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COMPARATIVE PCR ANALYSIS FOR DETECTION OF MYCOPLASMA INFECTIONS IN CONTINUOUS CELL LINES

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SUMMARY

Mycoplasma contamination of cell lines is one of the major problems in cell culturing. About 15–35% of all cell lines are infected with a limited number of mycoplasma species of predominantly human, swine, or bovine origin. We examined the mycoplasma contamination status in 495 cell cultures by polymerase chain reaction (PCR) assay, microbiological culture method, and deoxyribonucleic acid–ribonucleic acid (DNA–RNA) hybridization, and in 103 cell cultures by PCR and DNA–RNA hybridization, in order to determine the sensitivity and specificity of the PCR assay in routine cell culture. For those two cohorts, results for the three or two assays were concordant in 92 and 91% of the cases, respectively. The sensitivity (detection of true positives) of this PCR detection assay was 86%, and the specificity (detection of true negatives) was 93%, with positive and negative predictive values (probability of correct results) of 73 and 97%, respectively. PCR defined the mycoplasma status with 92% accuracy (detection of true positives and true negatives). The mycoplasma contaminants were speciated by analyzing the PCR amplification fragment using several restriction enzymes. Most of the cultures (47%) were infected with *Mycoplasma fermentans*, followed by *M. hyorhinis* (19%), *M. orale* (10%), *M. arginini* (9%), *Acholeplasma laidlawii* (6%), and *M. hominis* (3%). To sum up, PCR represents a sensitive, specific, accurate, inexpensive, and quick mycoplasma detection assay that is suitable for the routine screening of cell cultures.

Key words: cell culture; mycoplasmal contamination; PCR assay.

INTRODUCTION

Mycoplasma contamination of cell lines is by far the most frequently occurring problem in cell culture. Although well known for many yr, in most laboratories little effort is being made to prevent or cleanse infections of mycoplasma. About 30% of all cell cultures, varying from 15 to 80% depending on the source of the survey, are reportedly contaminated with mycoplasmas (Barile and Rottem, 1993; Fleckenstein et al., 1994). The high variations in the prevalence of mycoplasma infections reported seem to reflect the unequal distribution of contaminations in various laboratories, the variable awareness of the problem among the laboratory staff, and the quality of laboratory practice. Usually, either most or none of the cell cultures kept in a given laboratory are infected. However, infection often occurs with the same mycoplasma species, which demonstrates that mycoplasma infections are often spread from one culture to another. Hence, according to common sense, one should regard cell lines that are newly received from another laboratory with a certain degree of suspicion. Consequently, the cells should be kept in quarantine until the status of mycoplasma infection has been determined (Drexler and Uphoff, 2000). Mycoplasmas can persist in cell cultures as commensales for an indefinite time. For these reasons, fast and reliable methods are required to detect mycoplasma contaminations.

Because mycoplasmas are not detectable by routine cell culture technology, a number of methods have been developed to detect those contaminants with various efficiencies: histochemical stainings and electron microscopy are used infrequently to visualize mycoplasmas directly, whereas the direct demonstration of mycoplasmas by deoxyribonucleic acid (DNA) fluorochrome stainings (e.g., by 4′,6-diamidino-2-phenylindole [DAPI] or Hoechst 33258) still seems to be the most frequently applied method. The availability of various biochemical detection methods reveals the extent of the problem because these techniques can be used on many samples. Immunological methods are applied using polyclonal antisera or monoclonal antibodies against mycoplasmal components (Barile and Rottem, 1993). Using the latter assays, the sensitivity and specificity are highly increased compared with the other techniques.

Commercially available kits based on biochemical and immunological detection are still frequently used in cell culture. Molecular biological assays have been available for mycoplasma testing for some yr now, and include hybridization assays or a combination with other techniques (Johansson et al., 1990). But all the aforementioned detection techniques share one common drawback: it was shown, that a single test does not reveal all the mycoplasma contaminations in cell cultures (Uphoff et al., 1992b). This is the result of either the limited sensitivity or the limited specificity of the assays.

The polymerase chain reaction (PCR) technology is now widely used for analytical purposes, and several PCR applications were

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TABLE 1

OLIGONUCLEOTIDES USED FOR THE AMPLIFICATION OF 16S RIBOSOMAL DEOXYRIBONUCLEIC ACIDS OF DIFFERENT MYCOPLASMA SPECIES[a]

5′ Primers:	3′ Primers:
cgc ctg agt agt acg ttc gc	gcg gtg tgt aca aga ccc ga
cgc ctg agt agt acg tac gc	gcg gtg tgt aca aaa ccc ga
tgc ctg agt agt aca ttc gc	gcg gtg tgt aca aac ccc ga
cgc ctg ggt agt aca ttc gc	
cgc ctg agt agt atg ctc gc	
tgc ctg ggt agt aca ttc gc	

[a] The mycoplasma species covered by these primers have been described elsewhere (Wirth et al., 1994).

developed to detect mycoplasmas in cell cultures. But PCR is still a complex process, and sensitivity and specificity can be influenced by a number of different parameters (Uphoff and Drexler, 1999). Adequate controls have to be included to obtain reliable results.

The aim of the present study was to examine the reliability and applicability of the PCR technique in the routine detection of mycoplasma contamination. To that end, PCR data were compared with the classical microbiological cultivation assay (colonies on agar) and the molecular biological DNA–ribonucleic acid (RNA) hybridization assay (Gen-Probe). We present results of a long-term prospective investigation with the goal of determining the sensitivity and the specificity of the PCR method because this method offers some features that may render this assay advantageous compared with other techniques. PCR analysis, in general, represents an extremely sensitive and specific, very quick, reliable, and still overall inexpensive technique; however, despite the preeminent advantages of this diagnostic tool, certain disadvantages can possibly hamper interpretation of the data.

Materials and Methods

Culture of cell lines. The continuous cell lines were provided for accessioning to the cell lines bank or for research purposes by the original or secondary investigators (Drexler, 2000; Drexler et al., 2001). Cell lines were grown at 37° C in a humidified atmosphere of air containing 5% CO_2. The basic growth media (Life Technologies, Karlsruhe, Germany) were supplemented with 10–20% fetal bovine serum (Sigma Chemical Co., Deisenhofen, Germany). For growth factor–dependent cell lines, specific growth factors or conditioned media containing growth factors were added. For mycoplasma detection, the cell lines were cultured for at least 1 wk after thawing, and samples were taken after a culture period of at least 2 d without medium exchange. No antibiotics were added to the cultures.

Microbiological agar culture. Media for the detection of mycoplasma by microbiological culture were prepared according to the method previously described (Uphoff et al., 1992b). Cell culture supernatants (200 μl) were incubated in two different broth liquid media (Friis medium and PH medium) at 37° C. After 3–5 d, aliquots were pipetted onto the respective agar plates. Development of typical colonies ("fried egg" appearance) was observed under the inverted microscope.

DNA–RNA hybridization assay. The assay detects specifically ribosomal RNA sequences of a large panel of mycoplasma species, including those commonly seen in infected cultures by hybridization with a [^{3}H]-radioactively labeled probe. The test is commercially available (Gen-Probe; Gen-Probe Inc., San Diego, CA). To increase the sensitivity of the assay, the hybridization period was extended from the recommended 3 to 18 h. This assay was shown to be very reliable (Uphoff et al., 1992b).

Polymerase chain reaction. For the PCR assay, a modification of a previously described method was used (Hopert et al., 1993). One milliliter supernatant of the cell cultures was centrifuged for 6 min at 13,000 × *g*. The supernatants were discarded, and the pellets were washed twice with phosphate-buffered saline (PBS). After the second wash, the pellets were resuspended in 100 μl PBS and incubated for 15 min at 95° C. The DNAs were extracted with the Wizard DNA Clean-Up System (Promega, Mannheim, Germany) according to the instructions of the manufacturer (the DNAs were eluted from the column with 50 μl dH_2O at 80° C). One microliter of the DNA extract was applied for the PCR amplification. The PCR was carried out in a final volume of 25 μl in 1× PCR buffer (Qiagen, Hilden, Germany) containing 1.5 m*M* $MgCl_2$, 100 μ*M* of each deoxynucleoside triphosphate, 0.2 μ*M* of each oligonucleotide (Table 1), 1 μl internal control DNA, and 1.25 U *Taq* DNA polymerase. To perform a hot-start PCR, 15 μl of the reaction without the *Taq* polymerase was mixed, denatured at 95° C for 7 min, cooled to 72° C, and at this temperature, the Taq polymerase in 1× PCR buffer was added. The first annealing step was carried out for 2 min at 65° C, and the subsequent amplification step for 10 min at 72° C. After this initial cycle, 32 cycles of 95° C for 2 s, 65° C for 4 s, and 72° C for 8 s were run on the Applied Biosystems GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems, Weiterstadt, Germany). Ten microliters of the reaction mix was electrophoresed on a 1.4% agarose gel, stained with ethidium bromide, visualized under ultraviolet light, and photographed.

The internal control was prepared by cloning the PCR amplification product of *Acholeplasma laidlawii* into the pGEM-T vector (Stratagene, Amsterdam, The Netherlands), and amplified in *E. coli*. Using *Sfu* I, the resulting plasmid pMyco-GEM-T was linearized, and a 476-bp *Taq* I fragment from pHMSE-1 (Uphoff et al., 1994) was ligated into the *Sfu* I site. Using this plasmid, a 986-bp fragment is competitively amplified in the PCR reaction when *A. laidlawii* is the contaminating mycoplasma species and noncompetitively amplified when other mycoplasma species are present in the cell cultures.

Identification of mycoplasma species. The identification of the mycoplasma species, which occur predominantly in cell cultures, was carried out by digestion of the PCR products with various restriction enzymes (Table 2). One microliter of the appropriate 10× buffer and 1 μl of the respective restriction

TABLE 2

RESTRICTION FRAGMENT PATTERNS OF THE POLYMERASE CHAIN REACTION (PCR) AMPLICONS DIGESTED WITH VARIOUS RESTRICTION ENZYMES[a]

Mycoplasma species	*Asp* I	*Bam* HI	*Hae* III	*Hpa* II	*Sfu* I	*Xba* I
A. laidlawii	—[b]	—	—	—	436/81	—
M. arginini	—	—	—	—	—	266/253
M. bovis	303/213	469/47	—	—	—	—
M. fermentans	—	469/47	356/160	357/111/48	—	—
M. hominis	—	—	336/180	—	—	263/253
M. hyorhinis	303/213	—	—	—	—	263/253
M. orale	—	—	288/230	—	—	266/252

[a] The numbers represent the sizes of the restriction fragments of the PCR product that was digested with the respective restriction enzyme.
[b] —, not digestible.

enzyme were added to 8 μl of the PCR reaction mix containing the amplicon and incubated at 37° C for about 1 h. In a first round, three reactions were incubated in parallel using *Asp* I, *Hpa* II, and *Xba* I restriction enzymes. To distinguish between *Mycoplasma arginini* and *M. hominis*, the PCR amplicon was additionally digested with *Hae* III restriction enzyme. The restriction fragment patterns were determined by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Identification of mycoplasma species by enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay (ELISA) Mycoplasma Detection Kit (Roche, Mannheim, Germany) was used as an alternative assay for the identification of mycoplasma. It contains polyclonal antibodies raised against specific mycoplasma antigens of the species *A. laidlawii*, *M. arginini*, *M. hyorhinis*, and *M. orale*. The assay was carried out according to the recommendations of the manufacturer. Briefly, the antigens were immobilized by an antibody bound to the surface of a microtiter plate. To the detection antibodies conjugated with biotin, a streptavidin–alkaline phosphatase complex was bound in the next step. The alkaline phosphatase hydrolyzed the substrate 4-nitrophenyl phosphate to a yellow nitrophenyl product. Positivity was evaluated visually and with a microplate-reader (Titertek Multiskan, Flow, Eschwege, Germany).

Statistical evaluation. Using the agar colony formation and the liquid DNA–RNA hybridization (Gen-Probe) assays as the reference tests, the following statistical parameters (operating characteristics) were calculated from the set of available data—sensitivity: true positives/true positives + false negatives; specificity: true negatives/true negatives + false positives; predictive value of a positive result and of a negative result: true positives/true positives + false positives and true negatives/true negatives + false negatives; accuracy: true positives + true negatives/total number of cases.

Results and Discussion

For decades the detection of mycoplasma in cell cultures was hampered by the elaborateness and low sensitivity of the assays and the subjective interpretation of the results. This was shown for different methods, including staining procedures, and biochemical and immunological assays (Uphoff et al., 1992b). The introduction of molecular biological techniques represents a milestone in the development of reliable methods for the detection of contaminations in cell cultures. Various assays have been created employing numerous probes and taking advantage of different hybridization and visualization techniques.

Previously, we and others have published the application of a nested PCR method to detect mycoplasma species and even genera (*Mycoplasma* and *Acholeplasma*) that occur in human and animal cell cultures (Hopert et al., 1993). To cover these species, a mixture of primers was used to amplify the conserved 16S ribosomal DNA (rDNA) coding region. The primer sequences allow the detection of mycoplasma DNA but not any other bacterial DNA that may contaminate sample preparations or solutions used for the PCR. The method originally described was modified in several aspects in order to meet the requirements of routine screening. In the preliminary experiments, we could not detect PCR products in different cell cultures contaminated with nonmycoplasma bacteria. In a detailed analysis, Wirth et al. (1994) found no visible amplification product with DNA from various bacterial species.

(1) The second round of PCR with the nested primers was omitted. In standard applications, no significant differences in the results were observed between one round of PCR only and nested PCR. Mycoplasma-positive cell cultures were detected as positive in the first round of PCR, and negative samples were consistently negative employing nested PCR. Furthermore, applying a nested PCR increases the risk of transmission of first-round PCR products to the reagents used in the second amplification and potentially to those shared with the first round.

(2) An internal control DNA, exhibiting the primer sequence of *A. laidlawii*, was added to the PCR reaction in a limiting dilution. The amplified PCR fragment was significantly longer than the wild-type product because of an insertion of a plasmid sequence. The amplification of the internal control DNA demonstrated the integrity of the PCR reaction (Fig. 1).

(3) The application of crude sample aliquots, which were only treated with proteinase K and subsequently boiled for several min, was found to decrease the sensitivity of the PCR method substantially. This preparation may include unidentified inhibitors and may lead to false-negative results. Even the use of a dilution series of the sample is not always satisfactory. This was clearly demonstrated by the introduction of the internal control DNA. Hence, we introduced a DNA isolation and purification step by binding the crude DNA sample to DNA clean-up resins. This method increased the efficacy of the PCR reaction dramatically, as shown by the amplification of the internal control DNA.

(4) The duration of the PCR cycle steps was drastically reduced from 30 to 4 s for the denaturation, from 30 to 8 s for the annealing, and from 60 to 16 s for the amplification. This modification did not affect negatively the quality of the analysis; on the contrary, we found that the sensitivity was increased by shortening the steps. This improvement may be the result of conditions that are less detrimental for the Taq polymerase.

(5) To identify the species of the detected mycoplasma contaminants, we digested the PCR products with a series of selected restriction enzymes (*see* Table 2). The digestibility and the patterns of the resulting fragments indicated the given mycoplasma species. For the identification, only restriction patterns of the seven most abundantly occurring mycoplasma species, which together account for 99% of all contaminations in cell cultures (Bölske, 1988), were chosen.

Using PCR, we investigated the mycoplasma contamination status in a panel of 598 samples from 377 different cell lines. The PCR results were compared with those of two reference methods. All samples were simultaneously tested with the Gen-Probe assay, and most of them were tested with the microbiological culture method. The samples included supernatants from two different cohorts of cell cultures: (1) first-time evaluation of the mycoplasma status (mostly newly arrived cell lines), and (2) analysis of initially positive cell lines after antimycoplasma treatment. For 495 samples, all three detection methods were applied in parallel, whereas for 103 samples, only PCR and Gen-Probe were used. The three mycoplasma tests on 598 samples gave overall concordant results in 549 samples (92%, 457 mycoplasma negative, 92 mycoplasma positive) (Table 3). Among the 49 discordant results, only 15 were discordant negative and 34 discordant positive in the PCR analysis. All data taken together, the following statistical evaluation emerges: the sensitivity and the specificity of the PCR mycoplasma detection assay are 86 and 93%, respectively; PCR defines the mycoplasma status with 92% accuracy.

Regarding the identical results for all three assays, or for PCR or Gen-Probe only, the positive cases can be considered as correctly determined. This is corroborated by the finding that only one case was positive in agar and negative in both Gen-Probe and PCR. This exceptional case might have been a misinterpretation of pseudo-

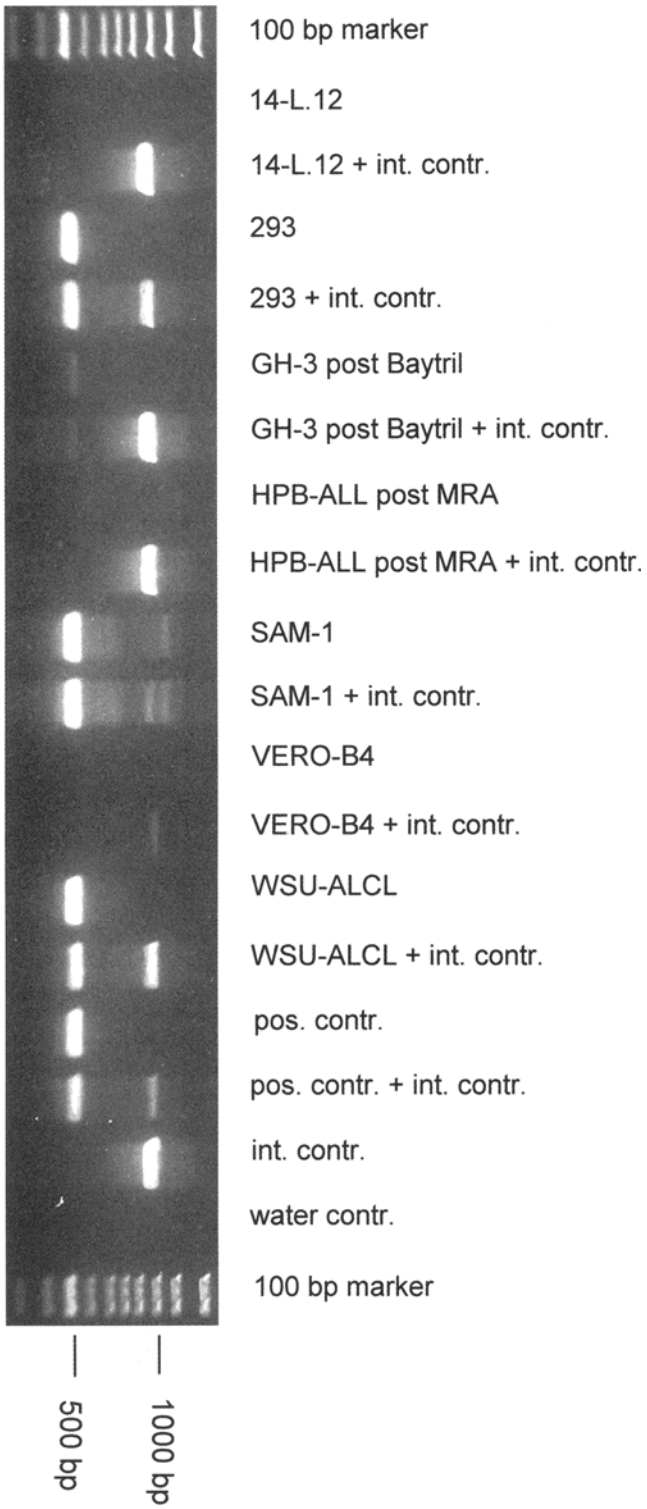

FIG. 1. Electrophoretic analysis of mycoplasma polymerase chain reaction (PCR) with and without internal control deoxyribonucleic acid (DNA) (int. contr.). Supernatants from seven different representative cell cultures were subjected to the sample preparation, and two PCR amplifications of each sample were run with the primer mixtures listed in Table 1. 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 with an inserted pHMSE1 fragment). Cell lines 14-L.12 and HPB-ALL post-mycoplasma removal agent (after antimycoplasma treatment) are clearly mycoplasma negative, and cell lines 293, SAM-1, and WSU-ALCL are clearly mycoplasma positive. Cell line GH-3 post-Baytril shows a sample after antimycoplasma treatment that remained weakly positive over several wk after treatment because of the residual mycoplasma DNA. In cell line SAM-1, the internal control DNA amplification was suppressed because of 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. pos. contr., positive control; water contr., water control; Mycoplasma Removal Agent (MRA).

TABLE 3

COMPARISON OF POLYMERASE CHAIN REACTION (PCR) MYCOPLASMA DETECTION WITH TWO REFERENCE METHODS

Statistical parameter[a]	Compared with Gen-Probe/agar	Compared with Gen-Probe	Total
Concordant PCR negative	382[b]	75	457
Concordant PCR positive	73	19	92
Discordant PCR negative	8	7	15
Discordant PCR positive	32	2	34
Sensitivity (%)	90	73	86
Specificity (%)	92	97	93
Predictive value of			
Positive result (%)	70	90	73
Negative result (%)	98	91	97
Accuracy (%)	92	91	92

[a] Concordant or discordant PCR results refer to the concordance or discordance with the two other mycoplasma detection assays employed here for comparison (Deoxyribonucleic acid–ribonucleic acid hybridization = Gen-Probe and agar colony formation = agar assay). Operatively, discordant negative or discordant positive results are considered here as false negative or false positive; however, neither of the two reference assays can be considered as the absolute "gold standard" of mycoplasma detection because these methods may occasionally produce false data.

[b] Number of cell lines.

mycoplasma colonies caused by colony formation of eukaryotic cells on the agar plate. Moreover, each assay is completely independent from the other assays, and no solutions are shared by two assays except for the supernatant to be tested. Thus, it would be unlikely to have false-positive results in all three assays because of contaminations. Regarding the concordantly negative samples, false-negative results in all three assays might only occur in samples with mycoplasma concentrations below the sensitivity threshold of the individual assay. Such low titers of mycoplasma are possible in cell cultures after antimycoplasma treatment or in primary cell material; the latter was not used for this study. Antimycoplasma-treated samples were therefore grown for at least 2 wk after treatment without antibiotics in order to give the contaminants the time to multiply to levels above the detection limits of all three assays.

Several publications have described studies comparing the PCR technique with other detection methods. Absolute concordance was reported by us in a previous publication (Hopert et al., 1993) on 42 different samples (29 positive, 13 negative), using the microbiological culture method as the reference method. Also, 100% concordance between PCR and microbiological culture or DAPI was described by Spaepen et al. (1992) on 54 cell cultures (9 positive) and Toji et al. (1998) on 8786 specimens with only 61 positive samples. On the other hand, Teyssou et al. (1993) found 82 concordant (36 positive, 46 negative) cases out of 86 cell culture sam-

ples (95%) using PCR, Hoechst 33258 DNA staining, culture, and ELISA. van Kuppeveld et al. (1994) tested 104 cell cultures (52 positive, three of them containing other bacteria) for mycoplasma contaminations and found an agreement between Hoechst 33258 DNA staining, microbiological culture, DNA–RNA hybridization, and PCR in 95 cases (91%). Further pertinent publications have been presented by Harasawa et al. (1993), Harasawa (1995), and Tang et al. (1999).

Regarding the discordant results, all possible combinations of results were seen here, albeit at various incidences. The majority of discrepant results (n = 14) were found in the categories PCR positive, and Gen-Probe and microbiological culture negative. One could assume that this might be because of the higher sensitivity of the PCR assay. But with regard to the source of the supernatants, 9 out of 14 samples were post-antimycoplasma treatment. The results were reproducible for more than 2 wk after treatment. Additionally, in most of these apparently false-positive cases, the PCR bands were much weaker than is normally found in untreated, continuously positive cultures. The PCR-based intensity of the bands remained constant over an extended period of time. This indicates that the mycoplasma concentration in the culture did not increase and that the positive results of the PCR might be truly false positive because of the residual mycoplasma DNA in the culture. The frequency of such false-positive results is 4% and underlines the need for regular and frequent testing of antimycoplasma-treated cell lines (for further details, *see* accompanying paper on mycoplasma treatment with antibiotics).

A second category of discordant data concerns the 11 samples that are PCR and microbiological culture positive, but Gen-Probe negative. These specimens can be regarded as false negative for Gen-Probe because the growth of mycoplasmas on agar is an unequivocal sign of contamination, and misinterpretations are relatively unusual for experienced observers. PCR and Gen-Probe–positive results account for seven samples (1%). This category is also likely to be truly positive because some mycoplasma strains were described as not culturable or difficult to grow on the media used for the general detection of mycoplasmas (McGarrity et al., 1985). In contrast, in the category of samples that are Gen-Probe positive but PCR and microbiological culture negative (n = 6), most cases are false positive, which might be the result of insufficient washings of the samples. False positivity of some samples was demonstrated by repetition of the assay. One sample turned out to be negative only in PCR but positive in Gen-Probe and microbiological culture assays. This case should be regarded as false negative in PCR. Because this was after antimycoplasma treatment, and the untreated culture was positive in PCR, this discordance might be caused by technical errors.

The two samples tested by PCR and Gen-Probe were only positive in PCR. These supernatants were both tested after treatment with antibiotics and later turned out to be negative also in PCR. Of the seven Gen-Probe–positive and PCR-negative specimens, six were retested and found to be Gen-Probe negative. One sample was not retested. Thus, at least six samples were detected as false positive by Gen-Probe in the first analysis.

Considering that various comparative results of the reference methods Gen-Probe and microbiological culture are likely to be false, the "real" accuracy of our PCR assay is likely to be even higher than the 92% calculated here (Table 3). Thus, although there is certainly not a single mycoplasma detection assay that is on the one hand, 100% sensitive, 100% specific, and 100% accurate and on the other hand, financially affordable and technically practical, PCR clearly appears to be the best tool for mycoplasma detection. For those workers wishing to cover the remaining percentages, it would be advisable to perform in parallel a second or even third mycoplasma detection assay.

In any event, for routine monitoring of cell lines at regular intervals, the PCR method is the assay of choice to check for mycoplasma contamination. The assay combines high sensitivity, specificity, and speed. We also suggest the replacement of the commonly used direct DAPI or Hoechst 33258 fluorochrome staining by PCR method. We found that the DAPI staining has several disadvantages compared with PCR: it is less sensitive; it is susceptible to the suboptimal conditions of the cell culture; inexperienced, and sometimes experienced, cell culturists arrive at discordant interpretations. Special care should be taken when cell cultures are treated against mycoplasma infections or are routinely cultured with antibiotics. These conditions seem to have a significant influence on the outcome of any detection assay, especially on the outcome of the PCR assay.

The use of PCR allows the identification of the mycoplasma species by digestion of the PCR products with specific restriction enzymes. The 16S rDNA PCR amplification products of the mycoplasma species, which are most commonly found in cell cultures (*A. laidlawii*, *M. arginini*, *M. bovis*, *M. fermentans*, *M. hominis*, *M. hyorhinis*, *M. orale*), were mapped by applying the sequences as available in the GeneBank database. Unique restriction sites were determined using the Mapping program of the GCG package (Genetics Computer Group, Madison, WI). The restriction patterns predicted by the program were verified using PCR amplification products of mycoplasma-type strains, kindly provided by Prof. Kirchhoff, Hannover, Germany, or from cell culture–contaminating mycoplasmas, which were identified applying the ELISA mycoplasma detection assay. This kit allows the identification of the mycoplasma species *A. laidlawii*, *M. arginini*, *M. hyorhinis*, and *M. orale*.

A typical PCR-based identification pattern is shown in Fig. 2. These control reactions proved that the resulting digestion patterns are suitable for speciating the mycoplasma. Employing this restriction fragment length polymorphism–related method, we identified the mycoplasma species of 79 PCR-positive samples. We detected an extraordinarily high incidence of *M. fermentans* contaminants (47%) (Table 4). Although many of the cell lines contaminated with *M. fermentans* were received from only a few laboratories, this finding confirms the observation that the incidence of *M. fermentans* contaminations increased over the last few decades. In 1992, we found that *M. fermentans* accounted for about 20% of the contaminations (Uphoff et al., 1992a), whereas Roulland-Dussoix et al. (1994) and Toji et al. (1998) identified 12 and 14% of the contaminations, respectively.

We suggest that most of the contaminations are spread from one infected culture to another either by sharing the same media and supplements or by direct infection caused by aerosoles and droplets (Drexler and Uphoff, 2000). This would account for the finding that many of the cell lines in one laboratory are infected with the same mycoplasma species and might also explain the heterogeneity of the results in the mycoplasma species distribution. For example, Roulland-Dussoix et al. (1994) found that more than 50% of the cell cultures are contaminated with *M. hyorhinis*. Nevertheless, the overall spectrum of species, which is predominantly found in cell cul-

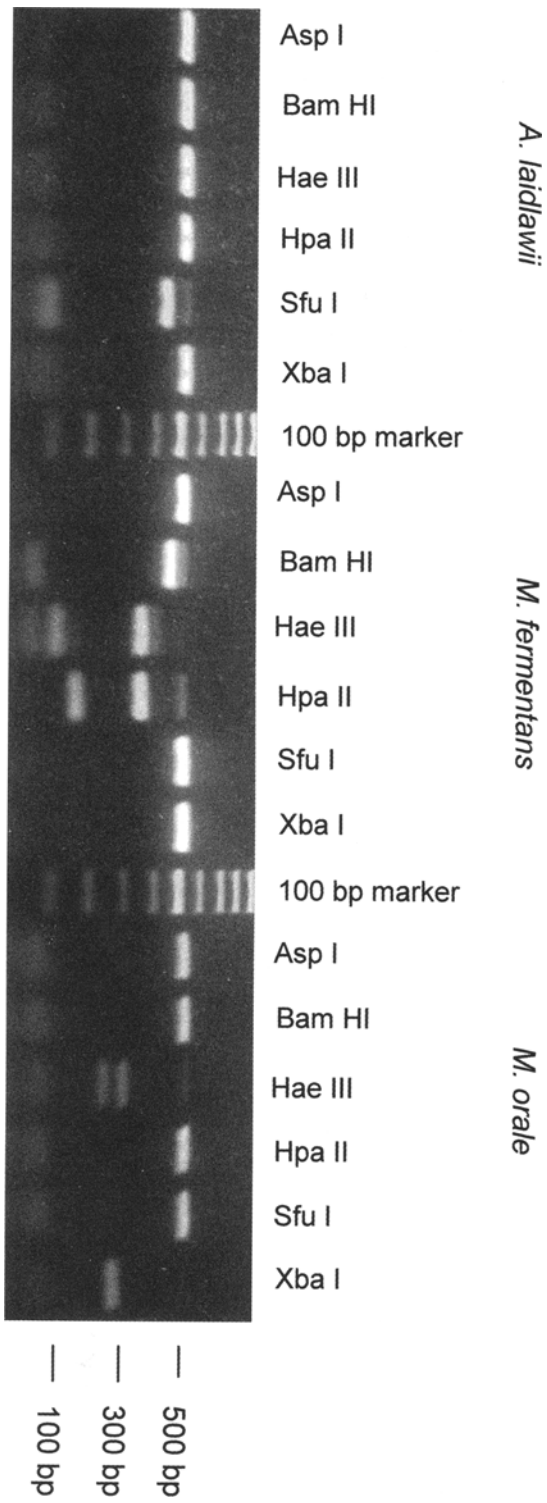

FIG. 2. Identification of mycoplasma species by restriction fragment length polymorphism analysis of polymerase chain reaction (PCR) products. The PCR products were digested with the annotated restriction endonucleases, and the resulting fragments were separated on an ethidiumbromide-stained agarose gel. The patterns were compared with the fragment sizes determined by sequence analysis (compare with Table 2). Shown here are representative examples of *A. laidlawii*, *M. fermentans*, and *M. orale*.

TABLE 4

IDENTIFICATION OF MYCOPLASMA SPECIES

Mycoplasma species	No. of identified samples (%)[a]
A. laidlawii	5 (6%)
M. arginini	7 (9%)
M. bovis	—
M. fermentans	37 (47%)
M. hominis	2 (3%)
M. hyorhinis	15 (19%)
M. orale	13 (16%)
Multiple infections	7

[a] Percentage among the single infections.

tures, is stable and is restricted to the afore-mentioned species. The distribution of the mycoplasma species in the present study was as follows: 47% *M. fermentans*, 19% *M. hyorhinis*, 16% *M. orale*, 9% *M. arginini*, 6% *A. laidlawii*, and 3% *M. hominis* (Table 4). Except for *M. fermentans*, the relative distribution of the species is similar to those found in other publications (reviewed in Drexler and Uphoff, 2000), with *M. hyorhinis* and *M. orale* as the predominant species followed by *M. arginini* and *A. laidlawii*. *Mycoplasma hominis* infections are detected with low frequency, and *M. bovis* is usually rather rarely seen. Seven cell lines were infected with more than one mycoplasma species.

In summary, we described and validated a genus-specific PCR assay for the detection of mycoplasma in cell cultures. We strongly recommend this PCR assay for the detection and monitoring of the frequently occurring mycoplasma infections in cell lines. This method is highly sensitive, specific, reliable, convenient, and can be completed within less than 5 h. Additionally, the assay is inexpensive because in many laboratories the requirements for performing PCR are already provided. Furthermore, the primers selected will not react with nonmycoplasma bacteria.

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