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Detection of Mycoplasma Contamination in Adipose Tissue Derived Mesenchymal Stem Cells

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Abstract

Problems: Contamination of cell lines and biological products is one of the major problems of cell culture techniques. Rapid detection of mycoplasma contamination in cell culture is an important part of controlled laboratory. In order to existence of infected cells, leading to unreliable results, the need for a reliable method for laboratories is unavoidable. Polymerase chain reaction (PCR), a technique of rapid, sensitive and specific detection of bacteria is considered as a precise method to detect contamination. The aim of this study was to evaluate the efficacy of PCR in the detection of contaminants in cell cultures and other biological products.

Experimental approach: In this study, PCR techniques were optimized by use of MGSO and GPO-1 primers and16SrRNA target gene. Also the utilized PCR method was evaluated in terms of sensitivity and specificity. Finally, a simple DNA extraction and PCR of 164 cell culture of adipose tissue derived mesenchymal stem cells were done.

Findings: A 715 bp product was amplified by the primers and was confirmed by sequencing. The Reviews feature, with none of the tested DNA was amplified products. This method has a sensitivity limit of 10 copies of the target DNA. No cross-reactivity with genomic DNA of other microorganisms was observed.

Conclusion: The results of this study indicate that molecular methods based on polymerase chain reaction (PCR) have great importance due to their innate features. The observed results in this study are based on accuracy, speed, sensitivity and high specification of PCR technique according to conserved and common sequence of 16srRNA to detect Mycoplasma contamination in cell cultures.
Keywords: ***Mycoplasma, PCR, contamination, molecular detection, cell culture***

Introduction

Mycoplasma are one of the newest contaminants of biological products and cell culture, and are consider as the destructive factor for biological results. These bacteria belongs to the Mollicutes class and is one of the smallest free-living microorganisms capable to self- replicating. (1-4). Because of the small size, Mycoplasmas can pass through 0.22 μm and 0.45 filters which normally are used for sterilizing cell culture reagents. Also they consist of just a cell membrane (without a cell wall), ribosomes and a 580kb genome. (5-6). Mycoplasmas are often slow-growing contaminants normally presenting in small numbers, but they may cause some problems in cultured cells (5-10).

Mycoplasmas have been identified as the contamination Source in most of the animal cell cultures (11). Identifying of mycoplasma contamination is an important part of quality control section in cell culture laboratories. Mycoplasma contamination of cell cultures is a problem that is seen in most cell culture laboratories that may remain unknown for a long time and subsequently affect cell proliferation, gene expression, and other cellular responses. (5). Mycoplasma contamination in cell cultures which are used in various researches will create many problems. In most cases, visual or microscopic identification of the pollution is impossible. Although mycoplasms affect cell metabolism and growth in culture medium, protein synthesis, secretion of cytokines, and even damage to DNA and RNA , they do not cause visible damages in cells. Mycoplasma contamination can be transmitted from bovine serum, laboratory staff, other contaminated cultures or cells taken from animals (1-2). The most identified Mycoplasma species in contaminated cell cultures include: Mycoplasma fermentans,

Mycoplasma hyorinis, Mycoplasma arginini, Mycoplasma orale and Achoplasma laidlawi (12-14). Mycoplasmas detection can be done by means of direct culture techniques of the organisms and some indirect assays such as: 1) DNA staining with the fluorescent dye 2) hybridization of nucleic acid 3) biochemical tests and 4) polymerase chain reaction (PCR) (7, 15). The comparison of PCR with the other methods (hybridization of DNA / RNA and bacterial cultures) indicates that the PCR method is more rapid and reliable. PCR method is a highly sensitive, appropriate technique for the detection of mycoplasma in cell culture. (5, 8). One of the important steps in PCR technique is to identify the target gene and to determine primers sequence. In most PCR methods, 16SrRNA sequences are used as the template sequences because this gene has some regions with commonly conserved sequences among Mycoplasmas (18-22). The aim of this study is to identify the mycoplasma genus in *mesenchymal stem cells* (MSCs) by molecular methods based on PCR and constant 16srRNA sequences fixed.

Materials and methods

The used Bacterial strains:

In this study the species which are belong to mollicutes has been used including Mycoplasma Pneumoniae(NCTC 10119) , Mycoplasma arginini, Mycoplasma hyorinis, Mycoplasma orale, Mycoplasma synoviae, Mycoplasma gallinarum (Razi 1346,1350), Mycoplasma gallisepticum (Razi 1355), Achoplasma laidlawi, Mycoplasma agalactiae(Razi 1343), Mycoplasma ovipneumoniae (Razi 1364), Mycoplasma ureaplasma urealyticum(Razi 1369).

Preparation of samples

The utilized Cell cultures were adipose derived mesenchymal stem cells which were cultured in stem cell laboratory of highly specialized Jihad daneshgahi infertility treatment center of Qom. 164 samples of *mesenchymal stem cells* have been checked for mycoplasma contamination.

DNA extraction and nucleotide sequences of primers

In this study we have used the boiling method for DNA extraction. Thus, 100 µl of the cell suspension with mineral oil placed in boiling heat and after Centrifuging in 12000 g for 10 minutes, supernatant was used as template in PCR testing (16).the target gene was 16srRNA of mycoplasma (21) which its primer sequences (designed by gene runner software) are as below:

Table 1. Primers sequences used in this study.

Primer name	**Sequence (5’-3’)**
GPO-1	*ACTCCTACGGGAGGCAGCATAG*
MGSO	*TGCACCATCTGTCACTCTGTTAACCTC*

PCR conditions

The required ingredients to perform polymerase chain reaction in a volume of 25 µl, were prepared as it is mentioned in below:
5 µl of template DNA, 1 µl of each one of forward and reverse primer , 2.5 µl of PCR buffer (10X) (sina clone) , 0.75 µl MgCl2 with mM50 concentration (sina clone) , 0.5 µl of dNTP mixture (10mM) (sina clone) and 0.4 µl of Taq Enzyme (sina clone) and 14 µl of twice distilled sterile deionized water in order to minimize the volume were used. The thermocycler was programmed for 40 cycles at 93 ° C for 20 s, 60 ° C for 20 s, and finally 72 ° C for 30 s. The PCR product with specified size (715 bp), with DNA ladder and positive and negative control, was assessed on the agarose gel 1.5% using ethidium bromide and UV light in transilluminator device.

Test Sensitivity and Specificity

In this test in order to examine the sensitivity of the used pair primers, we prepared serial dilutions of Mycoplasma arginini suspension with specific CFU, and finally PCR test was performed on the samples with eligible number.
Also the specificity test was done using DNA sample of some organisms like human, mouse, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus.

Results and discussion

In this study 132 samples consisted of *mesenchymal stem cells* were evaluated in which in 6.06% of examined samples (8 samples) mycoplasma contamination was detected. It was shown by use of sensitivity test that the applied primers in this study had 10CFU/ml sensitivity and high specificity in mycoplasma detection.
Using GPO-1 and MGSO Primers and DNA of Mycoplasma types like Mycoplasma Pneumoniae, Mycoplasma arginini, Mycoplasma hyorinis, Mycoplasma orale ,Achoplasma laidlawi and Mycoplasma aplasma urealyticum, PCR technique was optimized. The PCR assay with DNA of all tested mycoplasmas caused to generation of 715bp product (Figure 1). Evaluation of test sensitivity was done through the dilution of mycoplasma culture with the composer unit of the determined colony. It was shown that the sensitivity of this test is in limitation of 10 copies in the examined sample (Figure 2). By performing Specificity test, it was determined that utilized primers did not produce any unwanted product with DNA of non-mycoplasmic bacteria such as Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, and also DNA of human and mouse(Figure 3). Mycoplasma contamination in 164 samples consisted of *mesenchymal stem cells,* was assessed

by use of genus-specific PCR 8 contaminated samples (6.06%), through amplification of correct section, were detected (Figure 4). Another advantage of this research is Use of boiling method for DNA extraction instead of using commercial kits while this method is so quick and simple and can be done with low facilities.

Mycoplasmas can easily pass through 220 and 450 nm filters holes used in cell culture, and Therefore, they are considered as the most important contaminating factors in cell culture. These bacteria are some organisms invisible by use of optical microscopes, the smallest self-replicated polymorphic bacteria, 200 - 500nm in diameter, with no cell wall. Genetic and biochemical changes are the problems caused by infecting mycoplasmas which will lead to unreliable results. (24-22).

There are various methods applying to detect mycoplasma in cell culture. Identifying of these contaminating factors is performed by use of biochemical methods, serology, and staining by use of Fluorescent dyes (Hoechst and DAPI) and molecular techniques (24 - 23). In order to minimizing pseudo-results, in many studies the combination of two methods is used to detect of mycoplasma in cell culture. Combination of PCR with cell culturing is widely used to identify infected samples (22, 20, 7). Also in another study the combination of DNA staining technique and cultivation is used. Cultivation is a time-consuming method and has a high pseudo-negativeresults, as well as in DNA staining technique due to bacterial contamination interpretation of results is difficult (26,25,21,5,3).

It is shown that DNA staining techniques is a quick, highly specified and sensitive, among utilized techniques in detecting mycoplasma, including fluorescent, biochemical dyes and molecular PCR (25). In addition, there are still a number of strains of mycoplasma which are non-growing in microbial cultures or too hard to grow like Mycoplasma hyorinis. Microbial culture methods require a 1-4 weeks period in the laboratory. (25).
This technique should be able to identify at least five Typical species of mycoplasmas contaminating cell cultures Mycoplasma fermentans, Mycoplasma hyorinis, (Mycoplasma arginini), Mycoplasma orale and Achoplasma laidlawi that cause more than 95% of infection in biological products and cell cultures. Finding a rapid and sensitive method for the detection of mycoplasma contamination in cell cultures and biological products, is a particular important issue. The main objective of this study was to achieve a reliable and rapid method for the detection of mycoplasma in cell cultures. In our study, designed based on polymerase chain reaction (PCR), we could reach to at least sensitivity of 10 copies of target DNA molecule and also no cross-reactivity with other organisms genomic DNA, such as pseudomonas aeruginosa, human, Mycobacterium tuberculosis and Staphylococcus aureus. However, in some studies the identification range of Mycoplasma has shown between 1-100 Mycoplasmas, which is stated due

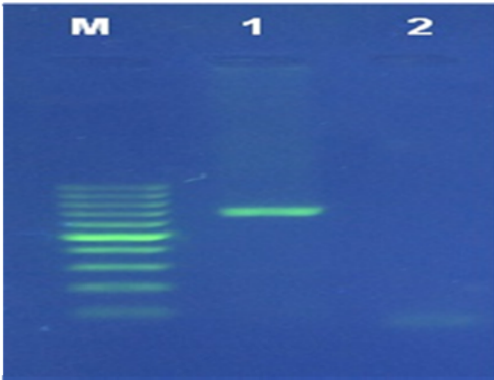

Figure 1. The optimized PCR test using GPO-1 and MGSO primers. Column M: DNA ladder 100 bp (fermentas). Column 1: positive control (DNA of mycoplasma arginini). Column 2: negative control (sterile water)(Agarose 1.5% and TBE 0.5× buffer).

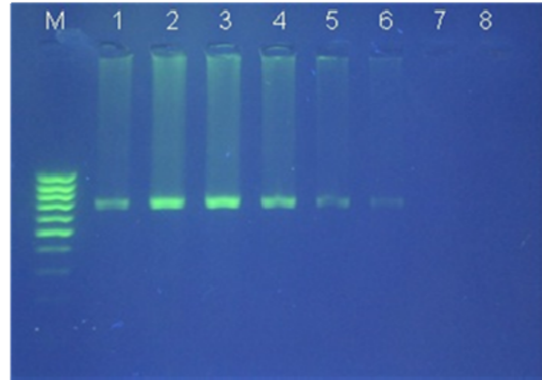

Figure 2. The test of assessing optimized PCR assay sensitivity. Column M: DNA ladder 100 bp (fermentas). Column 1: positive control (DNA of mycoplasma arginini). Column 2: 10^5 CFU/ml. Column3: 10^4 CFU/ml. column 4: 10^3 CFU/ml. column 5: 100 CFU/ml. Column 6:10 CFU/ml. column 7: 1 CFU/ml. Column 8: negative control (sterile water)(Agarose 1.5% and TBE 0.5× buffer).

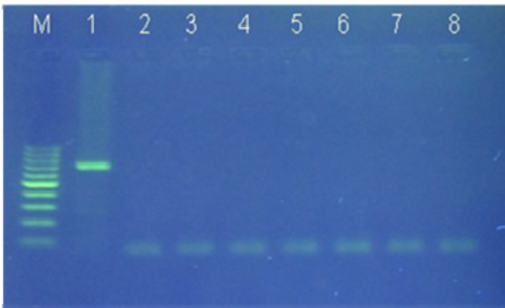

Figure 3: the specificity test of optimized PCR assay. Column M: DNA ladder 100bp (fermentase). Column 1: positive control (DNA of mycoplasma arginini). Column 2: DNA of human. Column 3: DNA of mouse. Column 4: mycobacterium tuberculosis. Column 5: pseudomonase aeruginosa. Column 6: Staphylococcus aureus. Column 7: Salmonella typhi. Column 8: negative control (sterile water)(Agarose 1.5% and TBE 0.5× buffer).

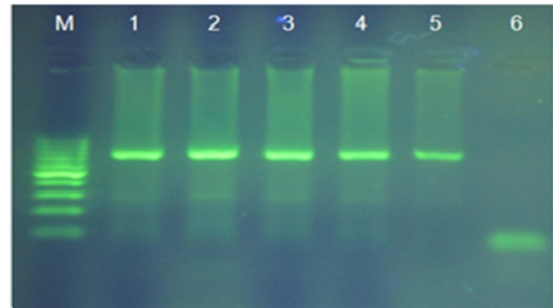

Figure 4: PCR amplification result of examined cell culture samples. Cell culture tested: column M: DNA ladder 100bp (fermentase). Column 1: positive control (DNA of mycoplasma arginini). Columns 2-5: contaminated samples. Column 6: negative control (sterile water)(Agarose 1.5% and TBE 0.5× buffer).

to Mycoplasma species, type and design of the primers, PCR target gene and PCR conditions (27-30). Thus, we selected the conserved and common parts of 16 srRNA as proliferative sequences. The other feature of this study is the way of DNA extraction, which is done by boiling method. The selection of these sequences is because they are frequent along genome and commonly constant among members of mollicutes. Because this method is simple, fast, and in addition does not use chemicals materials like chloroform to extract DNA has unique features.

Conclusion

Utilizing an appropriate method to detect Mycoplasmas is so important because conventional

methods due to limitations in time consumption, low sensibility and requirement to skill, do not have the adequate characteristics to be used in detection of Mycoplasma in cell cultures. These contamination can affect the biological characteristics of cells. However, molecular methods based on polymerase chain reaction (PCR) have great importance due to their innate features. The obtained results in our study are based on accuracy, speed, sensibility and high specification of PCR technique according to conserved and common sequence of 16srRNA to detect Mycoplasma contamination .

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