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Direct typing of human enteroviruses from wastewater samples

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A B S T R A C T

A RT-PCR approach for the direct detection and typing of human enteroviruses in the environment is described in this study. A semi-nested RT-PCR using COnsensus-DEgenerated Hybrid Oligonucleotide Primers (CODEHOP) designed from the *VP2* genome region has been developed for the direct typing of enteroviruses in clinical samples (Ibrahim et al., 2013). This CODEHOP/VP2 PCR strategy as well as the CODEHOP/VP1 technique described by Nix et al. (2006), were tested for the detection and typing of enteroviruses in wastewater samples. Virus particles were first extracted and concentrated from wastewater samples by using respectively beef extract and polyethylene glycol 6000, and the presence of enteroviruses was screened by a RT-PCR method using primers from the 5′-end non-coding region (5′NCR). Fifty-two of 172 samples (30.2%) were revealed positive by the 5′NCR method. From these 52 samples, only 19 samples (36.5%) were found positive by at least one of the two CODEHOP techniques, with the following distribution: VP1(+)/VP2(+) = 4 (7.7%), VP1(−)/VP2(+) = 13 (25%) and VP1(+)/VP2(−) = 2 (3.8%). These results illustrate that the direct typing of enteroviruses in environmental samples is insensitive, possibly due to the presence of large amounts of amplification inhibitors; however, the *VP2* method was found able to allow the direct detection and typing of *c.* one-third of the positive environmental samples.

1. Introduction

The presence of infectious agents in water environment is a key determinant for the evaluation of the level of microbiological pollution, with major impacts on the control of the fecal risk for human and animal health. For this purpose, the detection of coliforms is usually required but it is a poor reflect of viral contamination (Noble and Fuhrman, 2001). For evaluating the latter risk, different markers have been proposed, including notably bacteriophages, enteroviruses (EVs) and adenoviruses (Metcalf et al., 1995; Gantzer et al., 1998; Hot et al., 2003; Fong and Lipp, 2005).

EVs are small non-enveloped RNA viruses belonging to the *Picornaviridae* family and displaying a high genetic diversity (more than 100 genotypes distributed in four species in humans). They are highly resistant in the water environment that can retain their infectivity for months (Wetz et al., 2004; Fong and Lipp, 2005; Rajtar et al., 2008). The advantage of EVs as a marker of viral contamination of wastewaters is that some genotypes are relatively easy to cultivate in cell culture, which is still the reference method in terms of environmental surveillance (Ehlers et al., 2005). However, this technique is time-consuming, difficult to standardize and not easy to implement in all the environmental laboratories. During the last 10 years, molecular methods were proposed for supplanting cell culture in environmental samples, notably for the detection of EV genome (Metcalf et al., 1995; Bosch et al., 2008; Rajtar et al., 2008).

By contrast to cell culture, one of the limits of current molecular methods using primers located in the 5′ end non-coding region (5′NCR) of the viral genome, which is highly conserved within the *Enterovirus* genus, is the difficulty for further typing the EV sequences detected in environmental samples. Indeed, typing EV strains present in wastewaters may be an important goal, notably for tracing the presence of polio strains in areas where these agents are still in circulation (Hovi et al., 2012), but also for epidemiological purposes (*e.g.* establishment of a relationship between genotypes present in wastewaters and those responsible for epidemic episodes in humans) (Khetsuriani et al., 2010; Richter et al., 2011).

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In this study, a newly described typing method of EVs targeting the *VP2* region of the viral genome (Ibrahim et al., 2013) was evaluated for detecting and typing EVs in wastewater samples. By contrast to methods using primers located in the 5′NCR, this method permits the direct typing of EVs after sequencing of the amplicons. It was evaluated in comparison to a current PCR method targeting the 5′NCR and to another typing method targeting the *VP1* gene that can be considered as the gold standard for typing EVs (Nix et al., 2006).

2. Materials and methods

2.1. Virus recovery from wastewater samples

One hundred and seventy-two samples of wastewater were collected in 2009–2010 at a frequency of two samples per month from stations of water treatment in rural regions of the Monastir and Madhia governments, located in the East-Center part of Tunisia and covering a population of approximately 60,000 inhabitants. Two liters of wastewater were filtered through filter paper (Wathman® No. 1, Sigma–Aldrich, Sfax, Tunisia) using a vacuum pump. The resultant filtrate (100 ml) was added to 1% (v/v) of 0.05 M aluminum chloride and adjusted to pH 3.5 with HCl. The mixture was homogenized for 30 min then centrifuged at 2500 × *g* for 15 min at 4 °C. The pellet was resuspended in 100 ml of 10% beef extract (Oxoid, Basingstoke, UK) and adjusted to pH 7. The mixture was homogenized again and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was used for virus detection.

2.2. Concentration of virus suspensions

Virus particles recovered from wastewater samples were concentrated by precipitation with polyethylene glycol (PEG) 6000 (Prolabo, Fontenay sous Bois, France), as previously described (Lewis and Metcalf, 1988). In brief, suspensions were mixed with 5% (v/v) PEG 6000 and incubated at 4 °C overnight. The mixtures were then centrifuged at 10,000 × *g* for 45 min. The pellet was resuspended in 10 ml of 0.1 M phosphate buffer pH 7.2 and then filtered through a 0.22 μm Millex-GS membrane (Millipore, Molsheim, France). The suspension was either treated immediately or stored at −20 °C until use.

2.3. RNA extraction

For RNA extraction, 100 μl of the previous suspension were homogenized in 900 μl of TRI-Reagent (Sigma–Aldrich) and incubated for 5 min at room temperature. The mixture was centrifuged at 12,000 × *g* for 10 min at 48 °C to remove insoluble debris. The supernatant was then added to 200 μl of chloroform and incubated for 15 min at room temperature. After centrifugation at 48 °C for 15 min at 12,000 × *g*, the aqueous phase containing RNA was precipitated with 500 μl of isopropanol for 10 min at room temperature. After centrifugation as above, the pellet was washed once in 1 ml of 75% ethanol, dried in a speed-vacuum apparatus and suspended in 60 μl of diethylpyrocarbonate-treated water.

2.4. RNA amplification

2.4.1. 5′NCR

The primers NC1 and E2 (Table 1) used for the amplification of the 5′ untranslated region of the enterovirus genome were described by Rotbart (1991) and synthesized by Eurogentec (Seraing, Belgium). They generate a 152 bp PCR product. Synthesis and amplification of cDNA were performed in a single tube with the Superscript II OneStep RT-PCR kit (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions, in a total volume of 50 μl containing 1 mM of each primer, 10 μl of extracted RNA, 1 μl of RT-Taq polymerase, 25 μl of RT-PCR buffer, 0.5 μl of RNase inhibitor and 12 μl of sterilized bidistilled water. The RT reaction was performed at 50 °C for 30 min, followed by enzyme inactivation at 94 °C for 2 min. The mixture was then subjected to 38 cycles of amplification, consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 30 s at 68 °C. At the end of the reaction, a further extension step was performed at 68 °C for 7 min. The RT-PCR was carried out with a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Boston, MA). A positive control and a water control were included in each PCR reaction.

The amplified RT-PCR products were analyzed on a 3% Metaphor1 agarose gel (Invitrogen) and visualized under ultraviolet light after addition of ethidium bromide (Sigma–Aldrich). A 100-bp DNA ladder (Invitrogen) was used as size marker.

2.4.2. VP1 region

Ten microliters of extracted RNA were reverse transcribed into cDNA at 42 °C for 45 min using 200 units of SuperScriptIII reverse transcriptase and 2.5 ng/μl of random primers (Invitrogen) in the presence of 10 units of RNase OUT recombinant RNase inhibitor (Invitrogen). For the amplification step, the reagents and experimental conditions were those published previously (Nix et al., 2006). Table 1 shows the sequence and genomic location of the primers.

2.4.3. VP2 region

The reverse transcriptase step was the same as described for the *VP1* region. The amplification technique has been reported recently (Ibrahim et al., 2013). A first amplification step was performed by using two pairs of sense (AM11 and AM12) and antisense (AM31 and AM32) primers in order to obtain a fragment of 584 bp (Table 1). The CODEHOP PCR was performed using 2 μl of the first PCR product, 1.25 unit of HotStar Taq DNA polymerase (QIAGEN, Courtaboeuf, France), 40 pmol of primers NS20 and NS30 (Table 1), $MgCl_2$ 3 mM in a final volume of 50 μl. The Taq polymerase was activated by incubation at 95 °C for 15 min prior to 30 amplification cycles of 95 °C for 30 s, 65 °C for 45 s, and 72 °C for 45 s. The expected size of the amplicon was 379 bp (with reference to PV-1).

2.5. Template purification and sequencing

The amplicons of the *VP1* or *VP2* region were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Meylan, France) or the QIAquick gel extraction kit (QIAGEN), depending on the presence of single or multiple bands, respectively. The purified products were sequenced according to the Sanger method with the GenomeLab Dye Terminator Cycle Sequencing Quick Start kit according to the manufacturer's instructions (Beckman Coulter, Villepinte, France) as described (Ibrahim et al., 2013). The sequencing primers were NS20/NS30 and AN89/AN88 for the *VP2* and *VP1* region, respectively (Table 1). After purification of the amplicons with the Dye-Terminator Ex 2.0 kit (QIAGEN), the electrophoresis and analysis of DNA sequence reactions were performed with the automated DNA sequencer CEQ8000 (Beckman Coulter).

2.6. Sequence analysis and phylogeny

To determine the EV type, the sequences were compared to all the corresponding EV sequences available in GenBank database for the corresponding *VP1* or *VP2* capsid region by using BLAST software (Altschul et al., 1990). Nucleotide sequence homology of at least 75% was required for assignment to the same genotype (Oberste et al., 2000, 2004).