RAPID DETECTION OF POLIOVIRUSES IN ENVIRONMENTAL WATER SAMPLES BY ONE-STEP DUPLEX RT-PCR

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Abstract. This study describes the rapid detection of polioviruses in environmental waters by a simple reverse transcriptase-polymerase chain reaction (RT-PCR) using two primer pairs for differentiation of poliovirus from non-polio enteroviruses in a single reaction by a one-step method, combining RT and PCR in a single tube. The detection by agarose gel electrophoresis yielded 2 bands of 153-bp and 293-bp for poliovirus tested without the need for further hybridization. The detection sensitivity of this one-step duplex RT-PCR, as measured with RNA extracted by heat treatment from supernatant of infected cell extracts, was 10^{-1} 50% tissue culture effective doses ($TCID_{50}$). This assay was used to evaluate the ability of sample concentration by membrane filter-based adsorption and elution, and purification by a simple RNA isolation based on guanidine isothiocyanate-phenol-chloroform extraction; the system yielded a detection limit of 5 x 10^{-1} $TCID_{50}$ seeded in 5 liters of tap water. This protocol was applied to the poliovirus detection in environmental water collected from 2 communities in Bangkok, Thailand during February and May 1998. Of 100 samples tested, 2 water samples collected from the same open sewage pipeline at one location were positive for polioviruses and one sample collected from another sewage pipeline was positive for non-polio enterovirus while a further 97 water samples were negative for both polioviruses and non-polio enteroviruses. With poliovirus detection by cell culture technique, none of the 100 samples tested was positive for poliovirus type 1, 2 or 3. RT-PCR was more sensitive, rapid, simple and cost-effective than the cell culture technique since the two water samples which were positive for polioviruses by RT-PCR failed to be detected by cell culture. Sequence data of 293-bp amplicons from positive samples were compared with those of reference poliovirus strains in the Genbank and the EMBL databases and identity to the sequence of type 1 strain Sabin was found to be 99%.

INTRODUCTION

Enteroviruses in the environment pose a public health risk because these viruses can be transmitted via the fecal-oral route through contaminated water, and low numbers are able to initiate an infection in humans (Ward and Akin, 1984). The control of enteroviruses is especially important in surface waters, because they are not only used frequently for bathing but also serve as resources for drinking water. There is a clear need for periodic or even routine monitoring of water supplies for possible viral contamination, at least to develop a database of virus occurrence for the purpose of risk assessment analysis (Rose and Gerba, 1991). Among these waterborne enteroviruses, poliovirus is of importance in public health programs, as highlighted by the World Health Organization surveillance program for global eradication of poliomyelitis by the year 2000 (WHO, 1988). Direct detection of polioviruses in environmental specimens is of increasing importance as the poliovirus eradication campaign progresses, since relatively low levels of polioviruses have to be detected among the more abundant non-polio enteroviruses.

Current microbial standards used as safety criteria for water may not always be indicative of waterborne pathogens; in particular, the presence of enteric viruses is not always linked to the presence of *Escherichia coli* or other indicator organisms used in public health (Berg and Metcalf, 1978). Several studies have demonstrated that treated water supplies which are considered acceptable on the basis of levels of indicator bacteria may still contain viruses and other pathogens (Deetz *et al,* 1984). Conventional methods for enterovirus isolation from waters involve cell culture assays with subsequent typing by neutralization which are technically difficult, time-consuming, and expensive, and they have relied on animal cell cultures to detect only the culturable viruses, but it is inability to cultivate several waterborne enteroviruses of public health importance. To improve the detection sensitivity, ELISA assay (Steinman, 1981), radioimmunoassay (Hejkal *et al,* 1982), and nucleic acid hybridization (Zhou *et al,* 1991) have been used to identify

viruses from sewage or shellfish samples, but these assays require the use of radioisotopes or cumbersome procedures.

Recently, reverse transcriptase-polymerase chain reaction (RT-PCR) has been successfully used to detect enteroviruses in different aquatic environments (Chapman *et al*, 1990; Kopecka *et al*, 1993; Atmar *et al*, 1993; Egger *et al*, 1995). The decreased time and cost and the increased sensitivity of RT-PCR facilitate the detection of low numbers of target RNAs usually found in environmental samples and can circumvent the disadvantages of traditional cell culture methods. RT-PCR can be carried out either in two-step or one-step formats. In this study, we used the one-step RT-PCR method by combining RT and PCR in a single tube under conditions optimal for the RT and DNA polymerase without opening the tube. This simplifies the procedure for cross-sample contamination made it easy and convenient tool for rapid detection of poliovirus.

Since enteroviruses have several genomic regions which are very conserved among the various virus types (Toyoda *et al*, 1984). PCR primers specific for polioviruses have also been described, but they are not able to detect all poliovirus strains (Abraham *et al*, 1993). Several combinations of primer pairs need to be used simultaneously in order to obtain positive results. Recently, Chezzi (1996) developed a single-primer-set RT-PCR assay for the rapid detection of polioviruses in infected tissue culture fluids and clinical materials by using poliovirus-specific PCR primers located in the VP1-P2A region of the poliovirus genome. The technique was able to identify polioviruses and also to differentiate them from other enteroviruses within 24 hours, and to determine the genotypes of wild-type polioviruses by direct sequencing of the PCR products.

In this study, we apply the poliovirus-specific PCR primers located in the VP1-P2A region, and the general enterovirus primers located in the 5´ noncoding region, combined in a single tube one-step duplex RT-PCR assay for the rapid detection of both polioviruses and non-polio enteroviruses in environmental waters, and to use this assay to evaluate the ability of water sample concentration by membrane filter-based adsorption and elution method, and purification of RNA based on guanidine isothiocyanate-phenol-chloroform extraction to make the sample compatible with poliovirus detection by RT-PCR.

MATERIALS AND METHODS

Viral strains

Poliovirus type 1 (Sabin) was kindly provided by the Department of Microbiology, Siriraj Hospital, Mahidol University. Polioviruses type 2 and 3 (Sabin) were obtained from the National Institute of Health, Ministry of Public Health. Viruses were propagated in Buffalo green monkey kidney (BGM) cells growing in Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum. The 50% tissue culture infectious dose ($TCID_{50}$) per milliliter in the supernatants was determined (Lennette, 1969) and each supernatant was then adjusted to a titer of 10^6 $TCID_{50}$/ml. Poliovirus type 1 was generally used as a positive control in each run of RT-PCR assay and used in some experiments to seed water samples in order to evaluate the ability of sample concentration, purification and extraction methods.

Environmental water samples

A total of 100 water samples was collected from 2 communities in Dusit district, Bangkok during February and May 1998. A variety of water samples including 32 from taps and containers of households, 20 from Klong Sansap and Klong Padung Krungasem, and 48 from opening sewage pipelines with 2 samples for one place were collected. Each sample was collected in 2 bottles of sterile 1-4 liter glass container, carried in the ice box to laboratory and kept at 4ºC. After processing for concentration, the last volume was divided into aliquots and one was stored frozen at -80ºC until assayed for poliovirus by RT-PCR.

Virus concentration

Concentration of polioviruses from water was based on membrane filter adsorption technique, filter elution and reconcentration. A volume of 5 liters of clean water or 1 liter of turbid water was concentrated by passage through Millipore membrane filter as described previously (Kittigul *et al*, 1998). Briefly, water samples were adjusted to pH 3.5 with 0.1 N HCl, and 0.15 N aluminum chloride was added. Water samples were then passed through a cellulose nitrate membrane filter of 0.45 μm pore size for adsorption of viruses. Adsorbed viruses were eluated with the solution containing 2.9% tryptose phosphate broth and 6% glycine at pH 9.0. The eluate was further reconcentrated by Speed Vac concentrator (Savant Instruments, USA) to a volume of 4-5 ml, and penicillin-streptomycin, 10x

Hanks balanced salt solution and 10x nutrient broth were added and adjusted pH to 7.2 with 4 N HCl. An aliquot of 600 µl was mixed with fungizone (1 mg/ml) and used for isolation of 3 types of poliovirus by cell culture technique. Other aliquots of 300 µl were kept at -80ºC until assayed for poliovirus by RT-PCR.

Extraction of viral RNA

Conventional phenol-chloroform and commercial Trizol LS (Gibco, Life Technologies) were utilized for RNA isolation of viral genomic RNAs of poliovirus strains from infected cell extracts. The purity and yield obtained by both methods were compared and then the suitable method was used to extract viral RNA from all water samples. RNA extraction by conventional method followed the method described by Chezzi (1996). Briefly, 250 µl of infected tissue culture fluid was incubated on ice for 15 minutes with 50 µl of 5x lysis buffer [250 mM Tris-HCl (pH 8.3), 350 mM KCl, 25 mM $MgCl_2$, 2.5% Nonidet P-40], and then extracted once with phenol, once with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). The aqueous supernatant was mixed with 2.5 volumes of cold ethanol and 0.3 M sodium acetate and stored overnight at -20ºC. After centrifugation, the resulting pellet was dried and resuspended in 50 µl of RNase and DNase free distilled water.

RNA extraction by Trizol LS reagent was followed the instructions supplied with the kit. Briefly, 250 µl of water sample or infected tissue culture fluid was incubated for 5 minutes at room temperature with 750 µl of Trizol LS reagent. RNA was collected in the upper aqueous phase after mixing with 200 µl chloroform and centrifugation. A total of 500 µl of cold (-20ºC) isopropyl alcohol was then added to precipitate nucleic acids. After 10 minutes, the sample was centrifuged for 10 minutes. Isopropyl alcohol was removed and the pellet washed with 75% ethanol. The dried pellet was dissolved in 50 µl of RNase and DNase free distilled water and used immediately. The concentration of RNA was measured by spectrophotometer at OD 260 nm with the following conversion factor: 1 optical density (OD) unit of RNA = 40 µg/ml.

Preparation of poliovirus seeded water samples

Poliovirus type 1 prepared in 10-fold dilutions and each dilution was seeded in tap water (1 ml per 5 liters of water) and then followed the protocol described above for virus recovery, and 10 µl of the Trizol extracts from concentrated water samples was used for RT-PCR assay using the optimized condition of one-step RT-PCR with duplex primers.

Primers

Primers for amplification of enteroviruses (EV1 and EV2) are located in the conserved 5´ noncoding region of poliovirus type 1. They represent conserved sequences shared with several other enteroviruses, these primers generate an approximately 153-bp PCR product. For poliovirus-specific amplification, primers PV1 and PV2 were used, slightly different in design from those described (Chezzi, 1996). Our primers generated an approximately 293-bp PCR product. An upstream primer (PV1) was chosen from a conserved region of the poliovirus coding for the capsid protein VP1. A downstream primer (PV2) was based on those previously described (Rico-Hesse *et al*, 1987), located in the conserved region of the poliovirus genome coding for protease 2A. Optimal upstream and downstream primer sequences were checked for internal and 3´ end complementarity by using the oligonucleotide primer design and analysis software. The specificities of these primers were checked against the GenBank and EMBL data banks. The oligonucleotide primers, sequences and locations are given in Table 1.

One-step RT-PCR

Procedures were based on single-tube RT and PCR amplification using the Gibco BRL Superscript one-step RT-PCR system. Optimal conditions for RT-PCR using either primer set 1 (EV1 and EV2) or set 2 (PV1 and PV2), or both sets combined in a duplex RT-PCR reaction, were determined with the use of poliovirus type 1 RNA template at a concentration of 100 pg/µl. Reactions in a 50-µl final volume were assembled by mixing 25 µl of 2x reaction mix [2x buffer includes 2.4 mM $MgSO_4$, 400 µM dNTPs each, and 4 µg/ml BSA], 1 µl of enzyme mix [Supersrcipt II RT and recombinant *Taq* DNA polymerase in 20 mM Tris-HCl (pH 7.5 at 25ºC), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol (v/v), and stabilizer], 0.2 µM of each primer, and 10 µl of each sample. Fifty microliters of mineral oil was added on top of the mixture to prevent any sample loss during heating. The reaction mixtures were processed in a programable thermal cycler (480, Perkin-Elmer Cetus). The samples were incubated at 45ºC for 30 minutes; then 94ºC for 2 minutes followed by amplification for 40 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 1 minute; followed by one cycle of 72ºC for 7

Table 1
Primers for poliovirus and non-polio enteroviruses used in RT-PCR.

Primer region and map position[a]	Primer designation	Sequence (5´ – 3´)	Product length (bp)
5´-Non coding region			
Sen 444-463	EV1	5´ CCT CCG GCC CCT GAA TGC GG 3´	153
Ant 577-596	EV2	5´ ATT GTC ACC ATA AGC AGC CA 3´	
VP1 region			
Sen 3212-3231	PV1	5´ GTC AAT GAT CAC AAC CCG AC 3´	
P2A region			293
Ant 3485-3504	PV2	5´ AAG AGG TCT CTA CTC CAC AT 3´	

[a] Map position and sequence refer to poliovirus type 1 (Mahoney strain), complete genome. Accession J02281.

minutes. Positive and negative controls were included in each RT-PCR.

RT-PCR detection of polioviruses in seeded and environmental water samples

The optimized condition of one-step RT-PCR with duplex primers was performed with 10 µl of Trizol extracts from concentrated water samples. Positive and negative controls were included in each run. Positive control sample consisted of 1 ng purified RNA of poliovirus type 1 extracted by Trizol reagent or some experiment used boiled suspension of 10^2 $TCID_{50}$/ml, which were run simultaneously in RT-PCR to ensure satisfactory reagent quality and technique. Distilled water was run as a negative control in RT-PCR to ensure no viral or genomic contamination through the experimental procedures.

Agarose gel electrophoresis

After amplification, PCR products were analyzed by gel electrophoresis. Ten microliters of amplified products were electrophoresed at 10 V/cm for 90 minutes through 2.5% agarose gels (Promega) in 0.5x TBE buffer (1x TBE; 89 mM Tris borate, 2 mM EDTA, pH 8.2). Gels were stained with 0.5 µg/ml of ethidium bromide and visualized by UV transilluminator. Positive, negative, and reagent controls were always loaded to every gels. The presence of band of the expected size (153-bp for primer EV1-EV2 and 293-bp for primer PV1-PV2) was assessed by comparison with a molecular size marker (100 bp DNA Ladder, New England Biolabs, Inc, USA).

Detection sensitivity of duplex RT-PCR for polioviruses in cell extracts

To determine the limit of detection by single step duplex RT-PCR procedure, an end-point virus dilution determination was carried out using intact virions (poliovirus type 1). A 10-fold dilution of the virus from 10^6 $TCID_{50}$/ml was prepared, RNA was extracted from each dilution by boiling in water bath at 95ºC for 10 minutes and then placed in wet ice for at least 5 minutes, after centrifugation at 10,000*g* for 2 minutes, 10 µl of supernatant of each dilution were subjected to RT-PCR in a 50-µl reaction. Then 10 µl of the amplification products were electrophoresed through 2.5% agarose gel and visualized by ethidium bromide staining. The highest dilution yielding an amplicon with 2 bands of corrected sizes (153-bp and 293-bp) from a 10-µl sample volume was taken as the end-point.

Sequencing analysis of amplified products

The PCR products in the size of 293-bp was excised from 2.5% agarose gel following electrophoresis, and purified by using the Qiaex DNA Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's recommendations. Nucleotide sequencing was carried out by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli *Taq* DNA polymerase (Perkin Elmer) and the results were checked with an ABI PRISM 377 automated sequencer (Perkin Elmer). The sequences were compared with all the sequences of the Genbank and the EMBL database.

RESULTS

RNA extraction

To assess the quality of the isolated RNA by phenol-chloroform-isoamyl alcohol (PCI) and Trizol extraction, samples were resolved by gel electrophoresis on 1.5% agarose-formaldedyde gel in MOPS

buffer. A typical pattern was obtained for the RNA samples tested, indicative of undegraded RNA by both methods (data not shown). The isolated RNA had an $A_{260/280}$ ratio of 1.6 to 1.8. The average yields of RNA extracted from 250-µl volume of 10^6 $TCID_{50}$/ml with the PCI and Trizol extraction methods were 1.5 µg and 1.9 µg, respectively. The use of Trizol was found to be suitable and more practical than the PCI extraction, thus this method was used for RNA extraction from all concentrated water samples throughout this study.

One-step duplex RT-PCR

Fig 1 shows the result of one-step RT-PCR using single primer pairs or both primer pairs were combined in a duplex RT-PCR reaction, with the use of poliovirus type 1 RNA template at the concentration of 100 pg/µl or 1 ng per reaction (10 µl). A product consistent in size with that predicted is shown, *ie* 153-bp from the conserved sequence 5´ noncoding region of poliovirus type 1 shared with several other enteroviruses when using EV1-EV2 primers, or 293-bp from the conserved region of the poliovirus coding for the capsid protein VP1 and protease 2A when using PV1-PV2 primers, or both 153-bp and 293-bp when all primers were combined in a duplex reaction. The same result was also shown when using RNA extracted from poliovirus types 2 and 3.

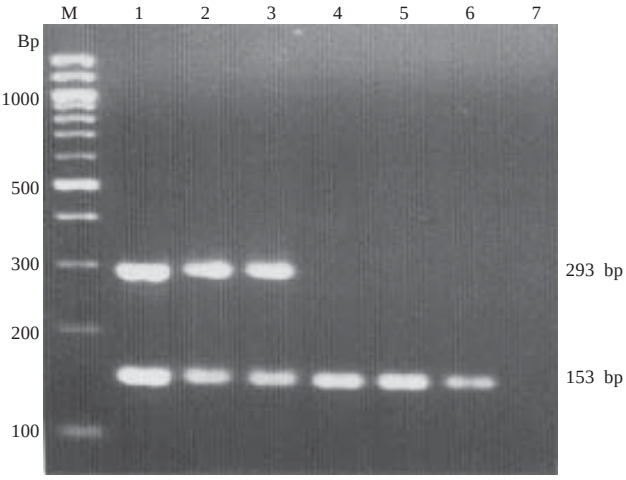

Fig 1–Agarose gel observed with PCR of poliovirus type 1, 2 and 3 (Sabin strain) with the primers were tested singly and in combination (duplex). In duplex RT-PCR with two primer pairs, two bands of 293-bp and 153-bp were shown (lanes 1 to 3). In single reaction with primer pairs EV1-EV2, one band (153-bp) was shown (lanes 4 to 6). Lanes 1 and 4, 2 and 5, 3 and 6, contained amplicons of poliovirus type 1, 2 and 3, respectively. Lane M, molecular weight marker (100-bp DNA Ladder). Lane 7, negative reagent control.

Detection sensitivity of duplex RT-PCR for poliovirus in cell extracts

The detection sensitivity is based on the greatest 10-fold dilution of a 10-µl volume of cell extracts giving RT-PCR amplifiable viral nucleic acid relative to the infectivity titer of the same extract expressed as $TCID_{50}$ per 10 µl. After the amplification products were electrophoresed through 2.5% agarose gel and visualized by ethidium bromide staining and yielding amplicon with 2 bands of corrected sizes. As shown in Fig 2, the detection sensitivity for cytopathic poliovirus in cellular extracts was 10^{-1} $TCID_{50}$.

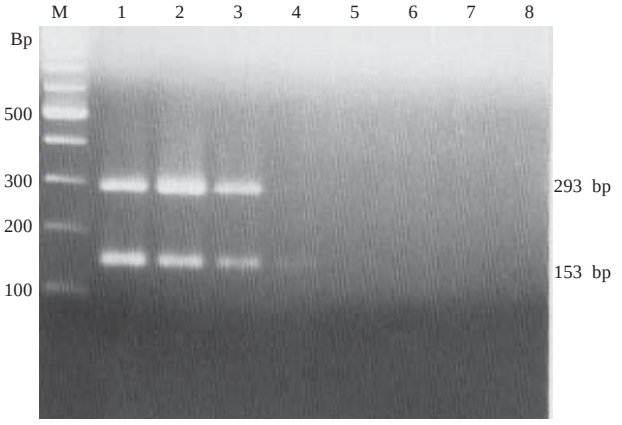

Fig 2–Agarose gel showing two bands of 153-bp and 293-bp amplification products of one-step duplex RT-PCR assay for detecting the minimum amount of poliovirus type 1 expressed as $TCID_{50}$ per 10 µl. Lane M, molecular weight marker (100-bp DNA Ladder). Lanes 1 to 7 contained poliovirus type 1 (in 10-fold dilution) from 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} $TCID_{50}$ per 10 µl, respectively. Lane 8, negative reagent control.

Evaluation of poliovirus recovery in seeded water samples

When the system for viral concentration by membrane filtration and RNA extraction by Trizol reagent followed by one-step duplex RT-PCR assay was tested with tap water artificially spiked with poliovirus vaccine strain, the results showed that all three dilutions seeded in water samples were positive for poliovirus; or poliovirus as little as

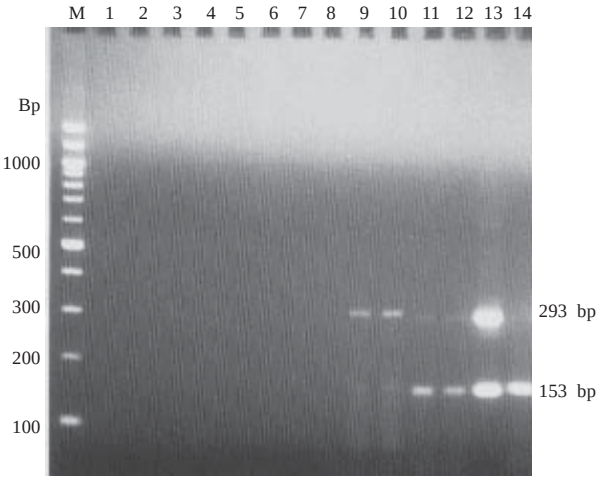

Fig 3–Agarose gel electrophoresis showing the first amplified product from one step duplex RT-PCR of 2 sewage samples that were positive for polioviruses (lanes 9 and 10) and 3 tap water samples seeded with poliovirus type 1 at 10^{-1}, 10^{2}, and 10^{5} $TCID_{50}$/ml (lanes 11, 12, and 14, repectively). Lanes 1 to 7 were negative samples. Lane 8, negative reagent control. Lane 13, poliovirus type 1 RNA positive control. Lane M, DNA size marker; 100-bp DNA Ladder.

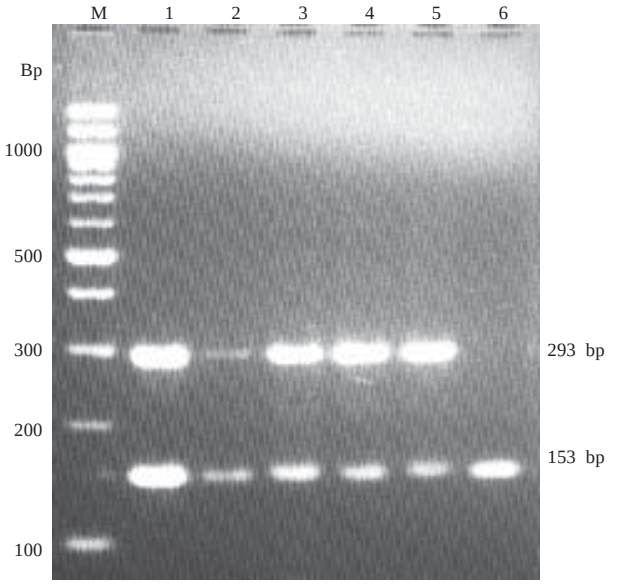

Fig 4–Agarose gel showing positive examples of poliovirus detection by duplex RT-PCR. Lanes 1 and 2 were poliovirus type 1 RNA in cell extracts at 10^{2} and 10^{0} $TCID_{50}$/ml. Lane 3 was poliovirus type 2 at 10^{2} $TCID_{50}$/ml. Lanes 4 to 6 were the second PCR amplified products of 3 environmental samples that were positive for polioviruses (lanes 4 and 5) and for enterovirus (lane 6). Lane M, DNA size marker; 100-bp DNA Ladder.

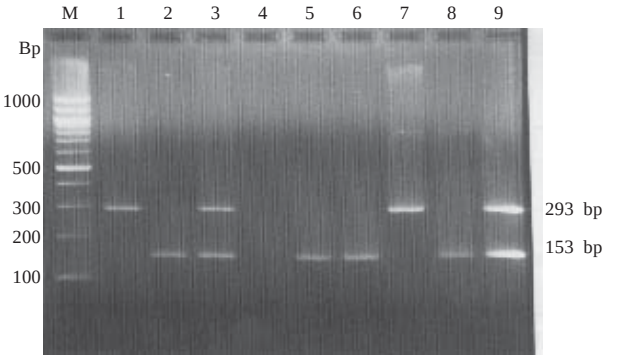

Fig 5–Band patterns on agarose gel observed with PCR of two environmental water samples, one was positive for poliovirus (lanes 1 to 3), another was positive for non-polio enterovirus (lanes 4 to 6) compared with band pattern of poliovirus type 1 (lanes 7 to 9) which used as positive control, the primers were tested singly and in combination with the primer pairs PV1-PV2 (lanes 1, 4, and 7), primer pairs EV1-EV2 (lanes 2, 5, and 8), and combination of both primer pairs in duplex RT-PCR (lanes 3, 6, and 9). Lane M, molecular weight marker (100-bp DNA Ladder).

5×10^{-1} $TCID_{50}$/ml seeded in 5 liters of water could be assayed by this system (Fig 3).

Detection of polioviruses in environmental water samples

When the same procedure was used to investigate the water quality of 100 samples collected from 2 communities. The results showed 2 bands of corrected sizes (153-bp and 293-bp) for polioviruses in 2 samples (2%) collected from the same opening sewage pipeline at one location, and one band of 153-bp for non-polio enteroviruses in 1 sample (1%) collected from the opening sewage pipeline at one location. The other 97 samples were negative for both polio- and non-polio enteroviruses. For poliovirus detection in all 100 samples by cell culture technique, none was positive for poliovirus type 1, 2 or 3. Two water samples which were positive for poliovirus by RT-PCR were failed to examine by cell culture because of a high fungal load interfere with the cell culture assay.

Since the amplicon bands of all 3 positive samples from the first amplification on agarose gel were less intense than those obtained when RNA extracted from cell culture supernatants (Fig 3), after they were confirmed by the second PCR with the same primers, these amplicons showed more intense bands of the corrected sizes (Fig 4). In

order to confirm the true positive results, concentrated water samples kept at -80ºC were subjected to the second extraction with Trizol and then 10 µl was used in RT-PCR with the primers tested singly and in combination. The PCR result is depicted in Fig 5 which shows the band patterns on agarose gel for two examples (one was positive for poliovirus, and another was positive for non-polio enterovirus) compared with the band pattern of poliovirus type 1 (Sabin) which was used in this study as positive control.

DISCUSSION

Currently, the standard methods for the detection of enteroviruses in environmental samples involves cell culture assays which are expensive and time-consuming. Results may take 2 weeks to be known. Another problem with cell culture assay is that environmental samples may contain materials that are toxic to the cells and more than one cell line is required in order to be able to identify all enteroviruses in water samples (Schmidt *et al*, 1978). PCR is an attractive method for the routine monitoring of enteroviruses in water samples because of it is faster, simpler and less expensive than conventional cell culture methods.

The amount of enteroviruses present in waters is highly variable, depending on factors such as the density of the population, the prevalence of infections in the community, the hygienic level and also the season (Kopecka *et al*, 1993). The levels of viruses of public health concern typically found in environmental and drinking water are low. For the detection of low numbers of target DNAs and RNAs, large volumes of water are concentrated into small volumes. Concentrating viruses usually involves numerous steps and requires considerable manpower. In this study, direct concentration of enteroviruses through a membrane filter-based adsorption, filter elution, reconcentration, RNA purification and RT-PCR amplification demonstrated a break-through in the detection of poliovirus. The efficacy of the protocol was established with tap water samples artificially spiked with vaccine strain and the result yielded a detection limit of 5 x 10^{-1} $TCID_{50}$ when seeded in 5 liters of water.

Reduced detection sensitivity has often been observed when RT-PCR was used directly to detect enteroviruses present in complex environmental samples such as sewage and sludge (Shieh *et al*, 1995). The technical problem is failed amplification due to inhibitors present in environmental samples which may interfere with RT-PCR used subsequently to detect viruses. The concentration method also effectively concentrates these substances. Many naturally occurring inorganic and organic solutes may be toxic to reverse transcriptase and polymerase enzymes, and even proteases and abundant RNases in environmental samples may also degrade RNA virus genomes before they can be amplified. In addition some organic compounds may bind magnesium ions and nucleotides required by polymerases. To deal with this problem, PCR inhibitors must be diluted, inactivated or removed from the samples. Various sample purification protocols have been developed in response to the type of environmental samples being tested. Some techniques are labor intensive, high cost, or incorporate multiple steps that may cause virus loss and subsequent decrease in sensitivity of detection. In addition, many of the reagents used for nucleic acid extraction or to prepare samples for PCR can inhibit PCR when present at contaminating levels (Rossen *et al*, 1992).

Poor detection sensitivity by RT-PCR in sewage samples or other high-solid fecal wastes was sometimes observed when using concentration steps commonly used for environmental samples, *ie* precipitation by polyethylene glycol and ProCipitate, solvent extraction and Sephadex G-200 spin column chromatography. To improve RT-PCR detection of enteric viruses in sewage and other fecal wastes, Shieh *et al* (1995) used a combination of these steps for sample processing to concentrate and purify virions followed by guanidinium isothiocyanate (GIT) extraction to remove inhibitors. By this protocol, GIT extraction eliminated sample inhibitory substances and increased the proportion of enterovirus positive samples from 3 to 7 of 11. Schwab *et al* (1996) also used the immunocapture method with subsequent heat release or guanidinium extraction of viral RNA; 9 of 11 samples were positive for enteroviruses in their study.

We applied this RNA extraction based on guanidine-phenol-chloroform by using a commercial "Trizol" reagent, a monophasic solution of phenol and guanidine isothiocyanate. This extraction reagent was utilized successfully for viral genomic RNAs in infected cell extracts and also in environmental samples, and was found to be suitable and more practical than the conventional RNA extraction by phenol-chloroform method. The tech-

nique can be used with small quantities of cells and obtained high-quality total RNA in high yield. The simplicity of this extraction method allows simultaneous processing of a large number of samples. This extraction method has been growing in popularity because it dramatically reduces the amount of time require to isolate RNA without sacrificing its quality. The entire procedure can be completed in one hour without the use of protease treatment, it is possible to complete extraction, amplification, and electrophoretic analysis in one day.

In addition, heat treatment by boiling also provided an effective method of liberating viral genomes for RT-PCR detection. Compared with other extraction of viral RNA, heat treatment is simple, economic, convenient, and rapid for extraction of poliovirus genomic RNAs in infected cell extracts. The detection sensitivity was established initially for 3 strains of poliovirus in infected cell extracts. When amplicons from boiled RNA were analysed through agarose gel comparing with the product of Trizol extracted RNA, the PCR results using RNA templates of the corresponding sample extracted by both methods were not different. However, RNA extraction from concentrated environmental samples could not be achieved by boiling since several inhibitory substances present in environmental samples interfere with RT-PCR. Heat treatment is the preferred method to liberate viral nucleic acid for RT-PCR detection only when the water sample is free of inhibitory substances (Ma *et al*, 1995).

In an effort to facilitate routine detection of polioviruses and their differentiation from non-polio enteroviruses, we combined the enterovirus-specific primers (EV1-EV2) and the poliovirus-specific primers (PV1-PV2) by using one annealing temperature at 50°C in a duplex RT-PCR. These primer pairs were chosen so that they can give rise to amplicons of different sizes (153-bp and 293-bp) which could be easily resolved from each other by gel electrophoresis. The positive PCR products with any primer set produced bright clearly visible bands of corrected size without non-specific reaction. Positive results were not observed in the reagent blanks included in these experiments. On the other hand, the negative PCR product did not produce any DNA band or nonspecific reaction as well. Since a set of amplicon with 2 bands of corrected sizes (153-bp and 293-bp) will be expected if a sample contain poliovirus, this obviates the use of either hybridization with poliovirus-specific probes or restriction enzyme analysis. The result can be achieved with 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining, thus rendered the technique straightforward and rapid. This protocol allows the result to be obtained within one day.

Especially when we used these primer sets together with the Superscript one-step RT-PCR system, it was found to be convenience since both reverse transcriptase and *Taq* polymerase enzymes are premixed with 2x buffer containing optimized Mg^{2+} and dNTPs in a single tube, just add RNA and primers and program thermal cycler. This simplifies the procedure, minimizes pipetting steps, saves time and reduces variability. Both reactions are in one tube, thus reduces handling and risk of sample contamination. With this system, the sensitivity of our duplex one-step RT-PCR as measured with RNA extracted from poliovirus suspension (based on 40 amplification cycles) was found to be 10^{-1} $TCID_{50}$. This high sensitivity and premixed format of the one-step duplex RT-PCR system made it an easy and convenient tool for rapid screening of poliovirus in water samples within one day.

When this assay was used to detect poliovirus in artificially seeded water samples; poliovirus as little as $5x10^{-1}$ $TCID_{50}$/ml seeded in 5 liters of water could be detected; and when applied to the detection of polioviruses in environmental samples, at least 2 water samples were positive for polioviruses and one sample was positive for non-polio enterovirus. The assay gave an additional advantage over traditional cell culture method since the poliovirus positive samples by RT-PCR failed to be detected by cell culture assay because of a high bacterial or fungal load in these samples that interfere with the cell culture assay, leading to low levels of virus multiplication and premature culture degeneration. However, amplicon bands obtained with RNA extracted from these samples were less intense than those obtained in normal samples tested from tissue culture supernatants, this may reflect the much lower numbers of virions in these samples. When these amplicons were confirmed by the second PCR, each yielded a strong positive signal with either poliovirus or enterovirus primers with the band sizes as expected. The specificity of the 293-bp poliovirus amplicon was also confirmed by sequencing with either primer PV1 or PV2 to confirm their identities and genotypes. Sequence data of these positive samples were compared with those of reference poliovirus strains and from all the sequences of the Genbank and the EMBL database and identity to the sequence of type 1 strain Sabin

was found to be 99%. All locations with the presence of polioviruses and enteroviruses are from sewage pipelines, this means strains of vaccine-like poliovirus were excreted by vaccinees into the surrounding sewage pipelines. The phenomenon of waterborne poliomyelitis may become important in the near future since population growth and scarcity of water in many parts of the world makes recycling of waste water necessary, the eradication campaign of WHO must consider the possibility that vaccine virus excreted by vaccinees will be reproduced forever (Knolle, 1995).

The specificity of the poliovirus-specific PCR primers in the VP1-2A region of the poliovirus genome had been previously described by Chezzi (1996) in 125 enterovirus isolates typed as poliovirus by neutralization assays and 38 isolates typed as non-polio enteroviruses by conventional techniques. In his study with this PV-specific primer set, an amplicon of 290-bp was obtained with all of the poliovirus-positive isolates but not with any of the non-polio enteroviruses. We also used the primer set in this VP1-2A region with a slightly different design from Chezzi in some base positions. The sense primer (PV1) was located in the conserved region of the poliovirus genome coding for the capsid protein VP1, while the antisense primer (PV2) located in the conserved region of the poliovirus genome coding for protease 2A has been shown to bind to a large number of both vaccine-like and wild-type polioviruses. These poliovirus-specific primers in the VP1-2A region span the region used for genotype determination, so that genotype analysis of wild-type polioviruses can be performed by direct sequencing of the PCR products (Chezzi, 1996).

In conclusion, this study showed that the water sample concentration by membrane filter-based adsorption and elution method, followed by purification by a simple single-step RNA isolation method based on guanidine isothiocyanate-phenol-chloroform extraction, followed by a duplex RT-PCR assay with 2 primer sets combined in a single reaction, with both RT and PCR combined in a single tube, could be used to rapidly detect polioviruses and to differentiate them from non-polio enteroviruses in environmental water samples. This protocol was found to be simple, rapid, cost-effective and convenient. It was possible to complete extraction, amplification, and electrophoretic analysis in one day without the need for back hybridization of the PCR products. We are continuing our studies in order to accumulate a larger body of data that will give a clearer indication of feasibility in using this protocol for the laboratory isolation of polio-enteroviruses and other enteric viruses in environmental and municipal drinking water.

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