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Quantification of Enterovirus RNA in Sludge Samples Using Single Tube Real-Time RT-PCR

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ABSTRACT

We have developed a quantitative RT-PCR method that can be used to determine the amount of enterovirus RNA in urban sludge samples. This method combines TaqMan[®] technology with the ABI Prism[®] 7700 real-time sequence detection system. We optimized a one-step RT-PCR that uses a dual-labeled fluorogenic probe to quantify the 5' noncoding region of enteroviruses. For accurate quantification of the number of copies, a Mahoney type 1 poliovirus RNA standard was designed and produced using genetic engineering. This fragment, quantified using the Ribogreen[®] method, was used in serial dilutions as an external standard. The method had a 7-log dynamic range (5×10^2 to 2×10^7). PCR inhibitors were removed by extracting viral RNA (after virus concentration) using the RNeasy[®] mini kit with added polyvinylpyrrolidone (PVP) and running the amplification reaction with a mixture containing PVP and T4 gene 32 protein. This real-time quantification of enterovirus RNA allows large numbers of samples to be screened. Its sensitivity, simplicity and reproducibility render it suitable as a screening method with which to characterize enteroviruses, the presence of infectious particles being subsequently confirmed by cell culture.

INTRODUCTION

There are generally two kinds of methods for detecting enteric viruses in environmental samples, depending on whether they are based on cell culture infectivity or on molecular detection methods such as PCR followed by nucleic acid hybridization (16). Compared to time-consuming cell culture assays, PCR is rapid, sensitive and specific. It is also capable of detecting non-cytopathogenic, slow-growing and rare viruses

in complex environmental samples such as sewage or sludge (20). Current amplification techniques are either qualitative (10) or quantitative with the measurement of the amount of product amplified as their end point (25).

In this study, we suggest a single-tube, one-step, real-time RT-PCR assay for the quantification of enteroviruses in sludge samples using TaqMan[®] technology (Roche Molecular Biochemicals, Meylan, France). This technology makes use of the 5'→3' nucleolytic activity of AmpliTaq[®] DNA polymerase and uses primer pairs in conventional amplification and a dual-labeled fluorogenic probe (5,13). Three major difficulties need to be overcome in the development of such a technique: (i) because enteroviruses are RNA viruses, RT-PCR needs to be optimized in a single step to keep manipulation time to a minimum; (ii) because treated sludge contains only small quantities of enterovirus, a highly sensitive technique is required; and (iii) the purest possible genetic material needs to be extracted because sludge samples contain numerous organic compounds (polyphenols and humic acids) and heavy metals that are liable to inhibit amplification enzymes (7,17,21).

The use of polyvinylpyrrolidone (PVP) in genome extraction and the T4 gene 32 protein during amplification enabled us to achieve a complete protocol, from the extraction of virus from sludge to the counting of genome copies. Here, we present the first results obtained with samples of natural and artificially contaminated sludge. We compare these results with measurements made using cell culture, which remains the reference technique.

MATERIALS AND METHODS

Residual Sludge

Three types of sludge were sampled from two waste-water treatment plants in Lorraine, France. Primary sludge was taken from the primary decanting ponds of the Nancy and Metz treatment plants; activated sludge and thickened sludge from a secondary decanting pond were taken from the Metz plant. Samples of thickened sludge (78% dry

solids) loaded with 7×10^4 and 7×10^5 pfu poliovirus 1 vaccine (Sabin) were used as controls.

Virus Extraction and Concentration Technique

Virus was eluted from sludge samples using the technique described by Ahmed and Sorensen (1). A volume of sludge providing 10 g (dry solids), to which 100 mL 10% beef extract (pH 9.0) (Oxoid, Basingstoke, UK) had been added, was stirred at 500 rpm for 30 min. The mixture was sonicated on ice (100 W, 0.9 s) for 5 min in 1-min steps, mixed again for 5 min and then centrifuged at $5000 \times g$ for 1 h at 4°C. The extract consisted of the supernatant, neutralized to pH 7.2. This extract was concentrated using polyethylene glycol (PEG) 6000 (Prolabo, Fontenay sous bois, France) and precipitated according to the Lewis and Metcalf technique (12). An 8% final concentration (w/v) PEG 6000 (in PBS, pH 7.2, bio-Merieux, Lyon, France) was added to the extract. After vigorous agitation, the mixture was stored overnight at 4°C and then centrifuged at $10\,000 \times g$ for 90 min at 4°C. The pellet was resuspended in 10 mL PBS. Finally, this concentrate was decontaminated by adding a one-third volume of chloroform (10-min centrifugation at $1500 \times g$) followed by 200 µL antibiotic cocktail (penicillin 100 000 IU/mL, colimycin 250 000 IU/mL, streptomycin 100 mg/mL and amphotericin B 0.5 mg/mL).

Quantification of Infectious Enterovirus Using Cell Culture

Quantities of the present infectious enterovirus were measured by inoculating the decontaminated concentrates in vitro into buffalo green monkey cell cultures in 96-well microplates. All cultures were inoculated in duplicate using 40 wells for each dilution (fivefold serial dilutions). Each well was filled with 50 µL inoculum and 200-µL nutritive medium [Eagle minimum essential medium (Eurobio, Les Ulis, France), to which 5% newborn calf serum had been added] containing 1.5×10^5 cells/mL. The cultures were incubated at 37°C in 5% CO₂ for five days. Virus density was

determined from the cytopathic effects observed after duplicate inoculation of cell layers with three successive dilutions of the sample. The cytopathic effect was confirmed by two serial passages. The mean viral concentration for each sample was estimated using the most probable number (MPN) method with software devised by Maul (15). Results were expressed in the most probable number of colony unit (MPNCU)/mL sample and then converted to MPNCU/10 g sludge (dry solids) to take into account any variations in sludge dryness.

Extraction of Viral RNA

Enterovirus RNA was extracted from 250 μ L concentrate using the RNeasy[®] plant mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions, with the following exception: the kit RLT buffer was modified by the addition of 2% (w/v) PVP 40 000 (Sigma, St. Quentin, France) (4,22,26).

Fluorogenic RT-PCR

To design the primers and probe for the TaqMan technology, the most constant genome region in enteroviruses, the 5'-noncoding region (5' NCR), was chosen. The software Primer Express was used and identified primer Ev1 [5'-GATTGTCACCATAAGCAGC-3'] (position 579–597), primer Ev2 [5'-CCCCTGAATGCGGCTAATC-3'] (position 451–469) and Ev-probe [5'-FAM-CGGAACCGACTACTTTGGG-TGTCCGT-TAMRA-Phosphor-3'] (position 532–557). These primers were tested in the GenBank[®] database and found to be pan-specific and to cover all enteroviruses present in GenBank.

The reaction mixture (final volume 25 μ L) was prepared in a single tube as follows: 1 $\times$ TaqMan buffer A, 5.5 mM MgCl₂ (both from PE Biosystems, Courtaboeuf, France); 500 μ M dNTPs (Roche Molecular Biochemicals); 500 nM reverse primer Ev1, 400 nM primer Ev2 (both from Genosys, Pampisford, UK); 120 nM Ev-probe (Eurogentec, Serraing, Belgium); 6% glycerol (Pro-labo); 1.7% PVP-25 (Serva, Paris, France); 1.5 μ g T4 gene 32 protein (Amersham Pharmacia Biotech, Orsay,

France); 5 UI MuLV and 2.5 UI AmpliTaq[®] Gold (both from PE Biosystems) and 10 UI RNasin[®] (Promega, Charbonnière, France). Twenty microliters of the reaction mixture were added to the PCR tubes containing 5 μ L RNA from one of the sludge samples or RNA from the constructed standard in serial dilution. Enterovirus RNA was reverse transcribed into cDNA (45 min at 50°C) and

the 147-bp fragment was amplified by PCR (45 cycles of 94°C for 15 s and 60°C for 1 min) on an ABI Prism 7700.

Enterovirus RNA Standard for Absolute Quantification

An RNA standard representing the 5'NCR of enterovirus RNA was synthesized in vitro as follows: cDNA from

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Mahoney type 1 poliovirus was amplified by PCR using primer Ev1Clon 5'-TGGC-CAATCGAATTCGCTTTA-3' (position 612–632) and primer Ev2Clon 5'-CTA-CATAAGGA TCCTCCGCC-3' (position 432–452) modified by the introduction of restriction sites (*Bam*HI and *Eco*RI). The 201-bp fragment was identified on a 1.5% agarose gel, excised, isolated from the gel and ligated into the polylinker of a pT7-blue vector (Novagen, Madison, WI, USA). The recombinant plasmid (pT7-EV) was transformed into *E. coli* (Novagen) and a positive colony was amplified in LB medium containing tetracycline and ampicillin. Plasmids were purified by alkaline lysis (2), linearized, purified by gel electrophoresis and extracted from the gel using a plasmid maxi kit (Qiagen).

Standard plasmids were quantified using spectrophotometric analysis. The sequence and orientation were verified by automatic sequencing. Linearized plasmid (1.5 µg) was used to perform an in vitro transcription reaction with T7 DNA polymerase (Roche Molecular Biochemicals) at 37°C for 2 h. After digestion with RNase-free DNase, the resulting RNA transcripts were purified with phenolchloroform and precipitated with isopropanol. The RNA transcripts were dissolved in sterile, RNase-free water and quantified by spectrofluorometric analysis using Ribogreen® (Interchim, Montluçon, France). The titer was 10^{10} copies/µL. Dilutions (five copies to 2×10^7) of the RNA standard were made in TE buffer containing RNasin. Aliquots of the diluted RNA stock were frozen immediately at -70°C. Each aliquot was used once only for a fluorogenic RT-PCR.

Analysis of Fluorescence Signals on the ABI Prism 7700

Real-time fluorescence measurements were taken and the threshold cycle (C_t) value for each sample was calculated by determining the point at which fluorescence exceeded a threshold limit (10 times the baseline SD). A standard graph of the C_t values obtained from the serially diluted external RNA standard was compiled. The correlation coefficient and the slope were calculated. C_t values from the sludge samples were plotted on the standard curve, and the

number of copies was calculated automatically by Sequence Detector v1.6. Samples were defined as negative if their C_t values exceeded 45 cycles.

RESULTS AND DISCUSSION

During the development of this method, the optimal conditions for RT-PCR were determined by amplifying a range of the standard RNA. A problem we encountered was that of sensitivity

(Figure 1A; detection limit = 1.6×10^4 copies) related to the secondary structures present in the 5' NCR of the enterovirus genome. For this reason, we decreased the RT/*Taq* ratio and used the T4 gene 32 protein, usually considered to be a ssDNA binding protein and a dsDNA helicase (11,3). Figure 1B demonstrates the benefits of adding this protein to the reaction mixture (in quantities of 1.5 µg/reaction) on the amplification yield of the standard RNA.

The detection limit, intra-assay and

Figure 1. Comparison of intensity and amplification quality in sludge extracts. These extracts contains seven successive fivefold dilutions of standard RNA (25 to 4×10^5 copies) in the presence and absence of T4 gene 32 protein (Fam C3-D3 = 25, Fam C4-D4 = 125, Fam C5-D5 = 640, Fam C6-D6 = 3.2×10^3 , Fam C7-D7 = 1.6×10^4 , Fam C8-D8 = 8×10^4 , Fam C9-D9 = 4×10^5). 1A: Amplification in the absence of T4 gene 32 protein, three dilutions are amplified. 1B: Amplification in the presence of T4 gene 32 protein, all dilutions are amplified.

Table 1: Enterovirus Quantification Measurements.

Samples	Results from RT-PCR (number of copies/10 g dry solids)	Results from cell culture (MPNCU/10 g dry solids)
control 1 (7×10^4 pfu)	1.5×10^6	4.9×10^4
control 2 (7×10^4 pfu)	1.4×10^6	3.5×10^4
control 3 (7×10^4 pfu)	8×10^6	3.4×10^4
control 4 (7×10^4 pfu)	1×10^6	1.4×10^4
control 5 (7×10^4 pfu)	7×10^6	2.2×10^4
control 6 (7×10^3 pfu)	2×10^5	1.6×10^3
control 7 (7×10^3 pfu)	3×10^5	3.6×10^3
control 8 (7×10^3 pfu)	1×10^5	1.7×10^3
1P	54 720	10
2P	230 400	2242
3P	115 200	326
4A	5664	107
5A	14 400	278
6A	<400	< 3
7T	14 400	< 3
8T	14 400	72
9T	< 400	< 3

Measurements use RT-PCR and cell culture in eight sludge samples artificially contaminated with 7×10^4 and 7×10^3 pfu poliovirus 1 and nine natural sludge samples of three different types (P = primary, A = activated, T = thickened).

interassay variability were determined in 10 replicates of tenfold dilution standards, from 5 to 5×10^6 RNA copies, amplified in eight independent assays. Intra-assay cvs varied between 6.34% and 0.74%, and interassay cvs between 4.51% and 0.66%. The values of 6.34% and 4.51% obtained with low RNA concentrations agree with those found in other TaqMan reactions (3.4% and 6.2%) (6,14). A variability of five copies of the standard is acceptable, and no negative result was obtained. The sensitivity of the RT-PCR is therefore five copies.

Other problems encountered were those caused by the complexity of the material, such as the residual sludge. Sludges contain numerous inorganic and organic compounds—humic acids and polyphenols, for example—which are liable to form complexes with nucleic acids and inhibit amplification enzymes. Figure 2 shows the presence of such inhibitor substances on the graphs fam-D1 produced by the Sequence Detector software when 8×10^4 copies of enterovirus RNA from a sludge sample

were amplified. PVP is described in the literature as forming complexes with phenolic compounds (9,26). For maximum effect, it was added both at the RNA extraction stage and to the PCR mixture (Figure 2, Fam-D6). The amount of PVP required in the RNA extraction process was fixed at 2% as in the cetyltrimethylammonium bromide (CTAB) extraction protocol (22), while the amount required in the amplification mixture was determined by adding various quantities between 1% and 2%, as did Koonjul et al. (9). The optimum PVP amount was found to be 1.7%.

The analytical sensitivity of our method, considering all extraction steps, is 2000 copies/10 g (dry solids) of sludge. However, lower numbers can be found because the linearity of the standard curve permits the interpolation of values below the lower limit of the dynamic range (14). Therefore, the theoretical detection limit of our RT-PCR can be adjusted to 400 copies/10 g (dry solids). This sensitivity is compatible with requirements accepted in the liter-

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Figure 2. Graph showing fluorescence intensity as a function of numbers of amplification cycles. Comparison of amplification intensity of the same sludge concentrate (10 μ L) containing 8×10^4 copies of RNA under different reaction conditions. Fam-D1: RNA extraction and amplification without PVP; Fam-D2: RNA extraction with PVP, amplification without PVP; Fam-D6: RNA extraction and amplification with PVP.

ature as suitable for the detection of enteroviruses in sludge from waste-water treatment plants (10,20,23,24).

This fluorogenic RT-PCR protocol was first evaluated on eight samples of artificially contaminated thickened sludge and then applied to nine natural sludge samples. The results, presented in Table 1, were compared to measurements made of virus quantities by means of cell culture. For the samples artificially contaminated with 7×10^4 or 7×10^3 pfu, the mean values as measured by RT-PCR and cell culture, respectively were 3.78×10^6 copies/10 g and 3.08×10^4 MPNCU/10 g ($n = 5$) or 2×10^5 copies/10 g and 2.96×10^3 MPNCU/10 g ($n = 3$) (Table 1). In the natural sludge samples, two samples were negative (< 400 copies/10 g) according to RT-PCR and three according to cell culture (< 3 MPNCU).

The quantity of genomic RNA measured was always larger than the number of infectious particles, being on average 150 times greater for natural sludge and 120 times greater for contaminated sludge. This can be explained by (i) the aggregation of infectious particles into clumps that, while consisting of several viruses, only cause one area of lysis in a cell culture; (ii) the variable effects of cell culture inhibitory substances that might still be present in the

concentrates; and (iii) the ability of RT-PCR to detect inactivated viruses. These results, which agree with those reported in the literature (8,18,19), show a sharp decrease in quantities of virus present from primary sludge through activated and thickened sludges.

The fluorogenic RT-PCR technique developed in this study allows real-time analysis of the amplification reaction and calculation of genomic RNA quantities at the start of the exponential phase of amplification. The fluorescence signal caused by cleavage of the TaqMan fluorogenic probe can only be generated if the target sequence has been amplified, which gives the reaction a high level of specificity. Our protocol is sensitive, reproducible and rapidly executed on multiple samples. These features make it an excellent large-scale screening tool for sludge from waste-water treatment plants and for monitoring sewage processing procedures, even though the presence of infectious particles must be confirmed by cell culture, as current regulations require.

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