Iranian J Virol 2017; 11(2): 14-18
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Original Article

Identification of RD and L20B Cell Lines Cross-Contamination by Multiplex SYBR-Green PCR in Poliovirus Laboratory

Nejati A[1], Tabatabaie H[1], Yousefi M[1,2], Mollaei-Kandelous Y[1,3], Shahmahmoodi Sh[1,4*]

1. Polio National Laboratory, Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, telephone number: +982188950595
2. Department of Virology, International Campus, Tehran University of Medical Sciences, Tehran, Iran.
3. Immunology Department, Iran University of Medical Science, Tehran 14716-13151, Iran.
4. Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Background and Aims: World Health Organization Global Polio Laboratory Network (GPLN) plays a critical role in the Global Polio Eradication Initiative. Cell culture methods (mostly RD and L20B cell lines) have been used for Enteroviruses and polioviruses isolation, respectively. Cross-contamination among L20B and RD cell lines causes the problem in accuracy of poliovirus surveillance and decreases the poliovirus detection. Therefore, validation of identity of cell lines purity is a vital part of cell culture in polio laboratory.

Materials and Methods: In this study, a multiplex SYBR-Green PCR based on Cytochrome b oxidase amplification was designed to L20B and RD cell lines cross-contamination.

Results: The conventional multiplex PCR performed on DNA extracted from L20B cells deliberately cross-contaminated with RD cells clearly showed not only the identity of L20B cell line but also the presence of contaminant RD cells.

Conclusions: The results indicated that the multiplex SYBR-Green PCR was reliable method to identity L20B and RD cell lines individually and also after deliberate cross-contamination.

Keywords: Multiplex SYBR-Green PCR Poliovirus, Cell line authentication.

Introduction

Poliovirus surveillance is very important to eliminate wild type poliovirus which is the main goal of Global Poliovirus Eradication (1). World Health Organization Global Polio Laboratory Network (GPLN) has designed standard surveillance protocols based on virus isolation and molecular typing. L20B (the source of mouse L cells) and RD (the source of Rhabdomyosarcoma human cell lines) cell lines which have been used as a diagnostic tool for poliovirus and Enterovirus isolation, respectively (2).

Cross-contamination of cell lines usually occurs as a result of poor handling laboratory practices in tissue culture (3). Cross-contamination among L20B and RD cell lines causes the problem in accuracy of poliovirus surveillance and decreases the poliovirus detection. Therefore, validation of identity of cell lines purity is a vital part of cell culture polio laboratory.

For identifying species-specific cross-contamination of cell cultures, several methods have been applied. Isoenzyme analysis, karyological examination, DNA fingerprinting and short tandem repeat (STR) profiling are widely used for cross-contamination evaluation (4). The use of Polymerase chain reaction (PCR) as a tool for species identification was first suggested by Stacey et al in 1992 (5). The Cytochrome b and Cytochrome c oxidase genes (Cyt b and Cyt c) of mitochondrial DNA (Mt DNA) vary by several per cent between species. By using specific primers, it is possible to detect specific species (6, 7).

*Corresponding author: Dr Shohreh Shahmahmoodi npl.iran@gmail.com.

In the present study, we describe a multiplex SYBR-Green PCR based on Cytochrome b oxidase amplification that can be used to L20B and RD cell lines cross-contamination. The results of this study showed that the multiplex SYBR-Green PCR was reliable method to identity of L20B and RD cell lines individually and also after deliberate cross-contamination.

Methods

Cell lines. L20B (the source of mouse L cells) and RD cells (the source of rhabdomyosarcoma human cell lines) were obtained from WHO. Both cells maintained at a 37°C incubator. After growing cells with confluent monolayers, the cells were dislodged from cell culture flask by trypsin (Biosera). Trypsinized cells were counted using a hemocytometer then adjusted 10^6 cell/ml.

DNA exteraction. For DNA extraction from 200 µl of cell suspension, genomic DNA extraction kit (Roche) was used according to the manufacturer's instruction. Quantity and quality of DNA was evaluated by nanodrop 1000 (Thermo fisher).

Primer designs and single PCR amplification. MP primer software (http:/ biocompute. bmi.ac.cn/MPprimer) was used to design PCR primers to Cyt b of mitochondrial DNA based on the downloaded mouse and human sequences from NCBI Ref-Seq nucleotide program. The primer features (sequences, melting temperature and product length) are listed in Table 1.

Deliberate cross-contamination of cell lines. To evaluate the sensitivity and specificity species-specific cross-contamination of multiplex SYBR-Green PCR, L20B and RD cell lines were deliberately cross-contaminated. For this purpose, 10000, 50000 and 100000 RD cells were deliberately added to 990000, 950000 and 900000 L20B cell line to 1%, 5% and 10% cell lines cross-contamination, respectively. The cross-contaminated cells were taken for DNA extraction and multiplex SYBR-Green PCR mentioned above.

Gradient PCR. To determine optimum annealing temperature, the gradient PCR was carried out. The PCR mixture was put in the PeqSTAR gradient thermocycler (Peqlab, Germany) with temperature ranging from 55 to 60°C. The best annealing temperature was indicated by agarose gel electrophoresis.

Multiplex SYBR-Green PCR. The multiplex SYBR-Green PCR was applied to detect the human and mouse mitochondrial DNA using specific human and mouse Cyt b primers in one reaction. Briefly, PCR were performed in the final volume of 25ul reactions mixtures containing 1µl extracted DNA, 12.5ul Fast SYBR® Green Master Mix (Thermofisher) and 10 pmol of each primer. The multiplex SYBR-Green PCR conditions were carried out as follows: 5 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C, and a default melting curve. The results of the final products (human Cyt b 101 bp and mouse Cyt b 122 bp) were observed by melting curve analysis.

Results

The primers targeting Cytochrome b oxidase genes in the multiplex PCR assay were designed based on the Mus musculus and Homo sapience origin reference mitochondrial DNA from NCBI. The best ranked primers from MP primer program were selected for more analysis. To determine the specificity, the candidate primers were checked by BLAST program from human and mouse genome. The BLAST analysis revealed 100% homology with the respective selected gene and no match to other unwanted genes.

Table 1: Primer sequences designed for this study

CYT-B			
Primer name	**Sequences(5' to 3')**	**tm**	**product**
Forward primer Homo	AACTTCGGCTCACTCCTTGG	59.96	**101**
Reverse primer Homo	GATGAAAAGGCGGTTGAGGC	59.83	
Forward primer Mus	CTTCATGTCGGACGAGGCTT	60.11	**122**
Reverse primer Mus	CCTCATGGAAGGACGTAGCC	59.89	

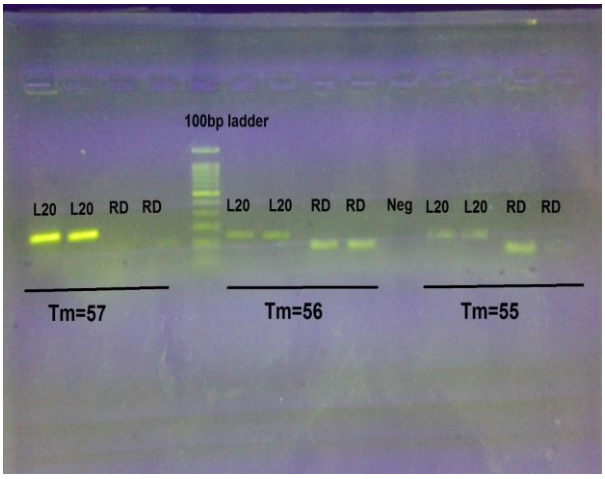


Fig. 1. The agarose gel electrophoresis of gradient PCR. As indicated, the best annealing temperature to detect both human and mouse target gene was performed at 560C. Each sample was performed in duplicate.

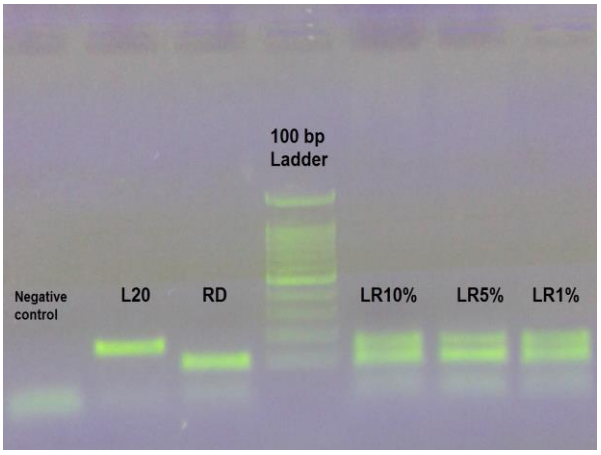


Fig. 3. Multiplex PCR. L20B cells deliberately cross-contaminated with 1%, 5% and 10% of RD cells clearly showed not only the identity of L20B cell line but also the presence of contaminant RD cells.

To determine optimum annealing temperature, the gradient PCR was carried out. The results showed that the best annealing temperature to detect both human and mouse target gene was performed at 56°C (Figure1).

detection limit of designed primers was carried out by single PCR with 10, 100 and 1000 RD and L20B cells. The results are shown in Figure 2.

The conventional multiplex PCR performed on DNA extracted from L20B cells deliberately

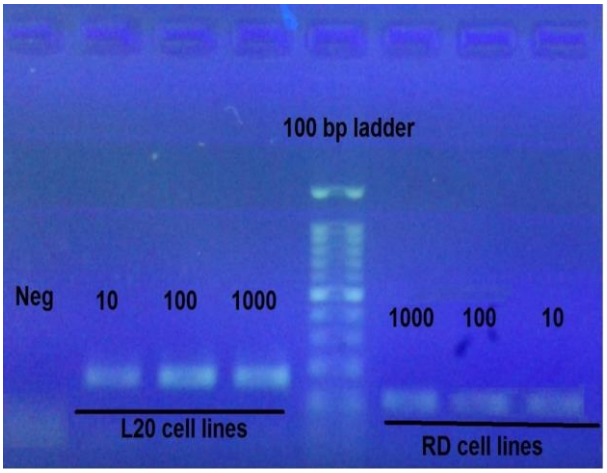


Fig. 2. Limitation detection of single PCR with 10, 100 and 1000 RD and L20B cells. As shown in the picture, the designed primers were sensitive enough in detection the lowest sample of both RD and L20B cell lines.

cross-contaminated with 1%, 5% and 10% of RD cells clearly showed not only the identity of L20B cell line but also the presence of contaminant RD cells (Figure3). The multiplex SYBR-Green PCR was carried out on separately L20B, RD cells and 1% L20B cells contaminated with RD cells and results are shown in Figure 4.

Discussion

The results of single PCR performed on DNA extracted from RD and L20B evidently showed that the primers were sensitive enough in demonstrating the lowest sample of both RD and L20B. This result also clearly demonstrated that RD and L20B cell lines available in our repository were free from cross-contamination.

Losi et al. used PCR-RLFP to determine the cross-contamination of 27 different cell types by targeting Cyt b gene (7). The results showed that in low levels of the genome, the sensitivity of targeting cytochrome oxidase b is much higher than Cyt c and 10% of the intercellular contamination is detectable by this method. In our study showed that multiplex PCR can be detected 1% L20B contaminated with RD cell lines.

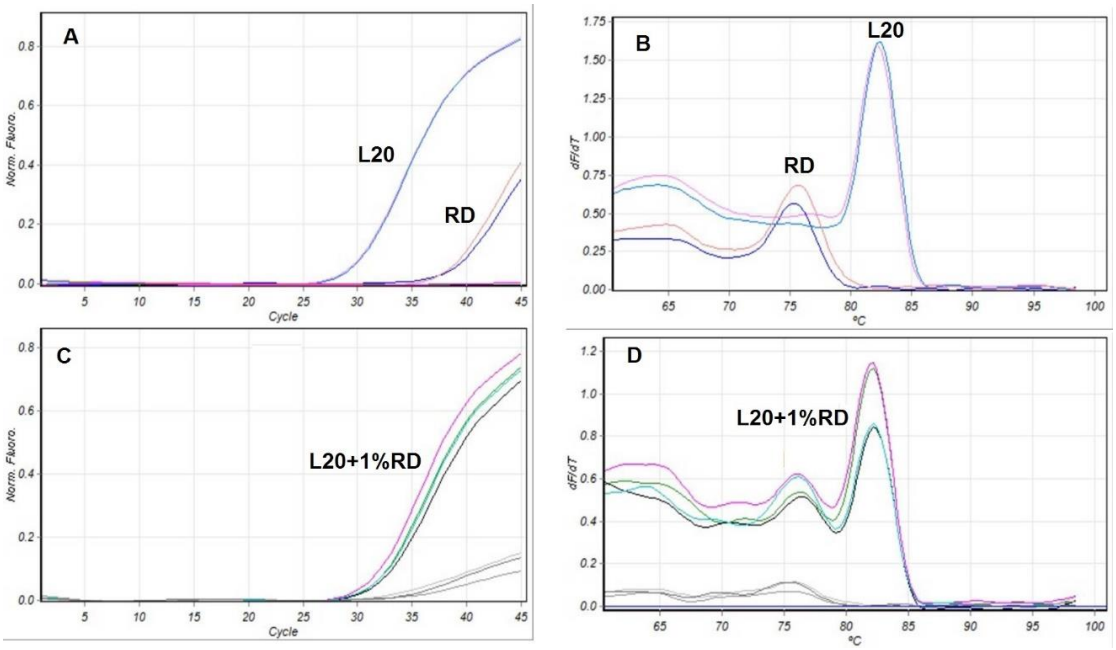


Fig. 4. Multiplex SYBR-Green PCR. L20 and RD distinctly detection with multiplex SYBR-Green PCR (A and B). L20+1%RD mix detection with multiplex SYBR-Green PCR (C and D).

Short tandem repeat (STR) profiling is used as a gold standard for cell line authentication (8). Since this method requires nucleotide sequencing and data analysis, causing very expensive and complicated. Therefore, this method is not proper for a Polio laboratory with a limited number of cell cultures. On the other hand, it is not economical to determine the L20B and RD cell lines cross-contamination by STR profiling method.

The multiplex SYBR-Green PCR technique was sensitive enough in demonstrating the presence of only 1% of contamination as strong product bands, both in L20B and RD cell lines.

This data clearly showed that multiplex SYBR-Green PCR based authentication could be suitable for other human and mouse cell lines. The sensitive, rapid and cost-effective nature of multiplex SYBR-Green PCR makes this method more suitable to other molecular techniques for cell line authentication. In addition, the multiplex SYBR-Green PCR technique could be used for establishing the other species identity of cell lines.

Conclusion

In recent years, new and highly accurate techniques have been used to determine cellular accuracy and cross-cellular contamination (9). By using the Luminex platform high-throughput method, Castro et al. introduced a high-tech method for cell authentication (10). In this method, the accuracy of 436 human cell lines were determined with a high sensitivity and specificity.

The present study indicated the effectiveness of the multiplex SYBR-Green PCR based amplification of mitochondrial Cyt b gene, in recognizing various individual and deliberately contaminated L20B and RD cell lines.

Acknowledgements

We acknowledge Iran National Polio Lab employees for their supports. This work was supported by School of Public Health, Tehran University of Medical Sciences. The authors declare that they have no conflict of interest.

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