Iranian J Publ Health, Vol. 38, No.3, 2009, pp.90-96 **Original Article**

Comparison of Integrated Cell Culture -RT-PCR & Cell Culture Methods for Detection of Enteroviruses

**M Kargar [1], S Sadeghipour [1], B Zarei Mahmoudabadi [1], R Nategh [2]*

[1]Dept. of Microbiology & Molecular Biology Jahrom Azad University, Jahrom, Iran
[2]Dept. of Virology, School of Public Health, Tehran University of Medical Sciences, Iran

(Received 11 Jan 2009; accepted 26 May 2009)

Abstract

Background: Generally, sewage exposed water could be potentially contaminated with enteroviruses. For this reason, enterovirus isolation from sewage specimens is one of the most sensitive indicators for virus circulation in the population. We evaluated the ICC-RT-PCR and cell culture methods for detection of enteroviruses in Tehran sewage system.

Methods: This research utilized 63 specimens provided through Grab sample method to concentrate by two-phase method and cultured in RD and HEp-2 cells, respectively. All specimens then were inoculated using sensitive cell cultures of RD and HEp-2. After 24 hours incubation at 36°C by means of Pan E.V primers and afterwards Pan P.V Primers along with specific sabin primers, RT-PCR was carried out on the cell culture specimens. Data were analyzed using SPSS Software (SPSS for and ANOVA test as well as Chi-square test.

Results: Out of 63 collected specimens, enteroviruses were isolated from 33 specimens (52.38%) and 41(65.01%) specimens which utilized cell-culture & ICC-RT-PCR methods respectively. Polioviruses were also isolated from 6 specimens.

Conclusions: Statistical analysis indicated that there was a significant relationship (0.05 level) between cell culture and ICC-RT-PCR methods to isolate enteroviruses. Further the sensitivity of ICC-RT-PCR method to detect enteroviruses less than 0.01 TCID50 was evaluated, which indicated that this method is acceptable and sensitive enough to detect enteroviruses in sewage.

Keywords: *Enteroviruses, Sewage, ICC-RT-PCR, Iran*

Introduction

Enteroviruses are among the *Picornaviridae* family and include polioviruses, coxcackieviruses A, B, echoviruses and new enteroviruses 68 to 71 (1). Enterovirus infection has also been associated with such chronic diseases as dilated cardiomyopathy and chronic myocarditis chronic fatigue syndrome, post poliomyelitis syndrome, insulin dependent diabetes mellitus and motor neuron disease (2). Enteroviruses can shed in feces for several weeks, so many excrete viruses can remain infectious for a long time in environment. Therefore, by detecting enteroviruses in environmental specimens and sewage, we can understand this virus circulation and the approximate ratio of contaminated persons in society (3). Human enteric viruses associated with waterborne illness include, enteroviruses, astroviruses, hepatitis A virus, hepatitis E virus, norwalk-like virus (NLVs), sapporo-like virus (SLVs), parvoviruses, rotaviruses and small round viruses (4, 5). Traditionally, enteroviruses have been isolated by cell cultures such as RD, HEp-2, L20B, VERO, BGM, Hela, Skco-1 and HT-29 (6). Although most enteric viruses can multiply on cell culture lines, but some of enteric viruses such as hepatitis A, norwalk viruses, rotaviruses and coxackievirus group A in cell culture do not easily create the cytopathic effects (CPE) (4, 7). In addition, no sensitive cell line for isolation of all identified serotypes of enteroviruses has been discovered yet, therefore direct detection of enteroviruses by molecular methods such as RT-PCR has been noticed by environmental virologists. However, due to existence of organic inhibitors in sewage, using this method give rise to many problems (2, 4, 8). Scientists have used Guanidinium isothiocyanate (GIT), Phenol-chloroform, Nucleic acid ad-

 *Corresponding author: Tel: +98 917 3149203, Fax: +987116275697, E-mail: mkaragarmicro418@yahoo.com

sorption and Lyophilization methods to remove inhibitors in order to detect enteroviruses in sewage system (9-12). In 1996, for the first time, Reynolds et al. proposed using the integrated cell culture-RT-PCR (ICC-RT-PCR) method to detect enteroviruses in sewage specimens during 24 h (13). Then other scholars used the ICC-RT-PCR method to detect enteroviruses, asteroviruses, adenoviruses and rotaviruses in surface water, river water, marine water and sewage (7, 14-17). In the previous studies, we used RD, L20B and HEp-2 cell lines and also two different concentrated methods, Pellet & Two-Phase and different methods to remove organic inhibitors for environmental surveillance of enteroviruses and polioviruses in Tehran and Sistan & Baloochestan sewage and surface water (18-21) .
The aim of this study was to compare ICC-RT-PCR and cell culture methods for detection of enteroviruses in sewage system of Tehran.

Material and Methods

Sampling and Treatment

Sixty three grab samples were collected approximately each month over a 1 yr period (December 2002 to November 2003) from the 6 main inlet of wastewater treatment plants in Tehran (Gheitariea, 11; Sahebgharaniea, 11; Zargandeh, 5; Ekbatan, 11; Mahalati, 12 and Shoosh, 13 specimens). Sewage waters (1L) arrived in the National Poliovirus Laboratory (NPL) within a few hours and then concentrated about 100- fold by a modification of Two- Phase method (22).

Cell cultures and Microneutralization assays

For isolation of polioviruses and enteroviruses, we used RD and HEp-2 cell lines. The amount of sewage inoculation for each cell culture tubes was 200 μl , and each specimen was inoculated to 2 cell culture tubes. After 7 d of incubation at 36 ˚C all tubes were studied using Inverted microscope for the appearance CPE and were identified them by microneutralization test according to standard protocols recommended by the WHO with pooled horse antisera against the most frequently isolated ECHO and Coxackieviruses have been prepared at the National Institute of Public Health and the Environment (RIVM), Bilthoven. Each box of RIVM enteroviruses typing antisera contains anti-enteroviruses pool A, B, C, D, E, F and G, and anti- Coxackie B virus pool and a trivalent anti-poliovirus pool (23).

ICC-RT-PCR

To apply this method at first, RD, HEp-2 cell lines for detection of enteroviruses were prepared and then 200 µl of each sewage specimen was inoculated to 4 cell culture tubes and was incubated at 36 ˚C for 24 h. Then RD and HEp-2 cell lines were mixed separately and after freeze & thawing they were centrifuged for 1 min in 6200 g and 5µl of the supernatant was isolated for RT-PCR test.
At first to caring out RT-PCR, specific primers including pan E.V, pan P.V and Sabin (I, II, III) for protected 5' end of enteroviruses were designed (23-25). Then, a mixture of A buffer (33.5 ml Tris- Hcl 1M, 8.5ml Sulfate ammonium 1M, 6ml EDTA 0.5M, 8ml Distilled water), B buffer [25µl A buffer,5µl dNTPs, 5µl pan E.V primer (Reverse), 5 µl Pan E.V primer (Forward), 150 µl Distilled water] and C buffer (13.75 $Mgcl_2$ 0.2 M, 0.7 µl DTT (1M), 6.9 µl RNase inhibitor, 3.6 µl AMV reverse transcriptase, 13.7 µl Taq DNA polymerase, 236.3 µl Distilled water) were prepared and kept at -20 ˚C. Afterwards, 19 µl of B buffer containing pan EV primer with 5 µl of cell culture supernatant were added to all tubes, which then were placed in Thermocycler (Biometric) at 95 ˚C for 5 min. Then, after cooling (for 5 min on the ice) 5 µl of C buffer added to each tube. Finally, by means of Thermocycler RT-PCR was carried out according to the following conditions:
I) Reaction to RT enzyme for 20 min at 42 ˚C and which was inactivated at 95 ˚C for 3 min.
II) PCR with Pan E.V primers under the following conditions: 95 ˚C, 45 s; 55 ˚C, 45 s; 70 ˚C, 45 s (for 36 cycles) and final extension stage for 5 min in 70 ˚C.
In addition, positive specimens with Pan E.V primers were assessed by Pan P.V specific primers.

Therefore, 19µl of B buffer containing Pan P.V primers along with 5µl of cell culture supernatant added to all tubes, which then placed in Thermocycler for 5 min at 95 ˚C. Then, after cooling the tubes on the ice, 5µl of the C buffer was added. Finally, RT-PCR was carried out under the following conditions:
I) Reaction to RT Enzyme, for 20 min at 42 ˚C, which was made, inactivated at 95 ˚C for 3min.
II) PCR with Pan P.V degenerate primers within this condition: 95 ˚C, 45 s; 42 ˚C, 45 s; 60 ˚C, 45 s (for 30 cycles).
Afterwards for Intratypical differentiation (ITD), between wild and vaccine viruses, all detected polioviruses, were studied with Sabin specific primers. Finally, 10µL of PCR product was transferred to the 2% gel containing Ethidium bromide which after electrophoresis, was investigated by UV transilluminator.

Statistical analysis

The data were then entered and analyzed using *SPSS* Software (*SPSS* for windows, 12 program) and ANOVA test and then Chi-square test. *P*-values less than 0.05 were taken to indicate statistical significance.

Results

In cell culture method out of 63 studied specimens in RD cell line from 28 specimens (44.44%) and in 36 specimens (57.14%) with ICC-RT-PCR method, enteroviruses have been detected. In addition, in HEp-2 cell line, using cell culture method from all studied specimens, in 14 specimens (22.22%) and from 29 specimens (46.01%) using ICC-RT-PCR method enteroviruses were detected. However, without taking into account the specific cell line, in ICC-RT-PCR method from 41 specimens (65.07%) and from 33 specimens (52.38%) with cell culture method, enteroviruses were isolated. The largest amount of enteroviruses was detected from Mahalati refinery with ICC-RT-PCR method and in cell culture method belonged to Shoosh refinery (Table 1). Non- typable enteroviruses (NTEVs), E_{11}, E_{25} and E_{13} were identified more frequently than other enteroviruses respectively with cell culture method. Also, with cell culture (15.87%) and in ICC-RT-PCR method (9.52%) polioviruses detected by means of sabin primers (Table 2). Out of all isolated polioviruses (Sabin Like=SL), one P1, one P2 and four P3 were detected. It was found that all of polioviruses related to vaccine strain (SL) and no cases depended on wild or vaccine derived polioviruses (VDPVs). As a whole there was a significant correlation (0.05 level) between the number of isolated enteroviruses from ICC-RT-PCR and cell culture in RD cell line (*P*= 0.01). Having applied SPSS 12 and ANOVA test and then Chi-square test to study the results of the study, it was found out that there is significant correlation (0.05 level) between ICC-RT-PCR and cell culture method (*P*= 0.004).

Table 1: Comparison of ICC-RT-PCR and Cell Culture methods in studied sites for detection of Enteroviruses

Methods	**Cell Culture (RD)**		**Cell Culture (HEp-2)**		**Cell Culture (Total)**		**ICC-RT-PCR (RD)**		**ICC-RT-PCR (HEp-2)**		**ICC-RT-PCR (Total)**	
Sampling site	**n (%)**		**n (%)**		**n (%)**		**n (%)**		**n (%)**		**n (%)**	
Gheitarea	3	(4.76)	0	(0)	3	(4.76)	5	(7.93)	4	(6.34)	6	(9.52)
Zargandeh	2	(3.17)	2	(3.17)	3	(4.76)	4	(6.34)	2	(3.17)	4	(6.34)
Sahebgharanea	2	(3.17)	2	(3.17)	3	(4.76)	6	(9.52)	4	(6.34)	7	(11.11)
Ekbatan	6	(9.52)	2	(3.17)	7	(11.11)	5	(7.93)	7	(11.11)	7	(11.11)
Shoosh	9	(14.28)	5	(7.93)	9	(14.28)	7	(11.11)	7	(11.11)	8	(12.69)
Mahalati	6	(9.52)	3	(4.76)	8	(12.69)	9	(14.28)	5	(7.93)	9	(14.28)
Total	28	(44.44)	14	(22.22)	33	(52.38)	36	(57.14)	29	(46.03)	41	(65.07)

Table 2: Comparison of ICC-RT-PCR and Cell Culture methods in HEp-2 and RD Cell lines for detection of Enteroviruses

Cell line	**RD**		**HEp-2**		**Total**	
Methods	**Cell Culture**	**ICC-RT-PCR**	**Cell Culture**	**ICC-RT-PCR**	**Cell Culture**	**ICC-RT-PCR**
Viruses	**n (%)**		**n (%)**		**n (%)**	
Poliovirus	9(14.28)	5(7.93)	7(11.11)	1(1.58)	10(15.87)	6(9.52)
N.P.E.V	25(39.68)	30(47.61)	7(11.11)	25(39.68)	27(42.85)	34(53.96)
Enteroviruses	28(44.44)	36(57.14)	14(22.22)	29(46.01)	33(52.38)	41(65.07)

Discussion

The cell culture standard method for detection of human pathogenic enteric viruses is time consuming and costly and it takes one month to confirm positive results and due to the existence of organic and inorganic inhibitors in sewage using this method faces has a lot of problems too. Nevertheless, owing to insufficient volume of reaction and natural inhibitors in sewage concentrated specimens direct RT-PCR method does not have necessary sensitivity for isolation of enteric viruses. In addition, this method cannot determine viral infectivity. Margolin et al. (26) and Wyn-Jones et al. (27) compared three methods, including ICC-RT-PCR, cell culture and RT-PCR for detection of enteroviruses, hepatitis A, rotaviruses in water and sewage specimens. They found that after 5 to 7 d of incubation the ICC-RT-PCR method was the best method for isolation of enteroviruses. Reynolds et al. (28) used the ICC-RT-PCR for detection of enteroviruses and hepatovirus in environmental specimens. By using of ICC-RT-PCR method and inoculation of > 10PFU enteroviruses to the cell culture they found positive results of PCR after 5 h in 4/5 of studied flasks and after 10 h incubation in all of the flasks. In addition, after 20 and 25 h, 1PFU and less than 1PFU enteroviruses were detected with ICC-RT-PCR method respectively. Besides, Cha-pron et al. (14) used integrated cell culture-nested PCR for detection of adenovirus type 40 & 41, astroviruses and enteroviruses in surface water samples. By the way, they incubated infected flasks for 5 d at 37 ˚C. Likewise, Lee et al. (29, 30) assessed cell culture- PCR assay based on A549 and BGMK cell lines to monitor infectious adenovirus, enteroviruses, reoviruses in water treatment plant systems and river water.

The aim of this study was to assess of ICC-RT-PCR method reliability for special and rapid detection of cytopathogenic and noncytopathogenic viruses in sewage specimens and a comparison with cell culture results. In this study after concentration of specimens with Two-Phase method, RD and HEp-2 lines were used. Our experience demonstrated that the sensitivity of RD was more than the HEp-2 cell line, but due to the isolation of different ranges of enteroviruses in each of these two cell lines, utilization of these two cell lines simultaneously for increasing the sensitivity of ICC-RT-PCR method was proposed. Rate of polio detection by ICC-RT-PCR was lower than the rate by cell culture, which can be justified on the low multiplicity of polioviruses at considered time (24 h) in ICC-RT-PCR methods. The sensitivity of ICC-RT-PCR method for detection of enteroviruses less than 0.01 TCID50 was invested too. It indicates the acceptability and sensitivity of this method for detection of enteroviruses in sewage. Detection of non- polio enteroviruses (NPEVs) in sewage specimens in the final stage of poliomyelitis eradication is the criterion of success for poliovirus environmental surveillance. According to WHO guidelines for environmental surveillance of polioviruses circulation in 2003 at least 30% of concentrated sewage from grab Samples should reveal NPEVs (31). In this research, out of all studied specimens

with using of Pan E.V primers in ICC-RT-PCR method from 41 specimens, enteroviruses were isolated, and after the assessment with Pan P.V primers in 6 specimens, polioviruses were isolated. At the end, with Sabin primers it was found that all isolated polioviruses were related to vaccine strain. This finding can also be another piece of evidence to confirm eradication of wild polioviruses and a suitable immune coverage in Iran.
In conclusion, because of higher sensitivity and necessary efficiency needed to remove organic inhibitors, the ICC-RT-PCR method can be a suitable way for environmental surveillance and epidemiological assessment of enteroviruses disease.

Acknowledgments

The authors offer their thanks & appreciation to the Scientific & Sanitary Research Institute affairs of the Tehran University of Medical Sciences for their financial & executive protection of this project. The authors declare that there is no conflict of interests.

References

1. Pallansch M, Roos RP (2001). Enteroviruses; Polioviruses, Coxackieviruses, Echoviruses and Newer Enteroviruses. In Filds. B.N et al (ed.), *Fileds virology*, 4th ed, Lippincott Raven., New Yourk, pp.: 723-76
2. Muir P, Kammerer U, Korn K, Mulders MN, Poyry T, Weissbrich B, Kandolf R, Cleator GM, Van Loon AM (1998). Molecular typing of Enteroviruses: current status and future requirements. *Clinical Microbiology Reviews*, 11(1): 202-27.
3. Warden PS, Ballester NA, Moore AE, Fontaine JH, Fallon KS, Margolin AB (1999). Detection of Enteric viruses in Archived ICR sample concentrates using and integrated cell culture-Nested PCR Technique. Research and Development. *Drinking Water*, 1-4.
4. Abbaszadegan M, Stewart P, Lechevallier M (1999). A strategy for detection of viruses in groundwater by PCR. *Appl Environ Microbiol*, 65(2): 444-49.
5. Miagostovich MP, Ferreira FF, Guimaraes F (2008). Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amozonia, Brazil. *Appl Environ Microbiol*, 74: 375-382.
6. Federal- Provincial- Territorial committee on Drinking water (2003). Virological Quality of Drinking water. *Draft Drinking Water Guidelines for Viruses*: 1-24.
7. Tougianidou D, Botzenhart K (1998). Molecular techniques for the detection of Enteroviruses in water. *OECD Workshop Molecular Methods for Safe Drinking Water*, 1-4.
8. Abbaszadegan M (2001). Advanced Detection of viruses and protozoan parasites in water. *Reviews in Biology and Biotechnology*, 1(2): 21-26.
9. Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid Guanidinium Thiocynate-phenol-chloroform extraction. Analytical Biochemistry, 162: 156-59.
10. Abbaszadegan M, Huber MS, Gerba CP (1993). Detection of Enteroviruses in groundwater with the Polymerase Chain Reaction. *Appl Environ Microbiol*, 59(5): 1318-24.
11. Berger M (1997). Movement toward making the Ground water (Disinfection) Rule a reality: 1-8.
12. Bosh A (1998). Human enteric viruses in the water environment: a miniseries. *Internal Microbiol*, 1: 191-96.
13. Reynolds KA, Gerba CP, Pepper IL (1996). Detection of Infectious Enteroviruses by an integrated cell culture-PCR procedure. *Appl Environ Microbiol*, 62(4): 1424-27.
14. Chapron CD, Ballester NA, Fontaine JH, Frades CN, Margolin AB (2000). Detection of Astroviruses, Enteroviruses, and Adenovirus Type 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture- Nested PCR procedure. *Appl Environ Microbiol*, 66(6): 2520-25.

15. Ballester NA, Rex AC, Coughlin KA (2004). Study of anthropogenic viruses in Boston Harbar, Charles River, cottage form CSO Treatment facility and deer island treatment plant 1995-2003. *MWRA Virus Report*, 15-57.
16. Fong TT, Lipp EK (2005). Enteric viruses of human and animals in aquatic environments; Health risks, detection and potential water quality assessment tools. *Microbiol Mol Biol Rev*, 69: 357-371.
17. Dale WC, Kim AD, John HP, Joan BR (2003). Pathogenic human viruses in coastal waters. *Clinin Microbial Rev*, 16(1): 129-43.
18. Kargar M., Sarijlou M, Tabatabaei H, Abbasian F, Shahmahmoudi SH, Holakuea K, Karimlou M, Nateghpour M, Sedighi H, Khavarinejad R, Mokhtariazad T, Nategh R (2004). A survey on non-polio enteroviruses (NPEVs) circulation in sewage system of Tehran by RD and HEp-2 cell lines using direct, Pellet and Two-Phase methods. *Scientific Journal of School of Public Health & Institute of Public Health Research*, 2: 37- 40.
19. Kargar M, Sarijlou M, Razavi SS, Tabatabaea H, Nategh R (2005). The assessment of NPEVs circulation in sewage and surface water of Sisten & Bloochestan in RD and HEp-2 cell lines and with use of Pellet and Two-Phase concentration methods. *Iranian Journal of Infectious Disease and Tropical Medicine*, 30(10): 33-40.
20. Kargar M, Sadeghipour S, Tabatabaei H, Sarijlou M, Ghodsi M, Nategh R (2006). Assessment of different methods to remove organic inhibitors in order to create a sensitive method for direct surveillance of enteroviral infectious diseases in sewage specimens. *Medical Sciences Journal,* 16(2): 69-77.
21. Kargar M, Tabatabaei H, Sarijlou M, Sadeghipour S, Ghodsi M, Nategh R (2007). Poliovirus environmental surveillance in sewage system of Tehran: isolation and identification of viruses by cell culture and microneutralization, and differentiation by ELISA and probe hybridization. *Hakim Journal*, 10(1): 43-49.
22. Hovi T, Stenvik M, Partanen, M (2001). Poliovirus Surveillance by examing sewage specimens, Quantitative recovery of virus after introduction into sewage at remove upstream location. *Epidemiology and Infection*, 127: 101-106.
23. World Health Organization (2001). Polio Laboratory Manual, under revision; available on the Internet at http://www.who.int/vaccines/en/poliolab/poliomanual%20Version1-May01.pdf.
24. C.D.C (1999). Poliovirus Diagnostic PCR, *Kuwait Polio ITD Workshop. EVS/ CDC* 11/02., 1-13.
25. Kilpatric DR, Nottay B, Yang CF, Yang SJ, Mulders MN, Holloway BP, Pallansch MA, Kew OM (1996). Group Specific Identification of Poliovirus by PCR using primers containing mixed Base or Deoxyinosine Residues at positions of codone Degeneracy. *Clinical Microbiol Journal,* 34(12): 2990-96.
26. Margoline A (1999). Viral Analyses. *Analytical Services*, 40(6): 1-3.
27. Wyn-Jones AP, Sellwood J (2001). Entric viruses in aquatic environment. *Appl Environ Microbiol*, 91: 945-62.
28. Reynolds KA, Gerba CP, Abbaszadegan M, Pepper IL (2001). ICC-PCR detection of Enteroviruses and hepatitis A virus in environmental samples. *Can J Microbiol*, 47: 153-157.
29. Lee HK, Jeong YS (2004). Comparison of total culturable virus assay and multiplex integrated cell culture-PCR for reliability of waterborne virus detection. *Appl Environ Microbiol*, 70(6): 3632-36.
30. Lee C, Lee SH, Han E, Kim SJ (2004). Use of cell culture-PCR assay based on combination of A 549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and en-

teroviruses in river water. *Appl Environ Microbiol,* 70(11): 6695-6705.
31. World Health Organization (2003). Guidelines for environmental surveillance of poliovi-ruse circulation, under revision; available on the Internet at: http://www.who.int/vaccines-documents /WHO/V&B 03.03.