Iranian Journal of Virology 2013;7(1&2): 61-3

Short Communication

A Nested RT-PCR Method for Confirming the Absence of all Three Types of Polioviruses in Primates and Human Cells Used in Vaccine Production

Salimi-Jeda A[1], Mohammadi A[1], Shahmahmoodi S[2,3], Foroughi A[1], Alirezaie B[1*]

1. Department of Human Viral Vaccine, Razi Vaccine and Serum Research Institute, Karaj, Iran.
2. Iran National Polio Laboratory, Virology Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
3. Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

A variety of cells, ranging from prokaryotic to eukaryotic cells, can be used for vaccine production. Although cell cultures are suitable substrate for production of biological products such as vaccines, they might be contaminated with adventitious agents and add them to final product unexpectedly which can imperil the safety and harmlessness of the vaccine. Contaminations of early polio and adenovirus vaccines with SV40 are good examples of such dangerous effect (1). As a consequence, many vaccinees have unknowingly been infected with these pathogens because of receiving contaminated vaccines. Major sources of contamination are all raw materials including virus seeds, cell banks, and animal products such as serum, BSA, and so on. So every sensitive cell culture might theoretically be contaminated with exogenous viruses in the laboratory or in vaccine production centers.

The vast majority of primate cells are susceptible to polioviruses due to containing the poliovirus receptor (PVR [a member of the immunoglobulin superfamily]), commonly known as CD155 (2). Therefore these cells can be contaminated in the site of production and the virus can spread to the other products as exogenous adventitious agent. The aim of this study was to describe a rapid and sensitive method for simultaneous detection of all three

***Corresponding author:** Behnam Alirezaie, MSc. Department of Human Viral Vaccine, Razi Vaccine and Serum Research Institute, Karaj, Iran.
Email: b.alirezaie@rvsri.ac.ir

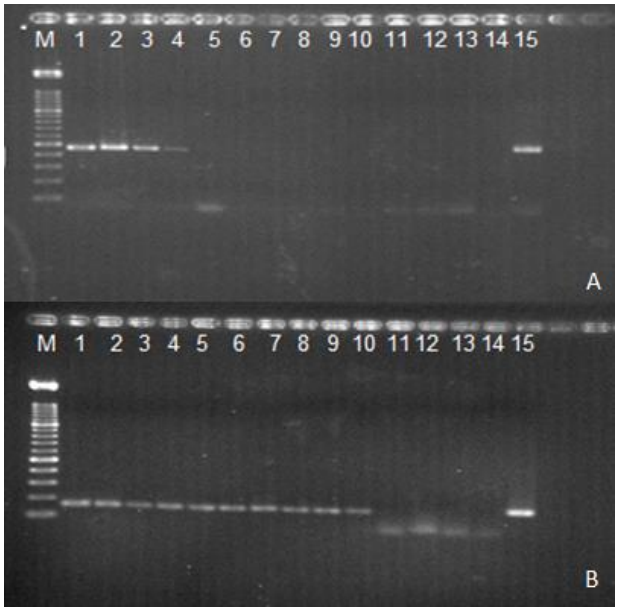

Fig. 1. Estimation of the sensitivity of RT-PCR (A) and nested RT-PCR (B) using serial dilution of PVs. The Sabin PVs containing $10^{7.5}$ $CCID_{50}$/ml was used. Virus was serially diluted by 1:10, and each dilution was subjected to RT-PCR and nested RT-PCR. From lane 1-12 a 10-fold serial dilutions of serotype 1 PVs. A 100 bp lader (Roche Applied Science, Mannheim, Germany) was used as DNA size marker (lane M). Negative controls (without template) were always negative (lane 13 and 14). The RT-PCR and nested RT-PCR assay on the commercial IPV (lane 15). The nested RT-PCR approximately showed the same results to two other types.

[DOI: 10.21859/isv.7.1.2.61]

Table 1. Comparison of time and dilution of appearance of typical Poliovirus CPE in five different cell cultures.

Poliovirus serotype	Cell type	appearance of CPE	
		Dilution[a]	Time (day)[b]
Type 1	Vero	10^{-6}	8
	HeLa	10^{-7}	8
	L20-B	10^{-8}	8
	MRC-5	10^{-6}	4
	Hep-2	10^{-7}	6
Type 2	Vero	10^{-6}	7
	HeLa	10^{-7}	9
	L20-B	10^{-7}	4
	MRC-5	10^{-7}	7
	Hep-2	10^{-7}	6
Type 3	Vero	10^{-6}	10
	HeLa	10^{-6}	3
	L20-B	10^{-7}	4
	MRC-5	10^{-6}	4
	Hep-2	10^{-6}	3

[a]The final dilution of virus which showed typical poliovirus CPE.
[b]The day of CPE appearance in the final dilution.

serotypes of poliovirus as adventitious agents in primate cells and some other virus seeds.

All three serotypes of poliovirus which were propagated in MRC-5 cell were used as positive control in this study. Virus cultivation and harvesting in MRC-5 cells was carried out as described previously (3). The concentration of viruses was determined using the endpoint dilution method (4). The DNAMAN (version 4.13) and Oligo® (version 5.0) software were used for sequence alignment and primer design, respectively. The most highly conserved regions in the genome of the three serotypes were identified (the 3D-3′ NCR [nt 6925 to nt 7392] of the PVs genome) and primers were designed for these region. Viral RNA was extracted from 200 µl of clarified virus suspensions using viral RNA extraction kits (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. After extraction of viral RNA, 8.2µl of extracted viral RNA was directly used in RT- PCR and the rest was stored at -80°C for later analysis. RNA was reversely transcribed into cDNA using first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) and random hexamers according to the manufacturer's recommendations. Synthesized cDNA was subsequently applied for nested PCR. For the first-step PCR two oligonucleotides (PV-7392- [ATTCGACTGAGGTAGGGTTAC] and PV-6925+ [CAAGGGCATAGATTTAGACCAC]) were used as primers enclosing a 468-bp fragment of the genome. For the second-step PCR, another set of oligonucleotides (PV-7173- [TCCTTCATTGGCATTACTGG] and PV-7014+ [TAGCCCAATCAGGAAAAGAC]) were applied as primers enclosing a 160-bp fragmen .The first-step nested PCR mixture (25 µl) contained 2.5 U Taq polymerase (Fermentas Life Sciences, Vilnius, Lithuania), 0.2 µM each primer (PV-7392- and PV-6925+), 1X buffer, 200 µM each dNTP, and 2.5 µl cDNA. The mixture in the microtubes were initially denatured at 95°C for 5 min and then subjected to 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 45 s. The second-step nested PCR mixture (25 µl) contained 1 µl of the diluted (1:100) product of the first step, 2.5 U Taq DNA polymerase (Fermentas Life Sciences, Vilnius, Lithuania), 1X buffer, and 200 µM each dNTP. The second-step PCR was

[DOI: 10.21859/isv.7.1.2.61]

performed with an initial denaturation at 95°C for 5 min and then subjected to 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 20 s. A polishing cycle as 72°C for 5 min was used for final extention. Amplicons were separated in 2% agarose gels with TAE buffer and visualized by ethidium bromide staining. A 100-bp DNA ladder (marker XIV [Roche Applied Science, Mannheim, Germany]) was used as molecular weight marker. Cell culture techniques were used in parallel to compare the sensitivity of this newly optimized method with traditional cell culture-based method. Five primate and human cells (Vero, HeLa, Hep-2, MRC-5 and L20-B) were infected with PVs and cultivated in DMEM media (Sigma-Aldrich). Cell cultures were observed daily for any sign of appearance of poliovirus CPE.

Each amplicon generated a sharp band at 468-bp and 160-bp using PV-7392-/PV-6925+ and PV-7173-/ PV-7014+ primer pairs, respectively. The specificity of each set of primers (PV-7392- /PV-6925+ and PV-7173-/ PV-7014+) used in this study was investigated. RNA samples extracted from other viruses (the Hoshino mumps virus strain, the Takahashi rubella virus strain, the AIK-C measles virus strain) were examined by this method. Neither the 468-bp nor the 160-bp DNA band was generated from other viruses.

The cell culture analysis revealed that the sensitivity of L20B cell to PVs was more than other cells (Table 1). In order to determine the limit of detection of the RT-PCR method 10-fold serial dilutions of all three types of poliovirus with known titers were prepared. The detection limit was calculated as 3.2 CCID50/ml, 316.3 CCID50/ml, and 0.003 CCID50/ml in the most sensitive cell culture (L20B), RT-PCR and nested RT-PCR, respectively (Figure 1 and Table 1). Limitation for the genome detecting nested RT-PCR was nearly 100-fold higher compared with the particle detecting the most sensitive cell culture-based method (L20-B). This difference may come about for detection of noninfectious particles by nested PCR. Using nested RT-PCR assay on the commercial IPV (IMOVAX POLIO [Aventis Pasteur, Lyon, France]) supports that this method could amplify the genome of noninfectious particles, too (Fig-1). Although one of the main limitations of nested RT-PCR assay is the detection of both infectious and non infectious particles, the differentiation between viable and inactive particles is not required for confirming the absence of the PVs as an adventitious agent.

RT-PCR technique using degenerate primers has been used to identify the genus and serotypes of PVs from clinical samples (5). In the present study, an attempt was made to describe a rapid and sensitive nested RT-PCR method without using degenerate primers for detection of all three serotypes of PVs. The results showed that the established nested RT-PCR method is sensitive and relatively rapid, which can be used for simultaneous detection of polioviruses in primate cells and virus seeds.

Acknowledgments

We thank from Department of Human Viral Vaccine in Razi Vaccine and Serum Research Institute for support and all collaborates who helped this study.

References

1. Montagnon BJ, Nicolas AJ, Fanget B, Peyron L. Comparison of sensitivity of VERO cell line versus primary monkey kidney cells in the detection of residual live polio virus during and after inactivation. Dev. Biol. Stand. 1981;47:151-5.
2. Minor PD. The polio-eradication programme and issues of the end game. J. Jen. Virol. 2012;93:457-74.
3. Alirezaie B, Taqavian M, Aghaiypour K, Esna-Ashari F, Shafyi A. Phenotypic and genomic analysis of serotype 3 sabin poliovirus vaccine produced in MRC-5 Cell substrate. J. Med. Virol. 2011;83:897-903.
4. Bey E, Golombick T. A comparison of three methods for titration of poliovirus vaccines. J. Virol. Methods. 1984;9:123-30.
5. Kilpatrick DR, Nottay B, Yang C-F, Yang S-J, Da Silva E, Pearanda S, Pallansch M, Kew O. Serotype-specific identification of polioviruses by PCR using primers containing mixed-base or deoxyinosine residues at positions of codon degeneracy. J. Clin. Microbiol. 1998;36:352-7.

[DOI: 10.21859/isv.7.1.2.61]