

Elimination of Mycoplasma Contamination in Cell Cultures

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Mycoplasma contamination remains a significant impediment to the culture of eukaryotic cells. For certain cultures, attempts to eliminate the infection are feasible alternatives to the normally recommended disposal of the contaminated culture. Here three antibiotic regimens for mycoplasma decontamination were compared in a large panel of naturally infected cultures: (i) a one-week treatment with the fluoroquinolone Mycoplasma Removal Agent (MRA), (ii) a two-week treatment with the fluoroquinolone ciprofloxacin, and (iii) three rounds of a sequential one-week treatment with BM-Cyclin, which contains a pleuromutilin and a tetracycline derivative.

These antibiotic treatments had a high efficiency of permanent cure: MRA 64%, ciprofloxacin 77%, BM-Cyclin 84%. Resistance to mycoplasma eradication was observed in some cell cultures: BM-Cyclin 5%, ciprofloxacin 14%, MRA 22%. Nearly all resistant contaminants that could be identified belonged to the species *M. arginini* and *M. orale*. Detrimental effects of the antibiotics were seen in form of culture death caused by cytotoxicity (in 9-13% of the cultures). Alterations of the cellular phenotypic features or selective clonal outgrowth might represent further untoward side-effects of exposure to these antibiotics.

Overall, antibiotic decontamination of mycoplasmas is an efficient, inexpensive, reliable, and simple method: 269/366 (73%) chronically and heavily contaminated cultures were cured, while 97/366 (27%) cultures could not be cleansed and were either lost or remained infected. It is concluded that eukaryotic cell cultures containing mycoplasmas are amenable to antibiotic treatment and that a cure rate of about three-quarters is a reasonable expectation.

Introduction

Mycoplasma contamination of cell culture systems continues to present major problems for basic research as well as for the manufacturing of bioproducts (1,2). Mycoplasmas affect virtually every parameter within the cell culture system (3). As mycoplasma infection of cell cultures might often persist for long periods of time without apparent cell damage (4), it is important to use one or several efficient detection methods (1,3,5,6). Mycoplasma-positive cell cultures are themselves the major source of infection (7). Thus, it was generally recommended that positive cultures should be discarded and replaced in order to prevent the spreading of the contaminant (2,3). If the culture is considered irreplaceable, it is possible to effectively eliminate the contaminant(s). Therefore, treatment of mycoplasma-positive cell cultures has become a practicable option (4).

A number of methods have been employed in recent years with mixed results. Procedures for elimination have included use of antibiotics, complement, and heterologous antisera; passage in nude mice; exposure to mouse macrophages; treatment with trypsin, S-bromouracil, and Hoechst 33258; culture in soft agar; and several other techniques

(2–4,8–16). However, few of these techniques produced satisfactory and consistent results. It became apparent that elimination of mycoplasmas from infected cell cultures is typically a time-consuming and often unsuccessful exercise posing the risk of secondary infection to other cultures (4). Obviously methods of mycoplasma decontamination should be simple, have minimal effect on cell growth, and not lead to loss of special cellular characteristics.

The most promising and still relatively simple technique appears to be antibiotic treatment. Here, in particular, it is important to closely monitor effectiveness of the clean-up relative to mycoplasma elimination and eukaryotic cytotoxicity (2). It has been suggested that the most efficient approach is to identify the contaminant by species and determine the antibiotic sensitivity of the infectant; then the mycoplasma-positive eukaryotic cell culture is exposed to several consecutive treatments with an antibiotic cocktail (17). However, for most scientists, cell culturing is only the means to an end. Most do not have the time, patience, or interest required for cumbersome trials. Therefore, new products to be used in predetermined protocols are marketed specifically for these purposes.

Currently the most often used antibiotics for mycoplasma decontamination are the two fluoroquinolones, Mycoplasma Removal Agent (MRA) and ciprofloxacin, and BM-Cyclin (4,11,18–23). However, these studies examined only limited numbers of treated cultures; furthermore, few comparative data are available. We summarize here our experience with antibiotic treatment (BM-Cyclin, ciprofloxacin, and MRA) of mycoplasma contamination in a large number of positive cell cultures.

Materials and Methods

Cell culture

Anti-mycoplasma treatment was performed in cell cultures from cell lines that were originally submitted to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German Collection of Microorganisms and Cell Cultures) and that were found to contain mycoplasmas. Cells were cultured under standard conditions at 37°C in a 5% CO₂-humidified atmosphere in the recommended culture medium supplemented with heat-inacti-

vated, mycoplasma-free fetal calf serum (5–20%) without any additional antibiotics. None of the cell lines were deliberately infected with mycoplasmas and thus represented chronically contaminated cultures. Cultures were passaged according to standard procedures.

Mycoplasma detection

All cell cultures were assayed for mycoplasmas with standard tests (5,6). Thus, the cultures were tested before and after treatment with the DAPI DNA fluorescence staining and the microbiological broth-agar colony assay. Most treated cultures were also analyzed with one of the following tests: RNA/DNA hybridization, an ELISA with specific polyclonal antisera (e.g., Mycoplasma Detection Kit, Boehringer Mannheim, Germany), the monoclonal antibody CCM-2 (R-Biopharm, Darmstadt, Germany), or a polymerase chain reaction using a mix of outer and inner primers (e.g., Mycoplasma PCR ELISA, Boehringer Mannheim, Germany). All assays were previously described in detail (5,6,24). Identification of mycoplasma species was

performed with an immunobinding assay on agar colonies and/or an ELISA (Boehringer Mannheim) using specific anti-mycoplasma antisera (5).

Mycoplasma elimination

Treatment with BM-Cyclin (Boehringer Mannheim) was carried out according to the manufacturer's instructions, using BM-Cyclin 1 for three days followed by BM-Cyclin 2 for four days; this alternating cycle was performed three times. The final concentrations of BM-Cyclin 1 (a pleuromutilin derivative) and BM-Cyclin 2 (a tetracycline derivative) were 10 µg/ml and 5 µg/ml, respectively. Ciprofloxacin was used for 14 days at 10 µg/ml. Mycoplasma Removal Agent was added to the culture medium for 8 days at a final concentration of 0.5 µg/ml. Following 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 mycoplasma contamination.

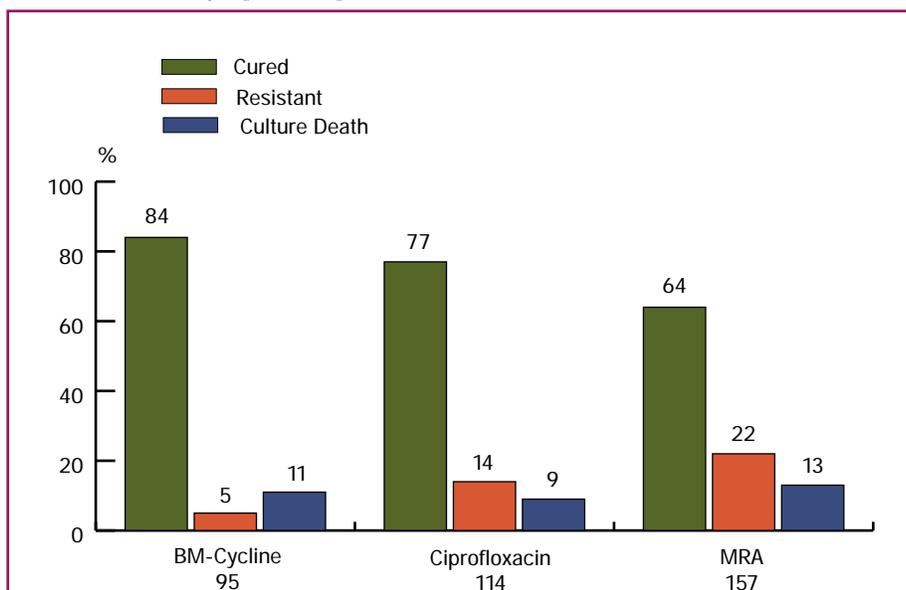


Figure 1 Outcome of treatment of mycoplasma-positive cell cultures with either BM-Cyclin, ciprofloxacin, or MRA. According to the reagent manufacturers' instructions, cells were treated for 1 week with MRA, for 2 weeks with ciprofloxacin, or for 3 weeks with BM-Cyclin, and then cultured in antibiotic-free medium (also without penicillin or streptomycin) for at least another 2 weeks prior to testing for residual mycoplasma contamination. All cultures were tested with DAPI DNA fluorescence staining and a broth-agar colony assay; 80% of the cultures were also investigated with a third method, either DNA/RNA hybridization (Mycoplasma Detection Kit), ELISA, monoclonal antibody CCM-2, or a polymerase chain reaction (Mycoplasma PCR ELISA). The results are shown as percent of cultures that were either cured or that remained mycoplasma contaminated (due to resistance) or that were lost during the treatment period (due to cytotoxicity). The number of cultures analyzed in each category is indicated (N).

Results and Discussion

In this study we set out to determine (i) whether chronically contaminated cell lines could be efficiently cleansed using antibiotic treatment, and (ii) whether the treated cultures remained free of mycoplasmas over long-term culturing, (*i.e.*, whether the contaminants were totally eradicated or only suppressed). The results of our attempts to remove cell culture mycoplasmas using either BM-Cyclin, ciprofloxacin, or MRA are summarized in Figure 1; mycoplasma infection was eliminated by MRA in 64%, by ciprofloxacin in 77%, and by BM-Cyclin in 84% of the cultures studied. Furthermore, the decontamination was total and permanent, as 14 days after treatment, no mycoplasmas were detected in cultures deemed to be cured. More than 50% of the cured cultures were retested for regrowth of mycoplasmas for up to 4 months following treatment; all cultures remained unequivocally negative.

Two aspects are of note when the results of the three different regimens are evaluated: (i) There was no selection on the cultures that were treated; attempts to eliminate mycoplasmas were undertaken in all positive cultures submitted to the cell bank from 1990–1995, using at least one of the antibiotic protocols; some cell cultures were exposed to two or even three treatments in parallel (see below); (ii) All cultures were treated under the same conditions, (*e.g.*, incubation, concentration of antibiotics, treatment protocol). Thus, our data are truly comparable.

The percentage of cultures with mycoplasma contaminants resistant to ciprofloxacin or MRA were similar (Figure 1). Although the species of contaminants were not identified in all cases, our data demonstrate clearly that *M. arginini* and *M. orale* account for the vast majority of resistant contaminants. Several cultures showed cross-resistance to ciprofloxacin and MRA; these cell lines contained mostly *M. arginini* or *M. orale*. This cross-resistance is not surprising because ciprofloxacin and MRA are both fluoroquinolones of similar structure (25). It should be added that consideration should be given to the possibility that a low level infection could persist undetected by standard methods. The possibility of recurrence may be more critical with adherent

cell lines. For such low-level persistent infection, the introduction of the PCR technology represents an outstanding tool in the battery of available mycoplasma detection assays (24).

Besides cure and resistance, the third type of outcome of the antibiotic treatment was loss of the culture, which occurred in 9–13% of the cases (Figure 1). Culture death was presumably caused by cytotoxic effects of the reagents. Although previous studies on mycoplasma-negative cell lines did not provide any evidence of antibiotic cytotoxicity on the eukaryotic cells (18), the situation is certainly different in chronically and heavily contaminated cultures, such as the ones treated here, because of the ensuing problems of poor cell growth and reduced viability. Ciprofloxacin has been reported to have an effect on intracellularly located topoisomerase II in human cells, inducing double-strand DNA breaks (26); however, in that study, ciprofloxacin was used at significantly higher concentrations (14–15 times the concentration used here) (27). Other reports described inhibitory effects of ciprofloxacin on hematopoietic cell growth (28–30). The long-term coculture of eukaryotic cells and mycoplasmas might lead to a sort of symbiosis whereby an abrupt termination might be detrimental to the cells. Further studies are required to elaborate the reason(s) why some cell lines are more susceptible to the cytotoxic effects of the antibiotics than others treated at the same concentrations. Possibly, cellular apoptosis might also play some as yet undefined role in this scenario.

Ideally, the anti-mycoplasma treatment method should be simple, efficient, and not have any adverse effects on the cell culture (4). The simplicity of antibiotic eradication is obvious, as mycoplasma-positive cell lines were cultured under the same conditions during the treatment period as prior to decontamination, only the reagents were added to the culture media. We noted that it is advantageous to increase the FCS concentration and to incubate the cells at higher densities. However, it must be pointed out that antibiotic mycoplasma decontamination might be laborious and time consuming: the duration of the treatment plus the minimum antibiotic-free post-treatment period ranged from 3–5 weeks

depending on the protocol used. It was suggested that it might be preferable to perform appropriate antibiotic sensitivity tests to maximize success (4). However, we believe that such susceptibility tests are unnecessary on a routine basis as they are time consuming and cumbersome.

A high efficiency, the second requirement, is illustrated by the percentages of successful outcomes: 64–84% of the cultures were permanently cleansed of the mycoplasmal contaminants. A review of the literature shows several similar results in the various series reported (4,11,14,18,23,31).

With regard to the last point, the possible adverse effects of the treatment on the cell culture, three unwanted developments must be considered: (i) cytotoxicity; (ii) loss of special cellular characteristics; and (iii) clonal selection of treated cells. A recent study found BM-Cyclin to be very cytotoxic to all 9 cell lines treated (23). While 8/9 cell lines were free of mycoplasma immediately at the end of the treatment period, all cultures were ultimately lost due to poor growth and extensive cell death (23). These latter data stand in clear contrast to the 11% culture death seen in our series (Figure 1). In another report, no cell toxicity was detected during BM-Cyclin treatment of 11 cell lines (22). Exposure to ciprofloxacin did not cause loss of cell culture in two series of 9 and 26 cases (11,21). No data have been published on cytotoxic effects of MRA during anti-mycoplasma treatment. Changes of specific cellular characteristics and/or accidental clonal selection have not yet been studied systematically in cultures exposed to mycoplasmal decontamination. Such an analysis is, however, urgently needed since antibiotics are becoming widely employed for mycoplasma elimination.

In this context, it is important to warn of the consequences of the unsolicited routine application of anti-mycoplasma antibiotics in the daily cell culture work: the emergence of resistant mycoplasma strains can surely be expected, and alterations of the eukaryotic phenotype in the long term are quite possible as well. It is further mandatory to use certified mycoplasma detection methods (6) at regular intervals in order to examine cleared cultures for

re-emergence of contaminants and to exclude re-infection.

In conclusion, the three antibiotic protocols BM-Cyclin, ciprofloxacin, and MRA proved to be valuable tools for the permanent mycoplasma eradication of contaminated cell cultures due to their effectiveness and simplicity in use. Possible drawbacks are resistance of certain mycoplasma contaminants and unwanted effects of the antibiotics on eukaryotic cells, such as culture loss caused by excessive cytotoxicity, alterations of cellular features, or selective clonal outgrowth. The 27% of cultures affected by the negative effects of antibiotic treatment in form of mycoplasma resistance or culture death were opposed by the high cure rate (about three-fourths) of all cultures treated. Amongst the antibiotics tested, BM-Cyclin was shown to remove mycoplasma with the highest efficiency and the lowest risk of resistance. ■

Product	Cat. No.	Pack Size
BM-Cyclin	799 050	37.5 mg (for 2 x 2.5 l medium)

Also Available	Cat. No.	Pack Size
DAPI	236 276	10 mg
Mycoplasma Detection Kit	1296 744	1 kit (25 tests)
Mycoplasma PCR ELISA	1663 925	1 kit (96 reactions)

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