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Original Article

Evaluation of BHK-21 Cell Line for Specific Viruses by Two Different PCR Methods

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Abstract

The selection of suitable cell cultures for use in the biological industry as well as research and diagnostic studies is critical. One of the factors affecting cell culture that can affect the results of studies is the contamination with viral agents. Therefore, efforts to preserve the health of cell cultures from contamination sound logical, and the use of virus-free cells is vital in research and diagnostic studies as well as in the manufacturing industries. For this purpose, it is crucial to use fast and correct diagnostic methods to detect the presence of critical viral contaminants in cells. Moreover, the use of susceptible diagnostic methods is also doubly important, especially in the case of contaminants that may remain hidden. Therefore, in this study, the BHK-21C5 cell line, one of the most widely used cells in the production and quality control of biological products and virological studies, was examined in terms of contamination with the most important viruses such as BVD and BLV. Detection of possible contaminants by using two-step RT-PCR to detect the 5′ UTR portion of the BVD virus. Moreover, Nested PCR was carried out to detect the BLV virus using the gp51 *env* gene region. Also, an Flk cell line and Hela cell line that were consistently contaminated with the BLV virus were used as positive controls in Nested PCR. Due to the absence of bands in the BHK-21C5 cell column and the bandwidth observed in the positive control column (BVDV) in the range of 283 bp, non-contaminating of the cell clone with the BVD virus was proved. Also, no band was observed in well related to BHK-21C5 cell, and no cell clone contamination with BLV was confirmed, and in wells related to the positive controls (Flk and Hela cells), the bands have seen in the range 444bp. So, the results showed that no obvious or covert viral contamination effect was observed in the cell clone studied. Hence, the use of this cell line seems unobstructed in the quality control and production of biological products and research and diagnostic studies.

Keywords: Cell, Viral Contamination, Nested PCR

1. Introduction

The cell cultures are used as an appropriate tool in research and diagnostic studies and in identifying drugs, production, and quality control of biological products (1). The importance of maintaining cells in a high-quality situation is substantial because, in the final products, sterility, purity, and even tumorigenicity are essential and, as the result of research studies, can be affected by the purity of cell cultures (2). One of the most important affecting the quality of cell cultures is the cross-contamination of the cells. Cell culture contamination can waste time, money, and efforts to develop the culture. In addition, these contaminants can lead to errors and incorrect results. Among cell culture contaminants, viruses are highly influential factors in cell quality. These agents can cause useless remedial products and potentially contaminate humans. Also, the prevalence of viral contaminations in cell cultures is

not unexpected. The determined regulations by utilizing more sensitive procedures aim to diminish the risk of viral contamination in useable raw materials (3).

According to previous studies, the most critical viral contaminants in cell cultures and the most effective agents are bovine viral diarrhea (BVD) and bovine leukemia viruses (BLV). BVDV and BLV viruses are members of the pestivirus and deltaretrovirus genus, the most critical viral agents that can cause economic losses worldwide (4). BVDV with non-cytopathic effects is considered a significant problem for fetal bovine serums in cultures media in cell cultures (5). The BLV virus is one of the viruses that can integrate and hide in the host cell genome. Hence, it is not possible to identify it by conventional methods. Also, the BLV virus can be transferred through natural and fresh animal products or human biological products produced using cell substrates (6).

Accordingly, some specified approaches should be attended to check for particular viruses that may exist in cell substrates (7). Therefore, finding a sensitive method to detect these viruses in cell cultures is significant. Amongst the beneficial diagnostic methods that can be handled in this context, PCR appeared to be one of the optimal and high-sensitivity procedures for this purpose (8). So, we can mention different practical PCR with their protocols and unique designs to detect contamination of cell cultures. BVD genome contains untranslated regions (UTRs) at both ends (5′ and 3′ UTRs). Pestivirus 5′-UTR includes several short ORFs of unknown function and has been predicted to form a highly structured RNA element that may serve as an internal ribosome entry site to initiate cap-independent translation of ORFs. The 5′-UTR is particularly attractive for viral differentiation because it is highly conserved among members within each pestivirus species (9). The BLV envelope (*Env*) glycoprotein, consisting of the gp51 outer membrane glycoprotein and the gp30 transmembrane glycoprotein, is directly involved in infectivity events, like the p24 major structural protein, which can elicit a robust immune response in infected cattle. The envelope glycoproteins of BVL play a crucial role in the virus life cycle; envelope proteins are responsible for cellular tropism because they contain the recognition site for the cell surface receptor required for virus entry and are the natural target for neutralizing antibodies (10).

BHK cell culture is one of the most important and widely used cells in research and diagnostic virological studies as well as in the industries of production and quality control of biological products, so this cell was selected to evaluate the presence of viral contaminants in cell cultures. So, Biological evaluation using high-precision control methods, careful checking, and control of the material can ensure the accuracy of the results obtained in experiments (11, 12).

Therefore, this study was designed to introduce two different PCR methods to evaluate critical viral contaminants in cell cultures and to evaluate cell cultures in laboratories working with a particular clone of BHK-21 cells.

2. Materials and Methods

For the assessment of the BHK-21C5 cell for two of the most critical viral contamination, BVD and BLV viruses, the following steps were taken (13):

2.1. BHK-21 C5 Cell Culture Preparation

In this study, the master cell of the BHK-21(C5) cell line was preserved in the nitrogen tank of the Razi Vaccine, and Serum Research Institute was used. This cell clone (C5) was prepared in our previous study and had excellent cellular properties. The frozen cell thawing process was performed rapidly to minimize ice crystals. Generation of solute gradients formed as the residual intracellular ice melted by placing a cryo tube in a water bath of 37 ° C. 1.5 ml cell seed of the BHK-21 cell line was transferred to the two 25 cm^2 cell culture flask; subsequently, 8ml DMEM media were added to culture slowly 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 37 ° C in 5% CO2. After cell monolayer formation, the cell is harvested,

centrifuged, and aliquoted in microtubes for the next step (2).

2.2. Evaluation of the Cell for BVDV

Before the beginning of the test, the microtubes and the sampler tips were immersed in the DEPC solution. The solutions were prepared according to the kit (Cinaclone) instructions as follows:

Polyadenine vials were dissolved in 0.4 ml of elution buffer, split into 50 µL volumes, and then transferred to the -20° C freezer. During the use, one of them melted with 5 ml of mixed binding buffer and was used. 20 ml and 40 ml of pure ethanol were added respectively to the inhibitor buffer vial and one of the wash buffer vials and were mixed well.

For RNA extraction from the BHK-21 C5 cell sample, the Nucleic Acid High Pure Extraction Kit (Cinaclone) was used. First, the microtube containing BHK-21 C5 cell clones was shaken. Then based on commercial kit procedures, the RNA was extracted and transferred to the freezer at -70 ° C (14).

After RNA extraction, two-step RT-PCR was used to amplify a part of the 5′ UTR gene of the BVD virus. The primers used are listed in table 1 (15).

Table 1. Primers amplifying fragments of the 5′ UTR gene of BVD virus by RT-PCR

No.	Gene title	Primer title	Primer Sequence 5′→3′	Expected fragment length (bp)
1	5′ UTR	BVDco-F	CAT GCC CAT AGT AGG AC	283
		BVDco-R	CCA TGT GCC ATG TAC AG	
2	5′ UTR	BVD1a-F	TCG ACG CCT TRR CAT GAA GGT	169
		BVD1a-R	CCA TGT GCC ATG TAC AG	
3	5′ UTR	BVD1b-F	TCG ACG CTT TGG AGG ACA AGC	169
		BVD1b-R	CCA TGT GCC ATG TAC AG	
4	5′ UTR	BVD2-F	CGA CAC TCC ATT AGT TGA GG	105
		BVD2-R	GTC CAT AAC GCC ACG AAT AG	

R: Reverse oligonucleotides F: Forward oligonucleotides

The purpose of this step was to prepare the cDNA from the purified extracted RNA for the portion of 5′ UTR, the BVD virus. For this purpose, primers that were lyophilized in powder form were dissolved with the amount of water mentioned in the brochure. Then, the required materials were poured into the tubes as follows, 2.25 µlit distilled water, 2 µlit M-MuLV buffer(5X), 1 µlit dNTPs (10 mmol), 0.5 µlit Primers (10 picomols), 0.25 µlit RNase inhibitor, 0.5 µlit M-MuLV reverse transcription enzyme, and 3 µlit RNA. Next, the microtubes were placed in a thermocycler. The thermocycler thermal program was 6 minutes at 56 $^{\circ}C$ and 4 minutes at 94 $^{\circ}C$ (16).

In order to amplify the cDNAs obtained in the previous step, the PCR reaction with the materials and values (8.4 µlit distilled water, 8.4 µlit Master mix, 0.6 µlit Primers (10 picomoles), and 2 µlit cDNA) was performed in a final volume of 20 µl according to the thermal program in table 2. The enzyme used for amplification at this stage was Taq DNA Polymerase (17).

Table 2. The thermal, thermal cycler program the second stage of the two-step RT-PCR reaction to replicate parts of the 5′ UTR gene of the BVD virus

Time	Temperature (C)	Number of cycles
10 s	94	30
15 s	50	30
The 30s	72	1
10 min	72	1

After that, 2% agarose gel was prepared and placed in TAE1X buffer. Ten microliters of each PCR product were mixed with 2 microliters of loading buffer 6x and were carefully injected into each gel well. The ladder100 pair (100 bp) was used, and 5 microliters of it were injected into one of the wells. The device voltage was set to 100 v. The gel documentation system was used to evaluate the result (18).

2.3. Possible Contamination of Cell Clones with BLV

Cinnagen DNA extraction kit was used to extract the cell DNA. Four pairs of primers have been ordered (Cinnaclone) to perform two Nested-PCR reactions to proliferate the gp51 *env* gene of the

BLV virus, according to table 3. The whole reaction was performed in aliquots of 50 µl, composed of 1 µl of the sample and the 49 µl reaction mixture. Two pairs of outer primers were used to amplify the specified sequence in the first PCR. The thermal thermocycler program for the Nested PCR reaction to replicate was as follows: Initial denaturation at 94 ◦C for 9 min, and the following steps were repeated 40 cycles, denaturation at 95 ◦C for 30 sec, annealing at 62 ◦C (outer primers) or 70 ◦C (inner primers) 30 sec for the first and second step and extension at 72 ◦C for 60 sec. The last step was a final extension at 72 ◦C for 4 min. For the second step, 1µl of the amplified specified sequence from the first amplification got the target of inner pairs of primers for annealing. The amplification of the portion about 444 bp related to gp 51 of the *env* gene was the purpose of the second PCR (19). In addition, an Flk cell line that is consistently infected with the BLV virus and a Hela cell line sample were used as positive controls. Therefore, DNA extraction of the BHK-21C5 cell, polymerase chain reaction (PCR), electrophoresis of PCR products for DNA observation, and purification of PCR products from electrophoresis gel was done in order (20).

Table 3. Primers amplifying fragments of the *env* gene of BLV virus by Nested PCR

No.	Gene title	Fragment	Primer sequence 5′→3′	Expected fragment length
1	*env*	*env* 5032-F	TCTGTGCCA AGTCTCCCA GATA	598 bp
		env 5608-R	AACAACAAC CTCTGGGAA GGGT	
2	*env*	*env* 5099-F	CCAACAAGG GCGGCGCCG GTTT	444 bp
		env 5521-R	GCGAGGCCG GGTCCAGAG CTGG	

3. Results

3.1. Possible Contamination Test of Cell Seeds to BVD Virus

After extracting the RNA from the BHK21C5 cell, preparation of the cDNA was performed to propagate the 5′ UTR portion of the BVD virus by two steps of RT-PCR. Then the produced cDNA was amplified by handling PCR and Taq DNA polymerase enzyme. In RT-PCR, no band in the BHK-21 C5 cell line column was obtained compared to the DNA marker and positive control. According to figure 1, due to the absence of bands in the BHK-21C5 cell column and the bandwidth observed in the positive control column (BVDV), non contaminating of the cell clone with the BVD virus was proved.

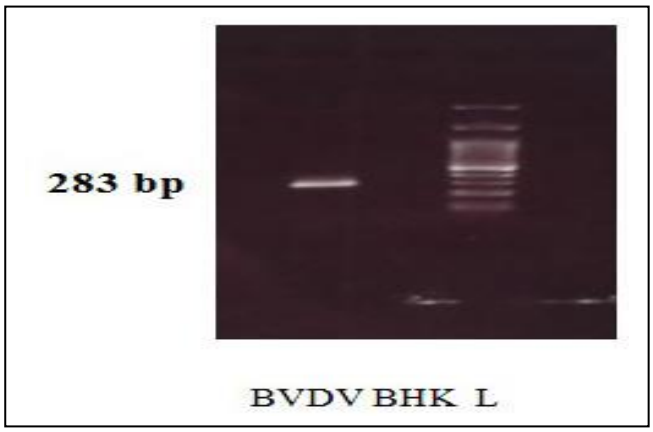

Figure 1. RT-PCR product electrophoresis BHK-21C5 cell clone extract

3.2. Possible Contamination Test of Cell Clone to BLV

Two Nested PCR reactions identified the BLV virus to proliferate the gp51 *env* gene region. The electrophoresis confirmed the purity of this cell line. In Nested PCR, no band was obtained from the propagation of the gp51 portion of BLV virus in this cell line column. As shown in figure 2, no band was observed in well 3 related to BHK-21C5 cell, no cell clone contamination was confirmed, and in wells 1 and 2 related to the positive controls (Flk and Hela cells), the bands seen in the range 444bp.

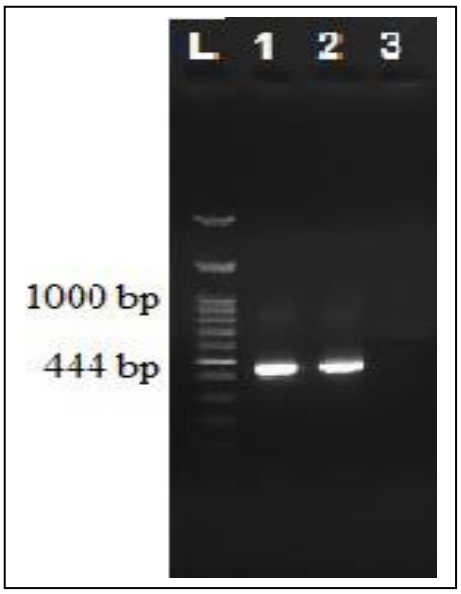

Figure 2. Electrophoresis of Nested products of Nested-PCR reaction to the proliferation of the 444 bp of Gp51 of *env* gene BLV: L: molecular weight marker 100 bp 1: FLK cell 2: Hela cell 3: BHK cell

4. Discussion

Among the viral contaminations, some specific viruses, such as BVD and BLV viruses in some contaminated cell cultures, have a non-cytopathic effect and, without changing the morphology or the rate of cell proliferation, can be propagated (21, 22). Gene fragments of this BVD virus, such as gag in DNA extracted from human body tissue, have been seen in contamination cases (23). Amongst the detection method of viral contaminations in cell culture, we can mention electron microscopy, retroviral reverse transcriptase, and nested PCR (13). In recent years, most of the findings related to the molecular biology of pestiviruses have been obtained using new methods (12). PCR transcriptase and indirect immunofluorescence detected BVD virus envelope glycoprotein in cell strains and fetal calf sera. The results showed levels of the BVD virus RNA genome in samples. This study by Real-time RT-PCR tried to detect 5′-UTR BVDV-1 in aerosol samples. The results represented a considerably higher positive detection rate of this virus than the conventional RT-PCR (24). Since the number of copies of the BLV virus is lower than the host genes, the use of high-sensitivity PCRs like Nested PCR seems logical (6). In 2020 Corredor-Figueroa, Salas (25) worked on identifying the existence of the BLV virus genotype in Jordan by using a portion of the *env* gene during the PCR and RFLP method and obtained a new genotype. In this study, they worked on the prevalence and molecular epidemiology of the bovine leukemia virus. Gene segments including *env*, gag, and tax were amplified. The results showed that these genotyping approaches were beneficial in determining the BLV genetic variability and diversity (25). In 2014 Buehring, DeLaney (26), Western blot analysis detected the structural proteins of gp51 and p24 virus in exosomes. The results demonstrated that the exosome as an intermittent pathway could play a role in virus contamination. This study on the determination of the BLV virus in human blood and antibodies produced by it via PCR and DNA sequencing. The results showed significant information about the transmission of the virus to humans and its prevention (26). Therefore, in this study, we tried to investigate the purity of the BHK-21C5 cell line contamination with some specific viruses. Among the viruses that often contaminate the cell culture media covertly and also have difficult identification in directly detecting, we could be mentioned to BVD and BLV viruses. For this purpose, the 5′ UTR portion of the BVD virus is propagated by two steps of RT-PCR. Then the produced cDNA was amplified by handling PCR and Taq DNA polymerase enzyme.

In RT-PCR, no band in the BHK-21 C5 cell line column was obtained compared to the DNA marker and positive control. Two Nested PCR reactions were used to proliferate the gp51 *env* gene region to identify the BLV virus. Because each of these procedures, according to their high sensitivity in evaluation, could show contaminants. Either way, the electrophoresis confirmed the purity of this cell line. In Nested PCR, no band was obtained from the propagation of the gp51

portion of the BLV virus in this cell line column. Since these viruses are often transmitted from animal products used in cell cultures, such as serum and animal raw materials, it is necessary to use methods that lead to the destruction and inactivation of viruses. Efforts always to perform sterility tests before using the material and use high-sensitivity viral diagnostic methods after using them have a significant impact on preventing contamination in biological products.

Authors' Contribution

Study concept and design: S. S. and F. Z.
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: F. Z.
Administrative, technical, and material support: S. S.

Conflict of Interest

The authors declare that they have no conflict of interest.

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