Detection and Treatment of Mycoplasma Contamination in Cultured Cells

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Background: Mycoplasmas, the smallest and simplest prokaryotes that reside in endosomes of mammalian cells, are widespread contaminants found in cell cultures. About 30% of all cell cultures, varying from 15 to 80%, are reportedly contaminated with mycoplasmas. Here, we present our experience in successfully detecting and treating mycoplasmal infection in various cell lines.

Methods: The nested polymerase chain reaction (PCR) detection and microscopic examination, including phase-contrast, fluorescent, as well as differential interference contrast, were used for detecting potential mycoplasma contamination of cell lines used in our laboratory. As soon as mycoplasma was identified, antibiotic treatment was initiated.

Results: Mycoplasmal contamination was detected in six of 15 cell lines using the nested PCR amplification of mycoplasma DNA, which was further demonstrated using 4, 6-Diamidino-2-phenylindole (DAPI) staining and fluorescent microscopy. Alternate treatment with two antibiotics, macrolide (tiamulin) and tetracycline (minocycline), effectively eliminated mycoplasma, which was validated by both PCR and microscopic studies.

Conclusions: The nested PCR using genomic DNA extracted from cultured cells as templates is a rapid and sensitive method for detecting mycoplasma contamination. Treatment with combined antibiotics can completely eradicate mycoplasmal infection from cultured cells. For the ease of use, PCR and/or DAPI staining appear suitable for detecting potential mycoplasmal contamination in laboratories that rely heavily on the cell culture system.
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Key words: **cell culture, mycoplasmal contamination.**

Mycoplasmas, the smallest (0.3-0.8 μm diameter)[1] and simplest prokaryotes that reside in endosomes of mammalian cells, are widespread contaminants found in cell cultures. About 30% of all cell cultures, varying from 15 to 80%, are reportedly contaminated with mycoplasmas.[2] The concentration can be as high as 107 colony-forming units (CFU) per milliliter.[3] Unlike other commonly

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prevalent bacteria, mycoplasmas are bound by a triple-layered membrane and lack of rigid cell walls, making them resistant to penicillin and its analogues.

Mycoplasmal infection may deprive host cells of arginine,[4] an essential amino acid which mycoplasmas use as an energy source, and alter incorporation of nucleic acid precursors into the host cells.[5,6] Attachment of mycoplasmas may cause host cells to leak and interfere with membrane-receptor function as well as signal transduction.[7] It may impose a deleterious effect on transient transfection which involves endocytosis of plasmid DNA.[8]

Mycoplasma-infected cells frequently show stunted, abnormal growth and 'moth-eaten' edges of the monolayer. Other cells may show cellular changes similar to nutrient deprivation which can be reversed by frequently changing the medium.[1] Unfortunately, not all contamination can be detected by examining the changes of morphology.

Many detection methods have been developed, including microbiological cultivation on broth and agar, fluorescence DNA staining using 4', 6-diamidino-2-phenylindole (DAPI) or Hoechest dyes,[9,10] nucleic acid hybridization with a probe specific for mycoplasmal rRNA,[11] enzyme-linked immunosorbent assay (ELISA) with mycoplasma-specific polyclonal antibodies,[12] immunofluorescence staining,[13] biochemical detection utilizing 6-methyl purine deoxyriboside (6-MPDR, an analogue of adenosine, which can be converted by mycoplasma to 6-methyl purine and 6-methyl purine riboside, both toxic to mammalian cells),[14] and polymerase chain reaction (PCR).[15,16] Among them, PCR is considered to be rapid and sensitive. Hopert et al. reported that PCR produced 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.[17] Comparisons among the four detection methods indicated that: (a) the microbiological culture is time-consuming, taking 1 to 4 weeks; (b) DNA fluorescent staining can be difficult to interpret due to the presence of contaminating bacteria or broken nuclei; (c) nonspecific signal interference may be caused by the presence of cross-reacting gram-positive bacteria in hybridization process; and (d) PCR can rapidly detect as few as 3-100 CFU/ml with good specificity.[18] Among different species of mycoplasma, PCR detected 20-180 CFU/ml reliably.[16] Most PCR methods employ primers that hybridize with 16S or 23S rRNA conserved to prokaryotes, and amplify the spacer region between 16S and 23S rRNA. The length and sequence of the spacer region, which differs from species to species, can be used to identify the contaminants by running an agarose gel or performing a second PCR.

Once contamination is detected, completely autoclaving the incubator and replacing with mycoplasma-free stock are recommended. However, when another mycoplasma-free stock is not available, eradicating mycoplasma from the infected cells is necessary. Mycoplasma eliminating methods can be classified into four types: (a) physical procedures, such as heat treatment or photo sensitizing; (b) chemical procedures, such as washings with ether-chloroform or culturing in medium containing 6-MPDR; (c) immunological procedures, such as treatment with specific anti-mycoplasma antisera or exposure to complement; and (d) chemotherapeutic procedures, such as antibiotic treatment in standard culture or soft agar cultivation.[19] The addition of antibiotics into the medium seems to be attractively simple and inexpensive, with the efficacy reportedly to be more than 75%.[20] Several anti-mycoplasmal agents, primarily quinolone and tetracycline, are commercially available. These reagents inhibit either nucleic acid synthesis or protein synthesis.[19] In the experiment by Fleckenstein,[21] all of the mycoplasma-positive cultures resistant to a first antibiotic could be cleaned up using a second treatment with a different antibiotic. From the comparison of several antibiotic regimens,[19-22] BM-cyclin (trade name), which is a combination of tiamulin and minocycline that both inhibiting protein synthesis, has shown to have better treatment efficiency than Sparflox, Enroflox, Ciprololx (a DNA gyrase inhibitor), or the mycoplasma removing agent that is a DNA gyrase inhibitor.

For years at our laboratory, we have been using more than a dozen cell lines for studying reproductive physiology and tumor biology. However, recent observations on the slow growth of the cells, quick acidification of the media, and poor efficiency of the transfection cautioned us about the potential contamination of mycoplasma in the cells. Here, we present

our experience in successfully detecting and treating mycoplasmal infection of various cell lines.

METHODS

Cell cultures

Cell lines that were screened in this study included human ovarian cancer cells (BG1, SKOV3, OVCAR3, BR, 67R, CaOV3), choriocarcinoma (JEG3, BeWo), breast cancer cells (MCF-7, T47D), endometrial cancer cells (KLE, RL-952), cervical cancer cells (HeLa), embryonal kidney cells (293), and Chinese hamster ovary cells (CHO). After being thawed, one part of the cell line stock was directly centrifuged then cultured, another part underwent DNA extraction. For cell cultures, cell lines (Table 1, all adherent cell lines), were cultured in 25 cm^2 culture flasks containing D-MEM/F-12 (Gibco BRL Life Technologies, #12400-024, Karlsruhe, Germany) medium with 10% fetal bovine serum (FBS; Gibco BRL, #16000-044), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL, #15140-122). All cultures were maintained at 37°C in a humidified incubator containing air and 5% CO_2.

DNA extraction

DNA of each cell line was extracted using QIAamp DNA Blood Midi Kit (Qiagen, #51183, Hilden, Germany) according to the manufacturer's instruction.

Nested Polymerase chain reaction (PCR)

Mycoplasmal contamination was detected using a PCR Mycoplasma Detection Set (Takara, #6601, Shiga, Japan). Briefly, the first PCR amplified the spacer region between the 16S and 23S mycoplasmal rRNA- the forward primer (5'-ACACCATGG-GAGCTGGTAAT-3') and reverse primer (5'-CTTCATCGACTTTCAGA-CCCAAGGCAT-3'). The second run of nested PCR was then performed using a forward primer (5'-GTTCTTTGAAAAC-TGAAT-3') hybridizing with a conservative sequence on the spacer region and a reverse primer (5'-GCATCCACCAAAAACTCT-3') hybridizing with 23S rRNA. Depending on the different mycoplasma species, the product lengths of the first PCR ranged from 369 to 681 bp, and from 145 to 237 bp for the second PCR. For each cell line, 200-900 ng of extracted DNA or 10 μg/ml of medium collected from the cells that had been cultured for at least 3 days were used as PCR templates. Negative controls were included in every PCR experiment to ensure the absence of contamination. Taq polymerase from Takara (Takara, #R001A), and a thermocycler (Applied Biosystems GeneAmp PCR System 9700; Perkin Elmer Applied Biosystems, Weiterstadt, Germany) were used. PCR products were analyzed using agarose gel electrophoresis (1.5% and 2% for first and second runs of PCR, respectively) and ethidium bromide staining.

4,6-Diamidino-2-phenylindole (DAPI) staining

The fluorescent DAPI dye (Sigma, #D-8417, Deisenhofen, Germany) was dissolved in water to make the 1 mg/ml stock. The working solution was freshly prepared by diluting the stock DAPI into 1 μg/ml with methanol. Cells cultured on chamber slides (Nunc, #154461, Wiesbaden, Germany) coated with fibronectin were rinsed once with the working solution, incubated with the working solution in 37°C for 15 minutes, then rinsed once with methanol. Slides were mounted with glycerol and examined under a fluorescence microscope with 340/380 nm excitation filter and LP 430 nm barrier filter (Olympus, BX50, Japan). Alternatively, a confocal microscope (Leica TCS SP2, Germany) was used to capture the isolated and overlapped images of differential interference contrast (DIC) and fluorescence.

Mycoplasma elimination

Two mycoplasma infected cell lines, BR and 67R, were treated with BM-cyclin (Roche, #799050, Mannheim, Germany) according to the supplier's recommendations. Briefly, BM-cyclin I (macrolide tiamulin) was added to a final concentration of 10 μg/ml for 3 days followed by BM-cyclin II (tetracycline minocycline) at 5 μg/ml for 4 days. The treatment cycle was repeated three times.

RESULTS

Detection of mycoplasmal contamination with PCR

Among the 15 cell lines analyzed (Table 1), six were detected as mycoplasma-positive, as shown by the amplified fragments of 488 bp and 211 bp in first and second runs of PCR, respectively (Fig. 1). These

Table 1. Nested PCR Results of Mycoplasma Detection in 15 Cell Lines Used in Our Laboratory.

Cell line	Cell type	PCR results using medium as template	PCR results using genomic DNA as template
BG1	Ovarian cancer	+/+*	+/+
67R	Ovarian cancer	-/+	+/+
SKOV3	Ovarian cancer	-/-	-/-
CaOV3	Ovarian cancer	-/-	-/-
BR	Ovarian cancer	+/+	+/+
OVCAR3	Ovarian cancer	-/-	-/-
MCF-7	Breast cancer	-/-	-/-
T47D	Breast cancer	-/-	-/-
HeLa	Cervical cancer	-/+	+/+
JEG3	Choriocarcinoma	-/-	-/+
BeWo	Choriocarcinoma	-/-	-/-
KLE	Endometrial cancer	-/-	-/-
RL-952	Endometrial cancer	-/-	-/-
293	Embryonic kidney cells	-/-	-/-
CHO	Chinese hamster ovary cells	-/+	+/+

* +/+: positive in the first run / positive in the second run of PCR; -/+: negative in the first run / positive in the second run of PCR; -/-: negative in the first run / negative in the second run of PCR.

Abbreviations: PCR: polymerase chain reaction.

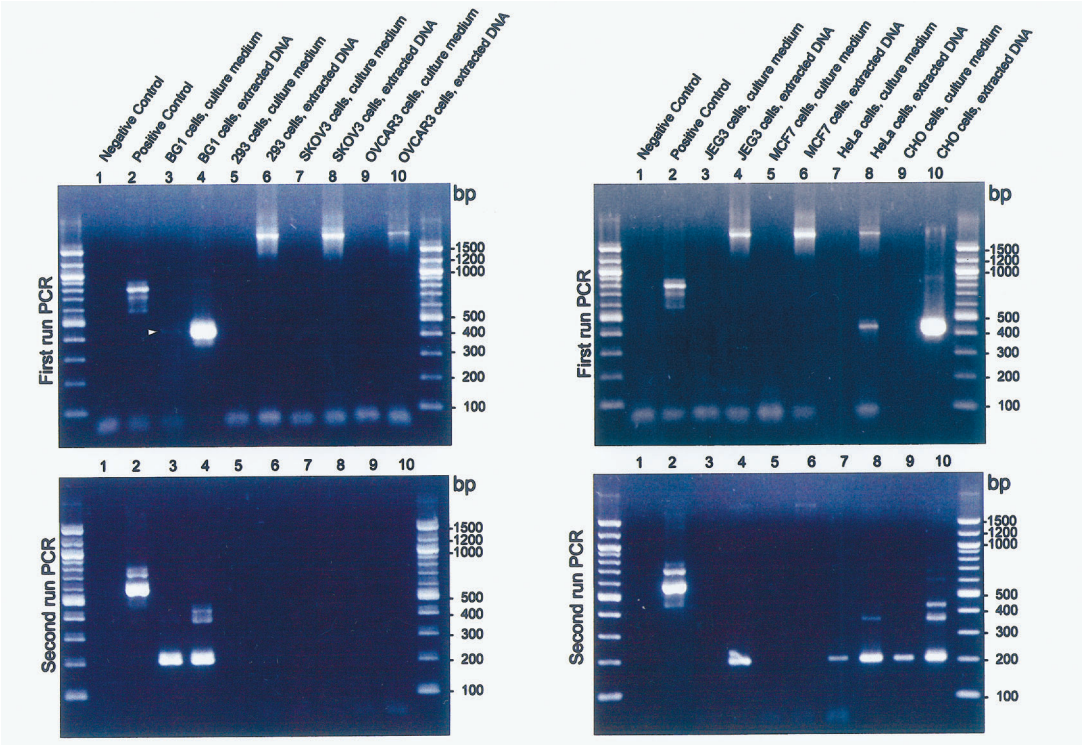

Fig. 1 Comparison of detection sensitivity using DNA derived from cells and culture medium. Eight pairs of culture media and extracted DNA from cell lines as indicated were used as templates for PCR. For the electrophoresis of the Upper and Lower panels, 1.5% and 2% of agarose gels were used, respectively. The presence of PCR products with a size of 488 bp in the first run of PCR and/or 211 bp in the second run revealed the existence of mycoplasma contamination. A very light band of PCR product amplified from the BG1 culture medium (lane 3 of the left upper panel) is marked with an arrow head for easy visualization. An additional 5 studied cell lines (CaOV3, T47D, BeWo, KLE, RL-952) that gave identical PCR results are not shown here.

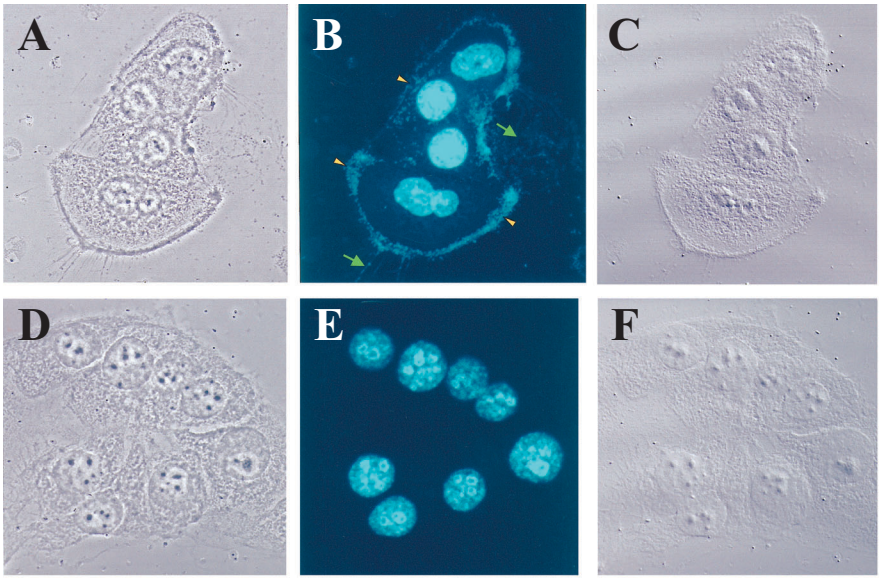

Fig. 2 DAPI staining of mycoplasmal DNA in the ovarian cancer BR cells before and after antibiotics treatment with BM Cyclin. BR cells before (A-C) and after (D-F) antibiotics treatment were cultured on glass chamber slides, stained with 1 μg/ml of DAPI in methanol, and examined under phase-contrast (A, D), fluorescent (B, E), and differential interference contrast microscopy (C, F) at the 400X magnification. Compared with non-infected cells (D, F), mycoplasma-infected cells (A, C) exhibited dense and elevated granularity. In the panel B, mycoplasmal DNA abundant in the cytoplasm of infected cells is marked by the yellow arrowheads. Some mycoplasma even leaked out of the host cells (marked by the green arrows).

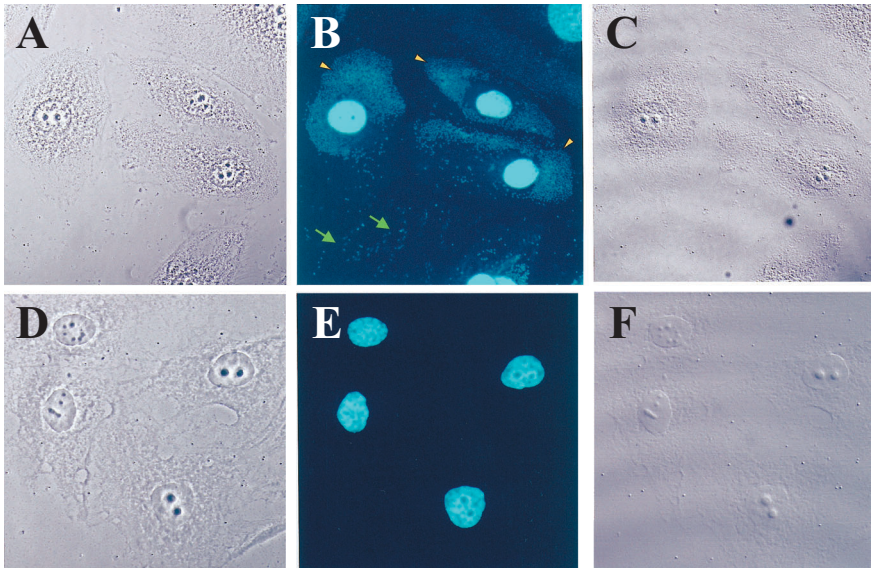

Fig. 3 DAPI staining of mycoplasmal DNA in the ovarian cancer 67R cells before and after antibiotics treatment with BM Cyclin. 67R cells before (A-C) and after (D-F) antibiotics treatment were cultured on glass chamber slides, stained with 1 μg/ml of DAPI in methanol, and examined under phase-contrast (A, D), fluorescent (B, E), and differential interference contrast microscopy (C, F) at the 400X magnification. Compared with non-infected cells (D, F), mycoplasma-infected cells (A, C) exhibited dense and elevated granularity. In the panel B, mycoplasmal DNA abundant in the cytoplasm of infected cells is marked by the yellow arrowheads. Some mycoplasma even leaked out of the host cells (marked by the green arrows).

results also indicated that all of the contaminated cell lines were infected with the same species, M. hyorhinis.

Elimination of mycoplasmas with BM-cyclin treatment

Before treatment, both cell lines showed severely inhibited growth. When stained with DAPI, mycoplasma DNA scattered in the cytoplasm was clearly observed. In the severely infected cells, mycoplasma even leaked out of the host cells (Figs. 2 and 3). The cell lines were examined using both DAPI staining and PCR before as well as after BM-cyclin treatment (Fig. 4). The DAPI staining demonstrated that treated cells were completely free of mycoplasma infection (Figs. 2 and 3). Nevertheless, the PCR results revealed residual amounts of mycoplasma DNA within the BR cells, as shown in

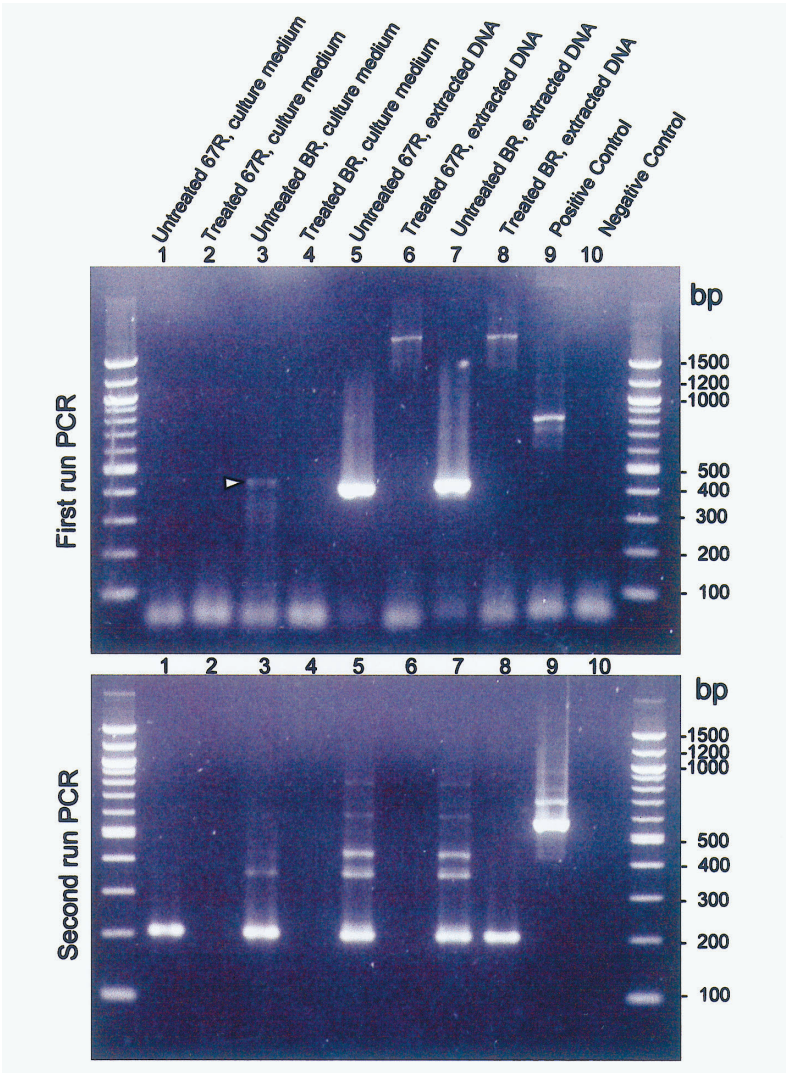

Fig. 4 Antibiotics treatment efficiently eliminated mycoplasma from infected cells. Both culture media and genomic DNA extracted from ovarian cancer 67R and BR cells, before and after treatment with BM-Cyclin, were used as templates for the nested PCR for mycoplasmal detection. A light band of PCR product amplified from the culture medium collected from untreated BR cells (lane 3 of the upper panel) is marked with an arrowhead for easy visualization. Noted in lane 8 of the lower panel was a 211 bp band, indicating the presence of residual mycoplasmal DNA in the genomic DNA extracted from the treated BR (discussed in the text).

lane 8 in the lower panel of Figure 4. The residual mycoplasmal DNA apparently belonged to dead mycoplasma that imposed no harm on the treated cells. The follow-up PCR using DNA extracted from the treated cells that were continuously cultured for 3 more weeks did not detected mycoplasma DNA anymore (data not shown).

DISCUSSION

Although more convenient, direct application of culture medium for PCR detection, as recommended by the PCR Mycoplasma Detection set (Takara), was not as sensitive as using extracted DNA as templates. When culture medium was used as the source of the PCR templates, no contaminations (67R, HeLa, CHO) were detected on any of the three cell lines until the second run of PCR. However, when DNA was used as templates, positive results were found on the first run (Fig. 1). Similarly, the culture medium of JEG3 cells showed negative PCR results but contamination was detected when the DNA was examined. When DNA was applied, most contamination was detected on the first run of PCR. However, very slight infections, such as that of JEG3, may not give a visible band (Fig. 1). Thus, a nested PCR is still essential for improving the sensitivity even when DNA is used.

We chose BM-Cyclin for mycoplasma elimination because it was reported to have better efficacy than other antibiotics. In our laboratory, three cycles of alternative treatment effectively eliminated mycoplasma from ovarian cancer 67R and BR cells (Fig. 4). The same success was achieved for other contaminated cells too (data not shown).

The general practice of managing mycoplasmal contamination in cultured cell lines was to discard the contaminated cells and replace with new ones, unless those cells could not be obtained elsewhere as for the BR and 67R ovarian cancer lines in our case. Resources of cell lines include American Type Culture Collection (ATCC, http://www.atcc.org) internationally or from Bioresources Collection and Research Center (BCRC, http://www.ccrc.firdi.org.tw) in Taiwan.

Some undesired side effects of antibiotic treatment may occur including[19] (a) strong cytotoxicity leading to loss of culture; (b) growth inhibition under treatment; (c) loss of special cellular characteristics; and (d) clonal selection of treated cells. During treatment, we observed that cell death occurred more frequently in cultures with low cell densities and when antibiotics were added before cells attached to the culture flask. Both situations made cells more susceptible to the cytotoxic effects of BM-Cyclin. Inhibited growth was also observed but it was manageable and reversible. The inhibition was minimal when cells were grown at a higher density. In addition, after three cycles of BM Cyclin treatment were completed, the cell growth rates resumed. Nevertheless, we still emphasize the importance of having back-up vials of cells in stock in case the antibiotic treatment kills the cells.

Concluded from these experiences, we suggest that a nested PCR using genomic DNA extracted from cultured cells as templates is a rapid and sensitive method to detect mycoplasma contamination and the treatment of BM-Cyclin can completely eradicate mycoplasmal infection from cultured cells. For the ease of use, PCR and/or DAPI staining appear suitable for detecting potential mycoplasmal contamination in laboratories that heavily rely on the cell culture system.

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細胞培養中黴漿菌污染的檢測及清除

容 萱 王師怡[1] 楊伊雯 薛丁瑋 楊薇儒 王子豪[2,3] 王馨世[4]

背 景： 黴漿菌 (Mycoplasma) 常污染細胞培養，是宿在哺乳類細胞之內小體 (endosomes) 中最小的原核生物。不同的研究報告指出，大約有15到80%的細胞培養遭受此菌污染。在本文中，我們報告在我們實驗室中篩檢與消除污染細胞之黴漿菌的成功經驗。

方 法： 巢複式聚合酶鏈結反應 (nested PCR) 與三種顯微檢視—相位差、螢光、差別阻礙位差 (DIC)，一起用來偵測可能的黴漿菌污染。一旦污染確認，我們就投予抗生素之治療，並追蹤治療效果。

結 果： 在15株細胞中，我們鑑定出6株細胞受到污染。以兩種抗生素—屬macrolide的tiamulin與屬四環素的minocycline治療三週，可有效地去除黴漿菌的污染。這些結果都經過巢複式聚合酶鏈結反應與螢光顯微鏡的雙重確認。

結 論： 從培養細胞中萃取基因體DNA，再用巢複式聚合酶鏈結反應偵測，是一種快速及靈敏的黴漿菌檢測法。而以上述兩種抗生素的複合治療能徹底消除污染培養細胞的黴漿菌。因爲容易操作，聚合酶鏈結反應及/或DAPI螢光染色適合經常使用培養細胞模式的研究室，用來檢測黴漿菌之污染。

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關鍵字： 細胞培養，黴漿菌污染。

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