

## Molecular detection of cell culture mycoplasmal contaminants in bovine serum

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Mycoplasmas are the most frequent contaminants of animal cell cultures. In different surveys, from 15 to 80% of cell cultures were reported to be contaminated with mollicutes (Uphoff *et al.* 2002). Mycoplasmas in cell culture not only affect physiological and biochemical parameters but are known to induce chromosomal instability in mammalian cells and inhibit cell growth. Thus, a sensitive, reliable and regular mycoplasma diagnosis is advisable for laboratories handling cell cultures.

*Mycoplasma* species (*M. fermentans*, *M. hyorhinis*, *M. orale*, *M. arginini*, *A. laidlawii*) most commonly contaminate mammalian cell culture (McGaritty *et al.* 1993). The detection methods differ in sensitivity and specificity. Traditional detection procedures like culture, electron microscopy, DNA staining with fluorescent dyes (e.g., by 4',6-diamidino-2-phenylindole [DAPI] or Hoechst 33258), immunobinding, enzymatic or serological tests are not sufficient for the simultaneous detection of major mycoplasmal contaminants (Mardassi *et al.* 2005). PCR based detection methods proven to be rapid and specific (Hu *et al.* 1995).

Present study aimed to detect common (*Mycoplasma arginini*, *M. orale*, *M. hyorhinis* and *M. fermentans*) cell culture contaminants using PCR.

Cell lines (Vero, MDCK, HeLa, CrFK, IBRS, BHK<sub>21</sub> and CHO) available in the department, were used supplementing growth media containing 10% foetal bovine serum. Genus specific 16s rRNA gene amplification was used to detect mycoplasma by RT-PCR. Total RNA isolation was extracted from trypsinized cells using TRIZOL as per Chomczynski and Sacchi (1987). First strand cDNA synthesis was carried out using cDNA synthesis kit as per manufacturer's protocol. RT PCR was carried out to amplify genus specific 16s rRNA gene. The genus specific primers used were Molli, My1 and My2 as described by Rawadi *et al.* (1995). PCR conditions were initial denaturation at 94°C for 5 min followed by

denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min for 30 cycles and final extension at 72°C for 7 min. The PCR products were analysed using 1.5% agarose gel.

Species specific identification of *Mycoplasma* was carried out using PCR by part of 16s -23s rRNA intergenic spacer region amplification DNA was extracted by treating the cells with extraction buffer and proteinase K. After incubation at 55°C for 4 h, the samples were treated with sodium acetate and equal volume of phenol: chloroform: isoamyl alcohol followed by centrifugation at 12 000 rpm for 15 min at 4°C. Equal volume of isopropanol was added to the aqueous phase and stored at -20°C overnight. Samples were centrifuged at 12 000 rpm for 15 min at 4°C and pellet was washed with 70% ethanol and was dissolved in 20µl of nuclease free water. The species specific primers for *M. fermentans*, *M. hyorhinis*, *M. orale* and *M. arginini* were used as reported by Hui Wang *et al.* (2005). PCR conditions were: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min for 30 cycles and final extension at 72°C for 7 min. The PCR products were analyzed using 1.5% agarose gel. PCR products were purified using extraction kit method.

**Nucleotide sequencing and BLAST analysis:** The purified samples were sequenced in genetic analyzer and the sequences were subjected to BLAST analysis to find out the homology percentage with other *Mycoplasma* strains already available in Gen bank.

**Phylogenetic analysis:** The sequences were aligned and phylogenetic tree was obtained using Clustal W software available at EMBL.

**Morphological changes in cell lines:** In the IBRS and CHO cell lines after sub culturing within 24 h the medium became yellow in colour without any turbidity and morphological changes such as elongation of cells and loss of cell sheet were noticed in comparison to control cells as shown in Figs 1a, b.

**Genus specific PCR:** Total RNA extracted from 7 cell cultures namely HeLa, MDCK, CRFK, IBRS, CHO, VERO and BHK21 were subjected to RT-PCR. The expected

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Fig. 1a. Normal cells.

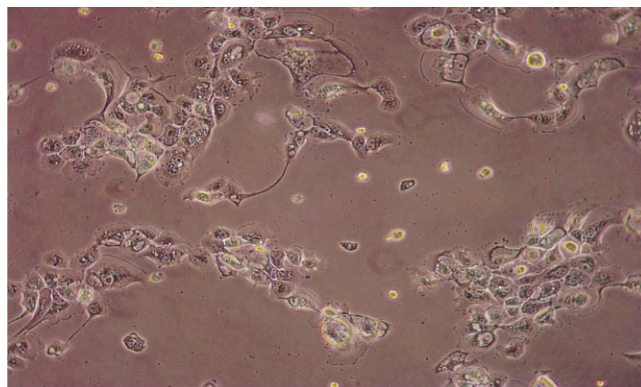


Fig. 1b. Infected cells.

amplicon size of 139bp was observed for IBRS and CHO cell line samples while other cultures showed negative results (Fig. 2).

**Species specific PCR:** A distinct band at 130bp was observed in ethidium bromide stained 1.5% agarose gel (Fig. 3). Then, the PCR products were purified and sequenced.

**Nucleotide sequencing and BLAST analysis:** Sequences obtained after sequencing were subjected to BLAST analysis. 100% and 98% homology to *Mycoplasma hyorhinis* was found for IBRS (Accession No GU564253) and CHO (Accession No GU564254) cell lines, respectively. Phylogenetic analysis indicated that our samples are very much closer to Spain and Brazil isolates.

The immunological detection by ELISA and biochemical detection using *Mycoplasma*-specific adenosine phosphorylase are limited by cross-reactivity and sensitivity, and require large numbers of *Mycoplasma* cells. Fluorochrome staining agents are complicated and hindered by nonspecific background of cell debris, media components, or mitochondrial DNA (Chen 1977). Despite limitations, culture is the method of choice, when other methods do not yield conclusive results. However, non-culturable *M. hyorhinis* strains elude such diagnostic tests (Sung *et al.*

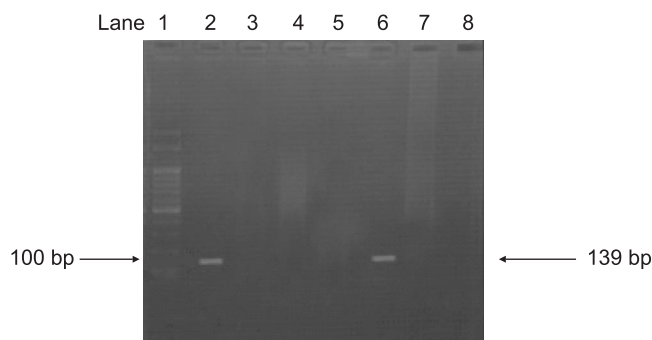


Fig. 2. Agarose gel electrophoresis for the demonstration of RT-PCR amplicon. Lane 1, 100bp ladder; Lane 2, IBRS cell line sample; Lane 3, HeLa cell line sample; Lane 4, CRFK cell line sample; Lane 5, VERO cell line sample; Lane 6, CHO cell line sample; Lane 7, MDCK cell line sample; lane 8: BHK21 cell line sample.

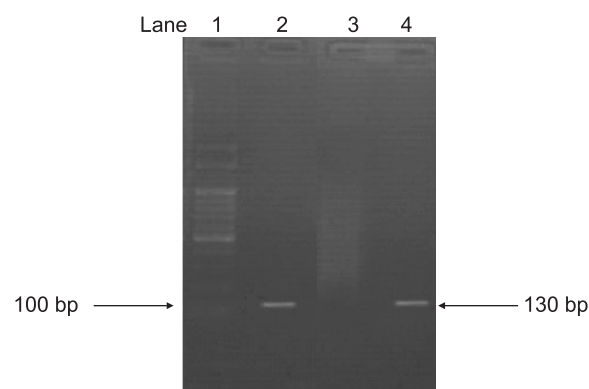


Fig. 3. Agarose gel electrophoresis for the demonstration of nested- PCR amplicon. Lane 1, 100bp ladder; Lane 2, IBRS cell line sample; Lane 4, CHO cell line sample.

2006). Recently a 16S rRNA-based mycoplasma group-specific PCR (Van Kuppeveld *et al.* 1994) was used to increase specificity. The 16S rRNA genes, 16S–23S rRNA intergenic spacer regions, and 23S rRNA genes have been widely used as targets to detect and identify many different types of bacteria (Clayton *et al.* 1995).

The method is also useful for detecting if more than one *Mycoplasma* infections are present in cell cultures. The lengths and sequences of the spacer region in the rRNA operon differ among *Mycoplasma* species and this heterogeneity can be used for species identification (Tang *et al.* 2000). The total spacer region for rRNA operon of *M. hyorhinis* is 447bp and in our study, a part of intergenic region of 130 bp was amplified i.e flanking 34 to 164 nucleotides. The spacer region lengths in the rRNA operon of *M. arginini*, *M. fermentans*, *M. orale*, *A. laidlawii* were 368bp, 490bp, 422bp, 440bp respectively. This indicates that there is nucleotide sequence variability among mycoplasmal species (Kong *et al.* 2001).

In this study RT PCR was carried out to amplify 16s rRNA gene to identify *Mycoplasma* genus as reported by Van Kuppeveld *et al.* (1992) and species specific identification of *Mycoplasma* was carried out by amplification of 16s–23s

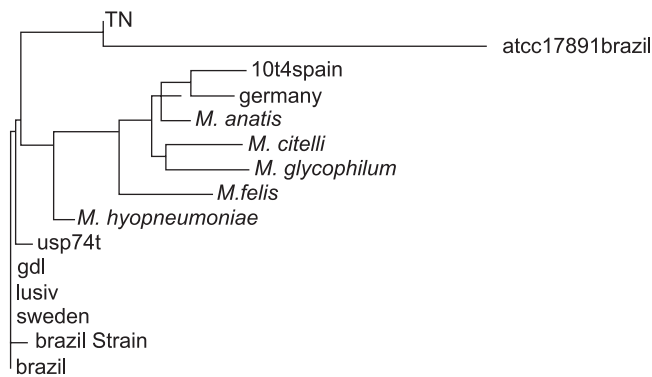


Fig. 4. Phylogenetic tree to indicate the close relationship of our mycoplasma sample with other isolates.

rRNA intergenic spacer region by conventional PCR as reported by Rawadi and Dusserget (1995).

The primary objective of this study was to standardize sensitive and specific method for detection of *Mycoplasmas* in cell culture, using PCR. Evolutionary conserved region of rRNA genes and intergenic spacer region were utilized as an amplification target, due both the abundance of usable nucleotide sequences and the size difference of the factor among many species.

Sequence analysis was done for further confirmation of PCR products to eliminate the non-specific amplification in PCR assay (Barry *et al.* 1991).

Previous studies showed that several species can be detected via 2-stage nested PCR. However, these procedures remain controversial with regard to their sensitivity. Optimization of primer sequences and the reaction conditions presented here enhanced the sensitivity and ensured the high degree of specificity of the PCR. It was revealed that the specificity of the method presented here was sufficient for the discrimination of mycoplasma contamination from other probable contaminants, including *E. coli* and *S. aureus*.

#### SUMMARY

Present study aimed to detect common (*Mycoplasma arginini*, *M. orale*, *M. hyorhinis* and *M. fermentans*) cell culture contaminants using PCR. The method was sufficient for the discrimination of mycoplasma contamination from other probable contaminants, including *E. coli* and *S. aureus*.

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