PCR-based detection and eradication of mycoplasmal infections from various mammalian cell lines: a local experience

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Abstract A total of 200 cell lines including different human, monkey, mice, hamster and rat cell types were examined for mycoplasma infection status. PCR assay using generic-specific universal primers showed that 40 (20%) of the cell lines are contaminated with mycoplasma. Employment of species-specific primers within these infected cell lines revealed infection with *M. hyorhinis* (42.5%),

M. fermentas (37.5%), *M. arginini* (37.5%), *M. orale* (12.5%) and *A. laidlawii* (7.5%). A number of the cultures were coinfecting with 2 or 3 different species. Contaminated samples were treated with BM-Cyclin, Ciprofloxacin and mycoplasma removal agent (MRA). Mycoplasma eradication was subsequently checked by PCR following 2 weeks continuous culture of treated cells in antibiotic free culture medium. Mycoplasmal infections were eradicated in 100, 70 and 42% of infected cell lines when the samples were treated with BM-Cyclin, MRA and Ciprofloxacin, respectively. However, 12% (BM-Cyclin), 62.5% (MRA) and 82.5% (Ciprofloxacin) of mycoplasma regrowth was observed 4 months after the treatment. Notably, the risk of spontaneous culture death was 17.5, 12.5 and 0% for BM-Cyclin, MRA and Ciprofloxacin, respectively.

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Introduction

Continuous cell lines are being widely used in variety of experimental researches. Among the various problems associated with cell lines, are contaminations. The two most common concerns in this respect are: (1) cross-contamination with unrelated cells and

(2) contamination with microorganisms, in particular mycoplasmas (Drexler et al. 2002).

Mycoplasmas form a distinct class of mollicutes within prokaryotes, as they lack a rigid cell wall (Drexler and Uphoff 2002). One mycoplasma cell can grow up to 10^6 colony forming units per milliliter within three to 5 days in an infected cell culture (Barile and Rottem 1993). Mycoplasmal infections can have a myriad of different effects on the contaminated cell cultures (Rottem and Naot 1998). However, they do not affect various cell lines in the same manner and to the same degree (Drexler and Uphoff 2002; Jung et al. 2003). More than 50 years have passed since Robinson and Wichelhausen (1956) reported the first isolation of mycoplasma from a cell culture. Since then, about 20 species have been isolated from various cell lines (Tang et al. 2000). While, the species *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. orale*, *M. salivarium*, *M. fermentans* and *M. hyorhinae* are detected in about 95% of cell cultures that accidentally were infected with these microorganisms, other species such as *Ureaplasma urealyticum*, *M. pneumoniae* and *M. pirum* are rarely detected in cell cultures (Boolske 1988; Tang et al. 2000). According to the literature, the percentages of these species may vary greatly among studies (Drexler and Uphoff 2002). These variations might be due to the limited number of studied cell types and geographical differences.

The first step to deal with mycoplasmal contamination is to detect and identify the contaminant species. In case of replaceable cells it is advisable to destroy the culture to prevent the hazard of mycoplasma dissemination. If the contaminated cells are not replaceable and eradication of the infection is to be considered it is important to reduce the risk of infection to other laboratory cultures by handling any affected cultures separately in a quarantine area. Morphological and biochemical assays for detection of mycoplasmas have been described elsewhere (Rottem and Barile 1993). However, very few contaminations can be detected by examining the morphological changes, which led to the development of many detection methods such as fluorescence DNA staining using 4,6-diamidino-2-phenylindole (DAPI) or Hoechst dyes (Jung et al. 2003), nucleic acid hybridization with a probe specific for mycoplasmal rRNA (Mattsson and Johansson 1993)

enzyme-linked immunosorbent assay (ELISA) with mycoplasma specific polyclonal antibodies (Drexler and Uphoff 2002), and polymerase chain reaction (PCR) (Kong et al. 2001).

In a comparative study by Uphoff and Drexler (2002), a PCR-based detection method was reported as the most rapid detection method, since it produces significantly fewer false-negative or false-positive results than DAPI DNA fluorescence staining, immunostaining with a monoclonal antibody, ELISA and DNA–RNA hybridization in solution. Moreover, PCR also showed better sensitivity when samples were diluted, perhaps due to minimizing PCR inhibiting factors present in template DNA (Jung et al. 2003; Hopert et al. 1993).

Although prevention measures have been reported elsewhere (Drexler and Uphoff 2002), it is not advisable to try mycoplasma treatment as a routine practice because of the low success rate and high risk of transmission of infection. A number of decontamination protocols have been employed in recent years with different efficiencies (Lincoln and Lundin 1990; Kotani et al. 1991; Fleckenstein and Drexler 1996). However, the most promising technique, which is relatively simple and inexpensive, appears to be antibiotic treatment (Fleckenstein and Drexler 1996). During antibiotic disinfection, it is important to closely monitor effectiveness of the clean-up relative to mycoplasma elimination and eukaryotic cytotoxicity (Lincoln and Lundin 1990). It has been suggested that the most efficient approach is to identify the contaminant at the species level and determine the antibiotic sensitivity of the infectant (Gardella and Del Giudice 1984). Then the mycoplasma positive eukaryotic cell culture is exposed to several consecutive treatments with an antibiotic cocktail (Fleckenstein and Drexler 1996). The aim of this study was to explore the status of mycoplasmal contaminations in a collection of human and animal cell lines deposited in the National Cell Bank of Iran (NCBI) and to determine the effect of different antibiotic treatment procedures. For this purpose, different cell lines from human, monkey, mouse, hamster and rat were chosen for analyzing their mycoplasmal contamination status and evaluating effectiveness of the most common antibiotic reagents for mycoplasma decontamination. Due to the fastidious nature of mycoplasma, it is recommended that infected cells are discarded unless they can not be

replaced. As such, infected cultures should be held separately from clean cultures, ideally in a quarantine laboratory.

Materials and methods

Culture of cell lines

A total of 200 cell lines including 40 different cell types (Table 1) from the National Cell Bank Iran (Pasteur Institute of Iran, Tehran, Iran) were analyzed in this study. Cell lines were grown at 37 °C in a humidified atmosphere of air containing 5% CO₂. The basic growth media were supplemented with 10–20% fetal bovine serum (Sigma, Deisenhofen, Germany). For growth factor-dependent cell lines, specific growth factors or conditioned media containing growth factors were added (Drexler et al. 2001). All supplements, such as serum, conditioned media and trypsin were mycoplasma negative as indicated by the suppliers. For detection of mycoplasma, the cell lines were cultured initially for at least 1 week after thawing, and samples were taken after a culture period of at least 2 days without medium exchange. No antibiotics were added to the cultures.

Mollicute strains

The following species were purchased from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC) and were used as control: *U. urealyticum* (NCTC 10177T), *M. genitalium* (NCTC 10195), *M. salivarium* (NCTC 10113), *M. pneumoniae* (NCTC 10119), *A. laidlawii* (ATCC 23206), *M. orale* (ATCC 23714), *M. hyorhinis* (ATCC 17981), *M. fermentans* (ATCC 19989), *M. hominis* (ATCC 23114), *M. arginini* (ATCC 23838).

Preparation of genomic DNA

Genomic DNA extraction from mollicutes species and DNA of each cell line were performed according to the standard protocols previously described by Tang et al. (2000) with slight modifications. Briefly, Mycoplasma cells from late log phase were harvested by centrifugation for 1 min at 12,000g. The pellets were resuspended in STE buffer (10 mM NaCl,

20 mM Tris HCl and 1 mM EDTA; pH 8.0) and incubated for 2 h at 37 °C with 1% (w/v) SDS and 40 µg of proteinase K (Promega, USA) per ml. Proteins were then precipitated by addition of equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). The total nucleic acids were then precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 96% (v/v) ethanol. The pellets were then washed with 70% (v/v) ethanol and air-dried for 15–30 min. Eventually, the pellets were resuspended in sterile distilled water containing RNase.

Oligonucleotides and PCR-based detection of mycoplasmas

The mycoplasmal infection status was investigated before and after antibiotic treatment using PCR-based method. A total of 11 species-specific primers suitable for the detection of 11 mollicutes, as well as a universal generic-specific primer capable of detecting all mycoplasma species (listed in Table 2) were used to target the conserved region of 16S rDNA intergenic spacer regions. The universal primers were degenerate having equal quantities of T and C nucleotides at position 13 (Y) of the sense and A and G nucleotides at position 20 (R) of the anti-sense primers, respectively. PCR reaction mixtures were prepared in a total volume of 25 µL containing, 1× PCR buffer, 50 µM each of dNTP, 15 pmol of each primer, 1 U Taq DNA polymerase, 1.5 mM MgCl₂, and 0.1 µg of mycoplasmal genomic DNA or 1 µg of DNA from cell line as template. Thermal profiles were as follows: initial denaturation at 94 °C for 3 min, 32 cycles consisting of denaturation at 94 °C for 60 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The annealing temperature of the universal primers was set at 55 °C and all other conditions were the same as the species-specific PCR. The PCR products were analyzed on 1% (w/v) agarose gel. DNA fragments were visualized with a UV transilluminator after being stained with ethidium bromide.

Antibiotic treatment of mycoplasmal contaminants

The infected cell lines were treated with three antibiotics. The treatment protocols using BM-

Table 1 PCR results of mycoplasma detection in 40 cell lines

Cell line name	Cell type	NCBI code	Ma	Mf	Mo	Mh	Al
MCF-7	Human breast Adeno carcinoma	C135	—	—	—	+	—
T-47D	Human breast ductal carcinoma	C203	—	+	—	—	—
RAJI	Human Burkitt's lymphoma	C127	—	+	—	—	—
NSO	Mouse myeloma	C142	—	+	—	—	—
HEP G2	Human hepatocyte carcinoma	C158	—	—	—	+	—
A-431	Human squamous carcinoma	C204	+	—	—	+	—
HFFF-PI6	Human fetal foreskin fibroblast	C170	—	—	+	—	—
Hep2	Human Larynx carcinoma	C144	—	—	—	+	—
MRC-5	Human foetal lung fibroblast	C125	+	—	—	—	—
Saos-2	Human osteogenic sarcoma	C453	+	+	—	+	—
McCoy	Synovial tissue fibroblast	C123	+	—	—	—	—
CCRF-CEM	Human Acute lymphoblastic leukemia	C105	—	+	—	—	—
PANC-1	Human pancreas duct epithelial carcinoma	C556	+	+	—	+	—
B95-8	Marmoset EBV transformed lymphocytes	C110	+	+	—	—	—
CHO	Chinese hamster ovary	C111	+	—	—	—	—
DAUDI	Human Burkitt's lymphoma	C112	—	—	+	—	—
BT-474	Human breast ductal carcinoma	C435	+	—	—	—	—
J1YOYE	Human Burkitt's lymphoma	C117	—	—	+	—	—
MDA-MB-468	Human breast adenocarcinoma	C208	+	+	—	+	—
HUT-78	Human cutaneus T cell lymphoma	C185	+	+	—	—	+
J774A.1	Mouse monocyte/macrophage	C483	+	—	—	+	—
LNCap-FGC-10	Human prostate cancer	C439	—	—	—	+	—
F3B6	Human × mouse heterohybridoma	C197	+	—	+	—	—
SP2/0-Ag14	Mouse myeloma	C129	—	+	—	+	—
Vero	Monkey kidney	C101	+	+	—	+	—
K562	Human CML	C122	—	—	—	—	+
WEHI-164	Mouse BALB/c fibrosarcoma	C200	—	—	—	+	—
BCL1 clone 5B1b	Mouse lymphoma	C551	—	—	—	—	+
SK-N-MC	Human neuroblastoma	C535	—	+	—	—	—
BW5147	Mouse thymoma	C542	—	—	—	+	—
STO	Mouse SIM fetal fibroblast	C537	—	—	—	+	—
CT26	Mouse colon carcinoma	C532	—	—	—	+	—
THP-1	Human acute Monocytic leukemia	C563	—	—	+	—	—
PC12	Rat adrenal fibroblast pheochromocytoma	C153	—	—	—	+	—
Seraphina	Human Burkitt's lymphoma	C102	—	+	—	—	—
LCL-PI 12	Human EBV transformed cord blood B cell	C178	—	+	—	—	—
HGF3-PI 53	Human gingival fibroblast	C502	—	—	—	+	—
HSF-PI 17	Human skin fibroblast	C193	—	+	—	—	—
B65	Rat nervous tissue neuronal	C134	+	—	—	—	—
P3X63Ag8.653	Mouse-myeloma	C109	+	—	—	—	—

Ma, *M. arginini*; Mf, *M. fermentans*; Mo, *M. orale*; Mh, *M. hyorhinis*; Al, *A. laidlawii*; (—), non-detected mycoplasma species; (+), detected species-specific mycoplasma; NCBI, National Cell Bank of Iran

Table 2 The sequences of oligonucleotide primers used for detection of mycoplasmas

Mycoplasma species	Primer sequence	Amplicon size
Universal primer	S: GTG GGG AGC AAA YAG GAT TAG A AS: GGC ATG ATG ATT TGA CGT CRT	425 bp
<i>M. arginini</i>	S: TGA TCA TTA GTC GGT GGA GAG TTC AS: TAT CTC TAG AGT CCT CGA CAT GAC TC	326 bp
<i>M. orale</i>	S: TGA TCA TTA GTC GGT GGA AAA CTA S: TGA TCA TTA GTC GGT GGA AAA CTA	325 bp
<i>M. hyorhinis</i>	AS: TAT CTC TAG AGT CCT CGA CAT GAC TC AS: AGG CAG TAT CTC TAG AGT CCT TAA CTT A	334 bp
<i>M. fermentans</i>	S: TGA TCA TTA GCT GAT GGG GAA CT AS: TCT CTT AGA GTC CTC AAC TAA ATG	324 bp
<i>M. genitalium</i>	S: ATA GAT ACT AGC TGT CGG AGC GAT AS: CCA ATT TAC ATT AGC AGT CTC GTT AA	335 bp
<i>A. laidlawii</i>	S: GAT GAG AAC TAA GTG TTG GCC ATA A AS: CGC TAG AGT CCC CAA CTT AAT GA	300 bp
<i>M. hominis</i>	S: ATC ATT AGT CGG TGG AGA ATC A AS: GCA GTA TCT CTA CTA GAG TCC TCA ACT TAAT	301 bp
<i>M. pirum</i>	S: TGG ATG TTA GAT GTC GGG GTA AA AS: GTT GGC AGT ATC GCT AGA CAA A	324 bp
<i>M. pneumoniae</i>	S: GAT ACT AGC TGT CGG GGC GAT AS: AAT TTG CAT TAG TAG CAG TCT CGC TAG	329 bp
<i>M. salivarium</i>	S: GAT CAT TAG TCG GCA GAG AAC TCG AS: TAT CTC TAG AGT CCT CGA CAT GAC TC	324 bp
<i>U. urealyticum</i>	S: CAT CAT TAA ATG TCG GCT CGA A AS: CGG TAG CAG TAT CGC TAG AAA AGC	323 bp

Cyclin, Ciprofloxacin and mycoplasma removal agent (MRA) have been described in detail elsewhere, with little modifications (Fleckenstein et al. 1994; Uphoff et al. 1992, 2002). Treatment with BM-Cyclin (Roche, Mannheim, Germany, cat. No. 799050) was carried out according to the manufacture's instructions. Samples were treated with BM-Cyclin I (10 µg/mL) for 3 days followed by BM-Cyclin II (5 µg/mL) for 4 days. This alternating cycle was performed three times. Ciprofloxacin (ICN, USA, cat. No. 199020) was used for 14 days at 10 µg/mL. Mycoplasma removal agent (Serotec, UK, cat. No. Buf035) was added to the culture medium for 10 days at a final concentration of 0.5 µg/mL. Following the treatment with these reagents, cells were cultured in antibiotic-free medium (also without penicillin, streptomycin or other commonly used antibiotics) for at least another 2 weeks prior to testing for residual mycoplasmal contamination. All of the cured cultures were retested for

regrowth of mycoplasmas for up to 4 months following the treatment.

Results

Detection of mycoplasmal contamination with PCR

Results obtained from PCR-based detection methods of mycoplasmas are summarized in Table 1. Infected cultures were initially identified using universal primers detecting all species of mycoplasma (Timenetsky et al. 2006). These cell lines were subsequently screened by species-specific primers. Specificity of these primers was initially verified using standard mycoplasma species provided by ATCC and NCTC, as described in the Materials and Methods. 30/40 (75%) of the infected cell

Fig. 1 Outcome of treatment of mycoplasma-positive cell cultures with three antibiotics including BM-Cyclin, Ciprofloxacin and MRA

cultures contained one mycoplasma species. Other samples were infected with two 5/40 (12.5%) or three 5/40 (12.5%) species. *M. hyorhinis* was detected in 17/40 (42.5%) of the studied samples, *M. fermentans* in 15/40 (37.5%), *M. arginini* in 15/40 (37.5%), *M. orale* in 5/40 (12.5%) and *A. laidlawii* in 3/40 (7.5%). *M. hyorhinis* and *M. fermentans* were present as single infecting agent in 9/40 (22.5%) and 8/40 (20%) samples, respectively.

Treatment with antibiotics

The results obtained from eradication experiments are summarized in Fig. 1. Mycoplasma infections were eliminated by Ciprofloxacin, BM-Cyclin and MRA in 42.5, 100 and 70% of the infected cell cultures, respectively. Furthermore, the decontamination was confirmed by PCR, as no mycoplasma was detected in treated cultures 14 days after completion of antibiotic treatment (Fleckenstein and Drexler 1996). However, the percentages of cultures which were found to be re-infected with mycoplasma after the period of 4 months were in the range of 12.5–82.5%. Besides curing and regrowth of mycoplasmas, the third type of outcome of the antibiotic treatment was loss of the culture which occurred in 12.5 and 17.5% for MRA and BM-Cyclin, respectively (Fig. 1).

Discussion

Mycoplasma is the most important and common source of cell culture infection, worldwide. Unlike

bacterial and fungal infections which are readily identified by microscopic and visual inspections, mycoplasma infected cultures are hardly, if ever, detected by these routine inspections. Infected cell lines are usually identified either by the use of mycoplasma-specific culture media or PCR technique.

Accidental infection of cell cultures with mycoplasmas, especially when unnoticed, usually invalidates the results of biomedical researches. The cell cultures may not die, but remain altered and inadequate for experimentation for a long period of time (Fleckenstein and Drexler 1996; Drexler et al. 2002).

Mycoplasma-infected cell lines are themselves the single most important source for further spreading of the contamination. This is due to the high concentration of mycoplasmas in infected cultures, and the prolonged survival of dried mycoplasmas (Drexler and Uphoff 2002). Operator-induced contamination is also a potential issue. Mycoplasmas spread by using laboratory equipment, media or reagents that have been contaminated (Drexler and Uphoff 2002).

In a study by Timenetsky et al. (2006), *M. orale*, *M. hyorhinis* and *A. laidlawii* were found to be the most frequent species in cell cultures. While Uphoff and Drexler (2002) observed most of their cell lines were contaminated with *M. fermentans*, in our study, however, the most frequent species was *M. hyorhinis* (42.5%), followed by *M. arginini* (37.5%), *M. fermentans* (37.5%), *M. orale* (12.5%) and *A. laidlawii* (7.5%).

M. hyorhinis, a common inhabitant of nasal cavity of swine, also accounts for a high proportion of the infections (Drexler and Uphoff 2002). *M. arginini* has a wide host range as they have been isolated from mammals, birds and insects (Drexler and Uphoff 2002). *M. fermentans* was believed to be associated with the development of AIDS in HIV-positive individuals in 1986. However, the species is now considered to be a normal inhabitant of the human urogenital tract (Blanchard and Montagnier 1994). Therefore, the increase in the frequency of *M. fermentans* in cell cultures can be explained by the growing use of human blood cells or tissues for primary culture (Timenetsky et al. 2006). The high incidence of the species *A. laidlawii* and *M. arginini* seems to be in direct correlation with the use of fetal or newborn bovine serum (Barile and Rottem 1993). *M. orale* inhabits the human oropharynx and in

addition to cell line cross-contamination may infect cell cultures through the aerosol generated by mouth pipetting (Razin et al. 1998), although this practice is now prohibited in most cell culture laboratories.

In the present study challenging results were obtained from eradication experiments, as each antibiotics showed advantages and disadvantages. For instance, mycoplasma infections were successfully eliminated using BM-Cyclin, but 17.5% loss of cultures was observed, spontaneously. We could manage to overcome this drawback by using Ciprofloxacin, but the percentage of successful eliminations was decreased down to 42.5%. Intermediate results were obtained when MRA was used where 70% of elimination and 12.5% loss in culture were observed (Fig. 1). According to the literature, BM-Cyclin showed no cytotoxicity on cultures in a study by Fleckenstein and Drexler (1996), while Somasundaram et al. (1992) observed high levels of cytotoxicity. However, our results showed moderate levels of cytotoxicity which does not support any of the previous reports. Although Ciprofloxacin showed no cytotoxicity effect in our study, it has been recently reported that it has an effect on intracellular topoisomerase II in human cells as well as ability to break double strand DNA (Gurbay et al. 2007). Our findings indicate that BM-Cyclin is the most suitable antibiotic for the eradication of mycoplasma compared with the other two antibiotics, in particular with regards to the low percentage of mycoplasma regrowth (12.5%) after 4 months of treatment. Ciprofloxacin and MRA showed 82.5 and 62.5% of regrowth, respectively (Fig. 1).

In summary, mycoplasmal infection status of 200 cell lines including different mammalian cell types deposited in the National Cell Bank of Iran was obtained using PCR-based assay that allowed detection and identification of mycoplasmal contaminants in different types of cell lines with high sensitivity. The contaminated samples were subsequently subjected to antibiotic treatment procedures. Analysis of the results obtained from each treatment indicated that BM-Cyclin is the most efficient antibiotic. It is not recommended to apply anti-mycoplasma antibiotics in a routine culture medium in order to prevent or eradicate already established mycoplasmal infection. It is highly advisable to discard all infected cultures, because of the risk of transmission infection to other clean cultures. Eradication in a separate

quarantine laboratory should be considered as a final resort, if a valuable cell line or a primary cell culture, is not replaceable. Our data provide encouragement for the development of eradication procedures that render cell lines mycoplasma free for extended periods of experimental work.

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