Authentication of RBK (Razi Bovine Kidney) Cell Line Derived from Primary Kidney Cell

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Abstract

RBK cell line derived from primary bovine kidney cell was established and introduced by Razi Vaccine and Serum Research Institute. RBK cell line has been turned into continuous cell line through serial passaging of primary cell. On the basis of the experiments conducted, RBK cell line demonstrates high sensitivity to viruses. As cell line identification is crucial to confirmation of the experimental results, and can attest to the validity and credibility of such findings. Misidentification of the cell line, misdiagnosis of its contamination or lack thereof, can lead to unreliable results as well as waste of resources. Forecasting is Important

for the value of a cell because it can determine the cell line's origin. The more explanation of a cell line origin, that cell is more valuable. In addition, contamination is a serious concern for cell culture because contamination can significantly affect the physiology and viability of cell lines. Therefore, based on standard guidelines, it is necessary to implement different assays for contaminant free and confirming the identity of cell lines.

For identification purposes, verification and authentication of a cell line, various methods must be applied simultaneously to a particular cell line; so, in this study, purification and identification of RBK cell line was investigated by molecular tests such as PCR, PCR-RFLP and real time PCR based on mitochondrial DNA. The present study provides RBK cell line authentication; In addition, the results demonstrated the RBK is free of adventitious agents such as Bovine Leukemia Virus (BLV) and Bovine Viral Diarrhea (BVDV). In consideration of the RBK cell line capability to unlimited proliferation, stability and sensitivity to viruses, the present paper introduces this cell line as a reliable tool for viral research, which can contribute to numerous important scientific advances.

Keywords: authentication, bovine, cell line, identity, PCR, PCR-RFLP, real time PCR, primary cell, RBK.

1-Introduction

One of the most important scientific discoveries over the past century is Hela cell line introduced by Dr. George Otto Gey (1951), and has been used in various

scientific researches. From 1951 on, cell lines have become more noticeable, and have begun to be used considerably in different areas of research, scientific studies as well as diagnosis (1). Today they play a key role in biological experiments, which are often conducted using cell lines. Consequently, a large variety of cell lines have been derived from different sources and expand in vitro to diagnose different infectious or viral diseases.

As validity and characterization of a cell line, which originates from a primary cell or cell line, is necessary; one of the most important elements for determining identity is isoenzyme analysis and molecular methods, which are used to study and determine the intra-species origin of the cells dedicated to research and scientific work. These are methods authorized by the World Health Organization, and constitute the WHO guidelines for identifying different cells and cross contamination prevention. Modern clinical virology emphasizes fast and accurate identification of viruses to control infection or treat diseases. As such, cell substrate is extremely important and affects the success of experiments; therefore, standardizing the cell lines to produce and conduct virology experiments can prepare the ground for developing modern cell culture.

As during a research, precision in results and proper operation depend on cell lines in vitro, identification of cell lines and detection of cell contamination are crucial for scientific researches and replicability of results. Generally, choosing the appropriate cell line for research is essential to obtaining reliable and relevant results. (2).

Earlier, we stated that RBK cell line has a high proliferation capacity, as well as high stability as an infinite cell; furthermore, our findings revealed that the average doubling time of the RBK cell line was 21 hours (3). $\mu=0.6931/TD = 0.33$

As a matter of fact, RBK cell line showed high sensitivity to virus, and results demonstrated the RBK can serve as a proper cell line to identify such viruses. In particular, The RBK cell growth rate is much higher than Vero or MRC-5 cell lines (3, 4, 5, 6).

Our results suggested the potential of RBK cell line as proper cell line for virology research, serving as a valuable tool for diagnostic tests and experiments.

At this current study, in order to authenticate the RBK cell line, cell identity and purity were determined by different methods: DNA fingerprinting, PCR-RFLP and real time PCR (Figure 1, 2, 3, 4).

2-Materials and Methods

2-1-DNA Fingerprinting

Determination and choice of cells: For the present study, six cells were chosen with the following specifications: The cell line from bovines' kidney (RBK) and The Madin-Darby bovine kidney (MDBK); the primary cells from lambs' kidney (LK); the cell line from pigs' kidney (IBRS-2); the diploid cell from human embryo (MRC-5); the cell line from African green monkeys' kidney (Vero); and the primary cells from goats' lymphocytes (GL) (Figure 2). Five of these cells are monolayer, can grow and attach at surface of flask (the cells of goats' lymphocytes are in suspension).

PCR: The above cells were used to perform PCR on the DNA extracted. 0.5µl of the PCR Master Mix, which contains dNTPs, PCR buffer, Mgcl2, and Taq DNA polymerase, 0.5µl of the F primer, and 0.5µl of the R primer (according to table 1, species-specific primers used for amplification of a part of cytochrome c oxidase subunit I (COI) gene), 5µl of DNA, and for each, and up to 25ml for each micro tube, deionized water added to every sample of 12.5µl. The heat cycle to amplify COI gene in the thermal cycler machine is as follows: The initial denaturation step performed at 95°C for 5 min, and then 30 to 35 cycles, including three stages of denaturing, annealing and extension was done in 1 min, then again denaturation at 94°C for 1 min, the stage of annealing at 53°C for 50 sec, and the stage of extension at 72°C were all performed. The final stage, the extension step was performed for 10 min at 72°C. At the end, the PCR products were kept at 4°C and PCR products were electrophoresed.

2-2-PCR-RFLP

Amplifying part of the cytochrome b (cyt-b) gene: To determine the cross-contamination and the cell line fingerprinting through PCR-RFLP, part of the cyt-b gene was amplified using the reverse and forward primers designed by Parson, which target specific segment of DNA (358bp). To perform PCR on the extracted DNA of the RBK and MDBK cell lines were used. 0.5µl of the PCR Master Mix, which contains dNTPs, PCR buffer, Mgcl2, and Taq DNA polymerase, 0.5µl of the F primer, and 0.5µl of the R primer, 5µl of DNA, and for each, and up to 25ml for each micro tope, de-ionized distilled water were added to every sample of

12.5µl. To develop cyt-b gene, the heat cycle in the thermal cycler machine was set as follows: First, DNA was denatured for 5 min at 95°C, and then 30 to 35 cycles, including three stages of denaturation, annealing and extension was done in 1 min, again denaturation at 94°C for 1 min, then annealing stage at 53°C for 50 sec, and the stage of extension at 72°C for 10 min were all performed to complete polymerization.

Restriction enzyme digestion of PCR products: In brief, The 358 bp product of cyt-b gene of RBK cell line was purified and digested with 6 restriction enzymes (Table 2 and 3). The amplified products from MDBK used as control was also digested with 6 restriction enzymes (Figure 3).

In this experiment, first 10µl of the PCR product is poured into a 0.5ml micro tube. DNA density must be between 0.1 to 0.5 µg. Then 3µl of the buffer was added. Later, 16µl of distilled water was added to microtubes except HinfI microtube (16.75µl of distilled water was added to HinfI micro tube). Water was heated to 37°C, and kept at 37°C incubator. Then 1µl of MboI, Rsa, HaeIII and AluI enzymes was added to each of microtube from 1 to 4, and then 0.25 of HinfI enzyme was added to tube 5. All microtubes were placed at 37°C incubator for 16 hours. 1µl of TaqI was poured into microtube 6, and kept at 65°C incubator for 16 hours. The volume of the reaction solution was 30µl. Digestion results were revealed using the electrophoresis gel.

2-3-Adventitious viruses assay

Viral nucleic acid extracted from RBK cell was analyzed for detection of the adventitious viruses in cell line (especially BVDV and BLV) and contamination in the following way:

2-3-1-Assay for BVDV

RNA extraction: According to kit instructions.

Two-step RT-PCR: After extraction of the RNA, the two-step RT-PCR method was used to amplify 5′UTR gene, i.e. part of the BVD. Primers used are introduced in Table 1.

cDNA synthesis: In this stage, to provide cDNA from the extracted RNA for amplification of 5′UTR gene, i.e. part of the BVD for a volume of 10µl (2.25% distilled water, 2µl M-MuLV buffer (5x), 2µl dNTPs (10mmol), 10.5µl reverse, 10.5µl forward of the primers (10pmol), 10.25µl of RNase Inhibitor, 10.5µl of Reversed Replication Enzyme M-MuLV, and 3µl of RNA) were poured into a tube, and then the microtube was placed inside a thermocycler. Thermal cycle for amplification of 5′UTR gene of cDNA is as follows: First, at 42°C for 60 min., then 94°C for 4 min.

PCR: To amplify cDNA obtained we need PCR reaction for 20µl of every sample: 8.4µl of the PCR master mix, 6.0µl of forward primer, 6.0µl of reverse primer, 20µl of cDNA, and 8.4µl of unionized distilled water were added to each microtube (Enzyme used for amplification in this stage was Taq DNA Polymerase). The Thermocycler heat cycle for the second stage of the Two-Step RT-PCR reaction for amplification of 5′UTR gene, i.e. part of the BVD is as follows: 30 cycles including 3 stages of denaturation, annealing, and extension over 55 sec. Denaturation at 94°C

for 10 sec., annealing at 50°C for 10sec., and finally extension for 30 sec. at 72°C. Ultimately, another cycle of 72°C for 10 min was performed to complete polymerization. Then the PCR products were electrophoresed.

Real Time PCR for BVDV: To do this, master mix (RealQ Plus 2X Master Mix for Probe, High ROX Amplicon, Denmark) was used. 9 various dilutions of cDNA obtained from RNA (10^6 copies in each ml) were prepared. The amplification reaction at 20ml volume was done using Corbet 6000 Real Time PCR at the following thermal pattern, which includes: 10µl of master mix, 5µl of cDNA, which is obtained from serial dilutions, 1µl of initiator, 6µl of probe, and 2.4µl of water. Here is the thermal cycle: 95°C for 15 min., and then 45 cycles including: 95°C for 15 sec., and 60°C for 60 sec. The sequence of the primers and probe as well as the procedure was done in accordance with OIE 2018 presented in Table 1.

2-3-2-Assay for BLV

Real Time PCR method on the nucleic acid obtained was performed. The sequence of the primers and probe as well as the procedure was done in accordance with OIE 2018 (Table 1). Here is the thermal cycle: 94°C for 60 sec., then 35 cycles including: 94°C for 60 sec., 60°C for 60 sec and 72°C for 60 sec. Ultimately, 1 cycle of 72°C for 5 min was performed.

3-Results

3-1-DNA fingerprinting

In order to determination and choice of right cells, COI, species-specific gene was amplified to analyze cell identity and lack of contamination with other cells (7). By

using specific primers designed for bovine species, a 102bp fragment of COI gene was amplified (Figure 2).

3-2-PCR-RFLP

In the present study, cell line identification and confirmation of the RBK Cell Species were done using the PCR and PCR-RFLP methods based on mitochondrial cyt-b gene. The PCR-RFLP has identified the animal species origin with 100% accuracy. After a segment of DNA cut into pieces with restriction enzymes, the fragments were compared. At the same time, lack of contamination of the RBK cell line was analyzed (Figure 3).

Using the PCR-RFLP technique, through analysis of restriction sites (through enzymes mentioned earlier) and Mitochondrial DNA (mtDNA) length, we managed to determine cell line contamination or otherwise with undesirable cell species in addition to identification of the origin of the cell species. This study confirms the origin of RBK cell line (bovine species), revealing that it is contamination- free. By relying on the test under investigation, it can be concluded that cell line identity is confirmed.

3-3-Adventitious viruses assay

RT-PCR result shows lack of contamination of RBK cell with BVD or BLV viruses. Representative fluorescence acquisition graph showed, negative control, BVD and RBK (Based on OIE, positive sample must be Ct value< 40). In this assay, BVD virus was used as the positive control with Ct= 21.45. Negative control samples

must have no Ct value. Based on the results of the experiment, RBK determined contamination-free with BVD virus. Real time-PCR to analyze contamination RBK cell line with BLV (Number of copies is according to RT-PCR for BLV contamination). Based on the OIE, cell infected with BLV or positive control must be Ct ≤ 40.95. In this experiment, BLV virus sample and FLK cell (infected with virus) were used as positive control. Moreover, negative control must be Ct > 40.95. Due to absence of Ct in the RBK, this cell is BLV contamination-free (Figure 4).

4-Discussion

In order to authentication of a cell line, confirmation of its origin and free-contamination are important and crucial to forecast the value of a cell line. Therefore, based on standard routines, it is necessary to establish a cell line origin and its purification than cross-contamination and interspecific contamination (2, 3,11,12,13, 14).

There are numerous reports on using isoenzymes, which analyze cell isoenzymes as well as assurance of their cleanness from impurities at different stages. Isoenzymes analysis not only useful in identification and contamination detection of the cell lines, it also constitutes part of the tests for acceptance of new cell line. However, it would be better if isoenzymes analysis is associated with karyotyping or molecular techniques (15, 16).

mtDNA is a powerful tool for tracing generations through mothers. Its power to discriminate among different species; mtDNA is an appropriate tool for discovering

genetic relations among populations, intraspecific and interspecific that appears similar in various animal groups.

As cell lines are valuable models and essential resources for research, the more information in terms of the origin of a cell line is important. Moreover, DNA barcoding is one of the best methods in identifying cells, species, contaminations, and their identification of conserved genes that set the code for mitochondrial proteins (13, 14, 18, 19).

Therefore, in this project, we investigated a multifaceted molecular approach from a mitochondrial genome perspective. In the present study, first to identify the species, a 102bp fragment of COI gene, which contains conserved sequences, was used (Figure 3. A-1).

DNA fingerprinting is a reliable tool for rapid identification of cell cultures and tracing cross-contaminations among different species. By comparison of their DNA profiles of cells can serve as a useful tool for researchers to identify the origin of a cell (17, 18).

In this research, a part of COI gene of RBK cell line was experimented and analyzed using species-specific primers for 6 cells of: GL, Vero, MRC5, IBRS-2, LK and RBK (Table 1) simultaneously, and the identity of the RBK cell line and its lack of contamination with other species were confirmed (Figure 2).

PCR-RFLP is done on the basis of mitochondrial DNA markers. With this method, cut the mtDNA using restriction enzymes to create fragments of varying lengths to be rapid and reliable markers that can be a powerful and valuable tool for genotyping and genetic fingerprinting to guarantee the precision and reliability of

the cell line used in research. Moreover, PCR-RFLP is a different isoenzyme analysis system for confirmation of species origin and lack of interspecies contamination (20, 21).

Among intergenic regions of mDNA, genes of COI, COII and cyt-b are more often investigated. Between mitochondrial genes, cyt-b gene has multiple advantages in identification and discovering cross- contamination of cell lines (8, 22).

In the current study, cyt-b was used to assess the PCR-RFLP identification power of the bovine species and its sensitivity to identifying interspecies and cross-contamination. Based on the results obtained, the cell samples have no cross-contamination with other species (Figure 3).

Real-time PCR is one of the techniques that most widely used target amplification and was developed to identify cell lines among species such as: mammalian, human, drosophila, pig and chicken cell lines (23, 24, 25, 26).

Real-time PCR method is also useful in assessing cell lines with adventitious agents such as viruses. This method utilizes special primers and probes, can use its special features to target the intended section reliably, and it serves as a sensitive test for identification of intended items in diluted solutions (23). Overall, Real-time PCR is a reliable and economical approach that can validate all research and diagnostic activities (27).

In this research, RBK cell line was studied for contamination with BLV and BVDV using the Real-time PCR method. Our results indicated lack of contamination with the above-mentioned viruses (Figure 4).

Considering that one of the key factors in isolating viruses is multiplication of viruses in cell cultures; selecting the proper cell line with high sensitivity to intended virus is crucial extremely important to getting the most reliable results. As such, in this project, based on the research done and the results obtained, RBK cell line enjoys contamination-free by adventitious agents. Overall, our investigations confirm high capability of RBK to replicate and stability. Therefore, RBK cell line can be used widespread in research, diagnosis, virus proliferation as well as quantitative control of the virus (Titration).

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6-Author's contribution

Study concept and design: M. Lotfi. Acquisition of data: M. Maghami, M. Lotfi, A. Mohammadi & F. Sotoodehnejadnematalahi. Analysis and interpretation of data: M. Maghami & M. Lotfi. Drafting of the manuscript: M. Maghami & M. Lotfi. Critical reversion of the manuscript for important intellectual content: M. Lotfi. Statistical analysis: M. Maghami.

7-Ethics

All experimental protocols and experimental animals were approved by the Biomedical Research Ethics Committee and evaluated by Research Ethics Committees of Islamic Azad University-Science and Research Branch (Approval ID: IR.IAU.SRB.REC.1402.235 , Approval date: 2023-09-04), and all experiments were carried out in accordance with relevant guidelines and regulations.

8-Conflict of interest

The authors declare that they have no conflict of interests.

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