# Original Article

# Detection of Mycoplasma Contamination Directly from Culture Supernatant Using Polymerase Chain Reaction

(polymerase chain reaction / PCR / One Taq Polymerase)

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**Abstract. Ensuring mycoplasma-free cell culture is of prime importance as they severely affect cellular characteristics leading to experimental artefacts and spurious results. Various methods persist for mycoplasma detection; out of the whole array of methods polymerase chain reaction (PCR) is the most favoured one because it is highly sensitive, specific and quick. The PCR-based detection procedure involves three steps: cell culture supernatant collection, DNA isolation, and PCR. We have modified this procedure so that cell culture supernatant can directly be used for PCR without the need for DNA extraction. This modification makes the procedure quicker and more sensitive because loss of mycoplasma DNA is prevented and this loss becomes more significant when the level of mycoplasma contamination is very low.**

## Introduction

Mycoplasma contamination of cell lines is one of the major problems of animal tissue culture. Approximately 5–15 % of the cell cultures are contaminated with mycoplasma (Young et al., 2010). Mycoplasma belongs to the class of Mollicutes, which represents a vast group of highly specialized bacteria that lack a rigid cell wall. Predominantly six species *Acholeplasma laidlawii, Mycoplasma arginine, M. fermentans, M. hominis, M. hyorhinis* and *M. orale* contribute to the majority of infections (Bolske, 1988; Kong et al., 2001). The major cause for mycoplasma contamination in tissue culture is improper handling or source of the tissue from which the cells are harvested (Uphoff and Drexler, 1999; Uphoff et al., 2012).

Mycoplasma-free cell lines are a prerequisite, as the contamination alters a great variety of cellular characteristics and can affect cellular parameters, often leading to experimental artefacts and spurious results. The lack of a rigid cell wall, reduced metabolic rate and long generation time makes it impossible to detect mycoplasma by microscopic observation. Several methods for detection exist, e.g., molecular biology techniques, biochemical and radioactive incorporation assays, electron microscopy, etc. Amongst all of the above-mentioned methods, polymerase chain reaction (PCR) is very sensitive and specific; it can detect different species of mycoplasma with minimum effort in terms of time and labour (Drexler and Uphoff, 2000). Uphoff and Drexler (1999) had mentioned the use of PCR primers targeting the 16S rRNA gene for detection of mycoplasma contamination. The sequence of the 16S rRNA gene is well conserved across the mycoplasma species, which makes it a good candidate for targeting. Detection is carried out using a mixture of oligonucleotides and electrophoresis is run in the presence of appropriate controls in order to rule out false-positive or false-negative results (Uphoff and Drexler, 1999). The method involves three steps: harvesting a small volume of culture supernatant, DNA extraction, and PCR.

The major limitation of this method occurs during the DNA extraction step, where a significant amount of mycoplasma DNA is lost, rendering a false-negative result if the level of contamination is very low.

A slight modification in the detection technique by direct utilization of cell culture supernatant for PCR instead of the DNA extraction step facilitates quick detection and increases sensitivity of the process for detecting very low levels of mycoplasma contamination.

## Material and Methods

### *Cultivation of cell lines*

In total four adherent cell lines were used for the study, out of which three were human dermal fibroblasts

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Abbreviations: PCR – polymerase chain reaction, qPCR – real-time PCR.

*Table 1. Oligonucleotide primers used in PCR for the detection of mycoplasma contamination*

| Forward primers | GC cont. | Melting temp. | Cell culture mycoplasma species | Amplicon |
|---|---|---|---|---|
| cgc ctg agt agt acg tcc gc | 60 % | 62.5 °C | *M. fermentans, M. bovis* | 518 |
| cgc ctg agt agt acg tac gc | 60 % | 62.5 °C | *Acholeplasma laidlawii* | 525 |
| tgc ctg ggt agt aca ttc gc | 55 % | 60.5 °C | *Ureaplasma spp* | 504 |
| tgc ctg agt agt aca ttc gc | 50 % | 58.4 °C | *M. gallisepticum* | 504 |
| cgc ctg agt agt atg ctc gc | 60 % | 62.5 °C | *M. arginini, M. hominis, M. hyorhinis, M. orale, M. pneumoniae* | 520, 522, 518, 520, 517 |
| cac ctg agt agt atg ctc gc | 55 % | 60.5 °C | *M. pulmonis* | 518 |
| cgc ctg ggt agt aca ttc gc | 60 % | 62.5 °C | *M. pirum* | 504 |
| **Reverse primers** | | | | |
| gcg gtg tgt aca aga ccc ga | 60 % | 62.5 °C | *M. arginini, M. bovis, M. fermentans, M. gallisepticum, M. hominis, M. orale, M. pirum, Ureaplasma spp.* | |
| gcg gtg tgt aca aaa ccc ga | 55 % | 60.5 °C | *M. hyorhinis, M. pneumoniae* | |
| gcg gtg tgt aca aac ccc ga | 60 % | 62.5 °C | *A. laidlawii* | |

Adapted from Uphoff et al. (2012)

and one C2C12 murine myoblasts. Human dermal fibroblasts and C2C12 myoblasts were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), 10% foetal bovine serum (FBS) and L-glutamine. All the cell lines were cultured for at least one week without antibiotics before performing the mycoplasma test. None of the cell lines was deliberately infected with mycoplasma.

### *Mycoplasma detection by PCR*

One ml of cell culture supernatant was collected in a 1.5 ml PCR-graded centrifuge tube from each cell line for further processing. Thorough mixing of the cell culture supernatant is essential before using it for PCR. Forward and reverse primers were mixed respectively at 5 µM concentration each in nuclease-free water and were aliquoted in small amounts and stored frozen at –20 °C. Sequences of primers are given in Table 1. One µl (from the oligonucleotide mixture) each of forward and reverse primers and 1 µl of cell culture supernatant (previously collected) was added to 12.5 µl of PCR master Mix (cat. no. M0484S, New England Biolabs, Hitchkin, UK) and the final volume was adjusted to 25 µl using nuclease-free water. Ten pg of internal control plasmid was added to the PCR reaction mixture and positive control was diluted 10 times, and 9.5 µl of the dilution was used. PCR was assembled as follows:

| PCR components | Concentration 1X / volume |
|---|---|
| 5 µM forward primers | 1 µl |
| 5 µM reverse primers | 1 µl |
| Master Mix | 12.5 µl |
| Internal control 10 pg/µl | 1 µl |
| Cell culture supernatant | 1 µl |
| Nuclease-free water | 8.5 µl |

Positive control was diluted 10 times and 9.5 µl of the diluted sample was used.

PCR amplification was carried out using the following parameters:

| Cycle type | Temp. | Time |
|---|---|---|
| Denaturation | 94 °C | 5 min |
| | | |
| Cycles | 35 | |
| Denaturation | 94 °C | 30 s |
| Annealing | 55 °C | 30 s |
| Extension | 68 °C | 60 s |
| | | |
| Final Extension | 68 °C | 5 min |
| Hold | 4 °C | 20 min |

Ten µl of PCR-amplified product was used for electrophoresis.

## Results

The mycoplasma detection was carried in the presence of appropriate controls to eliminate the possibility of false-negative and false-positive results. Internal control consisted of PCR-amplified product of *A. laidlawii* cloned into pGEM-T vector and amplified in *E. coli* (Uphoff and Drexler, 2002). The internal control amplified as a 986 bp fragment (upper band), while the mycoplasma-contaminated sample and positive control amplified as a 510 bp (lower band) fragment (Fig 1). The internal control was included to eliminate the possibility of false-negative result in the event where polymerase activity would be inhibited by media components. A series of dilutions of internal control followed by PCR were performed to determine the lowest concentration of internal control essential for obtaining a detectable level of the amplicon (data not shown).

Subsequently, various volumes (1, 2, 3, 4, 6 and 8 µl) of cell culture supernatant were used to determine the appropriate DNA concentration permitting maximum polymerase activity and minimal inhibition of Taq poly-

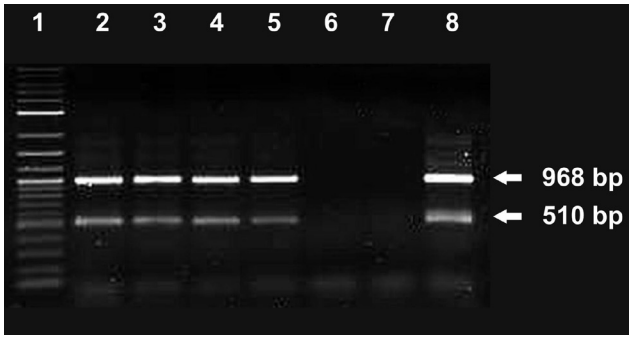


*Fig. 1.*

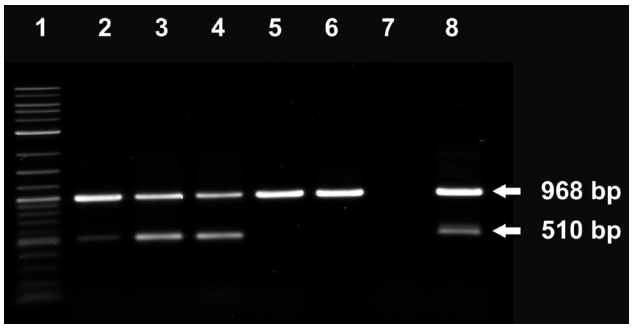


*Fig. 2.*

merase (Fig. 1). The tolerance limit of the One Taq polymerase enzyme was found out to be 4 µl of cell culture supernatant (Lane 6), while 1 µl of cell culture supernatant yielded maximum polymerase activity with a negligible inhibitory effect of media components (Lane 2). Care must be taken to ensure that the cell lines are not undergoing necrosis, because certain factors released during this process completely inhibit the polymerase activity.

The mycoplasma contamination (Fig. 2) was present in all three samples of human dermal fibroblasts (Lanes 2–4), while uncontaminated C2C12 myoblasts were free of contamination (Lane 5). The upper band, i.e. fragment corresponding to ~1000 bp, is the internal control (Lane 6), while the lower band, i.e. ~ 500 bp fragment, is the mycoplasma-specific band. Water control (Lane 7) and positive control along with internal control was run in Lane 8.

## Discussion

All commercially available mycoplasma detection kits rely on any one of the following detection principles: luminescence, PCR, qPCR, nucleotide labelled probes, or fluorescence. These kits are expensive when compared to the total number of tests that can be performed using a single kit. Our procedure requires purchase of the primers and PCR master mix, out of which the PCR master mix can be used for other routine PCR, thus reducing the cost of detection considerably.

Details of a few commercially available kits are as follows:

| Brand | Assay principle | Detection time |
|---|---|---|
| Lonza | Detection of the activity of two enzymes in mycoplasma | 20 min |
| Sigma-Aldrich | PCR | 2.5 h |
| Thermo-Scientific | Real-time PCR | 5 h |
| Thermo-Scientific | Fluorescent nucleic acid stain | 15–30 min |
| R & D | Detection of mycoplasma 16S ribosomal RNA via probe hybridization | 4.5 h |
| Invivogen | Detection of a protein secreted by mycoplasma | Overnight |

Our procedure can be scaled up or down as per the requirements, and since all the components are known, it makes it easy for troubleshooting; these features give an added advantage over other commercially available kits even though the detection time for our procedure is 2.5 h, which is longer compared to the few kits whose detection time is significantly less.

The modification applied to the previously mentioned method (Uphoff and Drexler, 1999) has made the detection technique more robust, sensitive and quick. The modified protocol is simple, helps to minimize the loss of the DNA template, and the results can be obtained in approximately two hours. Loss of template DNA is significantly reduced as it is directly released in the reaction mix after disruption of cell membrane caused by initial denaturation step. Hence, there is a minimal loss, which is not the case if the DNA extraction step is included.

We may conclude that this modified version of mycoplasma detection protocol is simpler, sensitive, quick and inexpensive.

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## References

Bolske, G. (1988) Survey of mycoplasma infections in cell cultures and a comparison of detection methods. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **269,** 331-340.

Drexler, H., Uphoff, C. (2000) Contamination of cell cultures, mycoplasma. In: *The Encyclopedia of Cell Technology*, eds. Spier, B., Griffiths, H., Scragg, E., pp. 609-627, John Wiley & Sons, Inc., New York.

Kong, F., James, G., Gordon, S., Zelynski, A., Gilbert, G. (2001) Species-specific PCR for identification of common contaminant mollicutes in cell culture. *Appl. Environ. Microbiol.* **67,** 3195-3200.

Uphoff, C., Drexler, H. (1999) Detection of mycoplasma contaminations in cell cultures by PCR analysis. *Hum. Cell* **12**, 229-236.

Uphoff, C., Drexler, H. (2002) Comparative PCR analysis for detection of mycoplasma infections in continuous cell lines. *In Vitro Cell. Dev. Biol. Anim.* **38**, 79-85.

Uphoff, C., Denkmann, S., Drexler, H. (2012) Treatment of mycoplasma contamination in cell cultures with plasmocin. *J. Biomed. Biotechnol.* **2012**, 267678.

Young, L., Sung, J., Stacey, G., Masters, J. (2010) Detection of mycoplasma in cell cultures. *Nat. Protoc.* **5,** 929-934.