Cytotechnology **39:** 75–90, 2002.
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Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention

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Received 7 July 2002; accepted 6 August 2002

Key words: antibiotics, cell lines, contamination, detection, elimination, incidence, mycoplasma, prevention

Abstract

The contamination of cell cultures by mycoplasmas remains a major problem in cell culture. Mycoplasmas can produce a virtually unlimited variety of effects in the cultures they infect. These organisms are resistant to most antibiotics commonly employed in cell cultures. Here we provide a concise overview of the current knowledge on: (1) the incidence and sources of mycoplasma contamination in cell cultures, the mycoplasma species most commonly detected in cell cultures, and the effects of mycoplasmas on the function and activities of infected cell cultures; (2) the various techniques available for the detection of mycoplasmas with particular emphasis on the most reliable detection methods; (3) the various methods available for the elimination of mycoplasmas highlighting antibiotic treatment; and (4) the recommended procedures and working protocols for the detection, elimination and prevention of mycoplasma contamination. The availability of accurate, sensitive and reliable detection methods and the application of robust and successful elimination methods provide powerful means for overcoming the problem of mycoplasma contamination in cell cultures.

Introduction

Continuous cell lines represent important tools for experimental research. Among the various problems associated with cell lines, the two most common concern contamination: (1) cross-contamination with unrelated cells and (2) contamination with microorganisms, in particular mycoplasma. Having collected a large number of human leukemia-lymphoma cell lines, we have had the opportunity to examine the incidence of these two problems (Drexler et al., 2002). Employing the two sets of parameters 'authentic *versus* false cell line' and 'mycoplasma-negative *versus* mycoplasma-positive cell line', we found that out of the 440 leukemia-lymphoma cell lines examined only some 64% of the cell lines were indeed authentic and mycoplasma-negative (Figure 1). It becomes furthermore clear that the problems of mycoplasma-positive and cross-contaminated cell lines are interrelated as 50% of the cross-contaminated cell lines were found to be also mycoplasma-positive whereas only some 23% of the authentic cell lines were mycoplasma-positive. While these data refer to a specific type of cell lines, similar results can presumably be obtained in an analysis encompassing all types of cell lines.

In this review article, we will discuss the problem of mycoplasma-contamination of cell lines, presenting a concise overview on the biology of mycoplasma, addressing incidence, sources and effects of an infection, suggesting efficient methods for its detection and elimination, and proposing steps to be taken to prevent the problem.

Biology of mycoplasmas

The mycoplasmas represent a large group of microorganisms which are all characterized by their lack of a rigid cell wall (Table 1). Therefore, the distinct class of *Mollicutes* within the prokaryotes was created.

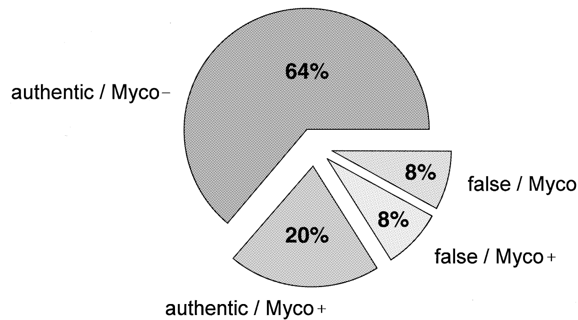

Figure 1. Percentages of false and mycoplasma-contaminated leukemia-lymphoma cell lines. Shown is an analysis of 440 leukemia-lymphoma cell lines for which data on these two parameters were available: parameters 'authentic/false' refer to cross-contamination of cell lines; parameters 'clean/Myco+' refer to the mycoplasma contamination status.

Table 1. Major biological features of mycoplasmas

- Lack of rigid cell wall
- Generation times of 1–9 hr
- Smallest self-replicating bacteria: 0.3–0.8 μm
- Filterable through 0.45 μm filter

For practical reasons, the trivial terms mycoplasmas and mollicutes are often used as synonyms. The first term for mycoplasmas was 'pleuropneumonia-like organisms' (PPLO). Specific details of the biology and taxonomy of mycoplasmas are described in specialist textbooks (Drexler and Uphoff, 2000; Razin and Freundt, 1984; Razin, 1991; Tully, 1992). Mycoplasmas are considered to be the smallest self-replicating organisms known at present. The small size of 0.3–0.8 μm in diameter and the flexibility of their cell membrane allow mycoplasmas to pass through commonly used anti-bacteriological filters with diameters of 0.45 μm.

Contrary to other bacteria, mycoplasmas grow very slowly, even under optimal conditions. The generation times usually range between one and three hours, but there are also generation times of up to nine hours; in addition, mycoplasmas have a relatively long lag phase. Therefore, it may take more than one week to obtain visible colonies on agar.

It has long been assumed that mycoplasmas exist only on the outside of the eukaryocytic cell membrane through cytadherence. However, studies in recent years have unequivocally demonstrated the intracellular location of certain mollicutes (notably *M. fermentans*, *M. genitalium* and *M. pneumoniae*), not only after phagocytosis by granulocytes and monocytes, but also in non-phagocytic epithelial cells. Furthermore, a new mycoplasma species capable of entering a variety of human cells *in vivo* and *in vitro* has been discovered and was named accordingly *M. penetrans* (Lo et al., 1992) (Figure 2). While the great majority of the infecting mycoplasma population is definitely located extracellularly, the intracellular location, even for only a short period, sequesters mycoplasmas and may protect them effectively from mycoplasmacidal therapies.

Table 2. Incidence of mycoplasma contamination

- 1% of primary cell cultures
- 5% of early passage cell cultures
- 15–35% of continuous human or animal cell lines

Incidence of mycoplasma contamination

Mycoplasmas were first isolated from a contaminated cell culture in 1956. One mycoplasma cell can grow to 10^6 colony forming units per ml within three to five days in an infected cell culture. Eukaryotic cell cultures contaminated with mycoplasma have titers in the range of 10^6 to 10^8 organisms per ml. Frequently, there are from 100 to 1000 mycoplasmas attached to each infected cell.

Primary cell cultures and cultures in early passage have been reported to be less frequently contaminated than continuous cell lines: primary cultures and early passage cultures on the order of 1 and 5%, respectively; continuously cultured cell lines in the range of 15–35% (Table 2). Several large series on tens of thousands of cell cultures analyzed over several decades (1960s–1980s) found an incidence of ca. 15%. Recent studies on a smaller series documented significantly higher infection rates of cell cultures, commonly in the range of 15–35%, but also as high as 65–80% (Barile and Rottem, 1993; Del Guidice and Gardella, 1984; Drexler et al., 2002; Hay et al., 1989; McGarrity et al., 1985).

The expanding application of cell lines in research and biotechnology (with the resulting exchange of non-authenticated and mycoplasma-positive lines between scientists) and the increasing use of certain antibiotics (mostly penicillin plus streptomycin which merely serve to mask but do not remove mycoplasmas)

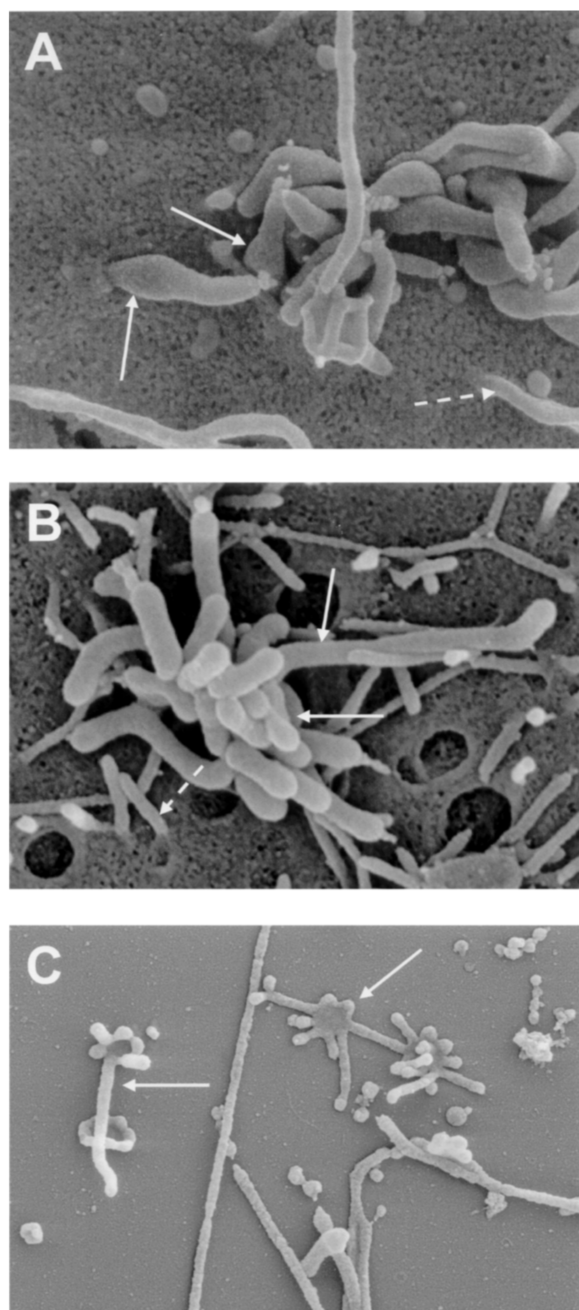

Figure 2. Electronmicroscopic photographs of mycoplasmas. Scanning electron micrograph of critical point-dried cell cultures infected with mycoplasma grown on coverslips. (A) *M. penetrans* on HELA cells; arrows indicate pear-shaped mycoplasmas penetrating the cell membrane; dashed arrow indicates microvilli of the eukaryotic cell; magnification 30 000×. (B) MDCK cell line infected with *M. arginini*; arrows indicate mycoplasmas, dashed arrow indicates microvilli; magnification 30 000×. (C) HELA infected with *A. laidlawii* (arrows); bacteria are adhered to the surface of the glass slide; magnification 20 000×. Photographs kindly provided by Dr. Manfred Rohde, GBF, Braunschweig, Germany.

Table 3. Most common contamination mycoplasma species

Species	Frequency	Natural host
M. orale	20–40%	Human
M. hyorhinis	10–40%	Swine
M. arginini	20–30%	Bovine
M. fermentans	10–20%	Human
M. hominis	10–20%	Human
A. laidlawii	5–20%	Bovine

in routine culture have presumably led to this increase in mycoplasma contamination of cell cultures.

Most common contaminating mycoplasma species

While more than 20 species have been isolated from contaminated cell lines, detailed investigations on the identity of the contaminating species showed that by far the largest portion of infections is caused by a relatively small number of *Mycoplasma* and *Acholeplasma* species: 90–95% of the contaminants were identified as either *M. orale, M. hyorhinis, M. arginini, M. fermentans, M. hominis* or *A. laidlawii* (Table 3). The individual percentages of these six species may vary greatly between studies.

Generally *M. orale*, which is the most common mycoplasma species in the oral cavity of clinically normal humans, also represents the single most common isolate accounting for 20–40% of all mycoplasma infections in cell cultures (Table 3). Other non-pathogenic mycoplasma species from the normal human microbial flora of the oropharynx that are seen in cell cultures are *M. fermentans* and *M. hominis*.

The bovine group of mycoplasmas accounts for about another third of all strains isolated from cell cultures. Here the most frequent infectants are *M. arginini* and *A. laidlawii*. These two species have a relatively wide host range as they are isolated from cattle, sheep, goat, etc. and from a variety of other mammals, birds, and insects. These cell culture contaminants are thought to derive from bovine sources as in the early days of cell culture (1950s–1970s) bovine sera were not routinely and as strictly screened for mycoplasma contamination as they are today. *M. hyorhinis*, a common inhabitant of the nasal cavity of the swine, accounts also for a high proportion of the infections.

Table 4. Sources of mycoplasma contamination

- Original (primary) tissue isolate (<1%)
- Culture reagents (predominantly fetal bovine serum)
- Laboratory staff
- Cross-contamination from infected cultures

Sources of mycoplasma contamination

Tissue specimens used to initiate cell cultures do not appear to represent the major sources of mycoplasma infection (Table 4). The frequency of infection in primary cell cultures is low, in the order of 1% (Barile and Rottem, 1993; McGarrity et al., 1985). Furthermore, murine species of mycoplasma only account for 0.5–1% within the panel of mycoplasmas isolated from contaminated cultures – despite the wide use of murine cell lines.

The high incidence of bovine mycoplasma species, predominantly *A. laidlawii* and *M. arginini*, implicates fetal or newborn bovine serum. Studies in the 1960s and 1970s showed that 25–40% of the serum lots provided by commercial suppliers were contaminated (Barile and Rottem 1993). While bovine serum contamination has certainly significantly decreased over the last decades due to appropriate efforts of the suppliers with regard to prevention and testing, serum lots absolutely free from mycoplasmas cannot be guaranteed.

Because the largest percentage of mycoplasmas found in cell cultures are of human origin, one may assume that laboratory personnel are one of the major sources of contamination. In laboratories with contaminated cells, most or all cultures are positive containing the same mycoplasma species (McGarrity et al., 1992). Mycoplasma-infected cell lines are themselves the single most important source for further spreading of the contamination. This is due to the ease of droplet generation during handling of cell cultures, the high concentration of mycoplasmas in infected cultures, and the prolonged survival of dried mycoplasmas. Operator-induced contamination is a multifaceted problem. Mycoplasmas are spread by using laboratory equipment, media, or reagents that have been contaminated by previous use in processing mycoplasma-infected cells.

Effects of mycoplasma contamination

Mycoplasma infections can have a myriad of different effects on the contaminated cell cultures. However, this multitude of different effects does not affect the various cells in the same manner and to the same degree (Table 5). Many mycoplasma species produce severe cytopathic effects while others may cause very little overt cytopathology. There can be qualitative and quantitative differences in the same parameter, depending on the infecting mycoplasma species, the culture conditions, the type of the infected cell culture, the intensity and duration of the infection, an additional infection with viruses, and other parameters. Thus, contaminations can interfere with virtually every parameter measured in cell cultures during routine cultivation or in experimental investigations. Consequently, the mycoplasmas in these cultures cannot simply be ignored or regarded as harmless bystander organisms.

Besides the loss of an important culture, in the worst case all experiments might be influenced by the infections and artefacts are produced. Because of the virtually unlimited number of reported mycoplasmal effects on cultured cells, only some of the most important parameters have been listed here in order to highlight the diversity of possible effects (Table 5).

Detection of mycoplasma contamination

Various detection methods

A vast array of techniques have been developed to detect mycoplasma contamination of cell cultures (Table 6). Most of these methods are relatively lengthy, involve subjective assessments and use measurements that are often quite complex in nature. The merits and disadvantages of these detection methods have been discussed in detail elsewhere (Drexler and Uphoff, 2000). Below we will present what is, in our hands, the most reliable and useful detection method using the polymerase chain reaction (PCR) together with the classical microbiological colony assay.

Ideal detection methods should be highly sensitive and specific, but also simple, rapid, efficient and cost effective. The evaluation of tests comprises the components validity and reproducibility pertaining to the statistical parameters (operating characteristics): sensitivity (detection of true positives), specificity (detection of true negatives), accuracy (detection of true

Table 5. Effects of mycoplasma contamination on cell cultures

- General effects on eukaryotic cells:
  - Altered levels of protein, RNA and DNA synthesis
  - Alteration of cellular metabolism
  - Induction of chromosomal aberrations (numerical and structural alterations)
  - Change in cell membrane composition (surface antigen and receptor expression)
  - Alteration of cellular morphology
  - Induction (or inhibition) of lymphocyte activation
  - Induction (or suppression) of cytokine expression
  - Increase (or decrease) of virus propagation
  - Interference with various biochemical and biological assays
  - Influence on signal transduction
  - Promotion of cellular transformation
  - Alteration of proliferation characteristics (growth, viability)
  - Total culture degeneration and loss
- Specific effects on hybridomas:
  - Inhibition of cell fusion
  - Influence on selection of fusion products
  - Interference in screening of monoclonal antibody reactivity
  - Monoclonal antibody against mycoplasma instead of target antigen
  - Reduced yield of monoclonal antibody
  - Conservation of hybridoma

positives and true negatives – combination of sensitivity and specificity), and predictive value (probability of correct result). Further aspects of the evaluation of a test are technical reproducibility and inter-observer reproducibility (concordance of interpretation of the results). Many detection methods rely on subjective reading and interpretation of the results which obviously requires training, experience and consensus (Uphoff et al., 1992c).

Traditionally, mycoplasmologists discerned direct and indirect detection methods (Uphoff et al., 1992a). While the term direct method referred to the classical microbiological colony growth of mycoplasmas on agar, indirect detection techniques included procedures that measure a gene product that is associated with mycoplasmas rather than with the mammalian cells in culture. Furthermore, tests may be performed directly on the specimen taken from a given cell culture or indirectly using the so-called indicator cell culture procedure whereby the specimen is inoculated into another cell culture known to be free of mycoplasmas (monkey cell line Vero and murine cell lines NIH 3T3 or 3T6 have been used with equal success). Use of an indicator cell culture promotes better standardization and allows appropriate positive and negative controls to be included in each assay. Mycoplasmologists advise identifying every cell culture mycoplasma isolate as identification presents a clearer picture of the nature of the infection and may be helpful in determining its source. Identification can be achieved with various immunological techniques (e.g. using species-specific antibodies in immunostaining or ELISA) or nowadays species-specific PCR primers. However, to the cell culturist, the chief concern appears to be whether the culture is free from contamination rather than specific identification of the contaminant.

Until the arrival of RNA hybridization and PCR, DNA fluorochrome staining (so-called DAPI and Hoechst stains) and microbiological culture had been regarded as the 'gold standards' for detection of mycoplasma contamination (Barile and Rottem, 1993; McGarity et al., 1985). While the classical methods certainly have kept their eminent place in the armamentarium of mycoplasma detection methods, the new approach to a highly sensitive, specific and rapid diagnosis of mycoplasmal infection is based on the development of gene or DNA probes which were first in-

Table 6. Various mycoplasma detection methods

- Histological staining:
  - Histochemical stains and light microscopy
- Electron microscopy:
  - Transmission electron microscopy
  - Scanning electron microscopy
- Biochemical methods:
  - Enzyme assays
  - Gradient/electrophoresis separation of labeled RNA
  - Protein analysis
- Immunological procedures:
  - Fluorescence/enzymatic staining with antibodies
  - ELISA
  - Autoradiography
- DNA fluorochrome staining:
  - DAPI stain
  - Hoechst 33258 stain
- Microbiological culture:
  - Colony formation on agar
- RNA hybridization:
  - Filter hybridization
  - Liquid hybridization
- Polymerase chain reaction:
  - Species-/genus-specific PCR primers
  - Universal PCR primers

troduced in the 1980s. The principle is simple as genes or genomic sequences, that are specific for a single species or a particular group of mycoplasma or universally for nearly all mycoplasmas, have been identified and synthesized; these probes are used for DNA or RNA hybridization. The more recent development of PCR enables the amplification of the target DNA in the specimen using specific synthetic oligonucleotides complementary to conserved rRNA sequences and increases the sensitivity by several orders of magnitude (Razin, 1994). Positive hybridization signals as dots on filters or scintillation counts and visual demonstration of the PCR amplicon in gels indicate the presence of the infectious agent. In the following, the classical colony assay and the modern PCR method will be presented in greater detail.

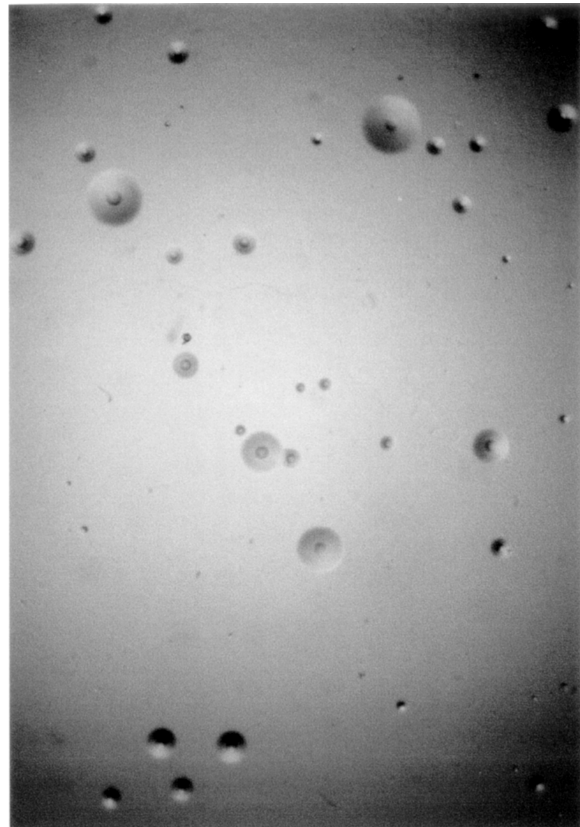

Figure 3. Detection of Mycoplasma Contamination by Colony Growth on Agar. Mycoplasmas (*M. arginini*) from the human suspension cell line U-937 were first enriched in a liquid broth medium and then plated on agar to allow for formation of characteristic colonies. Shown is a typical field of an agar plate incubated for one week. Note the characteristic 'fried- egg'-type mycoplasma colonies. For technical details see reference Uphoff et al. (1992). Microphotograph was taken with an inverted microscope using a x3.2 objective.

Microbiological colony assay

For decades the mainstay of mycoplasma detection was based on standard microbiological culture procedures. Specimens are inoculated into mycoplasma broth and onto agar. Anaerobic incubation is recommended as aerobic incubation yields a lower detection rate. Broths are transferred to agar plates after 4–7 days of incubation. Most mycoplasmas produce microscopic colonies (100–400 μm in diameter) with a 'fried egg'

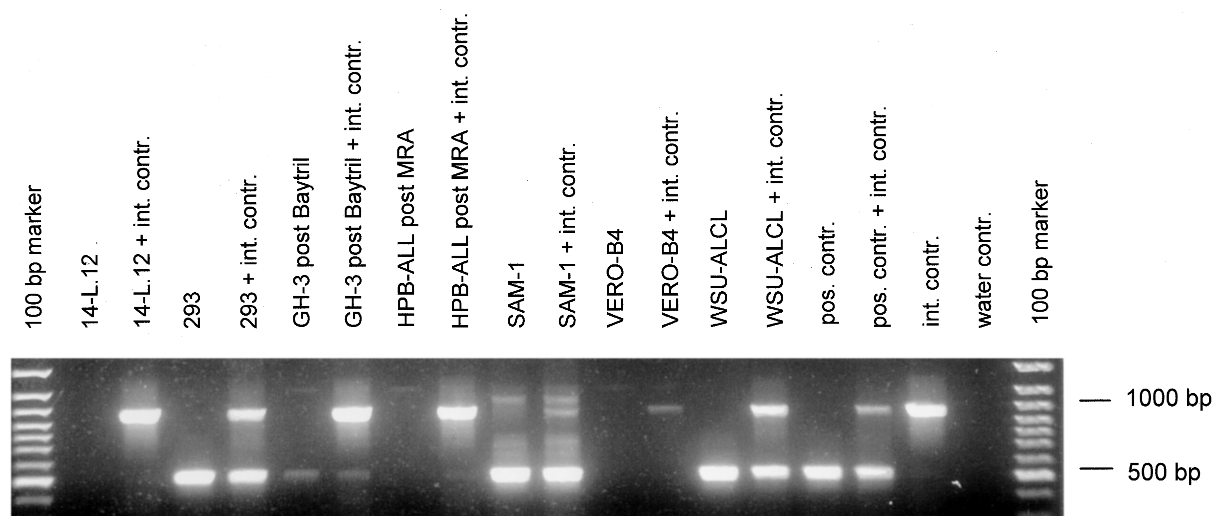

Figure 4. PCR detection of mycoplasma contamination. Electrophoretic analysis of PCR with and without internal control DNA (int. contr.). Supernatants from seven different representative cell cultures were subjected to the sample preparation, and two PCR amplifications of each sample were run with specific primer mixtures. One PCR reaction contained an aliquot of the sample only, and one reaction contained the sample and a control DNA as internal standard (PCR product of 986 bp). Cell lines 14-L.12 and HPB-ALL post-MRA treatment are clearly mycoplasma-negative, and cell lines 293, SAM-1, and WSU-ALCL are clearly mycoplasma-positive. Cell line GH-3 post-Baytril treatment is a sample that remained positive over several weeks after treatment because of residual mycoplasma DNA. In cell line SAM-1, the internal control DNA amplification was suppressed because of a strong mycoplasma contamination of the cell line. Cell line VERO-B4 should be repeated because the amplification of the internal control DNA was insufficient, presumably because of inherent PCR inhibitors. For technical details see reference Uphoff and Drexler (2002d).

appearance growing embedded beneath the surface of the agar (Figure 3).

This procedure has the advantage of ease of manipulation and visual recognition of colonies. Some artifacts are occasionally seen on agar after inoculation of cell culture specimens: colonies must be distinguished from cell clumps by their eventual increase in size; pseudocolonies (e.g. crystals, air bubbles) can be a problem for the inexperienced observer since they can increase in size and can actually be transferred. There are wide variations in size, morphology and speed of growth of the mycoplasma colonies isolated from different cell cultures. Colonies become usually detectable after an average of 3–6 days, but can appear later. 'Noncultivable' mycoplasmas were reported and were recognized as *M. hyorhinis*. These contaminants grow poorly or not at all on well-standardized broth and agar media. A large percentage of *M. hyorhinis* strains do not propagate at all on cell-free medium.

While the microbiological culture has the advantages of being inexpensive, highly sensitive with a high detection rate, and an established and important reference method, the disadvantages are the long incubation time, the need for a subjective and experienced interpretation, the need to maintain positive controls and the risk involved, and the fact that not all mycoplasmas can be successfully cultured.

Table 7. Detection of mycoplasma by PCR

Statistical parameter	All cell lines (n = 598)	Leukemia-lymphoma cell lines (n = 339)
Sensitivity	86%	96%
Specificity	93%	96%
Predictive value		
Positive result	73%	86%
Negative result	97%	99%
Accuracy	92%	96%

Sensitivity = detection of true positives; specificity = detection of true negatives; predictive value = probability of correct results; accuracy = detection of true positives and true negatives.

Polymerase chain reaction

The first reports on the application of PCR to diagnosis of mycoplasma infections appeared in 1989. Nearly all mycoplasmic 16S rRNA sequences have been determined and form the basis for a systematic phylogenetic

Table 8. Various mycoplasma elimination methods

- Physical procedures:
  - Heat treatment
  - Filtration through microfilters
  - Induction of chromosomal or cell membrane damage with photosensitizing
- Chemical procedures:
  - Exposure to detergents
  - Washings with ether-chloroform
  - Treatment with methyl glycine buffer
  - Incubation with sodium polyanethol sulfonate
  - Culture in 6-methylpurine deoxyriboside
- Immunological procedures:
  - Co-cultivation with macrophages
  - *In vivo* passage through nude mice
  - Culture with specific anti-mycoplasma antisera
  - Exposure to complement
  - Cell cloning
- Chemotherapeutic procedures:
  - Antibiotic treatment in standard culture
  - Antibiotic treatment plus hyperimmune sera or co-cultivation with macrophages
  - Soft agar cultivation with antibiotics

Table 9. Effective anti-mycoplasma antibiotics

Brand name	Generic name	Antibiotic category
– BM-Cyclin	Tiamulin	Macrolide
	Minocycline	Tetracycline
– Ciprobay	Ciprofloxacin	Quinolone
– Baytril	Enrofloxacin	Quinolone
– Zagam	Sparfloxacin	Quinolone
– MRA	?	Quinolone

analysis of mollicutes. Computer alignment studies of mollicute 16S rRNA sequences reveal regions with sequence variability or conservation at the species, genus or class level allowing for the selection of appropriate oligonucleotides (primers) for detection and identification of mycoplasmas. The highly conserved regions of the genes enable the selection of primers of wide specificity (‘universal primers’) which will react with DNA of any mycoplasma or even with the DNA of other prokaryotes; this is sufficient for detection of mycoplasma cell culture infection where the goal is just to screen the cultures for contamination. Besides the conserved regions of mycoplasmal 16S rRNA genes, the 16S-23S intergenic regions are quite useful for mycoplasma detection and simultaneous identification (Rawadi and Dussurget, 1995; Razin, 1994; Uphoff and Drexler, 1999).

As PCR can be performed with frozen and lyophilized material, this offers a means for a retrospective analysis and facilitates the transport, collection and storage of samples. The amplification may be performed as a single-step PCR (Figure 4) or as a two-step (nested) PCR; the latter approach increases the sensitivity and specificity considerably, but also increases the risk of contamination by DNA carryover. Southern blotting of PCR products and hybridization with a specific internal probe is another possibility to improve sensitivity. However, a very high sensitivity level may not usually be required in routine diagnosis as acute and particularly chronic cell culture mycoplasma infections contain a large number of organisms (commonly 10^6 or higher). The very high sensitivity may be of advantage at a very early phase of infection or under conditions where mycoplasma growth is suppressed, e.g. in the testing and monitoring of cell cultures post-treatment with antibiotics for elimination of the contaminants.

The detection limit using a set of nested universal primers was determined to be 1 fg mycoplasmal DNA which is equivalent to 1–2 genome copies of the 16S mRNA coding region (mollicute genomes carry only one or two rRNA gene sets). The ability to detect a single mycoplasma cell makes PCR the most sensitive detection method available, clearly more so than microbiological culture.

The high sensitivity of PCR may cause problems in producing false-positive results due to contamination with target DNA. Another possible problem are false-negative data caused by the inhibition of the *Taq* polymerase by components in the samples. However, once all PCR-related problems are properly addressed, single-step or double-step PCR are clearly superior to other mycoplasma detection methods in many respects as this method combines simplicity and speed with high specificity and extreme sensitivity, in addition to objectivity, accuracy and reproducibility (Hopert et al., 1993). In particular, PCR is not limited by the ability of an organism to grow in culture; in certain areas, this molecular nucleic acid amplification may eventually replace biological amplification (i.e.

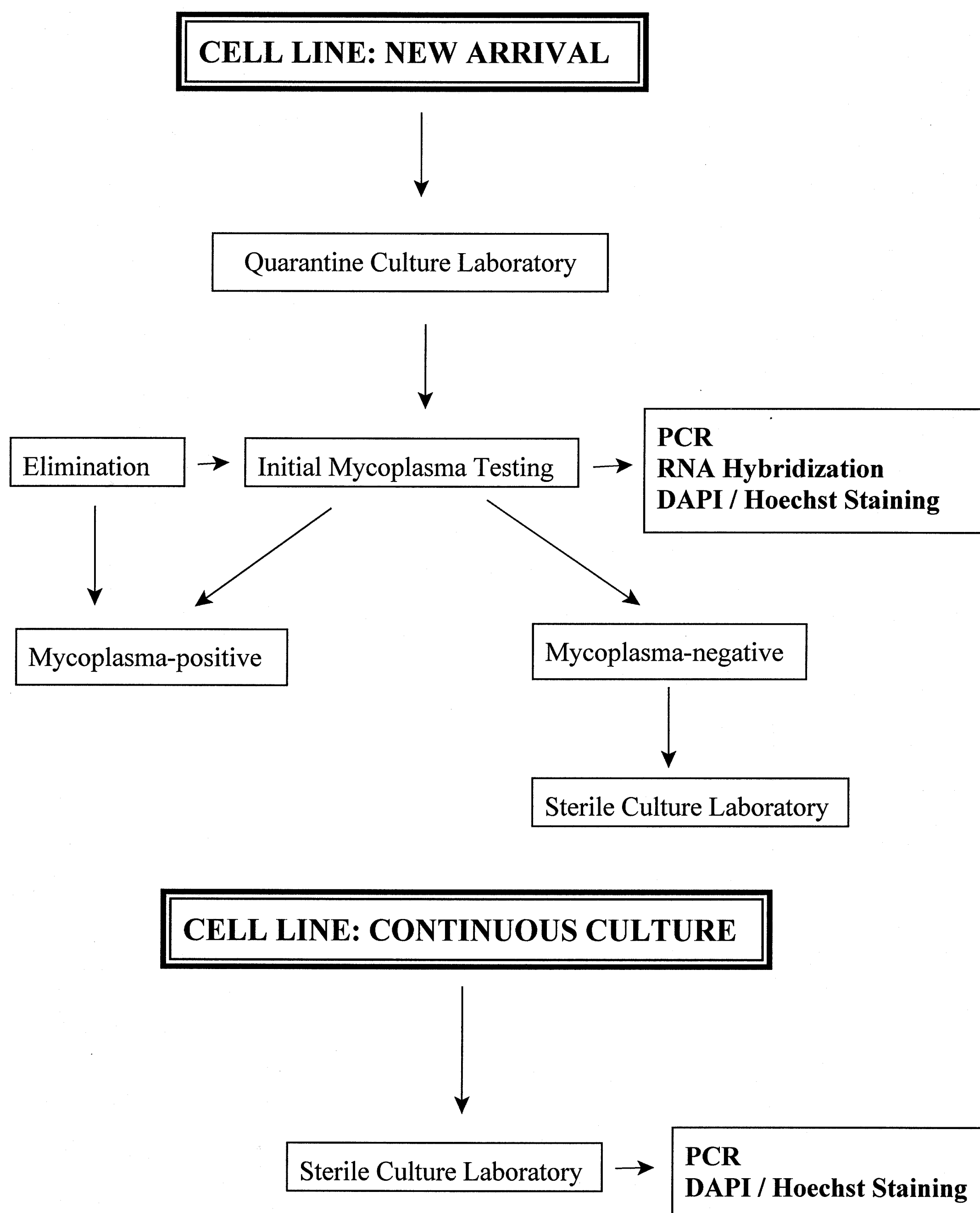

Figure 5. Routine detection of mycoplasma contamination. A flow chart for mycoplasma testing of newly arriving and continuously cultured cell lines is shown. Use of at least two different mycoplasma detection assays is suggested. PCR is best suited for determining the initial mycoplasma contamination status and for routine screening and continuous monitoring. It is useful to check continuous cell cultures at monthly intervals. For technical details see reference Uphoff and Drexler (2002d).

growth in artificial media), a feature of paramount importance considering the fastidious nature of mycoplasmas. Thus, PCR should prove to be the technique of the future for mycoplasma detection in cell cultures. Several PCR kits are commercially available, e.g. from the American Type Culture Collection (ATCC), Minerva Biolabs, Roche, Stratagene, Takara Bio.

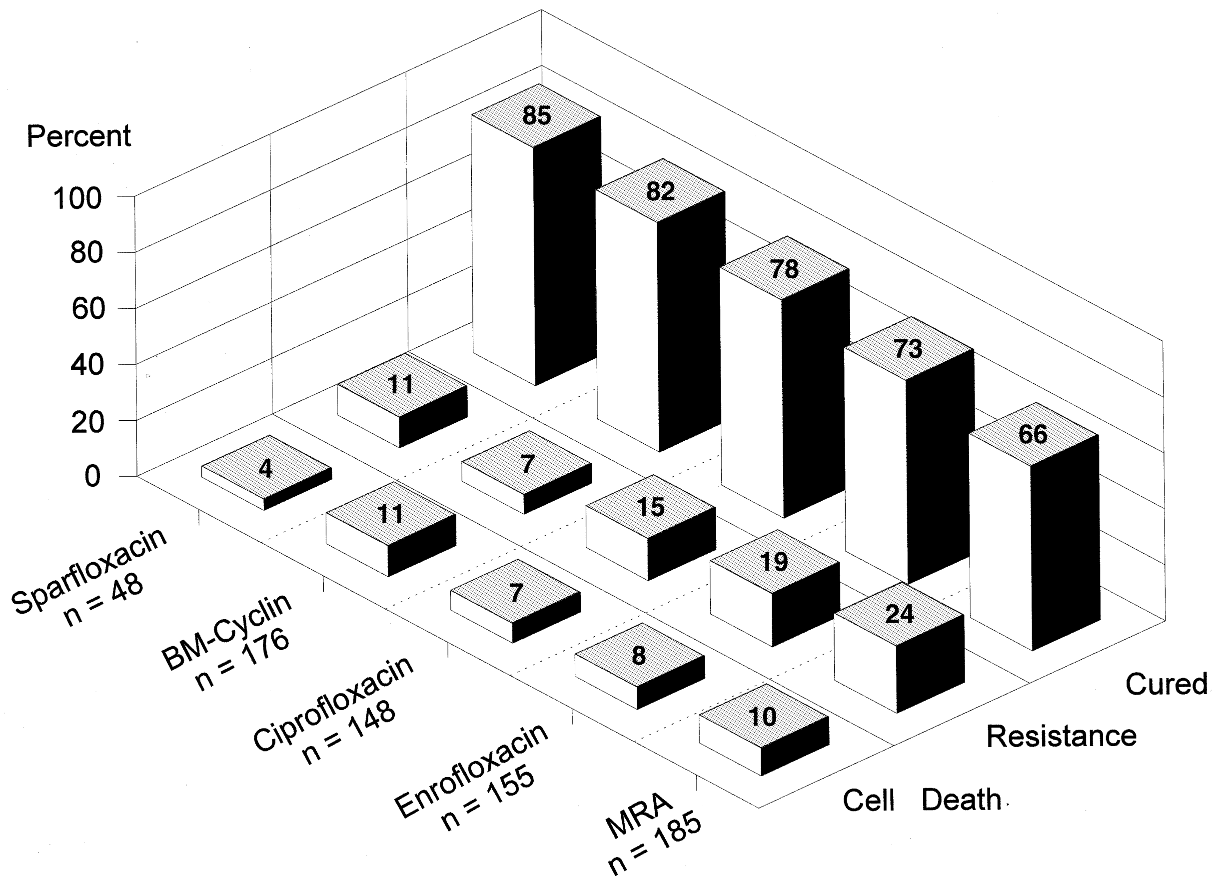

Figure 6. Treatment efficiency of anti-mycoplasma antibiotics. Shown are the outcomes of 712 treatments of 251 mycoplasma-positive cell lines with either BM-Cyclin, sparfloxacin, enrofloxacin, ciprofloxacin, or MRA. The results are presented as percentage of cultures that were either cured or that remained mycoplasma-contaminated (due to resistance) or that were lost during the treatment period (due to cytotoxicity). The number of cultures treated in each category is indicated (n).

In recent prospective studies, we examined the mycoplasma contamination status in two cohorts of 598 and 339 cell cultures by PCR assay, microbiological culture method, and DNA-RNA hybridization, in order to determine the sensitivity and specificity of the PCR assay in routine cell culture (Uphoff and Drexler, 2002a c). Results for the various assays in the two studies were concordant in 91 and 96% of the cases. In these two cohorts, the sensitivity of this PCR detection assay was 86 and 96%; the specificity was 93 and 96%; positive and negative predictive values were 73–86 and 97–99% (Table 7). PCR defined the mycoplasma status with 92 and 96% accuracy. Using a new technique, the mycoplasma contaminants were speciated by analyzing the PCR amplification fragment using a combination of restriction enzymes. Most of the cultures (47%) were infected with *M. fermentans*, followed by *M. hyorhinis* (19%), *M. orale* (16%), *M. arginini* (9%), *A. laidlawii* (6%), and *M. hominis* (3%).

In order to control the integrity of the PCR reactions and the preceding template preparation steps, it is essential to perform appropriate control reactions: these include internal, positive and negative control reactions. To that end, we have developped a technique whereby the internal control represents DNA which contains the same primer sequences but with an additional stretch of interspersed nucleotides, which results in a gel band of different size than the expected amplicon of the contaminant (Figure 4). This internal control is added to the PCR reaction in a limiting dilution to recognize any inhibitory components. A detailed protocol to establish the PCR method for the monitoring of mycoplasma contaminations in any laboratory has been summarized elsewhere (Uphoff and Drexler, 2002d). Figure 5 summarizes the hand-

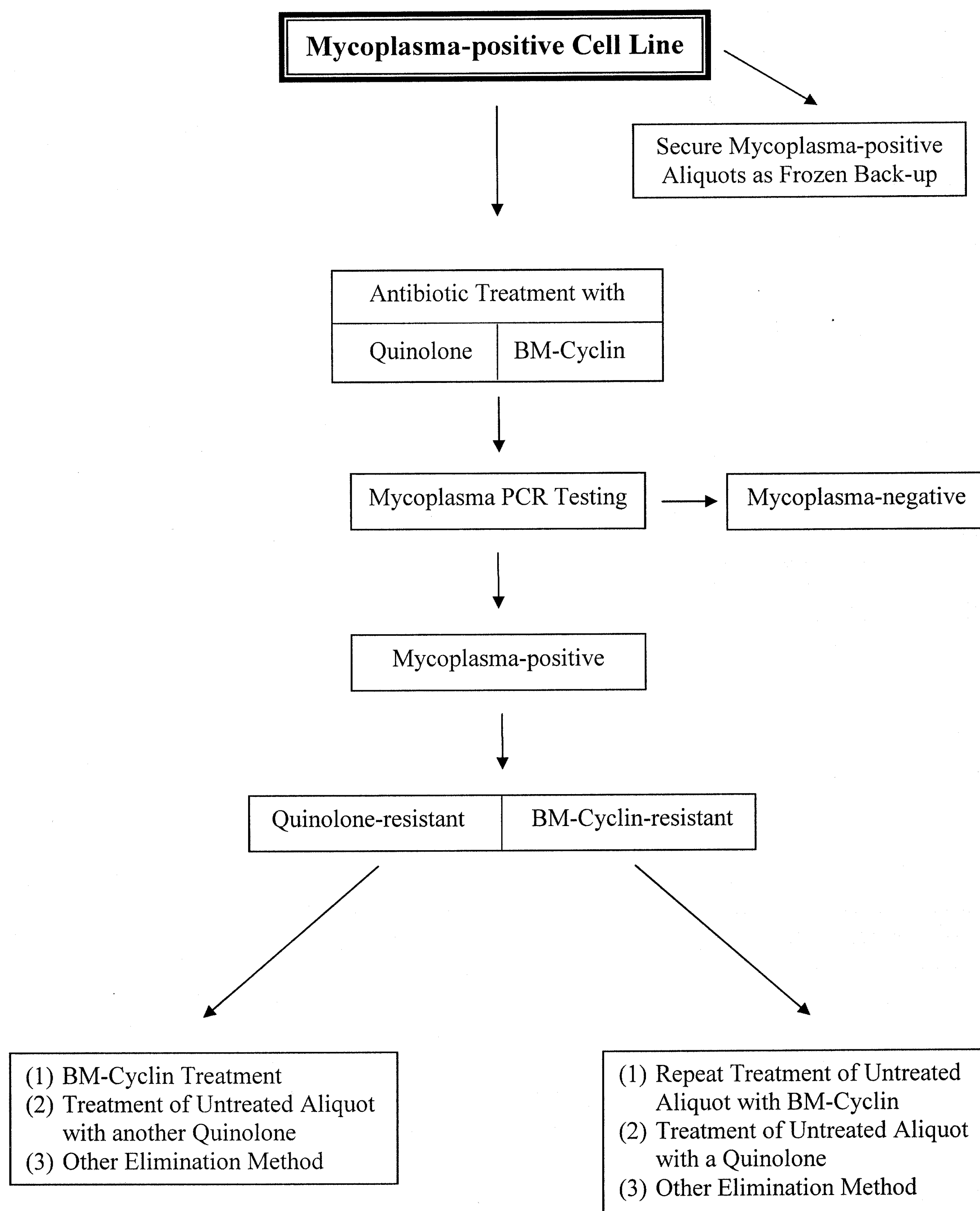

Figure 7. Recommended scheme for mycoplasma eradication. An arsenal of different antibiotics can be used to treat mycoplasma-contaminated cell lines with a high rate of expected success. We recommend (i) cryopreservation of original mycoplasma-positive cells as back-ups and (ii) splitting of the growing cells into different aliquots (e.g. in a 24-well plate). These aliquots should be exposed singly to the various antibiotics. It is important to point out that quinolone-resistant cultures could still be cleansed by BM-Cyclin. Post-treatment mycoplasma analysis and routine monitoring with a sensitive and reliable method (e.g. PCR) are of utmost importance. For technical details see reference Uphoff and Drexler (2002e).

ling of cell lines with regard to mycoplasma detection as performed at the DSMZ.

Elimination of mycoplasma contamination

Various elimination methods

Ever since mycoplasma contamination of cell cultures was first reported, attempts to develop methods for elimination of the mycoplasma have been made. It has been suggested that efforts to eradicate mycoplasmas from contaminated cells should be considered as a last resort (in order to prevent spread of the contaminant) and that it would be often far better to eliminate the problem completely by autoclaving the infected cultures and replacing them with fresh stocks known to be mycoplasma-free (Hay et al., 1989). However, all too often the cell line is not replaceable with a mycoplasma-free aliquot and purging of mycoplasmas from such cultures is a necessity.

Four general types of procedures have been used to eliminate mycoplasmas from infected cell cultures: physical, chemical, immunological and chemotherapeutic treatment (Table 8). Many of the methods were shown to be unreliable. Some techniques may apply to some, but not all mycoplasma species; some of them are too laborious or simply impractical. Elimination is typically time-consuming, often unsuccessful and poses risks of secondary infection to other cell cultures. Methods of elimination should ideally be simple and easy, rapid and efficient, reliable and inexpensive, have minimal effect on the eukaryotic cell and result in no loss of specialized characteristics; accidental cloning selection of treated cells also should not occur. However, there is clearly not a single method available that is both 100% effective and fulfills all the ideal requirements.

The effectiveness of some elimination methods has been investigated only in experimentally contaminated cell cultures, although experimentally infected cultures may not realistically reflect the laboratory situation since chronic infections certainly result in complex interactions between mycoplasmas and cells. If a clean-up is attempted, it is imperative to monitor closely the effectiveness of treatment relative to mycoplasma elimination and eukaryotic cytotoxicity. A variety of procedures have been described and utilized. Administration of antibiotics is by far the most common and efficient approach and will be discussed in greater detail below.

The physical, chemical and immunological methods are usually of restricted value since the mycoplasmas, although not detected for sometime afterwards, commonly reappear (low efficiency); some methods are time-consuming or have detrimental effects on the eukaryotic cells; other techniques are complex or impractical as they require extensive resources or special equipment and knowledge. Considering the various advantages and disadvantages of any elimination procedure, chemotherapeutic treatment appears to be superior to the other mycoplasma eradication techniques and thus the method of choice. The simple addition of another reagent to a cell culture is within the technical and financial capabilities of every cell culture laboratory.

Antibiotic treatment for elimination of mycoplasma

Mycoplasmas, which lack a cell wall and are incapable of peptidoglycan synthesis, are theoretically not susceptible to antibiotics such as penicillin and its analogues which are effective against most bacterial contaminants of cell cultures. However, it has been reported that several bacteriostatic antimicrobial agents inhibit the growth of mycoplasmas; thus they may not eradicate the contaminants, but simply suppress the full-blown picture of an infection and tend to mask the presence of mycoplasmas. A number of different antibiotics has been explicitly used for mycoplasma control (Table 9). The contaminant strains, however, often developed resistance to certain antibiotics which were thus completely ineffective. Other antibiotics (for instance some aminoglycosides and lincosamides) were moderately to highly effective in eliminating mycoplasmas, but only at concentrations which had detrimental effects on the eukaryotic cells, such as marked cytotoxicity.

Ideally, a basic procedure should involve isolating, speciating, and determining the antibiotic susceptibility of the contaminants to the arsenal of possible reagents to maximize success; then the cultures should be exposed to the effective antibiotics. However, this approach is extremely time-consuming, labor-intensive and requires certain expertise. It might be fair to say that few scientists (for whom a cell culture is normally only a means to an end) would use this complex approach and would prefer a quicker solution.

The pharmacological and clinical testing and application of mycoplasmacidal antibiotics has shown that tetracyclines are generally effective anti-myco-

Table 10. Prevention of mycoplasma contamination: Cell culture facility

- Facility should be designed and equipped for aseptic culture procedures.
- Certified laminar-flow biological safety cabinets should be used and their function should be regularly examined.
- Work surfaces should be chemically disinfected prior to and following work and thoroughly cleaned at regular intervals (monthly); effective non-toxic disinfectants are 70% ethanol for routine use and 2% phenol in 70% ethanol if contamination suspected.
- Incubators should be regularly controlled and cleaned (monthly).
- Discarded glass and plastic ware and spent media should be carefully disinfected.
- Cell culture materials should be properly disposed off by central sterilization.
- Effective house keeping procedures should be followed to minimize contamination of the environment (e.g. floor, sinks, faucets, water baths).
- Unauthorized persons should not be allowed entry.
- Animals should not be kept in the cell culture room.
- Laboratory should be kept clean.

Table 11. Prevention of mycoplasma contamination: Cell culture procedures

- Reliable mycoplasma detection methods should be established and performed.
- Medium components (especially serum) should be tested for sterility before use.
- Antibiotic-free media should be used whenever possible.
- Cell cultures should be obtained from reputable cell repositories.
- Incoming cell cultures should be kept in quarantine until proven sterile (or at least separated in time and space from sterile cultures).
- Mycoplasma testing should be performed at the time of arrival of the culture and at regular intervals (monthly).
- Mycoplasma-positive cultures should be immediately discarded (or cryopreserved) or treated with mycoplasmacidal measures.
- Strict aseptic techniques and good laboratory procedures should be followed.

plasmal agents; also quinolones were found to be highly effective against mycoplasmas. Of note are in particular the quinolones ciprofloxacin, enrofloxacin and an unpublished quinolone reagent available as Mycoplasma Removal Agent (MRA); the product BM-Cyclin combines the macrolide tiamulin (a pleuromutilin-derivative) and the tetracycline minocycline (Table 9) (Drexler et al., 1994; Uphoff et al., 1992b).

Tetracyclines inhibit protein synthesis by binding to subunits of ribosomes, thereby blocking peptide chain elongation. Tetracyclines inhibit both prokaryotic and eukaryotic ribosomal protein synthesis. Nalidixic acid is the prototype of a class of synthetic anti-bacterial agents, the fluoroquinolones, or simply quinolones. The mode of action of the quinolones involves the binding to and inhibition of the bacterial DNA gyrase, which is essential for DNA replication, transcription, repair and recombination. Despite their documented selectivity for prokaryotic enzymes, the quinolones may also exert an inhibitory effect on eukaryotic DNA polymerase α, topoisomerases and DNA deoxynucleotidyl transferases; the activity of these enzymes is especially high in rapidly dividing cells. Indeed, high doses of quinolones induced double-strand DNA breaks in human cells. Selectivity of the quinolones for the bacterial cell is at least partly due to the far greater sensitivity of the bacterial enzymes compared to the mammalian enzymes.

Our own data documented the effectiveness of several antibiotic treatment protocols which we have employed routinely in our cell lines bank (Fleckenstein et al., 1994; Gignac et al., 1991, 1992; Uphoff and Drexler, 2002b; Uphoff et al., 2002). On aggregate, 712 cultures from 251 chronically mycoplasma-positive cell lines were exposed to one of five antibiotic regimens (Figure 6). The mycoplasma infection was permanently eliminated by the various antibiotics in 66–85% of the cultures treated. Mycoplasma resistance was seen in 7–24%, and loss of the culture due to cytotoxically caused cell death in 4–11% of the cultures treated. Overall, 238 of the 251 mycoplasma-positive cell lines could be cured in a first round of antibiotic treatment with at least one regimen. Taken together, 95% of the mycoplasma-infected cell lines were permanently cleansed of the

Table 12. Prevention of mycoplasma contamination: Cell culturist

- Thorough washing and disinfection of hands prior to and following work.
- Prohibition of mouth pipetting.
- No unnecessary talking or traffic at the clean bench or in the immediate work area.
- Prohibition of eating, drinking, smoking and application of make-up in the laboratory.
- Protective clothing to protect both the culture and the culturist.
- Jewelry (rings, bracelets, wrist-watches) should be taken off; long hair to be tied back.
- Written laboratory records for every cell culture.
- Different medium aliquots for different cell lines.
- No pouring, but pipetting of medium from bottles or flasks.
- Handling of only one cell line at a given time.

contaminants by antibiotic treatment which validates this approach as an efficient and technically simple mycoplasma eradication method.

Besides cure, two other possible outcomes are loss of the culture and resistance. Culture death is presumably caused by cytotoxic effects of the reagents. Not unexpectedly, BM-Cyclin shows the greatest growth-inhibiting effect which may be either cytostatic or cytotoxic. Generally, one week after cessation of treatment, cell growth will return to normal. Cytostatic and cytotoxic effects of the antibiotics may be enhanced by the poor condition of cell cultures commonly found in chronically infected cells. This situation is clearly different from that of experimentally contaminated cell cultures. It was found that increasing the serum concentration and incubating the cells at higher densities was advantageous for the cell cultures.

With regard to resistance, the quinolones showed cross-resistance. This is not surprising given their basic structural similarity. Fortunately, sequential administration of BM-Cyclin to the same cells that were first exposed to a quinolone, can still result in eradication of the resistant infectant. Higher concentrations of the antibiotics may be more effective in purging mycoplasma-contaminated cultures by decreasing the rate of resistance, but this success would be counterbalanced by significantly higher cytotoxicity. It is not known whether the resistance of cell culture mycoplasmas to antibiotics is mostly acquired during treatment or exists already prior to exposure to these reagents. In any event, anti-mycoplasma antibiotics should be reserved for the specific situation of mycoplasma eradication in an irreplaceable infected cell culture and should not be routinely added to the culture medium as this will select resistant mycoplasma strains.

Anti-mycoplasma treatment conditions are certainly stressful to the eukaryotic cells. Thus, cells might no longer express the desired properties as a result of antibiotic administration. Outgrowth of a selected clone is another possibility. Some data suggest that cured cells generally preserve their characteristics. Still, any alterations to the cell lines induced by antibiotic treatment are obviously a matter of concern and require further detailed studies.

Taken together, the technically simplest alternative for mycoplasma decontamination with the most promising results is antibiotic treatment. A detailed protocol for the elimination of mycoplasma from infected cell lines with antibiotics has been published elsewhere (Uphoff and Drexler, 2002e). Figure 7 summarizes the treatment of mycoplasma-positive cell lines as performed at the DSMZ. The convenience of use and general availability of the reagents render it a reliable routine laboratory procedure. However, antibiotic mycoplasma elimination is laborious and time-consuming as the duration of the treatment plus the minimum antibiotic-free post-treatment period ranges from three to five weeks depending on the protocol used. Furthermore, special attention must be placed on possible cytotoxic effects or effects that alter the characteristics of the cell line.

Prevention of mycoplasma contamination

The prevention of mycoplasma contamination can be divided into three categories: cell culture facility, cell culture procedures, and operator technique (Uphoff and Drexler, 2001). While the measures proposed will not automatically prevent any mycoplasma infection, they will significantly decrease the probability of its occurrence. Such efforts are also of great importance

for the prevention of cross-contamination with other eukaryocytic cells which in the majority of cases appears to be the result of inadequate cell culturing as well.

Cell culture facility

Any sterile cell culture work should be performed in a vertical laminar-flow biohazard hood. It is important to disinfect all work surfaces before and after culture manipulations, including the various devices entering the laminar flow hood. Mycoplasmas are very sensitive to most disinfectants, but have shown extended survival in a dried state. Further critical points are summarized in Table 10.

Cell culture procedures

Cell culture laboratories should establish effective and regular mycoplasma testing procedures in the form of a routine screening program for all forms of microbial contamination, including mycoplasmas (Table 11). For mycoplasma screening, we recommend PCR analysis. Sera, media and supplements (and also cell lines whenever possible) should be purchased from reputable suppliers that adequately test for mycoplasmal contamination. All incoming cell lines should be quarantined until the contamination status is verified. Mycoplasma-free cultures should be segregated from infected cultures by time and place of handling. Reagents for the two sets of cultures should be separate. The general use of antibiotics is not recommended except in special applications and then only for short durations. Use of antibiotics may lead to lapses in aseptic technique, to selection of drug-resistant organisms, and to delayed detection of low-level infection by either mycoplasmas or other bacteria. Master stocks of mycoplasma-free cell lines should be frozen and stored to provide a continuous supply of cells should working stocks become contaminated. Actively growing mycoplasma-infected cell lines should be discarded or treated with mycoplasmacidal measures as quickly as possible in order to prevent lateral spread.

Operator technique

Strict adherence of the cell culturist to general aseptic culture techniques is a fundamental aspect in mycoplasma control. Cell culturists should continually be aware of the possibility of contaminating clean cultures with aerosols from mycoplasma-containing cultures which are handled in the same area. For example, the following procedures with liquid media generate droplets: pipetting, decanting, centrifuging, sonicating. These relatively large droplets settle into the immediate environment where they may remain viable for some time. Various further precautions that are necessary to minimize the risk of contamination are outlined in Table 12.

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