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Original Article

Identification of Iranian BHK-21-C5 Cell Line by Two Steps Polymerase Chain Reaction

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Abstract

Authentication of animal cell lines in cell banks is one of the most important programs regulated during cell culture and storage. This operation provides a thorough and beneficial document which can be advantageous for the functional use of animal cell lines. Therefore, various procedures are used to prevent misidentified cells, cross-contamination to other cell lines, and mislabeling errors leading to incorrect assessment. These contaminants can result in major financial disadvantages. One of the practical methods in this field is a molecular procedure which can demonstrate more accurate results. In the present study, the BHK-21 (C5) was characterized, and it was tried to determine the identity of BHK-21 (C5) as a continuous cell line by Polymerase chain reaction (PCR) molecular procedure in Iran. The cytochrome c oxidase I (CO1) gene was selected as a prevalent DNA fragment for the authentication of the BHK-21 (C5) cell line, along with six cell lines, including Chinese hamster ovary, Lamb kidney, Razi Bovine Kidney, Medical Research Council cell strain 5, Monkey Green Kidney, and Goat Lymphocyte. After amplification, PCR products were analyzed by agarose gel electrophoresis to ensure their accuracy. The results of characterization were indicated, cell viability was estimated to be about 92%, and a uniform cell culture was obtained. The doubling time and μ ratio equivalent were obtained at 20.5 h and 0.03, respectively. Sterility tests revealed that the cell seed was free of bacterial, mycoplasma, and mycobacterial infections. The results of molecular identification revealed that the identification of this cell line was approved and can be used in studies, diagnosis, production, and quality control of biological products.

Keywords: Authentication, BHK-21-C5 cell line, PCR, Cytochrome c oxidase I (CO1) gene

Identification de la Lignée Cellulaire Iranienne BHK-21-C5 par Réaction en Chaîne par Polymérase en deux Étapes

Résumé: L'authentification des lignées cellulaires animales dans les banques de cellules est l'un des programmes les plus importants réglementés pendant la culture et le stockage cellulaires. Cette opération fournit un document complet et bénéfique qui peut être avantageux pour l'utilisation fonctionnelle de lignées cellulaires animales. Par conséquent, diverses procédures sont utilisées pour empêcher les cellules mal identifiées, la contamination croisée avec d'autres lignées cellulaires et les erreurs d'étiquetage conduisant à une évaluation incorrecte. Ces contaminants peuvent entraîner des désavantages financiers majeurs. L'une des méthodes pratiques dans ce domaine est une procédure moléculaire qui peut démontrer des résultats plus précis. Dans la présente étude, la BHK-21 (C5) a été caractérisée, et on a essayé de déterminer l'identité de la BHK-21 (C5) en tant que lignée cellulaire continue par une procédure moléculaire de réaction en chaîne par polymérase (PCR) en Iran. Le gène

du cytochrome c oxydase I (CO1) a été sélectionné comme un fragment d'ADN répandu pour l'authentification de la lignée cellulaire BHK-21 (C5), ainsi que de six lignées cellulaires, y compris l'ovaire de hamster chinois, le rein d'agneau, le rein du bovin de Razi, Souche cellulaire 5 du Conseil de recherches médicales, rein vert de singe et lymphocyte de chèvre. Après amplification, les produits de PCR ont été analysés par électrophorèse sur gel d'agarose pour garantir leur précision. Les résultats de la caractérisation ont été indiqués, la viabilité cellulaire a été estimée à environ 92% et une culture cellulaire uniforme a été obtenue. Le temps de doublement et l'équivalent du rapport μ ont été obtenus à 20.5 h et 0.03, respectivement. Les tests de stérilité ont révélé que la graine cellulaire était exempte d'infections bactériennes, mycoplasiques et mycobactériennes. Les résultats de l'identification moléculaire ont révélé que l'identification de cette lignée cellulaire a été approuvée et peut être utilisée dans les études, la diagnose, la production et le contrôle de qualité des produits biologiques.
Mots-clés: Authentification, Lignée cellulaire BHK-21-C5, PCR, Gène du cytochrome c oxydase I (CO1)

1. Introduction

Animal cell lines are important resources for research and diagnostic applications (Liu et al., 2008). Cross-contaminated and misidentified cell lines during research continue as a major problem. Moreover, the use of cultures at high-passage levels contributes to the generation of inaccurate and misleading results (Hughes et al., 2007; Liu et al., 2008). Many laboratories that simultaneously cultivate various species of cell lines and conduct experimental medical and biological studies extensively use continuous cell lines as an appropriate model (Stacey, 2000). Therefore, it is indispensable to periodically monitor cell lines for identity and authenticity (Liu et al., 2008). Cell line identification has been carried out to specify the origination of various cell lines.

Apart from the recognition of the sources of different cell lines, the identification of any type of cross-contamination with other species requires authentication (Liu et al., 2003). A polymerase chain reaction (PCR)-based method seems to be an appropriate method for rapid identification and authentication of related cell lines (Liu et al., 2008). Therefore, the assessment of different species of animal cell lines and their authentication can be performed with the utilization of high accuracy PCR (Steube et al., 2003; Ono et al., 2007). Furthermore, this laboratory procedure is advantageous over conventional procedures since it requires rapid, low-cost, and minimal training for laboratory personnel (Liu et al., 2003).

The BHK-21 cell line was established in 1961 from the kidneys of 5 Syrian hamsters from litter number 21. Since then, this cell line has been a laboratory standard for the growth of countless viruses and the study of many biological processes (Hernandez and Brown, 2010). In the current research, it was strived to use a simple method for species identification and comparison. DNA barcoding system has been suggested as a promising approach for species authentication using the cytochrome c oxidase subunit 1 mitochondrial gene (cox1 or COI) as a standardized single molecular marker for the classification of animal species (Trivedi et al., 2016).

To ensure the non-contamination of the BHK-21 (C5) clone, it was necessary that the DNA of the clone, along with those of other cell lines, be used in PCR (Parodi et al., 2002). One of the DNA-based cell identity methods was approved in the present research. In this regard, to verify the identity of the BHK-21(C5) clone, the extracted DNA with a specific hamster primer and the DNA of five species cells with specific primers for Human, Monkey, Cattle, Sheep, and Goat were separately used in the PCR procedure.

It was expected that the desired fragment of DNA in each sample would be only replicated with its own specific primer. Consequently, after the electrophoresis of PCR products based on the presence or absence of the band and the size of the fragment, the origin of the

cell could be determined. In the PCR method used in the current study, the cytochrome C oxidase 1 (COI) was selected as a prevalent DNA fragment for the identification of the BHK-21 (C5) cell line. It exists in Iran and is used as a standard cell in research, diagnosis, quality control, and vaccine production.

2. Material and Methods

2.1. Cell Source

The present study made us of BHK-21(C5) continuous cell line (seed) that was preserved in the nitrogen tank of Razi Vaccine and Serum Research Institute. The frozen cell seeds thawing process was rapidly performed to minimize ice crystal growth. Solute gradients were formed as the residual intracellular ice melts by the placement of karyotype of received seed in a water bath of 37ºC.

2.2. Cell Culture

1.5 ml cell seed of BHK-21 cell line were transferred to the two 25-cm^2 cell culture flasks using a laminar flow hood with established sterile conditions. Subsequently, 8ml Dulbecco's Modified Eagle Medium (DMEM) was slowly added to the culture at the beginning and gradually a little faster diluting and nourishing the cells. In the next step, 1 ml of calf serum was added to the contents of each flask and incubated at 37ºC in 5% CO2 for 48 h (Freshney, 2011).

2.3. Cell Characterization

2.3.1. Viability

About 50 microliters of cell suspension were diluted by DMEM for counting the cells with neobar lam to assess cell viability (Strober, 2015).

2.3.2. Cell Morphology

The selected clone cell in cultured media was morphologically examined in terms of shape and arrangement of cells by an inverted microscope on a daily basis (Abercrombie, 1978).

2.3.3. Doubling Time

In order to calculate the cell doubling time, flasks were separately sampled twice (24-48 h), and counting was performed when cells were in a logarithmic phase based on the highest number of cells counted twice the counting interval (DeliveReD, 2012).

2.3.4. Homogeneity

The homogeneity or the quality and quantity of received cell seeds of the first and last cryovials after cultivating the cell seeds were investigated by cell shape and count (Freshney, 2011).

2.3.5. Sterility Tests

The cell seeds were investigated in a specific culture medium (Thioglycolate, TSB, PPLO, and Lowenstein-Jensen (LJ)) in terms of Microbial, Mycoplasma, and Mycobacterial contamination and at a specified time (Nema and Khare, 2012).

2.4. Cell Molecular Identification

2.4.1. Selection and Preparation of Cells

For this purpose, the BHK cell line was located along with six cell lines, including Chinese hamster ovary (CHO), Lamb kidney (LK), Razi Bovine Kidney (RBK), Medical Research Council cell strain 5 (MRC5), Monkey Green Kidney (Vero), and Goat Lymphocyte (GL). Each cell line was separately cultured in DMEM medium with 10% calf serum and kept in an incubator at 37°C with 5% CO2 for 24-48 h to achieve an exponential growth rate. All of these cell lines were anchorage-dependent, except for the GL cell line which is round and loosely attached. In the next phase, monolayers were trypsinized using trypsin 0.25% (sigma) and prepared high-concentration cell suspension with DMEM media (sigma). After transferring trypsinized cells and none-trypsinized GL to the centrifuge tubes, all of them were centrifuged at 1200 rpm for 5 min. Thereafter, all of the cellular sedimentations were washed three times with Phosphate-Buffered Saline (PBS) and then prepared for DNA extraction (Ramya et al., 2009).

2.4.2. DNA Extraction

In order to extract DNA from the ultimate cell clone, a DNA extraction and purification kit (Cinnagen, Tehran, Iran) was used according to the manufacturer's instructions.

2.4.3. Primers

Specific primers of the species were designed (Cooper et al., 2007) and ordered from Takapouzist company

for amplification of sequences of the cytochrome C oxidase I gene (COI). The validity of these primers was confirmed by Blast in the Gene Bank. The sequence of primer base was according to Table 1. Other species primers described in Table 2 were placed alongside the Hamster primer.

Table 1. Species-specific primer rows (Cooper et al., 2007)

Species	Sequence
Cricetulus griseus (ch.Hamster)	Cg-F- ACTAACCCGCTTCTTCGCATTC Cg-R- GCGTAGGCGAACGGAAGTATC

Table 2. Rows of other species primers (Cooper et al., 2007)

Species	Sequence $3'to5'$	Size (bp)
Capra hircus(goat)	F- ATATCAATCGGGTTTCTAGGATTTATT R- AGTTGGGATAGCGATAATTATGGTAGT	117 (bp)
Bos taurus (cow)	F- GCTATTCCAACCGGGGTAAAAGTC R- GAAAATAAAGCCTAGGGCTCAC	102(bp)
Ovis aris (sheep)	F- CGATACACGGGCTTACTTCACG R- AAATACAGCTCCTATTGATAAT	267(bp)
Homo sapiens(human)	F- TAGACATCGTACTACACGACACG R- TCCAGGTTTATGGAGGGTTC	391(bp)
Cercopithecus aethiops (Gr. Monkey)	F- CTTCTTTCCTGCTGCTAATG R-TTTGATACTGGGATATGGCG	222(bp)

2.4.4. PCR Amplification

Cinna gene kit was used to carry out PCR. In this regard, for each sample of 12.5 µl of the main mixture of Taq PCR Master Mix contains Taq DNA Polymerase, MgCl2, PCR Buffer, and dNTPs, 0.5 µl of forward primer and 0.5 µl of reverse primer, 5µl of DNA for each of them, and non-ionized distilled H2O was added to each microtube up to 25 ml.

In order to investigate the probable contamination of cell clones to other cells, six microtubes for BHK-21 (C5) cells were inserted separately with each of the six pairs of specific primers. For each cell, MRC5, LK, RBK, Vero, and GL were regarded as a microtube with a specific primer of any species, respectively. To control the Hamster primer, the CHO cell with Hamster source was used as a positive control. The microtubes

were centrifuged and transferred to the thermocycler, and the PCR reaction temperature cycle program was used to amplify the COI gene according to Table 3 (Cywinska et al., 2006).

Table 3. PCR reaction temperature cycle

Number of cycles	Time	Temperature(˚C)	Reaction	step
1	5 minutes	95	Denaturation	1
30-35	1 minute	94	Denaturation	2
	50 seconds	53	Annealing	
	50 seconds	72	Extensionor Elongation	
-	10 minutes	72	Extensionor Elongation	3
-	-	4	Maintenance	4

2.4.5. Electrophoresis

Fifty milliliters of 2% agarose gel solution was prepared. To this end, 1 gr of agarose powder and 50 ml of TAE 1X solution and SYBR Safe color was poured and heated to the degree that all the agarose crystals in the buffer were dissolved. Over time, the gel was fully polymerized at room temperature, and the agarose gel was completely closed. After the placement of the gel in the electrophoresis tank, 10 µL from each of the PCR products were mixed with 2 µL of loading buffer 6x and injected into the wells with speed and accuracy. Moreover, a well of gel was dedicated to pouring 5 µl of marker DNA (Lee et al., 2012).

2.4.6. DNA Product Analysis

After electrophoresis, the gel was transformed into a gel documentation system, and the DNA product was analyzed.

3. Results

3.1. Cell Characterization

Cell viability was estimated to be about 92% (Figure 1). After BHK-21 C5 cell culture, uniform cell culture was obtained (Figure 2). The cells were examined in a logarithmic phase and counted at regular intervals. The doubling time and µ ratio equivalent were obtained at 20.5 h and 0.03, respectively. Sterility tests revealed that the cell seed was free of bacterial, mycoplasma, and mycobacterial infections.

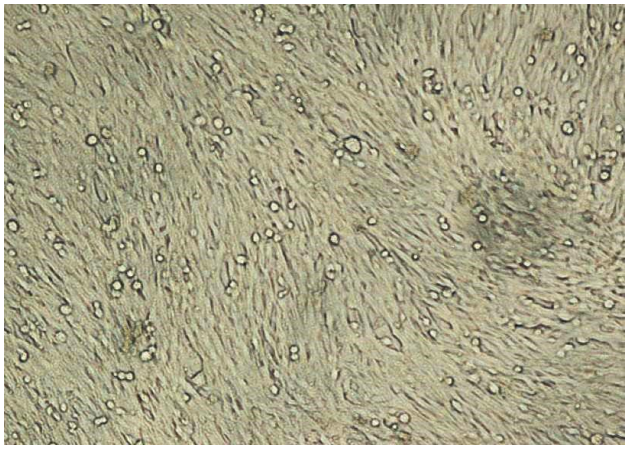

Figure 1. Monolayer of BHK cell (10X)

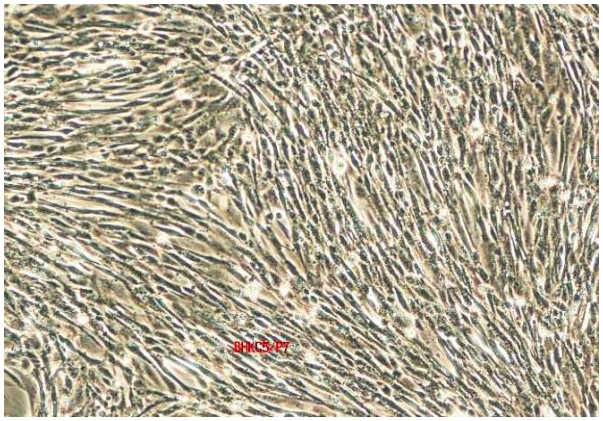

Figure 2. Homogeneity and uniformity of BHK cell (100X)

3.2. Cell Molecular Identification

The cell authentication was identified by amplification of the specific gene of species in terms of no variation in cells and absence of contamination in the cell bank. Figure 3 displays the gel electrophoresis of PCR products. DNA extracted from BHK-21 (C5) cell were amplified only with a primer designed for hamster strain. It was found that the amplified fragment length was approximately about 315 bp (lane 1), compared to the marker. Lane 2 demonstrated CHO cell lines with hamster primer (negative control). In addition, no band was observed in lanes 3-7 of DNA extracted from BHK-21 (C5) cell with primers of cell lines from five other species. Figure 4 illustrates the DNA extracted from each of the cell lines with their own specific primers, and the band size for each of the cell lines was specified next to the DNA marker.

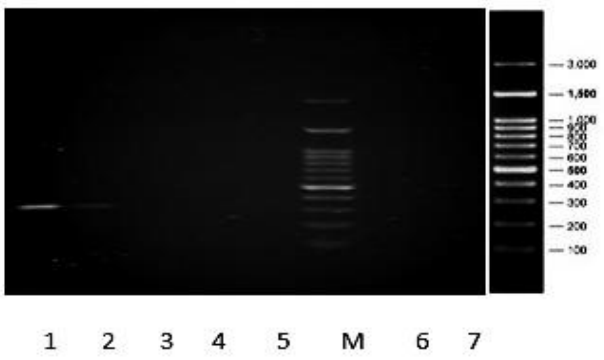

Figure 3. Electrophoresis of PCR products for mitochondrial cytochrome oxidase 1 (CO1) gene amplification in BHK-21 (C5) cell

Lane 1. BHK cell with hamster primer 315 bp**, Lane 2.** CHO cell with hamster primer, **Lane 3.** BHK cells with sheep primer, **Lane 4.** BHK cell with goat primer, **Lane 5.** BHK cell with monkey primer, **Lane 6.** BHK cell with human primer, **Lane 7.** BHK cell with bovine primer

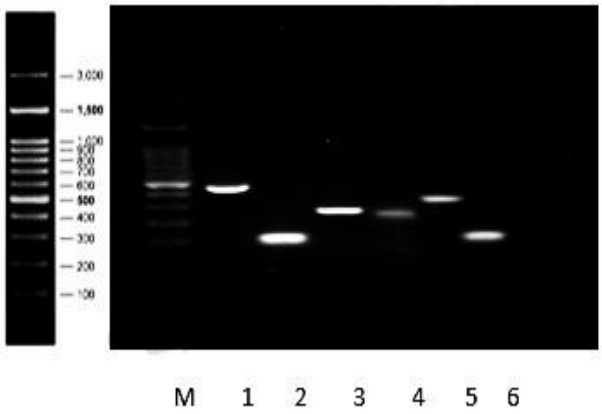

Figure 4. Electrophoresis of PCR products for the amplification of cytochrome oxidase (CO1) gene in different cells

Lane 1. PAE control cell with pig primer 460 bp, **Lane 2.** RBK cell with bovine primer 102 bp, **Lane3.** LK cell with 267bp sheep primer, **Lane 4.** Vero cell with 222 bp monkey primer, **Lane 5.** MRC5 cell with human primer 391bp, **Lane 6.** Goat lymphocyte cell with goat primer 117bp

4. Discussion

In biomedical research, cell lines are widely used as laboratory models. The identity of the cell line often determines the validation of the obtained data since it can be used as a substitute for the tissue of origin (ASN, 2010). A major problem posed to scientists in the field of quality control department over half a century has been the high frequency of inaccurate detection of cell lines and consequent inaccuracy of cell line sources. Moreover, this problem results in inaccurate identification of cross-contamination and culture of contaminated cells (ASN, 2010). In order to conduct research and clinical activities, it is necessary to ensure the quality and safety of the cell lines available in the cell banks (Cabrera et al., 2006). Nowadays, different methods are used to identify cell lines, such as karyotype analysis, immunoassay, isoenzyme analysis, **aloenzyme immuno-cytochemical technology**, DNA fingerprinting, and profile replication (Liu et al., 2008).

Two-step PCR was performed for the rapid identification of 14 cell lines from animal species by the mitochondrial ribonucleic acid gene. In the first

step, DNA fragments were amplified with universal pairs of primers, and in the second step, the amplification of DNA fragments was performed with specific primers of the previous step (Ono et al., 2007). The nested PCR technique was used to ensure the identity of the utilized cell lines and their use as cellular substrates for vaccine and bio-drug production. Mitochondrial DNA (Mt DNA) cytochrome b (Cyt b) gene which is more sensitive to the detection of contaminants at low concentrations was utilized (Ramya et al., 2009).

A PCR-restriction fragment length polymorphism (RFLP) assay was optimized. In order to identify cross-contamination and misidentification of cell lines in the cellular banking system, the PCR method was used to identify contaminants and significant differences between human and non-human cell lines (Mohammadi et al., 2016). In a similar study, 13 Combined DNA Index System (CODIS)-based short tandem repeats (STRs), along with gender determination gene, were used to identify 100 human cell lines in the National Cell Bank of Iran using the molecular PCR method. The obtained STR profiles were compared with other biosources of cell banks, and about 18% cross-contamination was observed in cell lines (Azari et al., 2007).

Some experts are of the belief that one of the precautionary measures that protect against the extension of incorrect cell lines is the development of a passing document that proved its authentication and history (Drexler et al., 2003). Validity testing is an obligatory necessity before and during the utilization of cell cultures (Steube et al., 2008). As an international cell repository, the present study aimed to provide a reproducible, rapid, and simple PCR method for the identification of animal species of cell lines most commonly used in cell culture laboratories(i.e, human, mouse, rat, and hamster) (Masters, 2000; Drexler et al., 2003).

Most cross-contamination in cell lines is caused by inferior cell culture due to the unintentional combination of two cell lines into a similar culture. In so doing, one of the cell lines gets replaced with another one after several passages due to differences in the growth rate of one of them. In addition, incorrect labeling of cells before the cryopreservation stage, which may occur during lab works, can bring about irreparable consequences. Therefore, an appropriate quality control investigation can be effective (Masters, 2000). The mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bioidentification system for animals (Hebert et al., 2003).

Continuous cell lines can also be used as cell models in biological production and research. Moreover, the unlimited proliferation characteristic of these cells will be only possible if the proper growth and seed conditions of the cell are gathered; therefore, they must be fully authenticated before usage. Consequently, in the present research, BHK-21 (C5) cell line was selected as continuous cell lines to be used in research, vaccine production, and vaccine quality control. Therefore, the cells should be characterized before storage in the cell bank. Authentication as an obligatory requirement before cryopreservation is one of the experiments in this regard.

The presence of cross-contamination with other cell lines and lack of proper reporting of cell line authentication can raise serious doubts about the results of studies and activities performed on the cell line. Cross-contamination in the present research signified this cell line is not misidentified with another one and is not polluted by microbiological agents since the financial consequences of them can be enormous. According to studies, PCR is one of the best molecular methods performed along with five other cell types using species-specific genes (COI).

It is important to compute the quantity of DNA required for PCR so that the determinate quantity produces non-specific products (other DNA fragments except the desired portion). Moreover, the low quantity of DNA samples reduces the accuracy of PCR reaction or it might be non- amplification of the desired fragment. The quality of the DNA sample is also

important so that the remaining compounds used in the DNA extraction step, such as phenol and EDTA, reduce the activity of the Taq-polymerase enzyme and fail to achieve the proposed result. Furthermore, the contamination of PCR with very little quantities of DNA from any other source may lead to the production of unexpected fragments due to the extreme sensitivity of this technique (Rahikainen et al., 2016).

Therefore, DNA was extracted from BHK-21 (C5) cell line for the amplification of cytochrome oxidase gene species, to verify cell identity with specific primers from other cell lines. The accuracy of nucleotide sequences of these primers was investigated by Blast analysis. Furthermore, the absence of cross-contamination with other cell lines was demonstrated. In addition, since the cytochrome oxidase C gene differentiates cell lines from each other, each of the cell lines with their own primers was examined in separate wells to properly validate the nucleotide sequences of each cell line with its own primers. Previous studies conducted in Iran have not proved the validity of this cell line when stored in a cell bank. Therefore, it has been attempted to make sure that its identity is used as a suitable cell line for research, diagnostic studies, vaccine manufacturing, and quality control.

Authors' Contribution

Study concept and design: S. S.
Acquisition of data: F. Z.
Analysis and interpretation of data: S. S.
Drafting of the manuscript: F. Z.
Critical revision of the manuscript for important intellectual content: S. S.
Statistical analysis: S. S.
Administrative, technical, and material support: S. S.

Ethics

We hereby declare, all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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