

## Quality Control of Biotechnological Inputs Detecting *Mycoplasma*

Cristiane Netto<sup>1,3\*</sup>, Vanete Thomaz Soccol<sup>2</sup>, Lya Sepulveda<sup>3</sup>, Gabriel Henrique Oliveira Garcia<sup>4</sup> and Jorge Timenetsky<sup>5</sup>

<sup>1</sup>Mestrado em Biotecnologia Industrial; Universidade Positivo; Curitiba - PR - Brasil. <sup>2</sup>Programa de Pós Graduação em Engenharia de Bioprocessos e Biotecnologia; Universidade Federal do Paraná; Curitiba - PR - Brasil. <sup>3</sup>Ourofino Saúde Animal; Ribeirão Preto - SP - Brasil. <sup>4</sup>Universidade Positivo; Curitiba - PR - Brasil. <sup>5</sup>Departamento de Microbiologia; Instituto de Ciências Biomédicas; Universidade de São Paulo; São Paulo - SP - Brasil

### 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 *Mycoplasma* 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  $\mu\text{m}$  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. hyorhinitis* 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. hyorhinitis* 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

\* Author for correspondence: Cristiane.Netto@ourofino.com

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. hyorhinitis*, donated by Jorge Timenetsky of University of São Paulo, were cultivated in 10 mL of SP4 broth at 37°C (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 -20°C 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 microrganisms, 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'- TGCACCATCTGTCACTCTGTAAACCTC - 3' were obtained from Invitrogen. PCR reaction contained 2.0 µL of buffer 10X, 1.5 mM of MgCl<sub>2</sub>, 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 94°C by 5 min, 35 cycles at 94°C 30 sec, 55°C during 30 sec, 72°C 30 sec and a final extension at 72°C during 5 min. A negative control (ultra-pure water) and a positive control (DNA of the references strains) were added to all amplifications.

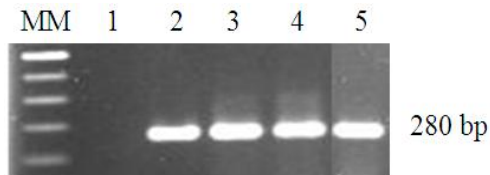
In order to determine the sensibility of the PCR reaction, dilutions from 50 ng to 0.003 ng of the DNA of *M. salivarium* were cultivated in SP4 broth medium and extracted through the phenol/chloroform method were used. To determine the specificity of the PCR, DNAs extracted through phenol/chloroform 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

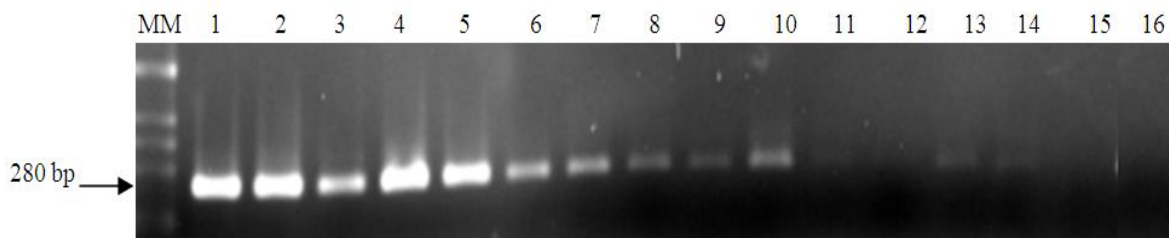
*Mycoplasma orale*, *M. salivarium*, *M. arginini* and *M. hyorhinis* grew in broth and in SP4 agar culture medium. All the species presented the expected product (280 bp) in the PCR (Fig. 1).



**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*; 5: *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,

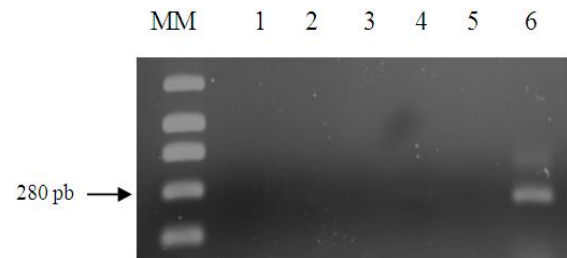


**Figure 2** – Electrophoresis in agarose gel (1.5%) of the PCR products of *M. salivarium* growth in SP4 broth and DNA extraction method using phenol/chloroform to determine the PCR reaction sensibility. MM: Molecular Mass Marker (100 pb); 1 – 15 different DNA concentration: 1: 50 ng; 2: 25 ng; 3: 12.50 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

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).

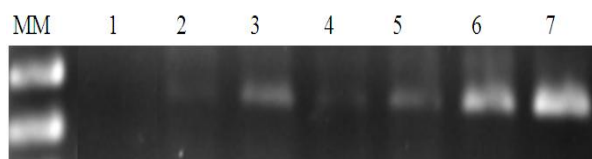


**Figure 3** – Electrophoresis in agarose gel 1.5% of the PCR products of *Mycoplasma salivarium* growth in SP4 broth and DNA extraction method using phenol/chloroform to determine the PCR reaction specificity. MM: Molecular Mass Marker (100 pb); 1: PCR negative control; 2: *Staphylococcus aureus* ATCC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

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 oligonucleotides 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; Timenetski 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.

## ACKNOWLEDGMENTS

Special thanks are due to the veterinary industry Ourofino Saúde Animal, its manager Silvia Barioni and its director Dan Artioli and to Dr. Jorge Timenetsky from the University of São Paulo and to Dr. Celso Caricati from Butantan Institute.

## REFERENCES

- Bashiruddin JB. Extraction of DNA from Mycoplasmas. In: MILES, R. & NICHOLAS, R. eds. *Mycoplasma Protocols: a Laboratory Manual*. New Jersey: Humana Press Inc; 1998.
- Domingues D, Nogueira F, Tavira L, Expoto F. Mycoplasmas: Que papel nas Infecções Humanas? *Acta Med Port*. 2005; 18: 377-384.
- Gopalkrishna V, Verma H, Kumbhar NS, Tomar RS, Patil PR. Detection of *Mycoplasma* species in cell culture by PCR and RFLP based method: effect of BMcyclin to cure infection. *Indian J of Med Microbiol*. 2005; 25(4): 364-368.
- Hay RJ, Macy ML, Chen TR. *Mycoplasma* infection of cultured cells. *Nature*. 1989; 339: 487-488.

- Hu M, Buck C, Jacobs D, Paulino G, Khouri H. Application of PCR for detection and identification of *Mycoplasma* contamination in virus stocks. *In vitro* cellular and developmental biology. *In Vitro Cell Dev Biol*. 1995; 31(3): 710-715.
- Kazemiha VM, Shokrgozar MA, Arabestani MR, Moghadam MS, Azari S, Maleki S, et al. PCR-based detection and eradication of mycoplasmal infections from various mammalian cell lines: a local experience. *Cytotechnology*. 2009; 61: 117-124.
- Kong F, James G, Gordon S, Zelynski A, Gilbert GL. Species specific PCR for identification of common contaminant mollicutes in cell culture. *Appl Environ Microbiol*. 2001; 67(7): 3195-3200.
- Lincoln CK, Lundin DJ. *Mycoplasma* detection and control. FCC Tools & Resources U. S. *Pharmacopeial Newsletter*. 1990: 20:1-3.
- Miyaki C, Pral MM, Gallina NMF, Rizzo E. *Mycoplasma* como contaminante de culturas celulares mantidas em laboratórios de instituições particulares e oficiais. *Rev Saúde Públ*. 1989; 23(1): 39-44.
- Nascimento CMO, Figueiredo CA, Timenetsky J. Sensitivity of Rabbit Fibrochondrocytes to *Mycoplasmas*. *Braz J Microbiol*. 2002; 33: 243-246.
- Ossewaarde JM, Vries A, Bestebroer T, Angulo AF. Application of a *Mycoplasma* group-specific PCR for monitoring decontamination of *Mycoplasma* infected *Chlamydia* sp. strains. *Appl Environ Microbiol*. 1996; 62(2): 328-331.
- Razin S, Yogeve D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol*. 1998; 62(4): 1094-1156.
- Rawadi G, Dussurget O. Advances in PCR-based detection of mycoplasmas contaminating cell cultures. *Genome Res*. 1995; 4: 199-208.
- Rottem S, Barile MF. Beware of mycoplasmas. *Trends Biotechnol*. 1933; 11: 143-151.
- Rottem S. Interaction of mycoplasmas with host cells. *Physiol Rev*. 2003; 83: 413-432.
- Smith A, Mowles J. Prevention and control of mycoplasma infection of cell cultures. Molecular and diagnostic procedures in mycoplasma. San Diego: Academic Press; 1996. p. 445-451.
- Sung H, Kang SH, Bae YJ, Hong JT, Chung YB, Lee CK, Song S. PCR-based detection of *Mycoplasma* species. *J Microbiol Korea*. 2006; 44(1): 42-49.
- Timenetsky J, Miyaki C, Mendes IF, Rizzo E. Identificação de micoplasmas pela inibição de culturas celulares. *Rev Saúde Públ*. 1992; 26(1): 17-20.
- Timenetsky J, Santos LM, Buzinbani M, Mettifogo E. Detection of multiple *Mycoplasma* infection in cell cultures by PCR. *Braz J Med Biol Res*. 2006; 39(7): 907-914.
- Tully JG. Mollicutes. In: Encyclopedia of microbiology. New York: Academic Press; 1992. p. 181-191.
- Uphoff CC, Drexler HG. Comparative PCR analysis for detection of *Mycoplasma* infections in continuous cells lines. *In vitro* cellular and developmental biology. *In Vitro Cell Dev Biol – Animal*. 2002; 38: 79-85.
- Van Kuppeveld FJM, Johansson KE, Galama JMD, Kissing J, Bölske G, Van Der Logt JTM, Melchers WJG. Detection of *Mycoplasma* contamination in cell cultures by mycoplasma group-specific PCR. *Appl Environ Microbiol*. 1994; 60: 149-152.

Received: May 09, 2014;  
Accepted: September 18, 2014.