



Development of a combined immunomagnetic separation and quantitative reverse transcription-PCR assay for sensitive detection of infectious rotavirus in water samples

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ABSTRACT

A quantitative and rapid detection method for rotavirus in water samples was developed using immunomagnetic separation combined with quantitative reverse transcription-polymerase chain reaction (IMS-RT-qPCR). Magnetic beads coated with antibodies against representative group A rotavirus were used to capture and purify intact rotavirus particles in both artificial and real environmental water sample matrix. Compared to extracting RNA using commercial kits and RT-qPCR assay, the developed IMS-RT-qPCR method increased the detection sensitivity by about one order of magnitude when applied in clean water, with a detection limit of 3.16 50% tissue culture infectious dose (TCID₅₀)/mL within 5 h. This method was compatible with various commonly used virus eluants, including beef extract (BE), beef extract with 0.05 M glycine (BEG) and urea arginine phosphate buffer (UAPB). The recovery efficiencies from various eluants using IMS-RT-qPCR are higher than that using direct RT-qPCR method, demonstrating the effectiveness of the IMS step for eliminating inhibitors in the eluant matrix. This method was also successfully applied to purify and detect rotavirus particles seeded in 10³-fold concentrated wastewater influent samples. It seemed to reduce the interference from complex sample background and increase the qPCR product reliability comparing to RT-qPCR method without the IMS step. The results indicated that IMS-RT-qPCR is a rapid, sensitive and reliable tool for detecting rotaviruses in complex water environments.

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1. Introduction

Outbreaks associated with waterborne viruses have been reported worldwide (Faustini et al., 2006; Karmakar et al., 2008; Maila et al., 2004; Maunula et al., 2005). It has been noted that more than 140 types of viruses have been found in environmental waters (Gutiérrez et al., 2007). Rotavirus is the most common cause for severe diarrhea in children worldwide, causing 74.9–208.5 million infections and about 600,000 deaths in children aged 5 years old and below (Glass et al., 2006; Stebbins, 2007). In China, rotavirus infections have been reported in many cities, including Beijing, Shanghai, Guangzhou and Hong Kong (Orenstein et al., 2002). The virus has been detected in various types of waters, including sewage, river water, ground water and drinking water (Kittigul et al., 2005; Kocwa-Haluch, and Zalewska, 2002). Hence, a rapid and reliable detection method is needed to monitor the presence of rotavirus in

environmental waters and it is the prerequisite for water quality control and public health protection.

Conventional methods for virus detection, such as the cell culture-based assay, have been used for risk assessment analysis. However, these methods are technically difficult, time-consuming and expensive. Moreover, they rely on animal cell cultures, rendering them only effective on culturable viruses and ineffective on viruses such as hepatitis A virus (HAV) and norovirus, which are difficult or not amenable to culture *in vitro*. Newly developed molecular methods, such as the polymerase chain reaction (PCR), have emerged recently as useful tools for the detection of viruses, due to their high rate and high throughput capacity, high specificity and sensitivity. However, PCR methods are susceptible to inhibitors widely existing in water samples and may consequently yield false negative results (Flekna et al., 2007). In addition, the results of PCR only demonstrate the existence of viral DNA or RNA rather than infective viruses (Espinosa et al., 2008; Moore, 1993).

Another problem with virus detection is the low level of viruses present in most environmental waters, which requires pre-concentration of water samples, typically by 10³–10⁶ times, before they can be analyzed. Unwanted contaminating materials present in water samples are consequently concentrated and will interfere with the

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analysis. For example, toxicants existing in the concentrates may kill the cells and therefore produce false negative results. The organic materials in the concentrates may inhibit PCR assays. Purification to remove interferences and inhibitors before the cell culture assay or PCR assay is critical for obtaining reliable results.

Immunomagnetic separation (IMS) uses magnetic beads coated with specific antibodies to capture target analyte (e.g., viral particles and microorganisms) bearing the corresponding antigens and subsequently separate them from the water matrix in a simple magnetic field. By sequestering the analyte with the magnetic beads, interfering and inhibiting materials in the water matrix can be removed by washing. The IMS method has proven to be effective in separating various types of pathogenic microorganisms, including *E. coli* O157 (Shelton et al., 2004), *Cryptosporidium* (Quintero-Betancourt et al., 2002), hepatitis A virus (Abd El Galil et al., 2005), and adenovirus (Haramoto et al., 2010) from different environmental water samples and in eliminating background interferences, including PCR inhibitors. Furthermore, the use of antibody–antigen affinity selection through IMS followed by PCR ensures that nucleic acid detected is derived from structurally intact (infectious) microorganisms and viral particles. A recent report by Hwang et al. (2007) demonstrated that IMS combined with quantitative real-time reverse transcription (RT)–PCR is an effective and sensitive detection method for enteroviruses in surface waters.

In order to evaluate the IMS for purification of rotavirus from complex environmental matrices (e.g., sewage concentrate) and develop a RT–qPCR method compatible with the IMS technique for rotavirus, we aimed to develop and optimize an IMS–RT–qPCR method for the detection of infectious rotavirus in environmental water samples. To evaluate its accuracy, sensitivity, susceptibility to water–matrix–interference, and compatibility with the eluants used in established filtration–elution concentration methods, the newly developed IMS–RT–qPCR method was compared with direct RT–qPCR method without IMS, which used a commercial kit to extract RNA from samples (denoted as RNA kit–RT–qPCR for reference), by applying them simultaneously against clean water (performed in double distilled water (ddH₂O)), 10³-fold sewage concentrate and three commonly used virus eluants.

2. Materials and methods

2.1. Virus strain and cells

The simian rotavirus was chosen as a surrogate for group A rotavirus to test and optimize the protocol. Rotavirus SA11 strain (group A, simian type G3) and MA-104 cells (HTB-37TM, ATCC) were generously donated by Professor Hong Meng from the Medicine Academy in Shandong Province, P.R. China. MA-104 cells were grown in Dulbecco's modified Eagle's medium (DMEM, HyClone, Beijing, China), supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1.7 g/L sodium bicarbonate. The SA11 rotavirus was propagated and grown in MA-104 cells as previously described (Estes et al., 1979). The cell cultures were frozen and thawed three times and centrifuged at 900×g for 5 min to remove the cell debris. The supernatant containing the virus (virus stock) was aliquoted in 1 mL volumes and stored at –80 °C until use.

2.2. Virus titration

The titration of rotavirus stock and dilutions was determined by both the 50% tissue culture infectious dose (TCID₅₀) assay (Distefano et al., 1995) and a modified plaque-forming unit (PFU) assay (Hansen et al., 2007), as previously described. Briefly, for the TCID₅₀ assay, MA-104 cells were seeded in 96-well plates (10⁴ cells/well) and incubated for 24 h in an incubator (under 5% CO₂ at 37 °C) to form monolayers in the wells. Serial 10-fold dilutions of SA11 rotavirus prepared in DMEM

were treated with 20 U trypsin/mL for 1 h in the incubator before they were added (100 µL) into the MA-104 cell monolayer and incubated for 2 h for virus adsorption. The virus solution in each well was then removed and replaced with 200 µL of DMEM containing 2% FBS. The plates were incubated for 5–7 days to observe cytopathic effect (CPE). The Reed–Muench equation was used to estimate the titer of the viral suspensions (Reed and Muench, 1938).

For the modified PFU assay, 100 µL of each serial SA11 dilutions was inoculated into 24-well plates with MA-104 monolayer cells. After 2 h of virus adsorption in the incubator, 2 mL of maintenance medium consisting of DMEM with 2% FBS was added to each well in the plates and they were incubated for 4 days. The maintenance medium was then replaced with 2 mL of 2×DMEM (final concentration) containing 2% agar and 1 µg/mL trypsin, permitted to solidify, and then followed by another 3 days of incubation. To enumerate the plaques, the MA-104 cell monolayers infected with SA11 were fixed overnight with 10% formaldehyde in normal saline and then stained with 0.1% crystal violet solution to determine the number of virus PFUs.

2.3. Preparation of immunomagnetic beads and Immunomagnetic separation (IMS)

Dynabeads® Protein A (1.3 g/mL stock suspension, Dynal Biotech, Oslo, Norway) were coated with antibodies against group A rotavirus (goat anti-rotavirus, Biodesign, Saco, USA) according to the manufacturer's instructions. Uncoated beads were pre-washed three times with sodium (Na)-phosphate buffer (0.1 M Na-phosphate, 0.01–0.05% Tween-20, pH 8.0). Antibodies with a concentration of ca. 1.1 mg/mL were added to the washed beads and allowed to bind for 30 min with gentle mixing. The unbound antibodies were removed and the antibody-coated beads were washed three times with Na-phosphate buffer. The coated immunomagnetic beads (IMBs) were then suspended in the phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and stored at 4 °C until use.

For IMS, 10 µL of IMBs (and 2.5 µL of Tween-20 when applied to ddH₂O samples) was added into each 1 mL sample and then incubated for 2 h at room temperature with gentle mixing. After the incubation, the IMBs were magnetized and the supernatant was removed, followed by three Na-phosphate buffer washes. The supernatant was completely removed at the last wash. The IMBs were then re-suspended in 20 µL of ddH₂O and transferred into a 200 µL PCR tube for RT.

2.4. Viral RNA extraction, RT, and qPCR

For quantification of viral genomes of the virus stock, viral RNA was extracted by heating 20 µL of virus stock dilutions (10-fold or 100-fold) at 95 °C for 5 min, and chilled on ice immediately after heating. The virus solution was used directly for RT. For the IMS–RT–qPCR assay, viral RNA was released from the IMBs by heating the IMB suspension at 95 °C for 5 min and chilled on ice immediately after heating. The IMBs were pelleted by centrifugation at 10,000×g for 2 min, and the supernatant containing viral RNA was used for RT immediately. For the RNA kit–RT–qPCR assay, a commercial kit was used for RNA extraction (QIAamp viral RNA Mini kit, Qiagen, Hilden, Germany) and it was performed with 1 mL sample volume (yielding the RNA extract in a 60 µL final volume) by following the manufacturer's instructions.

The RT reaction was performed in 10 µL volumes using a PrimeScriptTM RT Reagent Kit (TaKaRa, DaLian, China), according to the manufacturer's instructions. Two µL of viral RNA (extracted by the three methods presented above) was used for RT. Reverse transcription was carried out at 37 °C for 15 min and then 85 °C for 5 s to inactivate the enzyme. The cDNA obtained was used for qPCR afterwards or stored at –20 °C until use.

Quantification of viral genomes was conducted by qPCR. The primer pair, rota-sense (5'-CCTCACTTATACACTTTGCC-3') and rota-antisense (5'-TTCGCTTCGTCAGTTTGGCT-3'), was used to amplify a 318 bp region of the rotavirus viral protein (VP) 7 gene segment. The plasmid DNA carrying the same gene region was constructed in our laboratory (Hu et al., 2008). Briefly, qPCR was performed in 20 μ L volumes with a SYBR® Premix Ex Taq™ kit (TaKaRa, DaLian, China) in an iQ icycler (Bio-Rad, USA) following the manufacturer's instructions. The reaction mixture contained 4 μ L of either cDNA or known amount of plasmid DNA, 10 μ L of 2 \times SYBR buffer, 0.2 μ L of each primer (0.2 μ M final concentrations) and 5.6 μ L of PCR-grade water. The qPCR amplification was performed with 1 cycle of preheating at 95 °C for 10 s, 40 cycles of melting at 95 °C for 5 s, annealing at 59 °C for 20 s and extension at 72 °C for 30 s. The amplification was followed by a melting curve analysis with 71 cycles of dissociation; from 95 °C to 60 °C with a temperature ramp of 0.5 °C every 30 s. After the melting curve analysis, the products were held at 4 °C. Melting curve analysis showed the specific melting peak at 81.0 ± 0.5 °C. A linear regression equation was obtained for quantification of viral genome: $y = -3.3914x + 43.104$, where y is the threshold cycle number (C_T) and x is the logarithm of the starting quantity of rotavirus genome copy (GC). The detection range was between 3.6×10^2 to 3.6×10^{11} GC per reaction with a correlation coefficient (R^2) of 0.9958.

The total number of viral genomes in samples detected by either IMS-RT-qPCR or heat release-RT-qPCR (for quantifying viral genomes in the virus stock) was calculated by multiplying the result yielded by qPCR by a factor of 25 (2 μ L out of 20 μ L of viral RNA was used for RT, and 4 μ L out of 10 μ L of cDNA was used for qPCR). The total number of viral genomes in samples tested by RNA kit-RT-qPCR was calculated similarly as the qPCR result multiplied by 75.

2.5. Sewage concentration

Twenty liters of sewage (influent) was collected from Xiaohongmen Wastewater Treatment Plant (WWTP), Beijing, China. The water sample was concentrated according to methods reported elsewhere (Katayama et al., 2002; Li et al., 1998) with some modifications. Briefly, water samples were adjusted to pH 3.5 with 5 M HCl and were supplemented with AlCl₃ (final concentration of 1 mM) and 5 g SiO₂. Then the samples were stirred for 30 min to allow complete adsorption of viral particles to the SiO₂ particles and filtered through a membrane to collect the virus-SiO₂ complex. Thirty mL of H₂SO₄ (pH 3.0) was added and passed through to rinse out the cation, and the virus-SiO₂ complex was collected into a 50 mL tube with 18 mL NaOH (pH 10.5), and vortexed for 5 min to release the virus particles to the eluant. The SiO₂ particles, as well as the bigger particles, were removed by centrifugation at 4000 \times g, 4 °C for 10 min, while most colloidal and fine particles were left in the eluate to reduce loss of viral particles adhering to them. Two mL of 10 \times TE buffer (100 mM Tris HCl, 10 mM EDTA, pH 8.0) was added into the eluate that contained virus for neutralization. The virus concentrate (with a total volume of ca. 20 mL) was stored at -80 °C until use.

2.6. Comparison of IMS-RT-qPCR and RNA kit-RT-qPCR assays

To compare the efficiency and sensitivity of IMS-RT-qPCR with the previously reported RNA kit-RT-qPCR method, rotaviruses were seeded into ddH₂O in 1 mL volumes for final concentrations of 2×10^{-1} to 2×10^5 TCID₅₀/mL and tested by the two methods simultaneously. Statistical analysis was performed using SPSS 13.0 for windows.

2.7. Assessment of environmental sample matrix interference with IMS-RT-qPCR

To test the ability of IMS-RT-qPCR for reducing or eliminating the environmental interference from the virus concentrate, the 20 mL

sewage concentrate (prepared according to methods presented above and confirmed to contain no rotavirus by the cell culture assay) was distributed into four 10-mL tubes with 5 mL per tube, and were seeded with rotavirus for a concentration of 1.5×10^4 , 7.8×10^3 , 3.9×10^3 , or 1.5×10^3 TCID₅₀/mL, respectively. Virus concentration was examined by both IMS-RT-qPCR and RNA kit-RT-qPCR method (in duplicate) for comparison.

2.8. Compatibility of IMS with established virus eluants

To test the compatibility of IMS with established virus eluants, equivalent amounts of viruses (approximately 2.5×10^3 TCID₅₀/mL) were seeded into three commonly used organic virus eluants, namely 3% beef extract (BE, pH 9.5), 3% beef extract plus 0.05 M glycine (BEG, pH 9.5) (Zhang et al., 2007) and urea arginine phosphate buffer (UAPB, 1.5 M urea, 0.02 M arginine, 0.008 M phosphate, pH 9.0) (Jothikumar et al., 1993). Ten μ L of IMBs was added into each eluant and the amount of viral particles captured was quantified by IMS-RT-qPCR. Double distilled H₂O was used as a control. Parallel samples were also tested by the RNA kit-RT-qPCR assay for comparison. Experiments were done in duplicate.

3. Results

3.1. Correlation of TCID₅₀, PFU, and GC

The concentration of the virus in the stock, which was used to prepare dilution series for different tests, was measured by three methods, including the TCID₅₀ assay, the PFU assay, and RT-qPCR assay, in order to establish the correlation between TCID₅₀, PFU, and GC. The titer of the virus stock was determined to be 3.16×10^6 TCID₅₀/mL by TCID₅₀ assay and 1.8×10^6 PFU/mL by plaque assay. The concentration of viral genomes of the virus stock was determined to be 4.49×10^9 GC/mL by direct heating to release viral RNA and then subsequent RT-qPCR.

Based on the above results, for the virus stock of rotavirus SA11 applied in this study, 1 TCID₅₀ was equivalent to ca. 0.57 PFU and ca. 1.4×10^3 GC, and 1 PFU was equivalent to 1.76 TCID₅₀ and ca. 2.5×10^3 GC. Since one rotavirus particle contains only one genome, the number of viral particles is equivalent to the number of viral genomes (GC), i.e., 2.5×10^3 viral particles. According to Grinde et al. (1995), 1 PFU of rotavirus Wa strain equaled 1.2×10^3 viral particles (obtained by electron microscope), therefore, our results are consistent and are in the same order of magnitude as that reported by Grinde et al. (1995).

3.2. Comparison of IMS-RT-qPCR assay and RNA kit-RT-qPCR assay

Comparison of results with IMS-RT-qPCR and those with RNA kit-RT-qPCR showed that both methods performed equally well for detecting virus concentrations ranging from 2×10^2 to 2×10^5 TCID₅₀/mL (Fig. 1). Statistical data analysis shows that test results by the two assays have no significant difference (paired sample t-test, $P = 0.370$) at virus concentrations higher than 2×10^2 TCID₅₀/mL. However, at virus concentration of 20 TCID₅₀/mL, the RNA kit-RT-qPCR assay failed to yield positive results, while the IMS-RT-qPCR assay was able to quantify rotavirus at this level. Both assays failed to detect rotavirus with a concentration of 2 TCID₅₀/mL.

While previous study in our lab evaluated the correlation between the virus concentration detected with IMS-RT-qPCR and those with the TCID₅₀, the results showed that a linear correlation between results from the two assays with a correlation coefficient (R^2) of 0.9816 and the detection limit of IMS-RT-qPCR method is about 3.16 TCID₅₀/mL (Yang et al., 2009). Here the assay failed to detect 2 TCID₅₀/mL, indicating that concentration lower than 3.16 TCID₅₀/mL might be difficult to be detected by IMS-RT-qPCR.

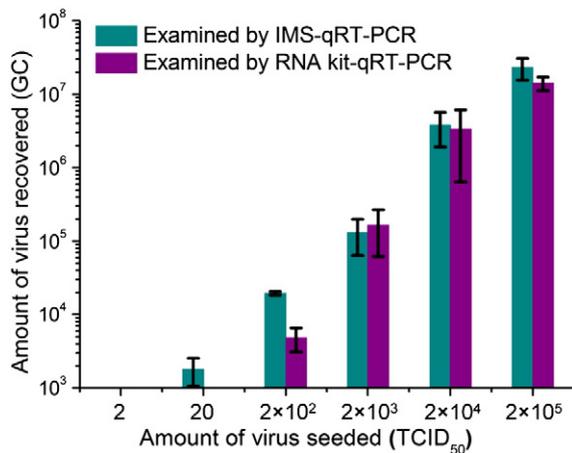


Fig. 1. Comparison of detection sensitivity between IMS-qRT-PCR assay and RNA kit-qRT-PCR assay.

3.3. Elimination of environmental sample matrix interference with IMS-RT-qPCR

As shown in Fig. 2, all seeded sewage concentrate samples yielded specific PCR product with both IMS-RT-qPCR and RNA kit-RT-qPCR methods. The melting curve analysis (Fig. 2a) showed that most of the samples tested by IMS-RT-qPCR yielded only the specific melting peak at 81 ± 0.5 °C, while samples at the lowest concentration of 1.5×10^3 TCID₅₀/mL yielded at least one non-specific melting peak and thus cannot be accurately quantified by qPCR. The main non-specific melting peak yielded by the three samples tested by IMS-RT-qPCR was around 74 °C, which also appeared in samples tested by RNA kit-RT-qPCR. It might result from the formation of primer dimers capable of binding the SYBR Green dye and causing the fluorescence. In comparison, as shown in Fig. 2b, all samples tested by the RNA kit-RT-qPCR assay yielded at least another non-specific melting peak in addition to the non-specific melting peak around 74 °C. These non-specific melting peaks (one around 68 °C and another around 84 °C) might be due to certain materials in the samples that would interfere with PCR and cause non-specific amplifications, or existence of SYBR Green dye associable materials from the samples.

Gel electrophoresis analysis (Fig. 3) showed that five out of eight samples tested by IMS-RT-qPCR (those had only one specific melting peak at 81 ± 0.5 °C) and all samples tested by RNA kit-RT-qPCR yielded the corresponding amplification band at the 318 bp position. The RNA kit-RT-qPCR assay seemed to be more sensitive than the IMS-RT-qPCR assay at lower concentrations (1.5×10^3 and 3.9×10^3 TCID₅₀/mL) in the gel analysis. However, compared to the bands yielded from RNA kit-RT-qPCR assays that were fraught with non-specific substances and blurred smear, the IMS-RT-qPCR method produced clearer and distinct bands, indicating the effective removal of environmental background interferences as described earlier in the melting curve analysis.

3.4. IMS compatibility with various virus eluants

Three different virus eluants commonly used in IMS-RT-qPCR method were examined and compared. The recovery efficiencies detected by the IMS-RT-qPCR method were also compared with that by the RNA kit-RT-qPCR method, in order to assess the effectiveness of IMS in removing inhibitory substances in the virus eluants. The results indicated that BE, BEG and UAPB showed higher recovery of virus than that performed in ddH₂O by IMS-RT-qPCR (Fig. 4), indicating that the organic matters present in these eluants did not inhibit IMS and that IMS was compatible with these widely used virus eluants. Samples

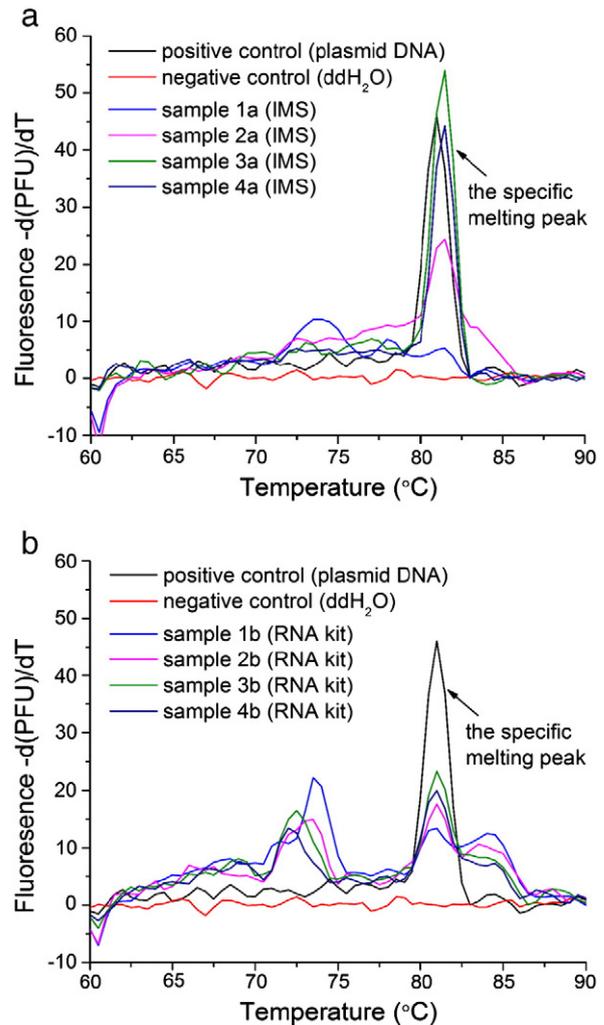


Fig. 2. Melting curves of samples examined by IMS-qRT-PCR (a) and RNA kit-qRT-PCR (b). The specific melting peak is at 81.0 ± 0.5 °C. Samples 1–4 referred the samples seeded with rotavirus at concentrations of 1.5×10^3 , 3.9×10^3 , 7.8×10^3 , and 1.5×10^4 TCID₅₀/mL, respectively. All the experiments were done at least in duplicate.

examined with RNA kit-RT-qPCR without the IMS step showed significant inhibition with BE and BEG eluants due to the failure of the RNA kit to remove organic matters/inhibitors from the eluants. In addition, the recovery efficiency of IMS-RT-qPCR in BE and BEG was higher than that obtained in ddH₂O. It suggests that BE and BEG are more suitable for rotavirus than ddH₂O.

4. Discussion

Immunomagnetic separation (IMS) relies on the specific antibodies coated on the outside of magnetic beads to capture target viruses and form virus-bead combinations, which are then separated out of samples in the presence of a magnetic field. It has proven to be effective in separating various types of viruses in different kinds of environmental waters and removing inhibitory substances in these samples (Abd El Galil et al., 2004; Gilpatrick et al., 2000). In our experiments, IMS exhibited high efficiency in eliminating interferences from environmental samples (Fig. 2) to facilitate less-sample-matrix-sensitive qPCR assay and consequently yield more reliable results. And it greatly reduced the adverse impact of organic matter present in virus eluants on PCR, as shown by the higher virus recovery efficiency of IMS-RT-qPCR than that of the RNA kit-RT-qPCR method (Fig. 4).

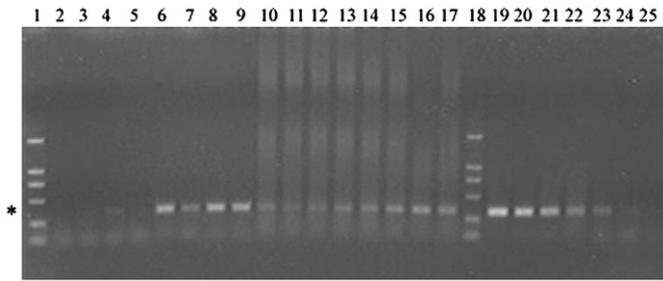


Fig. 3. Elimination of environmental sample matrix effects by IMS-qRT-PCR, in comparison to direct RNA kit-qRT-PCR method. One liter of sewage influent was concentrated into 1 mL (10^3 -fold), seeded with SA11 rotavirus at 1.5×10^3 , 3.9×10^3 , 7.8×10^3 , or 1.5×10^4 TCID₅₀/mL, and tested by IMS-qRT-PCR and RNA kit-qRT-PCR, respectively. The asterisk at the left indicates the 318-base position of the rotavirus amplicon. Lanes 1, 18, DNA ladder; lanes 2–9, tested by IMS-qRT-PCR, rotavirus at 1.5×10^3 (lanes 2, 3), 3.9×10^3 (lanes 4, 5), 7.8×10^3 (lanes 6, 7), 1.5×10^4 (lanes 8, 9) TCID₅₀/mL; lanes 10–17, tested by RNA kit-qRT-PCR, rotavirus at 1.5×10^3 (lanes 10, 11), 3.9×10^3 (lanes 4, 5), 7.8×10^3 (lanes 6, 7), 1.5×10^4 (lanes 8, 9) TCID₅₀/mL; lanes 19–24, rotavirus quantified plasmid DNA, starting quantity at 3.6×10^7 , 3.6×10^6 , 3.6×10^5 , 3.6×10^4 , 3.6×10^3 , 3.6×10^2 GC, respectively; lane 25, negative control.

The IMBs only capture intact and thus potentially infectious viral particles and rarely adsorb naked viral nucleic acid (Schwab et al., 1996). The IMS combined with PCR (IMS-PCR) for the detection of virus is able to examine viral antigens and viral nucleic acid simultaneously. Therefore, IMS-PCR is expected to be more reliable in the detection of potentially infectious viruses and afford relevant and valuable data for health risk assessment, especially for the viruses that are difficult or not amenable to culture *in vitro*. The results from our IMS-RT-qPCR assay were comparable with those obtained from accepted commercial RNA kit-RT-qPCR assay and they correlated well with those from the cell culture assay (correlation coefficient of 0.9816), indicating the validity of the method developed (Yang et al., 2009).

The detection limit of the developed IMS-RT-qPCR method is around 3.16 TCID₅₀/mL or 1.8 PFU/mL according to our measurement (Yang et al., 2009), which is comparable with the previously reported detection limits—10 PFU/mL for HAV through IMS-real time NASBA (Abd El Galil et al., 2005) and 1 PFU/mL for enteroviruses through IMS-real time RT PCR (Hwang et al., 2007). In addition, compared to the RNA kit-RT-qPCR method, IMS-RT-qPCR lowers the detection limit by approximately one order of magnitude when applied in clean

water. The improvement of sensitivity might be attributed to the higher efficiency of the IMS step combined with direct heating for recovering targeted viral particles than the commercial RNA extraction kit. There might be a certain amount of loss of viral RNA in the commercial RNA kit extraction procedure due to adsorption of RNA to the silica-membrane applied during RNA purification. The relatively minor loss of viral RNA as mentioned above might not be influential for detecting virus at higher concentrations, but would impact on detection limit when analyzing samples with very low virus concentrations. Furthermore, the IMS-RT-qPCR method we established requires only about 5 h (2–2.5 h for IMS, ca. 20 min for RT, ca. 2 h for qPCR) yielding quantitative results while the cell culture-based assay usually requires several days to weeks to obtain results and often the tests do not work due to bacterium/fungi contamination or cell deterioration. Thus, IMS-RT-qPCR is a more rapid and reliable method than traditional cell culture-based assay for the detection of viruses.

Previous investigations showed that IMS efficiency is affected by sample matrix. For example, Monceyron and Grinde (1993) reported that the detection limit of IMS-RT-PCR for the detection of HAV varied with the different background matrix of samples, showing 10^0 , 10^1 , 10^2 , 10^5 , and 10^3 TCID₅₀/mL for PBS/BSA, polluted fresh water, sea water, serum, and 5-fold diluted serum, respectively. Grinde et al. (1995) also observed decrease in sensitivity when applying IMS-RT-PCR against sea water for the detection of rotavirus, comparing to applying the method against fresh water or IMS buffer. Zhang et al. (2007) also showed that efficiencies of virus recovery using the IMS-RT-qPCR method varied with different kinds of water samples, indicating 0.15–0.72% for sewage samples, 11.15–43.25% for surface water samples, and 12.85% for tap water samples (actually untreated ground water). In this study, we further tested the IMS-RT-qPCR method against sewage concentrate and found that the detection limit of IMS-RT-qPCR for rotavirus increased to around 3.9×10^3 TCID₅₀/mL when performed in 10^3 -fold sewage influent concentrate compared to that obtained in ddH₂O (3.16 TCID₅₀/mL), indicating reduced virus recovery with IMS due to sample background matrix.

Possible causative factor for the virus recovery reductions in sewage samples was the colloidal and fine particles that were left with the virus in the eluate after the centrifugation step (see the sewage concentration protocol). It was observed in our experiment that the presence of residual particles in virus eluate might affect the separation of virus-IMB combinations in the IMS step, as they tended to enwrap IMBs and consequently diminish the magnetic power of IMBs. The reduced IMBs recovery consequently led to decreased virus recovery efficiency with the IMS step, and it may explain the seemingly higher detection limit of IMS-RT-qPCR method than the RNA kit-RT-qPCR method when applied to virus samples in concentrated sewage influent matrix (Fig. 3). We found that pipetting the sample multiple times to dissociate the particles from the IMBs could alleviate the impact to some extent. Another potential effect of colloidal and fine particles on IMS is competing with virus for the binding sites on the IMBs. However, effect on the affinity of IMBs (mediated by the antibodies coated on the outside of the beads) to the viral particles may be minor in our study, since excessive amount of IMBs were added to a sample so that there are sufficient affinity ligands coated on the outside of the IMBs for binding target virus particles, even though some of the ligands may be competed by the colloidal and fine particles. In our experiments, 10 μ L of IMBs (containing ca. $1-2 \times 10^7$ beads) were added into each 1 mL sample (containing $1 \times 10^4-1 \times 10^9$ viral particles), and amounts of ca. $2.06 \times 10^3-2.78 \times 10^8$ viral particles were recovered after IMS-RT-qPCR with similar recovery efficiency at both low and high virus concentrations, indicating that the probability of having colloids and fine particles competing with virus for binding sites and potentially inhibiting and reducing the virus binding efficiency is very small.

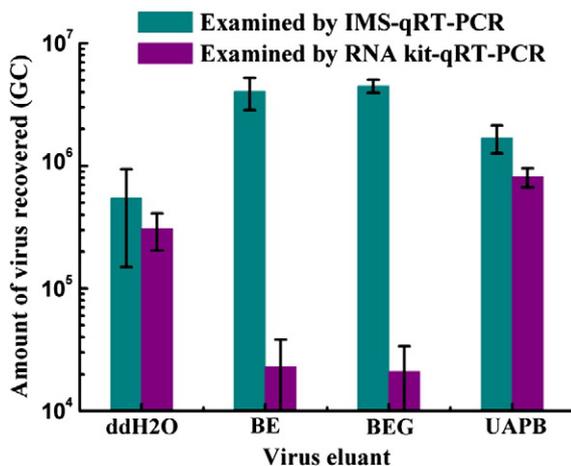


Fig. 4. IMS compatibility with three commonly used virus eluants. ddH₂O, double distilled water; BE, 3% beef extract, pH 9.5; BEG, 3% beef extract with 0.05 M glycine, pH 9.5; UAPB, urea arginine phosphate buffer (1.5 M urea, 0.02 M arginine, 0.008 M phosphate, pH 9.0).

Previous study by Kuhn et al. (2002) indicated that the IMS efficiency decreased dramatically when the pH was raised to 7.5 or lowered to 6.5 for detecting *Cryptosporidium* oocysts, hypothetically due to change in antigen-binding affinity. However, Jiskoot et al. (1990) reported that the amount and the antigen-binding capacity of antibodies studied hardly changed when incubated at pH 4.0–10.0 at 4 °C for 32 days. In our study, the IMS was performed after the virus concentration process which adjusts the pH to 7.0 (the H₂SO₄–NaOH eluant), or 9.0 (the UAPB eluant), or 9.5 (the BE and BEG eluant) and the authors found that IMS efficiency was not significantly affected with these three pH values.

We also observed that in samples with very low amount of organic matter (e.g., ddH₂O or tap water), a portion of IMBs tended to adhere to the tube wall and hence consequently reduced the IMS virus recovery efficiency. This is probably due to the relatively high hydrophobicity of the IMBs and tube wall in the absence of any organic matters. We found that adding Tween-20, a surfactant, into clean water samples before the IMS process with concentrations ranging from 0.01% to 0.5% can effectively prevent such adherence (e.g. the IMS virus recovery efficiency increased 1.4 times when adding 0.25% of Tween-20 (data not shown)). Thus Tween-20 was added when IMS-RT-qPCR was performed in clean water samples.

Another problem worthy caution when applying the IMS-RT-qPCR assay is the validity of heat release for extracting viral RNA. Heating is effective and convenient to release viral RNA as mentioned earlier, however, our experiments (data not shown) indicated that when the primers used were near the tail region of gene fragment, amplification of the target region may fail sometimes and the results were quite random. Although the reason for this phenomenon is not understood yet, it is possible that heating may cause certain damages to nucleic acid, and the tail of the gene fragment may be more susceptible to such kind of damages. And when these damages happen, it may result in failure of amplification although viral RNA or DNA may actually be present in the sample, rendering the test results false negative. Thus we suggest that primers used to amplify target region should be verified even though they are successfully applied combining with other genome extraction methods.

5. Conclusion

A rapid and sensitive IMS-RT-qPCR method for the detection of group A rotavirus in water samples was developed in present study. This method has higher sensitivity (>10 times) in comparison with other methods that do not have the IMS step when applied to clean water (ddH₂O) matrix. It can efficiently remove sample background matrix interference when applied to sewage concentrates, although the presence of large amount of colloidal and fine particles might reduce the virus recovery efficiency with the IMS step. The method is compatible with currently widely used virus eluants and has higher recovery efficiency than direct RT-qPCR methods for all the eluants tested, thus can be employed in detecting virus concentrated in these eluants. Furthermore, IMS-RT-qPCR exhibits comparable results with accepted RT-qPCR methods and showed a good correlation with the results from cell culture assay, indicating that the method is valid for the detection of infective rotavirus.

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