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# Quality Control of Biotechnological Inputs Detecting *Mycoplasma*

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## ABSTRACT

*The aim of this work was to study the Polymerase Chain Reaction (PCR) as a tool of quality control of bovine sera and cellular cultures used in the biotechnological industry. A total of 46 samples of bovine sera derived from two slaughterhouses and 33 samples of BHK21 cells derived from two biotechnological industries were evaluated using the primers GPO-3 (sense) and MGSO (antisense). The PCR technique sensibility analysis showed that 280 bp were amplified for the quantities of 50 ng to 0.006 ng of* Micoplasma *DNA. The primers specificity was confirmed in the test using* Staphylococcus aureus, Escherichia coli, Bacillus subtilis and Candida albicans; *except by the positive control, none of the samples showed amplification. The presence of* Mycoplasma *in bovine sera and in the cultures of BHK21 cells showed that 56.5 and 15.2%, respectively, were contaminated. Thus, it was possible to conclude that PCR was a fast and confident technique to detect mycoplasma and that it could be used to control the quality of immunobiological products and inputs, such as sera and cultures of BHK21 cells.*

**Key words:** PCR; bovine serum; BHK21 cells; mycoplasma, cellular culture

## INTRODUCTION

Mycoplasma belongs to the class *Mollicutes* and it differs phenotypically of other bacteria due to its reduced size and absence of cellular wall (Razin et al. 1998; Domingues et al. 2005). Due to its small size, it can pass through the filters with porosity from 0.45 to 0.22 µm that commonly are used to sterilize the reagents in industries and laboratories (Hay et al. 1989; Tully 1992). Cellular cultures contaminated with mycoplasma represent an artificial habitat for such microorganisms. Studies performed in many countries have demonstrated that from 10 to 87% of the cellular cultures were infected with mycoplasma (Kazemiha et al. 2009). The species *M. orale, M. salivarium, M. hyorhinis* and *M. arginini* are most found as contaminants (Miyaki et al. 1989; Hu et al. 1995; Timenetsky et al. 2006).

*Mycoplasma orale* and *M. salivarium* inhabit the human oropharynx and generally infect the cell cultures due to inadequate aseptic techniques. *M. arginini* has bovine origin and it is found in the cultures from the contaminated bovine serum. *M. hyorhinis* has porcine origin and it can contaminate the bovine serum in slaughterhouses when they are also used to slaughter swine (Rottem and Barile 1993; Smith and Mowles 1996; Razin et al. 1998). The contamination through mycoplasma in bovine cells and sera used

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to produce vaccine antigens or used in diagnosis can generate non-confident results and add potentially noxious effects to biotechnological products. Contaminations through mycoplasma cause alterations in the cellular metabolism, decreasing the cellular division rate through the interference in DNA, RNA and protein synthesis, chromosomal aberrations and death with the monolayer release (Timenetsky et al. 1992). Products, such as vaccines, medicines or monoclonal antibodies, manufactured based on such inputs should be disenabled, which causes prejudices to the pharmaceutical and biotechnological industries (Uphoff and Drexler 2002).

The tests to detect the contamination by mycoplasma are necessary to assure the confidence of the results and the quality of the biotechnological products (Uphoff and Drexler 2002; Timenetsky et al. 2006). As this kind of contaminant does not generate visual signals, because they do not cause turbidity, just specific tests enable its detection (Lincoln and Lundin 1990; Timenetsky et al. 1992; Hu et al. 1995; Ossewaarde et al. 1996). Among the different techniques to detect the contamination through mycoplasma, the histochemical staining, immunosorbent assays (ELISA), indirect immunofluorescence (IIFT), biochemical assays and polymerase chain reaction (PCR) are used (Kong et al. 2001; Uphoff and Drexler 2002; Sung et al. 2006; Gopalkrishna et al. 2007). PCR has been the chosen methodology to detect mycoplasma due to its high specificity, speed in the technique performance and the possibility of analyzing different samples at the same time (Rottem 2003).

This work aimed to study the use of PCR to detect *Mycoplasma* sp for the quality control of cellular cultures and bovine serum in a biotechnological industry and validating it testing the contamination by *Mycoplasma* sp in inputs used in the preparation of vaccines.

## MATERIAL AND METHODS

### Microrganisms Cultivation

The strains of *M. orale, M. salivarium, M. arginini* and *M. hyorhinis*, donated by Jorge Timenetsky of University of São Paulo, were cultivated in 10 mL of SP4 broth at 37ºC (Nascimento et al. 2002). The growth of the microrganisms was confirmed due to the acidification of the pH in the broth, absence of turbidity and production of colonies looking like 'fried eggs' in SP4 agar plates. Mycoplasma was frozen at -20ºC with 20% of glycerol (Sigma-Aldrich, St. Louis, MO and USA) till its use.

### Polymerase Chain Reaction

In order to extract the DNA of the microorganisms, the phenol/chloroform technique was used (Bashiruddin 1998). The DNA concentration of each sample was measured through the absorbance at 260 nm (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA). For PCR, the primers described by Van Kuppeveld et al. (1994) were used. The primer sense GPO-3 5'- GGGAGCAAACAGGATTAGATACCCT-3' and the antisense MGSO 5'-TGCACCATCTGTCACTCTGTTAACCTC - 3' were obtained from Invitrogen. PCR reaction contained 2.0 µL of buffer 10X, 1.5 mM of $MgCl_2$, 0.2 µM of each deoxyribonucleotide triphosphate (dNTP), 12.5 pmol of each primer (GPO3/MGSO*)*, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 50 ng of DNA (maximum volume of 8.0 µL) and ultrapure water to a final volume of 20 µL. The amplification was made in thermocycler (model Biocycler MG96) which one cycle at 94ºC by 5 min, 35 cycles at 94ºC 30 sec, 55ºC during 30 sec, 72ºC 30 sec and a final extension at 72ºC during 5 min. A negative control (ultra-pure water) and a positive control (DNA of the references strains) were added to all amplifications.

In order to determine the sensibility of the PCR reaction, dilutions from 50 ng to 0.003 ng of the DNA of *M. salivarium* were cultivated in SP4 broth medium and extracted through the phenol/chloroform method were used. To determine the specificity of the PCR, DNAs extracted through phenol/chloroform methodology from strains of *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Candida albicans* ATCC 10231 were used and the PCR was performed.

### *Mycoplasma* contamination detection

Forty six samples of bovine sera derived from slaughterhouses and 33 cultures of BHK21 cells derived from biotechnological industries were submitted to DNA extraction through phenol/chloroform method (Bashiruddin 1998) and then to PCR to detect the presence or absence of contamination by mycoplasma.

## RESULTS AND DISCUSSION

*Mycoplasma orale, M. salivarium, M. arginini* and *M. hyorhinis* grew in broth and in SP4 agar culture medium. All the species presented the expected product (280 bp) in the PCR (Fig. 1).

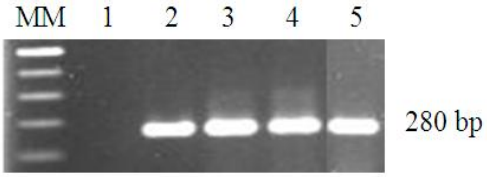


**Figure 1 -** Agarose gel electrophoresis at 1.5% of PCR products of mycoplasma grown in SP4 culture media. MM: Molecular Weight Marker (100 bp); 1: Negative Control; 2: *Mycoplasma orale*; 3: *M. salivarium*; 4: *M. arginini*; 6: *M. hyorhinis*.

According to Uphoff and Drexler (2002), to detect the mycoplasma based on PCR, it is possible to mention two main variants: the detection of any infection by mycoplasma (specific genus PCR), and the detection of just one species of mycoplasma (specific specie PCR).

In the study, specific genus was used and PCR employed the primers GPO-3 and MGSO to detect the contaminations by any species of mycoplasma. In the PCR technique, analytical sensibility test, the amplification of a 280 bp fragment for quantities from 50 ng to 0.006 ng DNA of mycoplasma was observed (Fig. 2).

The specificity of the primers used for *Mycoplasma* was confirmed, because except the positive control of mycoplasma, no amplification was observed for the DNAs extracted through the phenol/chloroform method of *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *B. subtilis* ATCC 6633 and *C. albicans* ATCC 10231 strains (Fig. 3).

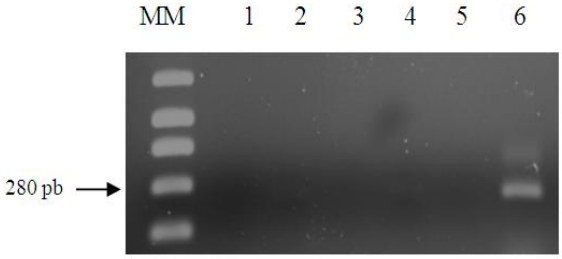


**Figure 3** – Electrophoresis in agarose gel 1.5% of the PCR products of *Mycoplasma salivarium* growth in SP4 broth and DNA extraction method using phenol/cloroform to determinate the PCR reaction specificity. MM: Molecular Mass Marker (100 pb); 1: PCR negative control; 2: *Staphylococcus aureus* ATCC 6538; 3: *Escherichia coli* ATCC 25922; 4: *Bacillus subtilis* ATCC 6633; 5: *Candida albicans* ATCC 10231; 6: PCR positive control (DNA *M. salivarium*).

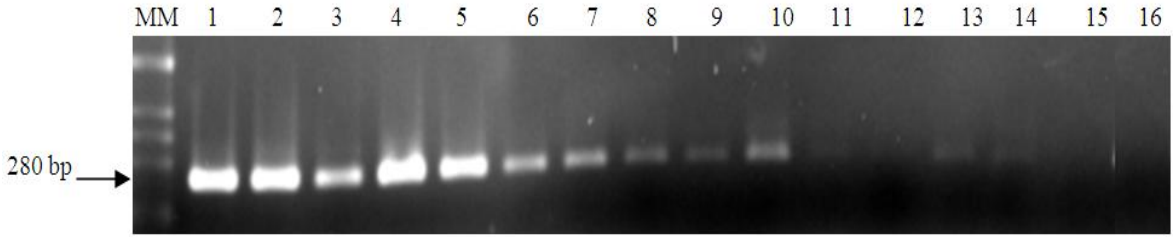


**Figure 2** – Electrophoresis in agarose gel (1.5%) of the PCR products of *M. salivarium* growth in SP4 broth and DNA extraction method using phenol/cloroform to determinate the PCR reaction sensibility. MM: Molecular Mass Marker (100 bp); 1 – 15 different DNA concentration: 1: 50 ng; 2: 25 ng; 3: 12.50 ng; 4: 6.250 ng; 5: 3.125 ng; 6: 1.562 ng; 7: 0.781 ng; 8: 0.391 ng; 9: 0.195 ng; 10: 0.098 ng; 11: 0.049 ng; 12: 0.024 ng; 13: 0.012 ng; 14: 0.006 ng; 15: 0.003 ng; 16: PCR negative control.

The PCR technique standardized in the study was highly sensitive, because it was able to detect the presence of mycoplasma DNA at 0.06 nanograms and it was also highly specific, because it did not amplify the DNA of other microorganisms' species that were commonly used in laboratories. Such method was fast and confident to detect different species of mycoplasma that contaminated cell cultures, which enabled its usage to control the quality of immunobiological products and inputs, such as sera and cultures of BHK21 cells.

Aiming to validate the consistency of the PCR methodology established to be used in industries as quality control of inputs, 46 samples of bovine

sera derived from two slaughterhouses and 33 samples of BHK21 cells derived from two biotechnological industries were evaluated to detect the possible contaminations by mycoplasma (Fig. 4). In most cases, the contamination was through mycoplasma derived from the animal sera, mainly from contaminated cattle and also from aerosols derived from the humans due to non-aseptic practices in the laboratory environments (Smith and Mowles 1996; Rottem 2003), justifying why such inputs were important to chose.

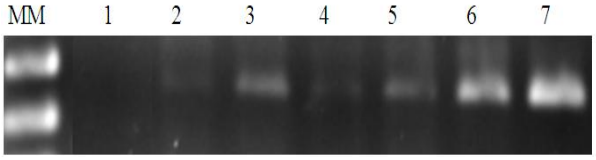


**Figure 4 -** Agarose gel electrophoresis at 1.5% of PCR products of cultures of BHK21 cells and bovine sera. MM: Molecular Weight Marker (100 pb); 1: Negative Control; 2 and 3: Samples of cultures of BHK21 cells; 4, 5 e 6: Samples of bovine sera; 7: PCR Positive Control (*DNA M. salivarium*).

Results showed that 56.5% of bovine sera and 15.2% of the cellular strains were contaminated (Table 1). According to Kazemiha et al. (2009), up to 87% of the cellular cultures could be contaminated by mycoplasma. The variation of the percentage of contamination found in the literature was related to the size of the studied samples population, contamination control practices and efficiency of the used detection assays (Rottem and Barile 1993; Kong et al. 2001).

**Table 1** - Percentage of samples of bovine sera and BHK21 cells contaminated by mycoplasma.

| | **Total of samples** | **number of samples contaminated by mycoplasma** | **% of samples contaminated by mycoplasma** |
|---|---|---|---|
| Bovine Sera | 46 | 26 | 56.5 |
| BHK21 Cells | 33 | 05 | 15.2 |

The test that detects the contamination by mycoplasma is necessary to assure the confidence of the results and biotechnological products quality control (Uphoff and Drexler 2002). PCR has been the chosen methodology due to the high specificity, sensibility and speed during the performance of the technique (Rawadi and Dussurget 1995). The specificity of such methodology occurs due to the usage of oligonucletides primers that are connected to the maintained region of the DNA that codifies the region 16S of rRNA or the intergenic regions of the rDNA 16S-23-S of mycoplasma (Uphoff and Drexler 2002; Sung et al. 2006; Timenetskt et al. 2006).

## CONCLUSIONS

Thus, it could be concluded that the PCR technique was adequate for the quality control of bovine serum and cultures of BHK21 cells and that the importance of preventing the contamination through such microorganisms should be highlighted as well as the implantation of quality control in the productive process such as detecting mycoplasma through PCR.

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