected by RT-qPCR (sewage) (Table 2).

The quantitative data of SaV genome copies obtained overall by RT-dPCR were not significantly different from that quantified by RT-qPCR as shown through the one-way ANOVA analysis. Comparing each sample separately, most of the cases again were not significantly different, except for two clinical samples (C2 and C3), two shellfish samples (S2 and S7) and four water samples (W2i, W3i, W3e, W4e) ($p < 0.05$) Also, to compare the precision of methods, the standard