

Simultaneous Detection of Selected Enteric Viruses in Water Samples by Multiplex Quantitative PCR

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Received: 8 December 2015 / Accepted: 3 March 2016 / Published online: 12 March 2016
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Abstract Human rotavirus (HRV), adenovirus (HAdV), and norovirus (HNV) are the most common causes of viral gastroenteritis in humans worldwide. Application of quantitative PCR (qPCR) coupled with reverse transcription (RT), known as RT-qPCR, has greatly improved detection sensitivity for enteric viruses, but it has been mostly used in monoplex mode where only a single virus type can be quantified per assay. Here, we report the development of a simple and specific multiplex RT-qPCR assay for simultaneous quantification of rotavirus, adenovirus, and norovirus in ground water, river water, and wastewater samples. For all three virus groups, the monoplex and multiplex RT-qPCR yielded virtually identical PCR efficiency, dynamic quantification range, and detection sensitivity, indicating the reliability of the multiplex RT-qPCR assay for simultaneous quantification of three viruses. The multiplex assay also accurately quantified the target genes of a small number (40 copies per PCR) in the presence of competing viral genes of up to 10^4 -fold more abundant. Specificity of the assay was estimated to be 100 % for all three viruses when tested against 19 common waterborne microorganisms. The results

showed that our multiplex RT-qPCR assay holds considerable promise in quantifying the three enteric viruses in environmental water samples.

Keywords Multiplex reverse transcriptase quantitative PCR · Water quality · Adenovirus · Norovirus · Rotavirus · Environmental water

1 Introduction

Human rotavirus (HRV), adenovirus (HAdV), and norovirus (HNV) are the most common causes of viral gastroenteritis worldwide (Jiang et al. 2014; Pang et al. 2013; van Maarseveen et al. 2010). They are non-enveloped viruses with RNA (HRV and HNV) or DNA (HAdV) genomes. These viruses represent a large part (26.7 %) of all gastroenteric pathogens that are isolated in Canada, but also the most under-reported enteric viruses (National Enteric Surveillance program in Canada 2012) likely due to the lack of a rapid and reliable monitoring tool. Norovirus infections via various environmental routes account for about 20 million annual cases of acute gastroenteritis, resulting in 570 to 800 deaths, in USA (US Centers for Disease Control and Prevention 2015). About 12 % of all recreational waterborne disease cases were attributed to norovirus and adenovirus infections in USA between 2011 and 2012 (Hlavsa et al. 2015). Although enteric viruses are usually present in small numbers in environmental water samples, such as ground water or surface water, their infectious doses can be low. For example, infectious

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doses for humans were estimated to be as low as 10 to 100 rotavirus particles and about 18 norovirus particles (Gray 2011; Hall et al. 2011).

The current standard method of enteric virus detection is based on viral infection and replication in a mammalian cell line (Rice et al. 2012). However, limitations of this method prevent it from being used in routine surveillance and monitoring of enteric viruses (Wong et al. 2012). These include the requirement of a specific cell line for each virus type, the propensity to underestimate infectious viruses from environmental samples, and being time-consuming and costly (Hamza et al. 2011). Furthermore, many viruses, including human norovirus, are not routinely cultivable in laboratories to date. To better protect public health from threats of these enteric viruses, it is crucial to develop a fast and accurate method of detection and quantification.

Application of real-time quantitative PCR coupled with reverse transcription (RT), RT-qPCR, has greatly improved detection sensitivity for enteric viruses (Butot et al. 2010; Jothikumar et al. 2005; Pang et al. 2013; Stals et al. 2009), but it has been mostly used in monoplex mode, i.e., to detect a single target. In contrast, the multiplex approach can be simpler for detection of multiple enteric viruses in the same sample, thereby reducing the cost and time of tests and allowing higher throughput analyses. However, previous attempts to detect HRV, HAdV, and HNV simultaneously (Jiang et al. 2014; van Maarseveen et al. 2010) did not compare the performance of the multiplex RT-qPCR assay to that of a monoplex counterpart. Furthermore, these assays were not fully validated for their applicability as routine surveillance tools; instead of utilizing absolute quantification capacity of qPCR assays, only qualitative information (i.e., presence or absence of target viruses) was tested for field samples.

The present study was undertaken to (a) develop a simple and accurate multiplex RT-qPCR assay to simultaneously quantify three common enteric viruses, norovirus GII, rotavirus, and adenovirus (strain 41), in water samples and (b) validate performance of the assay in terms of absolute quantification capacity, specificity of target virus detection, and reproducibility of monoplex RT-qPCR performance.

2 Materials and Methods

2.1 Cell Culturing Procedures and Microorganisms Tested

Rotavirus strain Wa (ATCC VR-2018, Manassas, Virginia, USA) was propagated in the MA104 cell line derived from African green monkey kidney epithelial cells (Arnold et al. 2009). The cell line was obtained from European Collection of Cell Cultures (Salisbury, UK). The cell culture was incubated in minimal essential medium with Earle's salts (MEM, pH 7.2; Life Technologies Inc., Burlington, ON, Canada) supplemented with 2 mM glutamine, 1 % non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 10 % fetal bovine serum (Life Technologies), and 0.075 % HCO_3^- . The cell culture was incubated at 37 °C with 5 % CO_2 . Adenovirus type 40 (strain Dugan, ATCC VR-931) and type 41 (strain Tak, ATCC VR-930) were kindly provided by Dr. M. Brown (University of Toronto, Toronto, ON, Canada). They were propagated in the HEK293 cell line (human embryonic kidney cells; Microbix Biosystems, Mississauga, ON, Canada) with MEM (pH 7.2) supplemented with 200 mM L-glutamate, 10 % fetal bovine serum, 7.5 % HCO_3^- , penicillin (10,000 U/mL), and streptomycin (10 mg/mL) (Brown 1990). The cell culture was incubated at 37 °C with 5 % CO_2 . Upon observation of cytopathic effects, such as changes in cell morphology or complete disintegration of cells, virions were released from host cells by 5 cycles of freezing (−80 °C) and thawing (room temperature). Particulate impurities were removed by centrifugation at 4800×g for 10 min. The supernatant layer was collected and stored at −80 °C until further tests. Norovirus RNA purified from clinical samples was provided by Dr. P. Jayaratne (Hamilton Regional Laboratory Medicine Program, Hamilton, ON, Canada). Nineteen common waterborne microorganisms, including our target viruses, were subjected to the nucleic acid extraction and cDNA creation procedures (for RNA viruses) as described below and tested for specificity of the developed qPCR assay. The following microorganisms were kindly provided by Dr. S. Weir (Ontario Ministry of the Environment and Climate Change, Etobicoke, ON, Canada): *Acinetobacter lwoffii* ATCC 17925, *Aeromonas hydrophila* ATCC 35654, *Bacillus subtilis* ATCC 6633, *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 11775, *Enterococcus faecalis* ATCC 19433, *Klebsiella pneumoniae* ATCC 13883,

Proteus vulgaris ATCC 6380, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *Staphylococcus epidermidis* ATCC 12228, *Cryptosporidium parvum* (environmental isolate), *Cryptosporidium muris* (environmental isolate), and *Giardia intestinalis* (environmental isolate). Enterovirus RNA was also provided by Dr. Jayaratne.

2.2 Sample Concentration, Propidium Monoazide (PMA) Treatment, and Environmental Sample Analyses

Water samples were concentrated by passing through positively charged alumina nanofiber NanoCeram[®] cartridge filters (Argonide Corp., Calgary, AB, Canada) as described by Karim et al. (2009) under a constant water flow rate of 1500 mL per min. Viruses adsorbed to the filter were soaked with 400 mL of an elution buffer (1.5 % beef extract and 50 mM glycine at pH 9.5) for 1 min and eluted. The first batch of collected eluant was fed back to the filter, used to soak the filter for 15 min and eluted for the second time. To the eluant, an equal volume of 16 % PEG 8000 (polyethylene glycol) (Sigma-Aldrich, Oakville, ON, Canada) was added and the eluant-PEG mixture was held stationary at 4 °C for 1 h to enhance flocculation (Vidovic et al. 2011). The mixture was concentrated by centrifugation at 15,000×g for 30 min at 4 °C. The pellets containing virus particles were resuspended in molecular biology grade water (HyClone Water; GE Health Care Life Sciences, Baie d'Urfé, QC, Canada) and particulate impurities were removed by passing through a 0.2-µm-pore size syringe filter (Whatman polyether sulfone membrane filter; GE Health Care Life Sciences). An aliquot of the filtrate containing viruses was treated with propidium monoazide (PMA; Biotium Inc., Hayward, CA, USA) as described below. Both PMA-treated and untreated portions were stored at -80 °C until nucleic acid extraction.

Infectious virus particles, defined as virions with intact capsids, were differentiated from non-infectious ones (i.e., virions with compromised capsids) by PMA treatment. PMA forms covalent cross-links with exposed nucleic acids (van Frankenhuyzen et al. 2011), either as free nucleic acids or nucleic acids originating from a virion with a compromised capsid, and thus prevents their amplification and quantification by qPCR (Fittipaldi et al. 2010; Parshionikar et al. 2010). Virus cultures or filtrates were treated with 10 µM (final concentration) PMA at room temperature (about 20 to

26 °C) for 10 min in the dark and subsequently on ice for 10 min while exposed to halogen light.

Untreated raw ground water samples (80 to 1020 L in volume depending on turbidity of water) from ten different locations in Ontario, Canada, were filtered on site through a NanoCeram[®] filter between July 24 and August 7, 2014. Four more environmental water samples [1000 L of ground water, 180 L of river water, and 1 L of wastewater (collected before and after disinfection with low pressure UV lamps)] were filtered between December 12, 2014 and January 13, 2015. The filters were eluted in the lab and processed according to the sample concentration procedures.

2.3 Nucleic Acid Extraction and Reverse Transcription (RT)

Concentrated water samples and virus cultures were subjected to nucleic acid extraction (both DNA and RNA) using the QIAamp MinElute Virus Spin Kit (QIAGEN Canada, Toronto, ON, Canada). Purified nucleic acids were stored at -20 °C before use. The RT reaction was carried out using the iScript cDNA Synthesis Kit (Bio-Rad Life Science, Mississauga, ON, Canada) with random hexamer primers. A typical RT reaction contained 4 µL of 5× reaction buffer, 1 µL of reverse transcriptase, and the extracted sample RNA (100 fg to 1 µg) in a final volume of 20 µL. The mixture was incubated in a thermal cycler (Model C1000, Bio-Rad Life Science) at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and then held at 4 °C. The RT product contained qPCR templates for adenovirus (genomic DNA), as well as for rotavirus and norovirus (cDNA). It was stored at -20 °C until further use for qPCR.

2.4 Real-Time Quantitative PCR (qPCR)

PCR primers and probes listed in Table 1 were designed for this study or modified from previous studies using AlleleID (ver. 4.0, Premier Biosoft, Palo Alto, CA, USA) to simultaneously quantify three viruses in multiplex mode. Three TaqMan probes (HRV-TP, HAdV-TP, and HNV-TP) were labeled at their respective 5'-ends with the reporter dyes 6-FAM (Life Technologies, Burlington, ON, Canada), VIC (Life Technologies), and JUN (Life Technologies), respectively, and tagged at the 3'-ends with a Minor Groove Binder-Non Fluorescent Quencher (Life Technologies). For norovirus, ORF2 (the junction of polymerase and capsid gene) of the genotype II was

Table 1 PCR primers and probes used to detect three enteric viruses

Virus	Primer/probe	Sequence (5' → 3')	Accession no	Position ^a	T _m	Reference
Human rotavirus	HRV-F ^{b1}	ACCAATCTACACATGACCCTCTATG	X81436	963–986	58.3	Pang et al. (2012) modified
	HRV-F2	ACCAATCTTACAGTAACCCCTCTATG	DQ146697	980–1003	58.8	Pang et al. (2012) modified
	HRV-R ^c	ACATAACGCCCTATAGCCATTT	X81436	1045–1067	58.6	This study
	HRV-TP ^d	AATAGTAAAAGCTAACACTGTC	X81436	992–1014	69.0	This study
Enteric adenovirus	HA _{AdV} -F	TCCGACCCACGATGTAACCA	D13781	370–389	59.1	Pang et al. (2012) modified
	HA _{AdV} -R	CACGGCCAGCGTAAAGCG	D13781	464–481	58.3	This study
	HA _{AdV} -TP	ACAGGTACACGCGACT	D13781	393–408	69.0	Pang et al. (2012) modified
Human norovirus	HNV-F	CCAATGTTTCAGATGGATGAGATTCTC	X86557	5009–5034	59.1	This study
	HNV-R	TCGACGCCATCTTCATTACACA	X86557	5080–5100	58.3	Kageyama et al. (2003)
	HNV-TP	ATCGCCCTCCACGT	X86557	5045–5059	69.0	This study

^a Oligonucleotide positions are based on the corresponding accession number

^b Forward primer

^c Reverse primer

^d TaqMan probe. All TaqMan probes were tagged with the Minor Groove Binder-Non Fluorescent Quencher at the 3' end

selected as the target for the qPCR assay. The non-structural protein 3 (NSP3) gene was targeted for qPCR detection of rotavirus species A (strain Wa). For adenovirus, the hexon structure gene of species F (type 41) was selected for qPCR. Specificity of the designed primers and probes was analyzed by BLAST search of the GenBank Database (National Center for Biotechnology Information, Bethesda, MD, USA).

Each qPCR assay contained a commercial PCR master mix (iQ Multiplex Powermix, Bio-Rad Life Science), primers (final concentration of 250 nM), and probes (final concentration of 100 nM) specific for each virus, and the template DNAs in the RT product (i.e., cDNA derived from norovirus and rotavirus as well as genomic DNA from adenovirus). The qPCR was run in a thermal cycler (iQ5 Multicolor Real-Time PCR Detection System, Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.5 Calibration and Quality Control of the RT-qPCR Assay

The assay was calibrated using standard plasmids containing the target gene sequences. The target gene sequences for three viruses were amplified with specific primer sets that were also used for the multiplex assay (Table 1). The amplified DNA was cloned into pGEM-T vector (Promega, Madison, WI, USA), which was transformed into competent *Escherichia coli* JM109 cells. Putative transformants were selected on LB agar plates containing 100 µg/mL ampicillin, 0.5 mM IPTG, and 80 µg/mL X-Gal. Each plasmid was screened for presence of the target gene by colony qPCR. For each qPCR run, standard quantification plasmids that were prepared in a series of 10-fold dilutions served as positive controls. Sample PCR assays were judged to be successful when the positive controls reproduced the known threshold cycles (Ct) along the dilution series. No Template Control (a qPCR control without template) was set up in duplicate for each PCR run by adding PCR grade water to the PCR mastermix in place of DNA template.

The inhibitory effect of sample matrix on RT-qPCR was estimated using an aliquot of nucleic acid extract for each field sample. A concentrated HRV RNA stock (~10⁵ HRV gene copies per 10 µL), which was more than 10² times higher than the highest environmental HRV level observed in this study, was spiked into the twofold serial diluents (no dilution, 1/2, 1/4, and 1/8) of the sample nucleic acid extract as well as into reagent-

grade water (Milli-Q Water Purification System; Millipore, Etobicoke, ON, Canada) to serve as a positive control. The spiked sample nucleic acid extract and positive control were subjected to RT-qPCR as described above. The Ct value of the positive control was compared to those obtained from the field samples. When a field sample exhibited a deviation of Ct value by >1.0 from that of the positive control, it was considered as a sign of PCR inhibition. Depending on the inhibition result, field samples were diluted up to 1/8 times with molecular biology grade water (GE Health Care Life Sciences) to reduce inhibitory effects prior to repeat RT-qPCR.

2.6 Concentration of Viruses from Water Samples and Estimation of Recovery Efficiency

Recovery of viruses during the sample concentration procedures was estimated by spiking HRV and HAdV41 cultures together in 10 L of reagent-grade water in triplicate and comparing the titer of the seeded viruses to that of the filtrates. Viruses were seeded at two different levels per 10 L of water: a high level at 2×10^5 (HRV) or 1×10^6 (HAdV41) gene copies, and a low level at 2×10^3 (HRV) or 1×10^4 (HAdV41) gene copies. Virus seeded water was subjected to the same concentration and recovery procedures and analyzed by multiplex qPCR as done for the environmental samples.

2.7 Assay Validation and Data Analysis

Performance of the multiplex RT-qPCR assay was validated in terms of absolute quantification capacity, specificity of target virus detection, similarity to monoplex RT-qPCR performance parameters, and absence of competition effect. Absolute quantification capacity was assessed from triplicate measurements of common qPCR parameters, such as detection limit, dynamic quantification range, and PCR efficiency. Detection limit was defined as the smallest amount of the qPCR standard that produced a positive qPCR signal at 95 % confidence level. Efficiency of qPCR was calculated from the slope of the standard quantification curve using the formula, $\text{efficiency} = 10^{(-1/\text{slope})} - 1$ (Bustin et al. 2009). Precision of qPCR quantification was estimated from the r^2 value of the standard quantification curve using test for significance of a regression line. Specificity was defined as how accurately the assay could differentiate the target genes from non target ones without

false-negative or positive results. All 19 microorganisms were subjected to the RT-qPCR assay in triplicate and specificity was calculated as the number of true negative qPCR divided by the number of true negative qPCR plus the number of false-positive qPCR (Lee et al. 2006).

Demonstrating that the multiplex RT-qPCR performance parameters are similar or identical to the monoplex RT-qPCR is an important step in validating a multiplex assay. This is necessary because multiplex assays tend to exhibit lower sensitivity and efficiency than its monoplex counterparts (Jiang et al. 2014; Stals et al. 2009) due to various non-specific reactions among complex RT-qPCR components. Similarity of the quantification parameters between the monoplex and multiplex qPCR was analyzed based on the slope and Y-intercept values of the regression lines using analysis of covariance.

To determine the extent of competition for PCR resources (e.g., dNTPs and Taq polymerase) in multiplex mode, 40 copies of a target virus gene was mixed with varying concentrations of another virus gene (0, 40, 4×10^2 , 4×10^3 , 4×10^4 , 4×10^5 copies per PCR). This experiment was set up in triplicate to simulate a situation in which a small quantity of the target virus (i.e., close to the detection limit of the assay) co-exists with competing background viruses at much larger quantities. The Ct value of the target virus gene was hypothesized to increase as the level of the background virus gene increased due to the competition effect. The Ct value change was analyzed using one-way analysis of variance.

3 Results

3.1 Absolute Quantification Capacity and Specificity of the RT-qPCR Assay

For each virus type, eight 10-fold serial dilutions (final concentrations of about 10^0 to 10^7 target gene copies/qPCR) of the corresponding qPCR standard were analyzed with the qPCR assay in both monoplex and multiplex modes. When the Ct values were plotted against the logarithm of the target gene copy numbers, all three qPCR exhibited an inverse linear relationship ($r^2 > 0.99$, $P < 0.01$ for all assays; test for significance of a regression line) (Fig. 1). The detection limits were about 37, 13, and 15 copies of the target genes for

HRV, HAdV, and HNV, respectively. Efficiencies of qPCR for HRV, HAdV, and HNV were observed to be 0.90, 0.90, and 1.00 in multiplex mode and 0.93, 0.92, and 0.98 in multiplex mode, respectively.

Specificity was evaluated against genomic DNA or cDNA derived from 19 waterborne microbial cultures of public health concern. The multiplex qPCR assay positively identified the target viruses without any false-negative reactions. HRV-specific qPCR exhibited a false-positive reaction with adenovirus type 40 DNA. However, the observed Ct value (38.2) was significantly larger than the minimum detection limit (Ct of 36.9 ± 0.44) of the HRV-specific qPCR, even though an excess quantity of HAdV40 DNA (equivalent to about 3×10^8 virions) was applied for the qPCR. Since quantitative information is more important than simple presence or absence of fluorescent signals in this study, the false-positive signal was not expected to influence quantitation capacity of the assay. HAdV 40 was also detected by HAdV-specific qPCR of the multiplex assay. When a comparable number of HAdV 40 and 41 genes was applied for the assay, the Ct value for HAdV 40 was substantially delayed (by about 6 Ct units) than that of HAdV 41. The present HAdV assay detected both types of adenovirus species F, with type 41 as the primary and type 40 as the secondary targets. Detection specificities of all three qPCR were estimated to be 100 % when tested against 19 waterborne microorganisms, as false-negative or false-positive signals were not observed.

3.2 Reproducibility of Monoplex qPCR Performance and Competition Effect in Multiplex Mode

For all three virus groups, standard quantification curves of the monoplex and multiplex qPCR fell virtually in a single line with very similar slopes and Y-intercept values, indicating a comparable PCR efficiency in either mode ($P > 0.1$ for all three viruses, analysis of covariance; Fig. 1). The multiplex and monoplex qPCR standard curves almost overlapped for HRV (mean Ct difference of 0.13), HAdV (mean Ct difference of 0.06), and HNV (mean Ct difference of 0.30).

In the competition test, all three viruses showed that 40 copies of the target viral gene were accurately quantified in the presence of up to 4×10^5 copies of a background gene (Fig. 2). The Ct values of the three viruses did not change significantly with increasing levels of background viruses ($P > 0.1$ for HRV, HAdV, and HNV; one-way ANOVA). Thus, the present multiplex assay is

expected to accurately quantify the target viruses present at a low concentration in the presence of higher levels of background viruses.

3.3 Estimation of Recovery Efficiency and Quantification of Enteric Viruses in Environmental Water Samples by Multiplex RT-qPCR

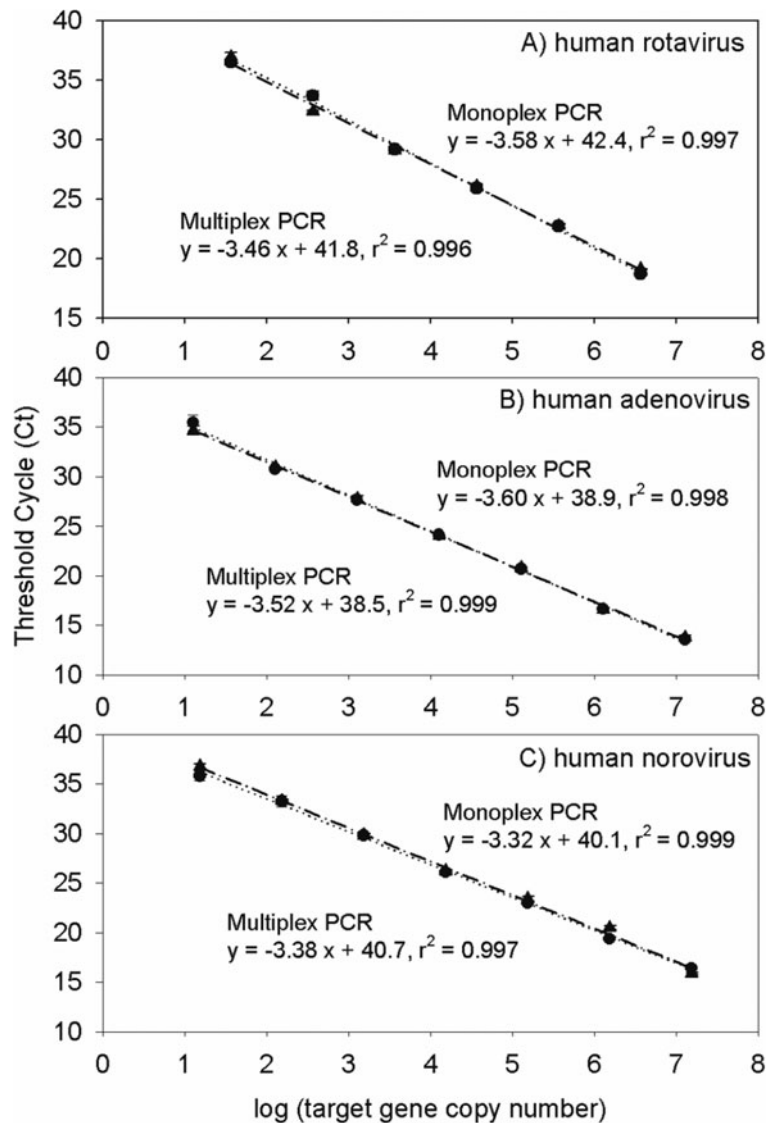
Recovery efficiency of HRV and HAdV41 seeded in 10 L of reagent-grade water at the high level was determined to be 21.0 ± 6.6 % (mean \pm SD, $n = 3$) and 24.7 ± 1.2 %, respectively. Viruses seeded at the lower level were recovered at an efficiency of 18.6 ± 6.7 and 16.4 ± 6.8 % for HRV and HAdV41, respectively.

From a total of 11 raw ground water samples, the multiplex RT-qPCR assay detected HRV and HAdV in three and seven samples, respectively, at levels of about 10^2 and 10^3 gene copies/L for both (Table 2). Norovirus was detected in only two samples at a level of about 10^2 gene copies/L. In the wastewater sample collected before UV disinfection, HAdV was found at about 10^6 gene copies/L, or 10^4 gene copies/L in PMA-treated sample. Both HRV and HNV were detected at about 10^4 gene copies/L, or 10^3 gene copies/L in PMA-treated samples. In PMA-treated samples after UV disinfection, HAdV was detected at about 10^3 gene copies/L, whereas HRV and HNV were not detected. The river water sample contained about 10^3 gene copies/L of HRV and HNV. The three viruses were not detected in the river water sample after the PMA treatment. The inhibitory effect of sample matrix on RT-qPCR was estimated as described in Section 2. Based on the inhibition result, field samples were diluted up to 1/8 times with molecular biology grade water to reduce inhibitory effects.

4 Discussion

For all three virus types, standard quantification curves exhibited only minor differences in quantification parameters between the monoplex and multiplex qPCR. The magnitude of differences observed in this study (mean Ct value shift of 0.06 to 0.30) was substantially smaller than those reported in previous studies (i.e., up to about 2 to 4 Ct shifts) (Stals et al. 2009; van Maarseveen et al. 2010). This suggests that the multiplex assay can reliably be applied for quantification of all three viruses in place of three individual monoplex qPCR within the quantification ranges tested. The

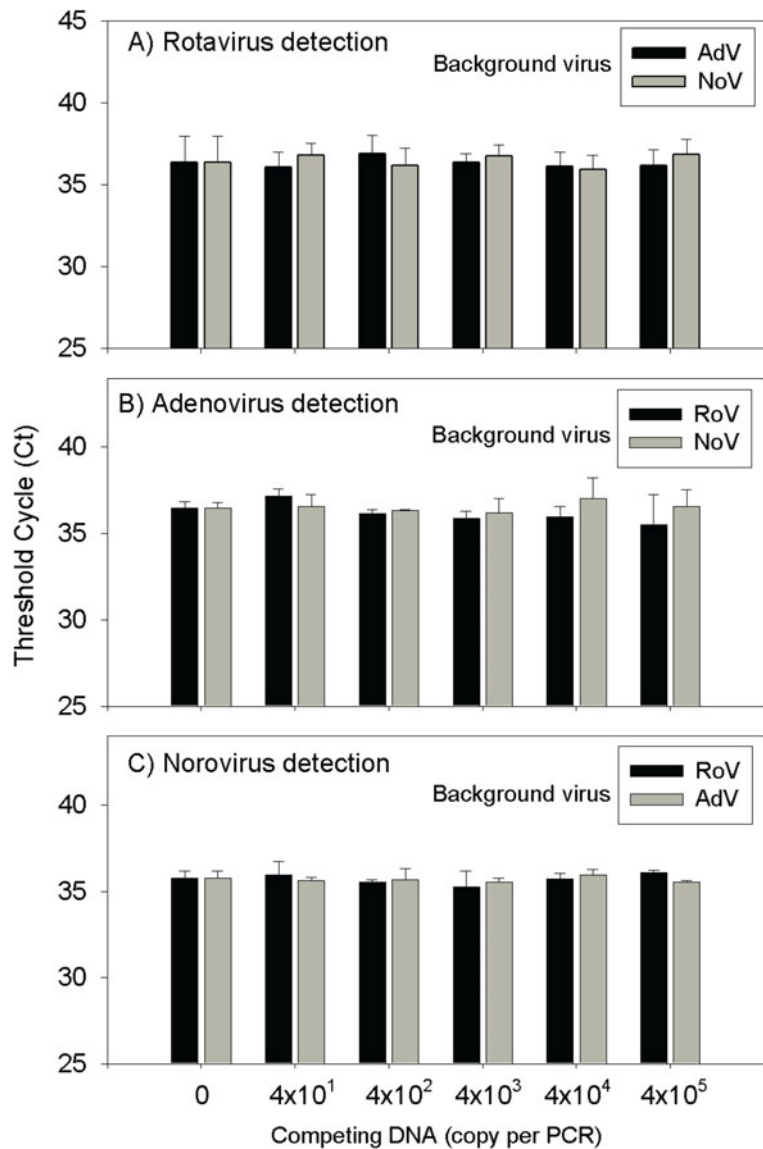
Fig. 1 Standard quantification curves of qPCR assays for human rotavirus (a), human adenovirus type 41 (b), and norovirus genotype II (c). Standard quantification plasmids were diluted by 10-fold series and quantified either in monoplex mode (●●●●●●), where each PCR contains a primer and probe set specific for a single virus group, or in multiplex mode (▲●●●●●), where each PCR contains three primer and probe sets specific for all three virus groups. Symbols and error bars represents mean \pm SD ($n=3$)



competition test showed that individual qPCR of the multiplex assay was limited by the quantity of virus-specific primers as designed, rather than by shared PCR resources, such as dNTPs or Taq DNA polymerase. In a water sample containing mixed enteric virus strains, the virus strain that exists in a larger number may exhaust the shared resources, causing suppression of PCR amplification for other virus strains before they can be amplified to detectable levels. Consequently, competition effect may result in false-negative test results or inaccurate quantifications. In this study, the presence of background virus strains present in 10^4 -fold larger quantity did not significantly inhibit PCR amplification of a target virus strain. In other multiplex qPCR studies on

enteric viruses, 10^2 copies of HRV and HAdV genes were accurately detected in the presence of 10^4 and 10^2 times more abundant background genes, respectively (Jiang et al. 2014). It was also reported that 10 copies of a HNV GII gene were accurately quantified in the presence of 10^2 times more abundant background virus genes, but the same qPCR was completely suppressed when the level of background genes was increased by 10^4 fold (Stals et al. 2009). Based on the outcome from our competition test and the monoplex and multiplex qPCR parameter comparison, we concluded that our target viruses were not underestimated in multiplex mode within the quantification range tested.

Fig. 2 The effect of PCR competition on quantification of a target virus. To determine the extent of PCR inhibition, 40 copies of a target gene were mixed with various concentrations of two competing genes (0, 40, 4×10^2 , 4×10^3 , 4×10^4 , 4×10^5 copies per PCR). The experiment was intended to accurately determine the known quantity of human rotavirus (a), human adenovirus type 41 (b), and norovirus genotype II (c) in the presence of background viruses at higher concentrations. Column and error bars represent mean \pm SD ($n = 3$)



In a previous qPCR study on enteric viruses, substantially reduced quantification capacity was observed in multiplex mode (van Maarseveen et al. 2010). This was likely due to incorporating too many degenerate bases in primers and probes, thereby increasing non-specific reactions among multiplex qPCR components. To reduce these side reactions, the number of degenerate bases in primers and probes was minimized in our assay design and two specific virus groups, HNV genotype II and HAdV type 41, were selected as type strains for gastroenteric norovirus and adenovirus, respectively. These strains were selected because, among the human-infecting noroviruses, the genogroup II is the most prevalent group globally, causing

an average of 96 % of sporadic infections by all gastroenteric noroviruses (Tran et al. 2013). Among the gastroenteric species F clones isolated from environmental and clinical samples around the world, serotype 41 is more abundant than serotype 40 by about an order of magnitude (Fong et al. 2010; Li et al. 2005; Sdiri-Loulizi et al. 2009).

As preceding steps to RT-qPCR, virus concentration procedures (e.g., filtration, elution, and flocculation) play an important role in recovering enteric viruses from water samples. The recovery efficiencies found in the present study were within the range reported in a previous study (Pang et al. 2012) where median recovery

Table 2 Three enteric viruses detected and quantified by the multiplex RT-qPCR assay in environmental water samples

Sample types (number of samples)	Parameters		Enteric virus groups		
			HRV	HAdV41	HNV
Ground water (11) ^a	Number of positive samples		3	7	2
	Target gene copy number/L. Mean \pm SD $n = 3$	Lowest	6.6×10^2 $\pm 2.0 \times 10^2$	2.1×10^2 $\pm 5.0 \times 10^1$	1.5×10^2 $\pm 2.1 \times 10^2$
		Median	1.4×10^3 $\pm 5.8 \times 10^2$	8.7×10^2 $\pm 2.6 \times 10^2$	N.A.
	Highest	1.7×10^3 $\pm 2.7 \times 10^2$	3.8×10^3 $\pm 3.5 \times 10^3$	1.7×10^2 $\pm 3.0 \times 10^1$	
River water (1)	Target gene copy number/L. Mean \pm SD $n = 4$	-PMA ^b	4.8×10^3 $\pm 5.5 \times 10^3$	N.D.	1.4×10^3 $\pm 4.7 \times 10^2$
		+PMA ^c	N.D.	N.D.	N.D.
Wastewater (pre-UV disinfection) (1)	Target gene copy number/L. Mean \pm SD $n = 4$	-PMA	3.8×10^4 $\pm 3.5 \times 10^4$	9.7×10^5 $\pm 9.4 \times 10^5$	1.7×10^4 $\pm 6.3 \times 10^3$
		+PMA	2.4×10^3 $\pm 4.8 \times 10^3$	3.5×10^4 $\pm 4.4 \times 10^4$	8.7×10^3 $\pm 1.7 \times 10^3$
Wastewater (post-UV disinfection) (1)	Target gene copy number/L. Mean \pm SD $n = 4$	-PMA	N.D.	3.9×10^4 $\pm 7.8 \times 10^4$	1.0×10^3 $\pm 1.5 \times 10^3$
		+PMA	N.D.	4.0×10^3 $\pm 6.0 \times 10^3$	N.D.

Samples were collected during July 24 to August 7, 2014 and December 12, 2014 to January 13, 2015 in Ontario, Canada

N.D. not detected, N.A. not available

^a Ground water samples were not treated with PMA

^b PMA untreated group, RT-qPCR only

^c PMA treated group, PMA treatment followed by RT-qPCR

efficiencies of 39 % (8 to 69 %) for HNV, 31 % (27 to 55 %) for HRV, and 26 % (13 to 52 %) for HAdV41 were observed when spiked at various titers in deionized water and concentrated by the procedures similar to ours. In the present study, the two titer seeding schemes were chosen to represent enteric virus levels in highly contaminated water samples (e.g., wastewater) and in less contaminated ones (e.g., ground water). The three enteric viruses were successfully detected and quantified using our RT-qPCR assay. However, it should be considered that recovery efficiencies of the spike tests may not accurately reflect those of field samples because of sample matrix effect and widely varying sample volumes.

The number of infectious virus particles can be overestimated by an RT-qPCR assay because naked nucleic acids and nucleic acids from capsid-damaged virus particles, as well as from infectious virus particles, can contribute to the RT-qPCR quantification (Knight

et al. 2013). To overcome this limitation, recent studies have focused on measuring capsid integrity as a surrogate marker for viral infectivity by coupling an RT-qPCR assay with a RNase pretreatment (Topping et al. 2009) or a nucleic acid-intercalating dye, such as PMA, pretreatment (Fittipaldi et al. 2010; Parshionkar et al. 2010). In this study, only a fraction of the total viral nucleic acids (about 3.6 to 51 %) was found to originate from virus particles with intact capsids for all three virus types tested. The PMA pretreatment can be useful in testing wastewater effluent quality since the efficiency of a disinfection tool at a wastewater treatment plant is often evaluated based on decrease of infectious virus particles.

5 Conclusions

A multiplex RT-qPCR assay was developed and shown to be reliable in quantification and specific identification

of three human enteric viruses through a series of validation tests. The assay allows for a fast, accurate, specific, and quantitative surveillance of common enteric viruses in environmental samples.

Acknowledgments The authors express gratitude to Ms. N. McLellan and C. Lofranco (U. of Guelph) and Laboratory Services Branch of Ontario Ministry of the Environment and Climate Change for their field sampling work.

Compliance with Ethical Standards

Funding This research was funded by the Ontario Ministry of the Environment and Climate Change Best in Science program (Project number 1314043).

Conflict of Interest The authors declare that they have no conflict of interest.

References

- Arnold, M., Patton, J.T., & McDonald, S.M. (2009). Culturing, storage, and quantification of rotaviruses. *Current Protocols in Microbiology*, (15), Unit 15C.3.
- Brown, M. (1990). Laboratory identification of adenoviruses associated with gastroenteritis in Canada from 1983 to 1986. *Journal of Clinical Microbiology*, 28, 1525–1529.
- Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, 611–622.
- Butot, S., Le Guyader, F. S., Krol, J., Putallaz, T., Amoroso, R., & Sánchez, G. (2010). Evaluation of various real-time RT-PCR assays for the detection and quantitation of human norovirus. *Journal of Virological Methods*, 167, 90–94.
- Centers for Disease Control and Prevention (2015). Norovirus: US trends and outbreaks. <http://www.cdc.gov/norovirus/trends-outbreaks.html>.
- Fittipaldi, M., Rodriguez, N. J. P., Codony, F., Adrados, B., Peñuela, G. A., & Morató, J. (2010). Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. *Journal of Virological Methods*, 168, 228–232.
- Fong, T. T., Phanikumar, M. S., Xagorarakis, I., & Rose, J. B. (2010). Quantitative detection of human adenoviruses in wastewater and combined sewer overflows influencing a Michigan river. *Applied and Environmental Microbiology*, 76, 715–723.
- Gray, J. (2011). Rotavirus vaccines: safety, efficacy and public health impact. *Journal of Internal Medicine*, 270, 206–214.
- Hall, A. J., Vinjé, J., Lopman, B., Park, G., Yen, C., Gregoricus, N., & Parashar, U. (2011). Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report*, 60(RR03), 1–15.
- Hamza, I. A., Jurzik, L., Überla, K., & Wilhelm, W. (2011). Methods to detect infectious human enteric viruses in environmental water samples. *International Journal of Hygiene and Environmental Health*, 214, 424–436.
- Hlavsa, M., Roberts, V. A., Kahler, A. M., Hilborn, E. D., Mecher, T. R., Beach, M. J., Wade, T. J., & Yoder, J. S. (2015). Outbreaks of illness associated with recreational water—United States, 2011–2012. *Morbidity and Mortality Weekly Report*, 64, 668–672.
- Jiang, Y., Fang, L., Shi, X., Zhang, H., Li, Y., Lin, Y., Qiu, Y., Chen, Q., Li, H., Zhou, L., & Hu, Q. (2014). Simultaneous detection of five enteric viruses associated with gastroenteritis by use of a PCR assay: a single real-time multiplex reaction and its clinical application. *Journal of Clinical Microbiology*, 52, 1266–1268.
- Jothikumar, N., Lowther, J. A., Henshilwood, K., Lees, D. N., Hill, V. R., & Vinjé, J. (2005). Rapid and sensitive detection of Noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Applied and Environmental Microbiology*, 71, 1870–1875.
- Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N., & Katayama, K. (2003). Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology*, 41, 1548–1557.
- Karim, M.R., Rhodes, E.R., Brinkman, N., Wymer, L., & Fout, G.S. (2009). New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. *Applied and Environmental Microbiology*, 75, 2393–2399.
- Knight, A., Li, D., Uytendaele, M., & Jaykus, L.-A. (2013). A critical review of methods for detecting human noroviruses and predicting their infectivity. *Critical Reviews in Microbiology*, 39, 295–309.
- Lee, D. Y., Shannon, K. E., & Beaudette, A. L. (2006). Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. *Journal of Microbiological Methods*, 65, 453–467.
- Li, L., Phan, T. G., Nguyen, T. A., Kim, K. S., Seo, J. L., Shimizu, H., Suzuki, E., Okitsu, S., & Ushijima, H. (2005). Molecular epidemiology of adenovirus infection among pediatric population with diarrhea in Asia. *Microbiology and Immunology*, 49, 121–128.
- Pang, X. L., Lee, B. E., Pabbarajua, K., Gabosd, S., Craike, S., Payment, P., & Neumann, N. (2012). Pre-analytical and analytical procedures for the detection of enteric viruses and enterovirus in water samples. *Journal of Virological Methods*, 184, 677–683.
- Pang, X. L., Preiksaitis, J. L., & Lee, B. E. (2013). Enhanced enteric virus detection in sporadic gastroenteritis using a multi-target real-time PCR panel: a one-year study. *Journal of Medical Virology*, 86, 1594–1601.
- Parshionikar, S., Laseke, I., & Fout, G. S. (2010). Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. *Applied and Environmental Microbiology*, 76, 4318–4326.
- Rice, E.W., Baird, R.B., Eaton, A.D., & Clesceri, L.S. (2012). *Standard methods for the examination of water and*

- wastewater, 22nd ed. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington DC.
- Sdiri-Loulizi, K., Gharbi-Khelifi, H., de Rougemont, A., Hassine, M., Chouchane, S., Sakly, N., Pothier, P., Guediche, M. N., Aouni, M., & Ambert-Balay, K. (2009). Molecular epidemiology of human astrovirus and adenovirus serotypes 40/41 strains related to acute diarrhea in Tunisian children. *Journal of Medical Virology*, *81*, 1895–1902.
- Stals, A., Baert, L., Botteldoorn, N., Werbrouck, H., Herman, L., Uyttendaele, M., & Coillie, E. V. (2009). Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus I. *Journal of Virological Methods*, *161*, 247–253.
- Topping, J. R., Schnerr, H., Haines, J., Scott, M., Carter, M. J., Willcocks, M. M., Bellamy, K., Brown, D. W., Gray, J. J., Gallimore, C. I., & Knight, A. I. (2009). Temperature inactivation of Feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction—a novel method for predicting virus infectivity. *Journal of Virological Methods*, *156*, 89–95.
- Tran, T. N. H., Trainor, E., Nakagomi, T., Cunliffe, N. A., & Nakagomi, O. (2013). Molecular epidemiology of noroviruses associated with acute sporadic gastroenteritis in children: global distribution of genogroups, genotypes and GII.4 variants. *Journal of Clinical Virology*, *56*, 185–193.
- van Frankenhuyzen, J. K., Trevors, J. T., Lee, H., Flemming, C. A., & Habash, M. B. (2011). Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. *Journal of Microbiological Methods*, *87*, 263–272.
- van Maarseveen, N. M., Wessels, E., de Brouwer, C. S., Vossen, A. C. T. M., & Claas, E. C. J. (2010). Diagnosis of viral gastroenteritis by simultaneous detection of adenovirus group F, astrovirus, rotavirus group A, norovirus genogroups I and II, and sapovirus in two internally controlled multiplex real-time PCR assays. *Journal of Clinical Virology*, *49*, 205–210.
- Vidovic, S., Aly, M., Flemming, C., Springthorpe, S., & Sattar, S. A. (2011). First evidence of genotypes Ad3a16 and Ad3a18 in North America, obtained by genetic analysis of infectious human adenovirus from wastewaters of two urban communities in Canada. *Applied and Environmental Microbiology*, *77*, 4256–4259.
- Wong, K., Fong, T.-T., Bibby, K., & Molina, M. (2012). Application of enteric viruses for fecal pollution source tracking in environmental waters. *Environment International*, *45*, 151–164.