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Full Length Research Paper

Cell cultures contaminations by mycoplasmas

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The contamination of cell cultures with mycoplasmas can have different cytogenetic effects, usually adhere to cell but, depending on the species, these bacteria deplete the nutrients of cell cultures and interfere with the response of these cells when challenged experimentally. The objective of the present study was mycoplasmas detection in cell cultures came from biomedical laboratories. The cell cultures were screened for mycoplasmas by using of microbiological culture and Polymerase Chain Reaction (PCR). Primers AR1 and AR2 were used for amplification of a 301-bp fragment from mycoplasmas DNA. Mycoplasmas were detected by culture and PCR in 68/88 (77%) and 78/88 (88.7%) samples, respectively. Mycoplasmas detection between the microbiological culture and PCR showing significant differences ($p < 0.05$).The use of two methods has been the most recommended strategy to minimize false results.

Key words: Mycoplasmas, cell culture, contamination, nested PCR.

INTRODUCTION

Mycoplasmas (class Mollicutes) are small prokaryotes that can pass through 0.22 µm filters and lack the rigid peptidoglycan cell wall and are bound by a single membrane, the plasma membrane. Wall-less bacteria were first described 110 years ago, and now over 190 species, widely distributed among humans, animals, insects and plants, are known (Razin et al., 1998; Rottem and Naot, 1998). Most human and animal Mollicutes are *Mycoplasmas* and Ureaplasma species of the family *Mycoplasmataceae*. Because mycoplasmas have an extremely small genome, these organisms have limited metabolic options for replication and survival (Maniloff, 1996). Owing to their limited biosynthetic capabilities, most mycoplasmas are parasites exhibiting strict host and tissue specificities. The mycoplasmas enter an appropriate host in which they multiply and survive for long periods of time (Rottem, 2003).

Contaminations of cell cultures with microbial organisms as well as with viruses or other eukaryotic cell lines are a major problem in cell culture-related research. Cell cultures are widely used in both biomedical and biotechnological research centers and industry, as well as for diagnostic test in hospitals (Kong et al., 2007; Volokhov et al., 2008). Contaminating mycoplasmas may alter host cellular characteristic, enzyme patterns; cell membrane composition, chromosomal abnormalities and the induction of cytopathogenic changes have been described (Van Kuppeveld et al., 1994; Mariotti et al., 2008). Contamination with microbial organism or cross-contaminations with other eukaryotic lines may to diminished cell growth and could result in loss of the original culture. The concentration of mycoplasmas in infected cultures can be as 10^7 colony forming units per ml and contaminations is not obvious, either macroscopically or microscopically; thus, routine mycoplasmas testing is essential for any cell culture laboratory. Many of the testing procedures developed so far time-consuming, expensive, inconclusive and unsuitable for screening large number of test specimens (Störmer et al., 2009; Kumar et al., 2008). The aim of the present study was mycoplasmas detection in cell cultures came from biomedical laboratories.

MATERIALS AND METHODS

Cell cultures

The cell cultures used in this study came from biomedical laboratories. The cell cultures were sent to the Mycoplasmas Laboratory of the Microbiological Research Center, Instituto de

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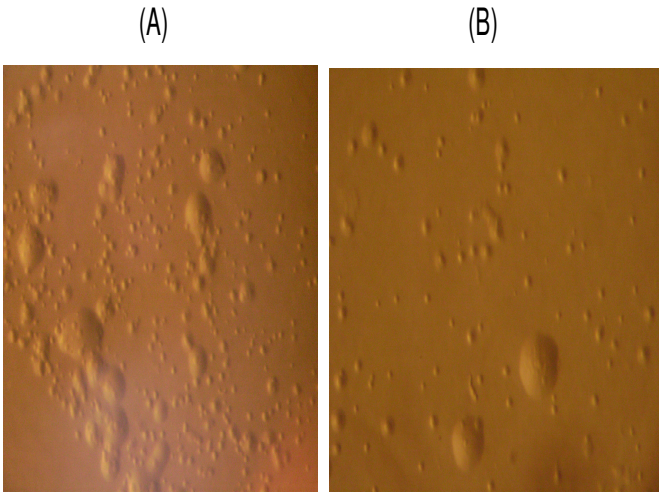

Figure 1. Characteristic colonies "fried egg" of mycoplasmas. Isolated mycoplasmas from Vero (A) and MRC-5 (B) cell cultures, growing on SP-4 agar. Magnification, X40.

Ciencias de la Benemérita Universidad Autónoma de Puebla, for screening of mycoplasmasl contamination, either for routine monitoring or because it was suspected that the cell lines were contaminated. The broad variety of cell cultures tested included Vero, Hep-2, RK-13, MRC-5, MDBK, L929, 3T3L1 and BHK-21, a total of 88 cell culture samples were studied.

Mycoplasmas detection methods

The cell cultures were screened for mycoplasmas by using of microbiological culture and PCR. Cell cultures were grown in the absence of antibiotics for 3 - 4 days and washed with PBS.

Microbiological culture

Each cell samples was diluted 10^{-1} to 10^{-3} in 2 ml SP-4 broth and 5 µl of each dilution was inoculated onto solid medium. The cultures were incubated for 20 days at 37°C under aerobic conditions. The microorganism was presumptively identified based on alterations in the pH of the broth in the absence of turbidity, production of fried egg colonies.

Nucleic acid isolation and PCR analysis

About 10^5 cells were suspended in 1 ml of lysis buffer containing: 10 mM Tris HCl (pH 8.5), 100 mM KCl, 2.5 mM $MgCl_2$, 1% Tween-20, 1% Triton X-100 (Research Organics, Cleveland-Ohio, USA) and 120 µg ml^{-1} of proteinase K (Promega, Madison-WI, USA). The cell suspensions were incubated at 55°C for 20 min followed by 95°C for 15 min. In a PCR reaction, cell lysate corresponding to about 10^4 cells was used for amplification.

Primers AR1 sense and AR2 anti-sense were used for amplification of a 301-bp fragment from mycoplasmas DNA. They were selected from a highly conserved sequence of 16S rRNA gene of mycoplasmas after a comparison of nucleotide sequence of 30 different species. The sequence of the sense primer is 5´ ATG RGG RTG CGG CGT ATT AG 3´ and the anti-sense primer is 5´ CKG CTG GCA CAT AGT TAG CCRT 3´. Symbol K stands for mixed nucleotides of G and T, and R stands for A and G. PCR was carried out in a total volume 25 µl which includes: 10 mM Tris HCl (pH 8.5), 200 µM each of dNTP´s, 50 mM KCl, 3.5 mM $MgCl_2$ (Research Organics, Cleveland-Ohio, USA), 2.5 U of Taq polymerase (Promega, Madison-WI, USA) and 0.3 µM of each respective primer (GibcoBRL, Grand Island-New York, USA). The thermal profile included an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. Aliquots of the amplified product (20%) were electrophoresed through 2% agarose gels and DNA bands were visualized by UV transilluminator after ethidium bromide staining, and then photographed (Sidhu et al., 1995).

Mycoplasmas DNA preparation

The following mycoplasmas ATCC strains were used as control: *Mycoplasma fermentans* PG-18, *Mycoplasma penetrans* GTU-54 and *Mycoplasma hominis* PG-21. The cultures obtained were resuspended in 1 ml of lysis buffer and incubated for 1 h at 60°C to lyse and deproteinize the cells. Proteinase K was inactivated at 95°C for 10 min and samples were allowed to cool at room temperature.

Statistical analysis

Comparison between microbiological cultured and PCR were made using Student T test, and were performed using INSTAT version 2.0 Software and differences were considered significant at the $p < 0.05$.

RESULTS AND DISCUSSION

One of the more important sources of Mollicutes contamination in eukaryotic cell culture systems comes from *Mycoplasmas* or *Acholeplasmas* species present in such cell cultivation. Although significant improvement in eradicating Mollicutes from serum supplements has come about with ultrafiltration (passage through pore diameters of 35 - 45 nm) of some commercial fetal bovine serum products, the cost and other availability factors have frequently limited the use of such products.

Mycoplasmas were detected by culture in 68/88 (77.3%) of the cell culture samples, the cultures mycoplasmas presented characteristic grown colonies "fried egg" (Figure 1) and shifted the pH of the broth without turbidity.

Primers AR1 and AR2 were used to amplify a 301-bp fragment from at least 30 different mycoplasmas species including the ones most commonly found in cell cultures. PCR using primers to detected Mollicutes revealed the presence of targeted DNA in 78/88 (88.7%) samples (Figure 2). Ten (12.8%) samples were positive by PCR and negative by culture. Mycoplasmas detection between the microbiological culture and PCR showing significant differences ($p < 0.05$). A comparison of the PCR technique with the microbiological culture indicated that the PCR is a rapid, sensitive, and efficient method. Culturing mycoplasmas can take 1 to 4 weeks and can be difficult because of a requirement for special growth

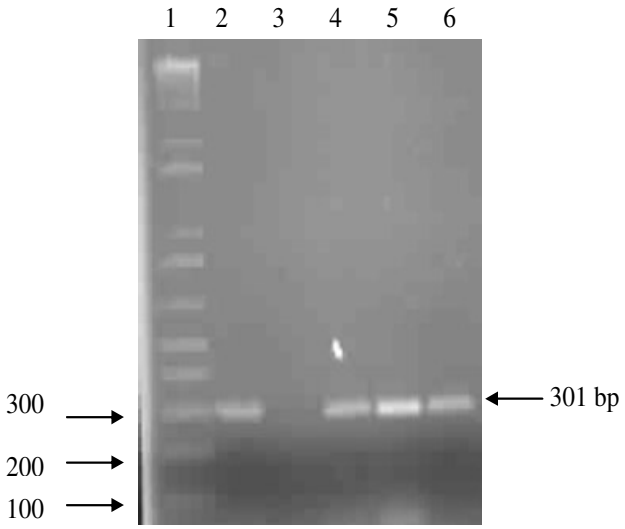

Figure 2. Detection of mycoplasmas contamination in various cell cultures. Lane 1, 1-kb ladder; lane 2, positive control; lane 3, reagent control; lanes 4-6 from a contaminated Vero, Hep-2 and L929 cell cultures, respectively.

Table 1. Comparison of the results of microbiological culture and PCR analysis.

Cell cultures	Methodologies culture		Analysis PCR	
	Positive*	Negative	Positive*	Negative
Vero	28	10	31	7
Hep-2	10	0	10	0
RK-13	2	4	5	1
MRC-5	6	0	6	0
MDBK	8	2	10	0
L929	8	4	10	2
3T3L1	2	0	2	0
BHK-21	4	0	4	0
Total	68	20	78	10

* Significant difference ($p < 0.05$).

conditions (Del Giudice et al., 1980; Hopps et al., 1973; Pruckler and Ades, 1995). Table 1 shows the general results obtained for the samples evaluated by culture and PCR. The mycoplasmas reference strains grew on liquid and solid medium and were confirmed by PCR.

In this study, the microbiological culture technique produced negative results for ten cell cultures that were positive as determined by the PCR technique. Retesting of the original ten cell cultures revealed again positive amplification by the PCR, which indicates that DNA carryover contamination may be excluded.

The detection of mycoplasmas species in cell cultures remains a problem, despite the substantial improvements that have been made in recent years in biochemical, immunological and molecular biological methods (Sung et al., 2006; Uphoff and Drexler, 2004). Although each of these methods is associated with certain advantages, these immunological procedures are often limited by the presence of intra-species cross-reactivity. Also, the cultivation of these species tends to be time-consuming and difficult to achieve, due to the requirement of fastidious conditions for their growth. Moreover, the majority of currently available detection procedures are not sufficient for the simultaneous detection of the major mycoplasmas species contaminants commonly encountered in *in vitro* cell cultures (Wirth et al., 1994; Loudová and Novosad, 2004).

PCR-based methods for detection of certain DNA regions of the mycoplasmas genome have proven both rapid and specific. However, the degree of sensitivity of these techniques tends to be somewhat low, as the nucleotide sequences of primers tend not to perfectly match the target DNA from different species. Primers have, however, been specially designed to target the conserved regions of the 16S rRNA gene or the 16S-23S rRNA regions encountered in mycoplasmas species. These procedures have been associated with a sensitivity of the between 5 and 100 organisms (Harasawa et al., 1993; Sidhu et al., 1995; Garner et al., 2000).

The diversity of mycoplasmas species in the same cell culture indicates the occurrence of different initial infections sources. The subculturing of a cell culture among laboratories over time due to successive sharing may explain the detection of multiple mycoplasmas species (Uphoff and Drexler, 2002; Uphoff and Drexler, 2005).

In conclusion, mycoplasmas is a common contamination of cell culture samples, a frequent source for mycoplasmas contaminations is contaminated cell culture medium ingredients or the experimenter. Mycoplasmas can cause impairment of many cellular functions such as inhibition of cell proliferation, protein biosynthesis, and alteration of immunological reactions, microarray gene expression profiles and virus replication. Due to their small cell size, mycoplasmas are difficult to detect and lack a cell wall some antibiotics (e.g. ampicillin) are not effective to suppress mycoplasmas growth. Culture methodologies in combination with PCR are the most widely recommended strategy to minimize false results.

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