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Best Viral Elution Method Available for Quantification of Enteroviruses in Sludge by Both Cell Culture and Reverse Transcription-PCR

S. MONPOEHO,[1] A. MAUL,[2] B. MIGNOTTE-CADIERGUES,[3] L. SCHWARTZBROD,[3] S. BILLAUDEL,[1] AND V. FERRÉ[1]*

Laboratoire de Virologie, CHU Hotel Dieu, Nantes,[1] I.U.T, Department of Statistics and Data Processing, Metz,[2] and LCPME-Virology, Faculty of Pharmacy, Nancy,[3] France

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The aim of this study was to select one or several virus extraction techniques that enable simultaneous detection of enterovirus genomes and infectious particles in different types of urban sludge. Eight techniques were compared by using 16 different liquid and solid sludge samples. The numbers of infectious enteroviruses in cell cultures were determined by using the most-probable-number method. The enterovirus genome was quantified by a single-tube reverse transcription-PCR using TaqMan technology. The results were statistically analyzed by Friedman's test, a nonparametric test for analysis of randomized block data using only ranks in terms of extraction technique efficiency. Two techniques seemed to yield higher viral titers as determined by simultaneous detection by cell culture and PCR. The first involved a 10% beef extract solution at pH 9 and sonication; the second involved a 0.3 M NaCl–7% beef extract solution at pH 7.5 followed by Freon treatment. In solid sludge, no significant differences were observed among the eight techniques tested. Both of the best techniques can be used for simultaneous detection of infectious enterovirus particles and genomes in any type of urban sludge.

The methods used to detect enteroviruses in environmental samples are of two general kinds, those based on cell culture infectivity and those in which molecular detection methods are used, such as PCR followed by nucleic acid hybridization (13). Environmental samples, especially urban sludge, contain numerous organic and inorganic compounds (humic acids, polyphenols, heavy metals) which are toxic and cause lysis in cell cultures. These compounds are also liable to form complexes with nucleic acids and inhibit amplification enzymes (9, 15, 18). The results of cell culture analysis and PCR therefore depend on the efficacy with which the viral extraction technique used removes such compounds. The aim of this study was to select one or several of eight previously described viral extraction techniques which would allow simultaneous cell culture and reverse transcription (RT)-PCR analyses for quantification of enteroviruses in sludge samples. We hoped to identify a screening method applicable on a large scale which was based on a real-time genomic quantification technique and allowed confirmation of infectivity with the same viral sludge concentrate. The efficiency of elution was evaluated by counting infectious enteroviruses (most-probable-number cytopathogenic units [MPNCU]/10 g of dry matter) and quantifying enterovirus genomes (number of copies per 10 g of dry matter) by a fluorogenic RT-PCR method developed in our laboratory (14).

MATERIALS AND METHODS

Residual sludge. Four types of sludge (16 samples) were obtained from two wastewater treatment plants in Lorraine (Nancy and Metz, France). At the Nancy plant, biological sludge produced during treatment of wastewater undergoes mesophilic anaerobic digestion at 37 to 38°C for 15 to 20 days, while at the Metz site sludge is also thickened, dehydrated, and packed. Primary sludge was obtained from the primary decanting ponds of the Nancy and Metz treatment plants. Activated, thickened, and digested sludge from a secondary decanting pond was obtained from the Metz plant. The dry matter content of each sludge sample was determined after the sample was desiccated by incubation at 105°C for 24 h, and the dry matter contents ranged from 3 to 4% for primary sludge, from 0.4 to 0.6% for activated sludge, from 1.6 to 2.3% for thickened sludge, and from 24 to 33% for digested sludge.

Virus extraction techniques. (i) Virus elution. Virus was eluted from quantities of sludge equivalent to 10 g of dry matter by using eight previously described techniques. The differences between techniques included differences in the pH of the elution solution, the homogenization method, and the use of sonication.

Technique 1 was described by the Environmental Protection Agency (5) for extraction of viruses from sludge. In this technique, a 0.05 M $AlCl_3$ solution was added (1%, vol/vol) to a 12-g (dry weight) sludge sample, and the pH was adjusted to 3.5 with 5 M HCl. The mixture was then stirred at 500 rpm (New Brunswick Scientific) for 30 min. After centrifugation at 2,500 × *g* for 15 min at 4°C, the pellet was suspended in 100 ml of buffered (pH 7) 10% beef extract (LP029B; Oxoid). Then the mixture was stirred at 500 rpm for 30 min and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant, adjusted to pH 7.2, constituted the extract.

Technique 2 was described by Albert and Schwartzbrod (2) for extraction of enteroviruses from sludge. Each sludge sample (10 g [dry weight]) was first centrifuged at 1,500 × *g* for 15 min, and the pellet was suspended in 360 ml of a 0.1 M borate solution (pH 9) containing 3% beef extract (LP029B; Oxoid). The mixture was then stirred at 500 rpm for 15 min prior to sonication on ice (100 W, 0.9 s) for 1 min. After centrifugation at 10,000 × *g* for 45 min at 4°C, the supernatant, adjusted to pH 7.2, constituted the extract.

Technique 3 was described by Soares et al. (17) for extraction of enteroviruses from sludge. In this technique, a 0.05 M $AlCl_3$ solution was added (1%, vol/vol) to a 10-g (dry weight) sludge sample, and the pH was adjusted to 3.5 with 5 M HCl. The mixture was then stirred at 500 rpm for 30 min and centrifuged at 2,500 × *g* for 15 min at 4°C. The pellet was suspended in 500 ml of pH 7 buffer (3.15 g of Na_2HPO_4 per liter, 0.15 g of citric acid per liter) containing 3% beef extract (LP029B; Oxoid). The mixture was homogenized by agitation for 30 min and centrifuged at 15,300 × *g* for 10 min at 4°C. The supernatant, adjusted to pH 7.2 if necessary, constituted the extract.

* Corresponding author. Mailing address: Laboratoire de Virologie, CHU Hotel Dieu, 9 quai moncousu, 44035 Nantes, France. Phone: (33)-2-40084101. Fax: (33)-2-40084139. E-mail: sbillaudel@chu-nantes.fr.

Technique 4 was derived from protocols used by Chung et al. (4) and Grabow et al. (8) to elute enteroviruses from shellfish. To each sludge sample (10 g [dry weight]) 350 ml of a 0.05 M glycine–0.14 M NaCl solution (pH 7.5) was added. The mixture was homogenized with an Ultra-Turrax homogenizer at 9,500 rpm for 1 min. Then 100 ml of Freon was added. After homogenization with the Ultra-Turrax homogenizer at 9,500 rpm for 1 min, the mixture was centrifuged at 1,500 × *g* for 20 min at 4°C. The supernatant, adjusted to pH 7.2 if necessary, constituted the extract.

Technique 5 was a technique used by Alouini and Sobsey (3) to elute enteroviruses from shellfish. First, 50 ml of 0.3 M NaCl was added to a 10-g (dry weight) sludge sample, and the preparation was homogenized at 9,500 rpm with the Ultra-Turrax homogenizer for 1 min. Then 350 ml of a 7% beef extract (LP029B; Oxoid)–0.3 M NaCl solution (pH 7.5) was added to the mixture, and the pH was adjusted to 7.5 if necessary. The mixture was homogenized with the Ultra-Turrax homogenizer at 9,500 rpm for 1 min, and 100 ml of Freon was added. The mixture was then homogenized again with the Ultra-Turrax homogenizer at 9,500 rpm for 1 min prior to centrifugation at 5,000 × *g* for 20 min at 4°C. The supernatant, adjusted to pH 7.2, constituted the extract.

Technique 6 was a technique used by Ahmed and Sorensen (1) to extract *Bacillus fragilis* phages from sediments. To a sludge volume that provided 5 g of dry matter 45 ml of 10% beef extract (pH 9; LP029B; Oxoid) was added, and the mixture was stirred at 500 rpm for 30 min, sonicated on ice (100 W, 0.9 s) for 5 min with 1-min bursts, mixed for 5 min, and then centrifuged at 5,000 × *g* for 1 h at 4°C. The supernatant, adjusted to pH 7.2, constituted the extract.

Technique 7 was a technique used by Jofre et al. (10) to extract *B. fragilis* phages from marine sediments. A 750-ml portion of a 0.25 M glycine solution (pH 9.5) was added to a 10-g (dry weight) sludge sample. The mixture was stirred at 500 rpm for 2 h at 4°C and then centrifuged at 5,000 × *g* for 1 h at 4°C. The supernatant, adjusted to pH 7.2, constituted the extract.

Technique 8 was a technique used by Tartera and Jofre (20) to extract f2 bacteriophages from sediments. First, 100 ml of a buffer solution (pH 7.2) containing (per 1,000 ml of distilled water) 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 10 ml of 0.1 M $MgSO_4$, and 10 ml of 0.1 M $CaCl_2$ was added to a 5-g (dry weight) sludge sample. The mixture was stirred at 500 rpm for 2 h at 4°C and then centrifuged at 5,000 × *g* for 1 h at 4°C. The supernatant, adjusted to pH 7.2, constituted the extract.

(ii) Virus concentration. For virus concentration we used polyethylene glycol 6000 precipitation as described by Lewis and Metcalf (11); 8% (wt/vol) polyethylene glycol 6000 (in a phosphate solution at pH 7.2) was added to each extract. After rigorous agitation the mixture was kept at 4°C overnight and then centrifuged at 10,000 × *g* for 90 min at 4°C. The pellet, suspended in 12 ml of phosphate buffer (pH 7.2), constituted the concentrate; as a final step the pellet was decontaminated by adding 0.33 volume of chloroform.

Cell culture. Infectious enteroviruses were counted by inoculating decontaminated concentrates into in vitro buffalo green monkey cell cultures in 96-well microplates. All cultures were inoculated in duplicate by using 40 wells for each dilution. Each well was filled with 50 μl of inoculum and 200 μl of nutritive medium (Eagle minimum essential medium [Eurobio] with 5% newborn calf serum) containing 1.5×10^5 cells/ml. The cells were incubated at 37°C in 5% CO_2 for 5 days.

Viral density was determined from the cytopathogenic effects observed after duplicate inoculation of cell layers with three successive 10-fold dilutions of a sample. After confirmation by transfer of 50-μl portions of the supernatants to new microplates, the mean viral concentration of the samples was estimated by using the most-probable-number method with software described by Maul (12). Thus, each viral concentration was determined from a combination of the positive responses observed in the 40 wells inoculated for each of three successive 10-fold dilutions. The final result for each sample analyzed was expressed as the geometric mean of the concentrations calculated for two independent replicates.

The results were expressed in MPNCU per milliliter of concentrate and then converted to MPNCU per 10 g (dry weight) of sludge to account for sludge dryness.

Viral RNA extraction. Enterovirus RNA was extracted from 250 μl of concentrate with an RNeasy plant mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. However, a modified lysis buffer containing 2% (wt/vol) polyvinylpyrrolidone PVP 40,000 (Sigma, St. Quentin, France) was used (6, 19, 21).

Flurogenic RT-PCR. To design the primers and probe used for the TaqMan technique, the most constant genome region in enteroviruses, the 5′ noncoding region, was chosen (14). The software Primer Express was used, and this software identified primer Ev1 (5′-GATTGTCACCATAAGCAGC-3′; positions 579 to 597), primer Ev2 (5′-CCCCTGAATGCGGCTAATC-3′; positions 451 to 469), and probe Ev-probe (5′-FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA-phosphor-3′; positions 532 to 557).

The reaction mixture (final volume, 25 μl) was prepared in a single tube and contained 1× TaqMan buffer A (Perkin-Elmer, Courtaboeuf, France), 5.5 mM $MgCl_2$ (Perkin-Elmer), each deoxynucleoside triphosphate (Roche, Meylan, France) at a concentration of 500 μM, 500 nM reverse primer Ev1 (Genosys, Pampisford, England), 400 nM primer Ev2 (Genosys), 120 nM Ev-probe (Eurogentec, Serraing, Belgium), 6% glycerol (Prolabo, Fontenay-sous-bois, France), 1.7% polyvinylpyrrolidone 25 (PVP-25; Serva, Paris, France), 1.5 μg of T4 gene 32 protein (Amersham, Orsay, France), 5 IU of murine leukemia virus reverse transcriptase (Perkin-Elmer), 2.5 IU of AmpliTaq Gold (Perkin-Elmer), and 10 IU of RNasin (Promega, Charbonnière, France). Twenty microliters of the reaction mixture was added to a PCR tube containing 5 μl of RNA from one of the sludge samples or RNA from the standard constructed for serial dilution. Enterovirus RNA was reverse transcribed into cDNA (45 min at 50°C), and the 147-bp fragment was amplified by PCR (15 s at 94°C and 1 min at 60°C) for 45 cycles with an ABI Prism 7700 (Perkin-Elmer).

Analysis of fluorescence signals with the ABI Prism 7700. Real-time fluorescence measurements were obtained, and the threshold cycle (*Ct*) value for each sample was calculated by determining the point at which fluorescence exceeded a threshold limit (10 times the baseline standard deviation). A standard graph of the *Ct* values obtained with a serially diluted external RNA standard was prepared. *Ct* values obtained from the sludge samples were plotted on the standard curve, and the number of copies was calculated automatically by the software Sequence Detector. Samples were considered negative if their *Ct* values exceeded 45 cycles.

Statistical analysis. A statistical analysis of each quantification result was performed by using Friedman's test (7), a nonparametric test of randomized block data using only the ranks of the extraction techniques in terms of their efficiency. If the null hypothesis was rejected (i.e., the techniques were not equivalent), the next step was to rank the extraction techniques by their relative efficiencies.

RESULTS AND DISCUSSION

The results obtained with the eight extraction techniques for four types of sludge are shown in Table 1. The theoretical coefficient of variation characterizing the most-probable-number method when 40 wells per dilution level are inoculated is approximately 20%. To analyze the results statistically, we decided to use nonparametric methods, mainly because of their robustness and because they would allow much easier handling of the large amount of censored data present. Friedman's test was therefore performed and produced the following results.

Cell culture. When the cell culture results obtained for the liquid sludge samples. (samples 1 to 9) were examined, the efficiencies of different techniques were found to differ significantly (Friedman's test, $P = 0.004$); the most efficient techniques were techniques 2, 5, and 6. The results obtained for the solid sludge samples (samples 10 to 16), however, did not allow any differences in the efficiencies of the eight techniques studied to be distinguished (Friedman's test, $P > 0.1$). When all of the samples analyzed were considered as a whole, the efficiencies of the eight elution techniques differed significantly (Friedman's test, $P = 0.011$). The most efficient technique was found to be technique 5, followed by techniques 6 and 2.

RT-PCR. When the RT-PCR results obtained for the liquid sludge samples (samples 1 to 9) were examined, no significant differences between techniques were observed (Friedman's test, $P = 0.082$). A tendency was nevertheless perceptible; techniques 3, 5, and 6 seemed to yield higher viral titers. The results obtained for the solid sludge samples (samples 10 to 16), on the other hand, did not allow any differences in the efficiencies of the eight techniques studied to be distinguished (Friedman's test, $P > 0.1$). When all of the samples analyzed were considered as a whole, no ranking of the eight elution

TABLE 1. Quantification of enterovirus eluted by eight techniques from 16 samples of different types of sludge[a]

Sample or parameter	Enterovirus concn[a]															
	Technique 1		Technique 2		Technique 3		Technique 4		Technique 5		Technique 6		Technique 7		Technique 8	
	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture
Primary sludge (3–4% dry matter)																
Sludge 1	13,440	20	<400	11	2,448	<3	172,800	<3	67,200	50	54,720	10	57,600	<3	<400	<3
Sludge 2	24,960	95	43,680	352	115,200	159	254,400	154	384,000	434	230,400	326	144,000	35	249,600	136
Sludge 3	292,800	1,067	100,800	984	18,240	1,201	230,400	696	336,000	1,744	115,200	2,242	201,600	400	172,800	850
Activated sludge (0.4–0.6% dry matter)																
Sludge 4	<400	131	8,160	<3	52,800	<3	<400	<3	52,800	48	<400	107	25,440	<3	47,040	<3
Sludge 5	<400	<3	576	64	4,800	<3	<400	<3	<400	60	14,400	278	<400	<3	<400	<3
Sludge 6	<400	<3	<400	<3	6,240	<3	<400	<3	576	<3	5,664	<3	480	<3	5,664	<3
Thickened sludge (1.6–2.3% dry matter)																
Sludge 7	8,640	9	<400	45	18,240	11	<400	21	<400	10	14,400	<3	<400	<3	<400	23
Sludge 8	<400	<3	<400	15	48,000	<3	23,040	<3	1,440	<3	14,400	72	<400	<3	<400	<3
Sludge 9	<400	<3	<400	<3	3,888	<3	10,080	<3	52,800	<3	<400	<3	<400	<3	60,480	<3
Digested sludge (24–33% dry matter)																
Sludge 10	127,000	<3	219,000	<3	<400	<3	<400	<3	173,000	132	<400	<3	8,640	84	<400	90
Sludge 11	<400	<3	74,900	<3	<400	<3	<400	<3	54,100	11	115,000	5	<400	<3	121,000	<3
Sludge 12	115,000	6	<400	<3	<400	<3	<400	<3	69,100	<3	86,400	<3	<400	<3	<400	8
Sludge 13	121,000	<3	69,100	<3	63,400	<3	74,900	<3	63,400	<3	156,000	<3	74,900	<3	<400	<3
Sludge 14	63,400	<3	<400	<3	<400	<3	80,600	<3	115,000	<3	<400	<3	<400	<3	74,900	<3
Sludge 15	104,000	<3	<400	<3	<400	<3	<400	6	<400	<3	74,900	6	57,600	<3	<400	<3
Sludge 16	144,000	36	184,000	<3	<400	<3	4,490	<3	<400	<3	<400	<3	<400	<3	<400	<3
Statistical ranking																
Samples 1 to 9																
Sum Rank	30	40.5	25.5	51	50.5	37	44	33	53.5	51.5	45	52.5	35	24.5	40.5	34
Rank[b]	2	5	1	6	7	4	5	2	8	7	6	8	3	1	4	3
Samples 10 to 16																
Sum Rank	43.5	34	34.5	26.5	19	26.5	29.5	30.5	34.5	36	37	34	27.5	29.5	26.5	35
Rank[b]	NC[c]	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Samples 1 to 16																
Sum Rank	73.5	74.5	60	77.5	69.5	63.5	73.5	63.5	88	87.5	82	86.5	62.5	54	67	69
Rank[b]	NC	5	NC	6	NC	2	NC	2	NC	8	NC	7	NC	1	NC	4

[a] RT-PCR results are expressed in numbers of copies per 10 g (dry weight) (lower limit of detection, 400 copies/10 g [dry weight]), and cell culture results are expressed in MPNCU per 10 g (dry weight) (lower limit of detection, 3 MPNCU/10 g [dry weight]).

[b] Ranks were assigned by assigning the higher value to the better technique.

[c] NC, not classified (the critical probability corresponding to Friedman's test was greater than 5%).

techniques was possible (Friedman's test, $P > 0.1$). The fact that it was impossible to rank the eight techniques may have been due in part to the low levels of contamination of the sludge samples and in part to the extreme heterogeneity of the samples. Moreover, even if viruses are inactivated during extraction, they remain detectable by PCR, which puts the different techniques on the same footing. The different techniques therefore appear to have the same capacity to extract all viruses (dead and alive) but not the same capacity to extract live viruses. Overall, the best results for both culture and PCR analyses were obtained with techniques 5 and 6, in which 0.03 M NaCl–7% beef extract (pH 7.5) and 10% beef extract (pH 9) solutions, respectively, were used. No significant differences were observed among the eight techniques with solid sludge samples (samples 10 to 16), and techniques 5 and 6 seemed to yield higher viral titers for either quantification method and all types of sludge. Our preference is for technique 6, which does not require Freon and is therefore free of the environmental disposal problems posed by this ingredient.

The quantities of enteroviruses detected varied considerably from one sample to another. For primary sludge, for example, the quantities varied from <3 to 2.24×10^3 MPNCU/10 g (dry weight) and from <400 to 3.84×10^5 copies/10 g (dry weight). The mean quantities detected in primary sludge were 457 MPNCU/10 g and 1.37×10^5 copies/10 g. The equivalent values were 29 MPNCU/10 g and 9.36×10^3 copies/10 g in activated sludge, 9 MPNCU/10 g and 1.06×10^4 copies/10 g in thickened sludge, and 7 MPNCU/10 g and 4.8×10^4 copies/10 g in digested sludge. From primary sludge to activated sludge, therefore, the quantities of virus decreased by a factor of 16 in terms of infectivity and by a factor of 15 in terms of genomes. Then from activated sludge to thickened sludge they decreased by a factor of 3 in terms of infectivity but remained unchanged in terms of genomes. Finally, from thickened sludge to digested sludge they decreased slightly, by a factor of 1.3, in terms of infectivity and increased by a factor of 4.5 in terms of genomes. The values for quantities of infectious particles agree with those published previously. Thus, in 10 g of primary sludge, Hu et al. (C. J. Hu, R. A. Gibbs, G. E. Ho, P. Phillips, and I. Unkovich, 3rd Int. Conf. Appropriate Waste Manag. Technol. Dev. Countries, 1995) detected 1.6×10^4 IU, Soares et al. (17) detected 330 MPNCU, and Pederson (16) detected 3.9×10^3 PFU. These values demonstrate the extreme heterogeneity of sludge samples. We also showed that there are considerable decreases in the quantities of viruses in terms of both infectivity and genomes from primary sludge to the other types of sludge, and again our values were consistent with those published previously. Hu et al. (Hu et al., 3rd Int. Conf. Appropriate Waste Manag. Technol. Dev. Countries), Pederson (16), and Soares et al. (17) detected 2.9 IU/10 g, 7.9 PFU/10 g, and 16 MPNCU/10 g, respectively, in digested and dehydrated sludge. However, no change in the genomic viral load—or even a slight increase—was found from activated sludge to digested sludge. This could be explained by denaturation of the viral capsid during elution and by the ability of RT-PCR to detect inactive or dead particles or particles that are difficult to culture. This was also shown by the differences between the results obtained by PCR and the results obtained by cell culturing. Indeed, 41 samples were found to be PCR positive but culture negative. Given the fragility of RNA genomes, it is very likely that the genomes detected in these cases were protected by the capsids of damaged viruses or were noncytopathogenic. Nevertheless, 12 samples were found to be PCR negative but culture positive. These results could be explained as being due to the presence of PCR inhibitors.

In conclusion, the purpose of this study was to test several methods for extracting virus particles in sludge which make use of cell culture and fluorogenic RT-PCR. The latter technique can be used for a large number of samples and has proved to be quite rapid, sensitive, and reproducible compared to the cell culture method. Of the eight extraction methods studied, two in which solutions based on beef extract were used seemed to yield higher viral titers with both cell culture and PCR techniques. Technique 6 allows a sludge testing protocol to be set up which is rapid and capable of determining quantities of viral genomes and subsequently the proportion of infectious particles by using the same sludge sample concentrate. This could prove to be very useful in strategies in which sludge samples from wastewater treatment plants are screened and the infectivity of any genome detected is confirmed if necessary.

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