Development of a Luminex assay for the simultaneous detection of human enteric viruses in sewage and river water

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A B S T R A C T

Real time PCR (qPCR) is increasingly being used for viral detection in aquatic environments because it enables high specificity and sensitivity of detection. However, the limited number of fluorescent reporter dyes restricts its multiplex application. In this study, a multiplex Luminex assay was established for the simultaneous detection of human adenovirus (HADV), human polyomavirus (HPyV), enterovirus (EV), rotavirus (RV), norovirus GI (NoVGI) and norovirus GII (NoVGII). Different river water and wastewater samples were tested for the viruses using both qPCR and the multiplex Luminex xMAP assay. HADV and HPyV were the most abundant in all environmental samples. HADV was detected in all river water and wastewater samples, and HPyV was detected in 79% of river water and 95.8% of wastewater samples. The multiplex xMAP assay revealed high specificity and no cross-reactivity. Using the multiplex Luminex assay, the viral detection rates in river water samples were lower than the rates obtained by qPCR for all viruses. Conversely, in wastewater samples, the viral detection rates were the same for both methods. In addition, the analytical sensitivity of the monoplex Luminex assay was comparable to or lower than qPCR. Results suggest that the multiplex Luminex assay could be a reliable method for the simultaneous detection of viral pathogens in wastewater.

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1. Introduction

Waterborne human pathogenic viruses are of much concern to public health in both the developed and developing worlds. Enteric viruses are the most suited for transmission via water due to their release in large quantities in the feces of infected persons and having a prolonged survival in water (Bosch, 1998). Despite the progress in water and wastewater treatment technology, enteric viruses are frequently detected in water (Pina et al., 1998; Hot et al., 2003; Jiang and Chu, 2004; Choi and Jiang, 2005; Pusch et al., 2005; Haramoto et al., 2008; Lee and Kim, 2008; Hamza et al., 2009a,b; Mena and Gerba, 2009; Rodriguez-Diaz et al., 2009; Haramoto et al., 2010; Hamza et al., 2011; Wyn-Jones et al., 2011). Surface water can be contaminated with enteric viruses from a variety of sources; however, the discharge from wastewater treatment plants into surface water is the main source of water pollution. The viral load may vary according to the efficiency of the wastewater treatment process, geographical area, season, hygiene and sanitary conditions (Wyn-Jones and Sellwood, 2001). Most water-transmitted viral infections occur asymptotically or, more frequently, cause diarrhea, self-limiting gastroenteritis, respiratory infections, conjunctivitis, hepatitis, aseptic meningitis, encephalitis and paralysis.

A wide variety of analytical methods are available for viral detection in environmental water samples (Mattison and Bidawid, 2009). Molecular methods such as the polymerase chain reaction (PCR) and real time PCR (qPCR) have the highest sensitivity and specificity for determining viral contamination in water; therefore, they are the most commonly used in environmental virology (Mattison and Bidawid, 2009). Multiplex PCR is the choice for the simultaneous detection of many targets of interest using more than one set of primers (Chamberlain et al., 1988). The sensitivity of multiplex PCR could be reduced as a result of interference between the primer sets, but this effect can be minimized by optimizing primer design. Multiplex PCR was used for the simultaneous detection of more than one virus in environmental samples (Fout et al., 2003; Lee et al., 2005). In addition, a multiplex qPCR that utilizes different reporter dyes can provide for the specific detection of simultaneous targets (Kirs and Smith, 2007; Wolf et al., 2010). However, the limited number of

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fluorescent reporter dyes, the number of fluorescence acquisition channels available in the PCR instruments and the potential of optical interference between a subset of fluorescent chemicals can restrict the utility of real-time PCR used for that purpose (Huang et al., 2011).

Luminex xMAP technology offers a novel platform for high-throughput pathogen detection using antibody- and nucleic acid-based assays. The Luminex system is a flexible analyzer based on the principles of flow cytometry. For nucleic acid detection, DNA is amplified with a biotin-labeled primer, and the amplimers are hybridized to capture probes bound to microspheres. The microspheres are filled with different relative concentrations of an infrared dye and a red dye to create 100 beads, each with a unique spectral identity. Therefore, in theory, this method can be used for the multiplex detection of up to 100 analytes in a single microplate well. Microfluidics controls the flow of the microspheres through the path of two lasers with different wavelengths, which respectively determine the spectral identity of the bead and the R-phycocerythrin that is conjugated to the capture probes. The Luminex assay has been primarily used for a variety of clinical applications (Dunbar et al., 2003; Opalka et al., 2003; Croft et al., 2008; Pabbaraju et al., 2011; Taniuchi et al., 2011; Liu et al., 2012a,b). In addition, it has been proposed for environmental applications such as the detection of 16S ribosomal DNA from contaminated wells (Spiro and Lowe, 2002), the detection of marine phytoplankton (Ellison and Burton, 2005) and the detection of fecal bacterial indicators in river water (Baums et al., 2007).

In this study, a Luminex xMAP assay was established and its potential use for viral monitoring in water and wastewater was assessed. A multiplex Luminex assay was designed to simultaneously detect six human enteric viruses, human adenovirus (HADV), human polyomavirus (HPyV), enterovirus (EV), rotavirus group A (RoV A), norovirus GI (NoVGI) and norovirus GII (NoVGII), in surface and wastewater samples, as well as murine norovirus (MNV) as an external control.

2. Materials and methods

2.1. Collection and concentration of environmental water samples for viral analyses

A total of 24 water samples (101 each) were collected from the Ruhr River in the North Rhine Westphalia region, Germany. Additionally, 24 influent wastewater samples (51 each) were collected from four wastewater treatment plants in the Ruhr district. The samples were concentrated to approximately 5 ml using the virus adsorption elution method as previously described (Hamza et al., 2009a).

Briefly, water samples were spiked with murine norovirus (~1 × 10⁶ genome equivalents) as an external control to determine the efficiency of the enrichment, extraction and amplification steps. The samples were adjusted to pH 3.5 with 1 N HCl and a negatively charged HA membrane (0.45 mm pore size, 142-mm diameter; Millipore) was used for viral adsorption. A non-organic elution buffer (0.05 M KH₂PO₄, 1.0 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2) was used for recovery of the adsorbed viruses. The eluate was concentrated by overnight precipitation with 12.5% polyethylene glycol 6000 + 2.5% NaCl at 4°C. The concentrated water samples were stored at −20°C until analysis. The overall recovery rates of the concentration process and the assay detection limits have been estimated previously for river water for HADV, HPyV, echovirus 11, norovirus, and φX174 (Hamza et al., 2009a).

2.2. Viral nucleic acid extraction

Viral RNA and DNA were co-extracted from 200 μl of the concentrated virus suspension using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Standard precautions to avoid contamination were taken. Total RNA and DNA were extracted, suspended in 50 μl elution buffer, and stored at −20°C.

2.3. DNA and RNA standards for q(RT-)PCR and Luminex assay

To obtain representative positive control DNA and RNA standards, PCR fragments from HADV5, JCPyV, RoV, NoVGI, NoVGII, MNV and Echo11 were obtained using the primers listed in Table 1. It should be noted that the HPyV primers used are specific only for JCPyV and BKPyV and not other human polyomaviruses. Each PCR fragment was cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA, USA), by the TA cloning strategy according to the manufacturer’s protocol. The plasmid was purified using a QiAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The concentration of the purified plasmid DNA was determined by using the QuantiT dsDNA HS Assay (Invitrogen, Carlsbad, CA, USA) and fluorescence was measured using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). To produce RNA standards for EV, NoVGI, NoVGII, and rotavirus, the AmpliScribe T7 transcription kit (Epicerent Technologies, Madison, WI, USA) was used following the manufacturer’s instructions. After the in vitro transcription reaction, the mixture was treated with DNase to remove the template DNA. RNA was subsequently purified using the ammonium acetate precipitation method according to the protocol of the AmpliScribe transcription kit. The purified RNA concentration was measured by the QuantiT RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and the Qubit Fluorometer. The number of molecules of standard RNA/DNA added to the PCR reactions was calculated from the experimentally determined concentration of standard RNA/DNA and its molecular weight.

2.4. Recovery rate of MNV from river and wastewater samples

In early experiments (n = 3), the recovery rate of MNV in spiked water samples was determined using the one-step quantitect multiplex RT-PCR kit (Qiagen, Hilden, Germany) and two-step RT-PCR, including cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) followed by PCR using the quantitect probe PCR kit (Qiagen, Hilden, Germany). To identify the occurrence of PCR inhibition, nucleic acid templates were diluted 1:50 and compared to the same samples without dilution.

2.5. Virus detection using monoplex qPCR

First-strand cDNA was produced by reverse specific primers with 2 μl of viral RNA standard or 5 μl of extracted RNA from environmental samples in a 20 μl reaction mixture containing 200 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and following the manufacturer’s instructions. Real time PCR was performed using the Quantitect probe PCR kit (Qiagen, Hilden, Germany), containing 0.25 μM of each forward and reverse primer, 0.1 μM of TaqMan probe and 2 μl of cDNA or 5 μl of viral DNA. The NoVGI and GI primer concentrations were 0.125 μM. The Rotorgene 6000 cycler system (Corbett Research, Sydney, Australia) was used for amplification and detection, and data analysis. The temperature conditions for the qPCR were optimized as follows:

(i) AdV/HPyV/EV/MNV: 95°C for 15 min, 45 cycles of 95°C for 15 sec, 60°C for 1 min
(ii) RoV/NoVGI/GII: 95°C for 15 min, 5 cycles of 95°C for 15 sec, 56°C for 1 min; 40 cycles of 95°C for 15 sec, 60°C for 1 min.
Table 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Oligo</th>
<th>Primer/probe sequence (5'-3') ¹</th>
<th>Size (bp)</th>
<th>Target gene</th>
<th>Reference</th>
</tr>
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<tr>
<td>HAdV</td>
<td>AQ1</td>
<td>[Bnn]GCCCCAAGCTCTATTACATGCACACATC</td>
<td>132</td>
<td>Hexon</td>
<td>Modified</td>
</tr>
<tr>
<td></td>
<td>AQ2</td>
<td>GACGAGTGGGGGTTCTATCCATC</td>
<td></td>
<td></td>
<td>(Henn et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>[Amc12]TGGCAAGCAGCCGGGTCATGATCAGCACGGA</td>
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</tr>
<tr>
<td>HPyV</td>
<td>PV-F</td>
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<td>223</td>
<td>VP1</td>
<td>Modified</td>
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<tr>
<td></td>
<td>PV-Back</td>
<td>[Bnn]GGGACACCTCTTGGAAAGACAG</td>
<td></td>
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<td>(Biel et al., 2000)</td>
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<tr>
<td>EV</td>
<td>F</td>
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<td>5'-UTR</td>
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<td>R</td>
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<td></td>
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<td>JLV1</td>
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<td>96</td>
<td>ORF1-ORF2</td>
<td>junction</td>
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<td></td>
<td>JLV1R</td>
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<tr>
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<td>COG2R</td>
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<td>ORF1-ORF2</td>
<td>junction</td>
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<td>VP6</td>
<td></td>
</tr>
<tr>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>probe2</td>
<td>[Amc12]JAGATGGCGCTCTGTTCCACA</td>
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<tr>
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<td>93</td>
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<tr>
<td></td>
<td>MNV-TM2</td>
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<td></td>
<td></td>
<td>(Muller et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>[Amc12]GCGGGTTTTCTGGCGACTTCGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Human adenovirus (HAdV); human polyomavirus (HPyV; JCpyV and BkPyV); enterovirus (EV); norovirus genogroup I and II (NoVGI, NoVGH); group A rotavirus (RoV A); murine norovirus (MNV).

One primer was labeled with Biotin (Bnn) and the probes were labeled with Amino C12 linker (AmC12) on the 5'-end.

² R, A or G; S, G or C; Y, C or T.

2.6. Multiplex PCR for virus detection using Luminex xMAP

The assay was initially performed in singleplex reactions and then multiplexed. After confirming that all of the individual primer pairs allowed for amplification of all seven target viruses in a single PCR reaction, forward and reverse gene-specific primers were mixed together. Either the forward or the reverse primers were biotinylated on the 5'-end. Then, 2 μL CDNA and DNA template were mixed in a 40 μL multiplex reaction containing 0.25 μM of each primer and 20 μL of 2x Multiplex PCR mastermix from the Qiagen multiplex PCR plus kit (Qiagen, Hilden, Germany). A gradient PCR was used to select the best annealing temperature for the simultaneous amplification of all target viruses. The optimal temperature conditions were 95 °C for 15 min, 45 cycles of 94° for 30 s, 57 °C for 90 s, 72 °C for 30 s and a final extension at 72 °C for 10 min.

2.7. Carbodiimide coupling of amine-modified oligonucleotides to carboxylated microspheres

Different sets of carboxylated fluorescent microbeads were obtained from Luminex Corp. (Austin, TX), and oligonucleotide probes for target viruses were assigned to individual bead sets. Each probe sequence represented the reverse complement to the target region of the biotinylated PCR product. Each probe was modified with 5’ amino modifier C12 (AmC12) to enable coupling to the carboxyl group located on the microsphere. The primers and probes are listed in Table 1. The probes were coupled to the beads according to the manufacturer’s recommended coupling protocol.

The uncoupled bead stock was vortexed and sonicated for 20 s and 5 x 10⁶ beads were transferred to a 1.5 mL LoBind microcentrifuge tube. The tube was placed in a magnetic separator for 60 s to allow the beads to separate. Then, the supernatant was removed and the beads were re-suspended in 50 μL of 0.1 M MES (2[N-Morpholino] ethanesulfonic acid), pH 4.5, with vortexing and sonication (20 s each). Next, 0.5 nmol probe and 2.5 μL of fresh 10 mg/ml EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) were added to the mixture, vortexed and incubated for 30 min at room temperature in the dark. After the 30 min incubation, a fresh solution of 10 mg/ml EDC in dH₂O was prepared and the EDC addition and incubation steps were repeated. The microspheres were then washed with 1 mL of 0.02% Tween-20, the beads were separated by a magnetic separator, the supernatant was discarded and a second washing step was performed using 1 mL of 0.1% SDS. The tube was placed in a magnetic separator for removal of the washing buffer, and the beads were resuspended in 100 μL of TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8). The probe-coupled beads were counted using a hemocytometer and were stored in the dark at 4 °C until use.

2.8. Hybridization of biotinylated PCR amplicons to Luminex probes and analysis

Biotinylated PCR products were hybridized to probe-coupled beads in 96-well plates and were detected using streptavidin–phycoerythrin. A working microsphere mixture consisted of seven types of beads, each coupled to a unique gene-specific capture probe from the viral panel. A working microsphere mixture was prepared by diluting coupled microsphere stocks to 150 microspheres of each set/μL in 1.5 x tetramethylammonium chloride (TMAC) hybridization solution (4.5 M TMAC, 0.15% sarkosyl, 75 mM Tris-HCl, pH 8, 6 mM EDTA, pH 8). The working beads were mixed with a vortexer and were sonicated for 20 s. Then, 33 μL of the bead mixture was added to each reaction. The sample reactions contained 7 μL of biotinylated PCR product and 10 μL of TE, pH 8. For a control, 17 μL of TE, pH 8, was added to a background well. The reaction was mixed gently by pipetting several times. Five different hybridization temperatures (48 °C, 53 °C, 50 °C, 56 °C, 58 °C) and different incubation times (15, 30, 45, 60, 90 min) were tested in triplicates to identify the optimal hybridization conditions. The optimal hybridization temperature and time were found to be 95 °C for 3 min followed by 50/53 °C for 60 min. After hybridization, the solutions were pelleted by placing the plate
in a magnetic separator for 1 min. The supernatant was carefully discarded to eliminate unbound PCR products. The hybridized amplicons were fluorescently labeled by re-suspending the pellet in 75 μl of 1× TMAC solution containing a freshly prepared solution of 4 μg/ml streptavidin-R-phycoerythrin. The plates were incubated in the dark at the same hybridization temperature for an additional 5 min. Finally, the reactions were placed into the Luminex 200 (Luminex Corporation, Austin, TX) for bead enumeration and quantification of phycoerythrin fluorescence of each bead. For each bead type, 100 microspheres were analyzed, representing 100 replicate measurements (Diaz and Fell, 2005). Data analysis was performed using xPONENT3.1 software and fluorescence was expressed as median fluorescence intensity (MFI), which was background corrected. The cutoff value for a positive result was set as twice the MFI value of the negative control (TE buffer).

2.9. Detection limits of q(RT-)PCR and Luminex assay

To determine the sensitivity of the assays, duplicates of 10-fold serial dilutions of the DNA/RNA standards of each virus were tested by monoplex qPCR, monoplex Luminex assay and multiplex Luminex assay. The detection limit was defined as the dilution containing the fewest copies of viral genome that still gave a positive result for all replicates.

3. Results

3.1. Specificity of Luminex assay

The specificity of the multiplex Luminex assay was tested in duplicates against seven target viruses (HAdV, HPyV, RoV A, NOVG1, NOVGII, EV, MNV), and three other viruses (pepper mild mottle virus, bovine polyomavirus, human bocavirus) that are in high abundance in surface water (Hundesa et al., 2006; Hamza et al., 2009b, 2011). TE buffer was used as a blank control. The tests showed that the Luminex assay was able to detect all target enteric viruses, with no positive cross-reaction signals with non-specific target viruses (Fig. 1).

3.2. Sensitivity of qPCR and multiplex Luminex assay

The sensitivity of the Luminex assay and qPCR was determined using 10-fold serial dilutions of each DNA/RNA standard. To determine the sensitivity of the qRT-PCR assay, standard curves of crossing point (Cp) values versus the number of copies were generated by analyzing duplicates of 10-fold serial dilutions of the DNA/RNA standards.

The sensitivity of qRT-PCR as genome copies per reaction was calculated to be 25 for NoVGII, 12.5 for NoVG1, 50 for EV, 5 for RV-A, 5 for MNV, 5 for HAdV, and 10 for HPyV. The sensitivity of the multiplex Luminex assay was lower than singleplex qPCR. The multiplex Luminex assay had a higher detection limit, of 10^5 for HAdV and HPyV and 10^2 for RoV, NoVG1, NoVGII, EV and 10 for MNV (Fig. 2). However, the sensitivity of the singleplex Luminex assay was equivalent to or lower than that obtained when each of the targets was amplified and detected in singleplex using qPCR. Furthermore, the relationship between virus concentration and real-time Ct was more linear than that of virus concentration and cMFI (data not shown).

3.3. Intra-assay repeatability and inter-assay reproducibility of the multiplexed Luminex-based assay

Assay precision was examined using triplicates within an experiment and three different experiments. The coefficient of variation (CV) ranged from 1.3 to 8.4% within the run and from 4.6 to 10.3 between runs (Table 3). Reproducibility was obtained by a single operator over 10 days.

3.4. Recovery rate of MNV and PCR inhibitor

To determine the inhibitory effects of extracted viral nucleic acids on RT-PCR or qPCR, 5 μl of undiluted NA (extracted from the environmental water samples) or the same volume of a 1:50 dilution was used in the RT-PCR reaction. Although the one-step qRT-PCR kit includes optimized components that allow both reverse transcription and PCR amplification to take place in the same reaction mix, direct analysis of MNV in river and wastewater samples yielded a mean recovery of 9.5% and 15.7%, respectively. Therefore, a dilution factor (1:50) was required to relieve enzymatic inhibition during PCR analysis (Table 2). For the two-step RT-PCR kit, the mean recovery efficiencies of MNV were 61% from river water and 42.7% from wastewater samples. Efficiency data were not used to correct the concentration of indigenous viruses.

3.5. Performance of multiplex Luminex assay on environmental samples

To assess the environmental performance of the multiplex Luminex assay, target viruses were examined in 24 concentrated river water and wastewater samples by qPCR compared to the multiplex Luminex assay. Using qPCR, all river water and wastewater samples were positive for HAdV (6.9 × 10^2–5.0 × 10^4, 9 × 10^2–1 × 10^3 gen equiv/l), HPyV was detected in 19 river water samples (1.3 × 10^2–5.0 × 10^4 gen equiv/l) and 23 wastewater samples (3 × 10^3–2.1 × 10^4 gen equiv/l). RNA viruses were less abundant; EV was detected in 14 river water samples and 4 wastewater samples. NoVGII was detected in 12 river water samples and 10 wastewater samples. Only 3 river water samples were positive for RoV and no wastewater samples were positive for RoV. All river water and wastewater samples were negative for NoVG1. Compared to qPCR, the multiplex Luminex assay gave lower detection rates in river water for all viruses (Fig. 3). The detection rates were 16/24 for HAdV, 11/24 for NoVGII, 6/24 for EV, 3/24 for polyomavirus and 1/24 for RoV. Norovirus GI was detected in one sample, which was a discrepancy with the qPCR results. Viral detection in wastewater with the Luminex assay was similar to that with qPCR, most likely due to a higher viral load in wastewater than river water (Fig. 3). Although PCR-based detection assays are prone to inhibition by substances present in environmental samples, the inhibitory effect was relieved by using the two-step RT-PCR protocol, and all samples were positive for MNV.

4. Discussion

Various enteric viruses, including enterovirus, rotavirus, norovirus and adenovirus, are the major etiological agents of water-borne disease outbreaks and are frequently detected in surface water using molecular-based methods, such as PCR or qPCR (Mattison and Bidawid, 2009). Different viruses have been proposed as indicators of viral contamination in water, including adenovirus and polyomavirus (Leclerc et al., 2000; Hot et al., 2003; Albinalna-Gimenez et al., 2009; Hamza et al., 2009b, 2011; McQuaig et al., 2009). Due to less concordance between published reports, the ideal indicator is provided by the identification of the viral pathogen itself (Bosch, 1998; Jurzik et al., 2010). Accordingly, multiplex detection is always required to cover a broad range of pathogens in one test.

Probe-based assays provide rapid and sensitive results and are widely used for viral detection; however, they are largely used in a singleplex format. To our knowledge, the Luminex xMAP
technology has not been utilized before for viral detection in environmental virology. A commercial gastrointestinal pathogen panel (GPP kit) is available from Luminex for the simultaneous detection of viruses, bacteria and parasites causing gastroenteritis (Mengelle et al., 2013; Navidad et al., 2013). However, only three enteric viruses (norovirus, rotavirus and adenovirus 40/41) can be identified by the GPP kit. Here, we established a multiplex detection assay based on Luminex xMAP technology for simultaneous detection of human enteric viruses in environmental water samples. The performance of the multiplex Luminex assay was compared to monoplex qPCR via the detection of six human viruses (HAdV, HPyV, EV, RoV A, NoVGI and NoVGII) and MNV (extrinsic control) in river and wastewater samples. The specificity of the assay was tested against different targets in a multiplex format.

Using qPCR, HAdV was detected in all samples and HPyV was detected in 79% of river water and 95.8% of wastewater samples.

<table>
<thead>
<tr>
<th>Water type</th>
<th>One-step qRT-PCR</th>
<th>One-step qRT-PCR 1:50 dilution</th>
<th>Two-step RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean recovery (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>River water</td>
<td>15.7% (8.2–20.3%)</td>
<td>31.9% (23.9–42.7%)</td>
<td>61% (47.4–80%)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>9.9% (9.3–10.7%)</td>
<td>31.7% (30.2–33.3%)</td>
<td>42.7% (33–52%)</td>
</tr>
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</table>
The high abundance of HAdV and HPyV in environmental samples is consistent with previous studies that showed high dissemination rates of these viruses in different aquatic environments (Pina et al., 1998; Boiffil-Mas et al., 2000; McQuaig et al., 2006; Hamza et al., 2009a, 2011; Mena and Gerba, 2009; Haramoto et al., 2010). The frequent detection of HAdV in surface water could be attributed to the establishment of latent infections with viral shedding for weeks. Similarly, HPyV is characterized by viral persistence in the kidney as judged by viral excretion in the urine (Dörries, 2002).

As shown in Fig. 1, the multiplex xMAP assay revealed high specificity and no cross-reactivity. However, the assay was not as sensitive as qPCR for the identification of viral contamination in river water samples. Analysis of river water samples using the multiplex Luminex xMAP assay underestimated the detection rate of all viruses (Fig. 3). The difference in the detection rates obtained by both methods might be due to the higher sensitivity provided by qPCR. In addition, multiplex amplification may reduce the sensitivity of the Luminex xMAP assay (Fig. 2).

In contrast, all wastewater samples that were positive via qPCR were also positive by the multiplex Luminex assay, suggesting that multiplex Luminex assay is sensitive enough for rapid monitoring of a large panel of enteric viruses in wastewater samples.

Although the detection limit of the multiplex Luminex assay described here is higher than qPCR, the assay is still more sensitive than a previously described protocol (Liu et al., 2012b) for the simultaneous detection of different human enteric viruses. Liu et al. (2012b) reported detection limits between 10³ and 10⁴ of viral nucleic acids per reaction, whereas the multiplex Luminex assay was one order of magnitude lower.

Our findings are consistent with Pabbaraju et al. (2011), who showed that under a low viral load, qPCR has a much better sensitivity than commercially available kits for the multiplex Luminex xTAG respiratory virus panel (RVP), which is approved by the United States Food and Drug Administration. We found that the detection limit of the Luminex assay was only comparable to or lower than qPCR under monoplex amplification, as shown in Fig. 2. On the contrary, it was reported that the sensitivity of the

### Table 3

<table>
<thead>
<tr>
<th>Target</th>
<th>Repeatability MFI CV%</th>
<th>Reproducibility MFI CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAdV</td>
<td>938 1081 1096 8.4</td>
<td>938 1081 1096 1000 1004 961 1038 825 854</td>
</tr>
<tr>
<td>HPyV</td>
<td>1967 1799 1798 5.3</td>
<td>1967 1799 1798 1624 1638 1594 1688 1528 1456</td>
</tr>
<tr>
<td>EV</td>
<td>1561 1702 1716 5.1</td>
<td>1561 1702 1716 1542 1545 1539 1614 1326 1344</td>
</tr>
<tr>
<td>NoVGI</td>
<td>1203 1383 1315 7.0</td>
<td>1203 1383 1315 1319 1273 1277 1262 1221 1212</td>
</tr>
<tr>
<td>NoVGI</td>
<td>2448 2581 2507 2.7</td>
<td>2448 2581 2507 2335 2381 2349 2361 1931 1924</td>
</tr>
<tr>
<td>RoV A</td>
<td>1172 1096 1126 3.4</td>
<td>1172 1096 1126 990 993 962 991 890 865</td>
</tr>
<tr>
<td>MNV</td>
<td>2183 2243 2202 1.3</td>
<td>1979 2088 1952 2080 2010 2033 2202 2243 2183</td>
</tr>
</tbody>
</table>

Human adenovirus (HAdV); human polyomavirus (HPyV); enterovirus (EV); norovirus genogroups I and II (NoVGI, NoVGI); group A rotavirus (RoV A); murine norovirus (MNV).

Median fluorescence intensity (MFI). Coefficient of variation (CV).
multiplex Luminex assay was equivalent to that obtained when each of the targets was amplified and detected in singleplex, and the limit of detection was equivalent to qPCR used to detect seven intestinal parasites in fecal material (Taniuchi et al., 2011). Although it is difficult to compare our data to Taniuchi et al. (2011), it should be noted that they conducted the PCR reaction in a 3–plex format, one with specific primers for the protozoa and one with specific primers for the helminthes, while we performed a 7-plex PCR format.

5. Conclusions

The Luminex system provides high throughput multiplex detection of multiple targets in a single microplate well. Although the assay was associated with the specific detection of viral pathogens and detection limits that were better than previous reports, it may underestimate the presence of viruses in surface water, particularly when a low virus titer is expected. However, the multiplex Luminex assay was as sensitive as qPCR for viral detection in wastewater samples. Therefore, it could be a reliable method for the simultaneous detection of viral pathogens in wastewater, which, in turn, reduces the costs of assays, as well as time and labor.

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