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PCR-Based Detection of Mycoplasma Species

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In this study, we describe our newly-developed sensitive two-stage PCR procedure for the detection of 13 common mycoplasmal contaminants (*M. arthritidis, M. bovis, M. fermentans, M. genitalium, M. hominis, M. hyorhinis, M. neurolyticum, M. orale, M. pirum, M. pneumoniae, M. pulmonis, M. salivarium, U. urealyticum*). For primary amplification, the DNA regions encompassing the 16S and 23S rRNA genes of 13 species were targeted using general mycoplasma primers. The primary PCR products were then subjected to secondary nested PCR, using two different primer pair sets, designed via the multiple alignment of nucleotide sequences obtained from the 13 mycoplasmal species. The nested PCR, which generated DNA fragments of 165-353 bp, was found to be able to detect 1-2 copies of the target DNA, and evidenced no cross-reactivity with the genomic DNA of related microorganisms or of human cell lines, thereby confirming the sensitivity and specificity of the primers used. The identification of contaminated species was achieved via the performance of restriction fragment length polymorphism (RFLP) coupled with *Sau*3AI digestion. The results obtained in this study furnish evidence suggesting that the employed assay system constitutes an effective tool for the disagnosis of mycoplasmal contamination in cell culture systems.

Keywords: Mycoplasma, molecular diagnosis, nested PCR, rDNA

Infections with mycoplasma species can induce a variety of problems in living organisms and in *in vitro* cell cultures. Many species that can exist as commensal organisms are associated with certain diseases, but mycoplasma infections are often subtle or subclinical in nature. These non-apparent infections tend to be quite insidious, as they may affect a variety of biochemical and genetic aspects of the infected cells, thereby resulting in unreliable experimental results and the possible transmission of diseases (McGarrity and Kotani, 1985; Balrile and Rottem, 1993; Harasawa *et al.*, 1993). Therefore, it is necessary to establish a routine diagnostic protocol for mycoplasma infection in order to ensure reliable research results, as well as the safety of commercial biological products. However, the detection of mycoplasmic species in cell cultures remains a problem, despite the substantial improvements that have been made in recent years in biochemical, immunological, and molecular biological methods.

Methods available for the diagnosis of mycoplasmal infection include cultural determination, DNA fluorochrome staining, enzyme-linked immunosorbent assays, immunofluorescence, biochemical assays, and DNA probe assays (Clyde *et al.*, 1984; Hopert *et al.*, 1993; Razin, 1994; Harasawa, 1995). Although each of these methods is associated with certain advantages, these immunological procedures are often limited by the presence of intra-species cross-reactivity. Also, the cultivation of these species tends to be time-consuming and difficult to achieve, due to the requirement of fastidious conditions for their growth. Moreover, the majority of currently available detection procedures are not sufficient for the simultaneous detection of the major mycoplasma species contaminants commonly encountered in *in vitro* cell cultures (Uphoff *et al.*, 1992; Wirth *et al.*, 1994; Loens *et al.*, 2002; Yoshida *et al.*, 2002; Mardassi *et al.*, 2005). In order to circumvent those limitations, many nucleic acid technology-predicated procedures have been developed. PCR-based methods for the detection of certain DNA regions of the mycoplasma genome have proven both rapid and specific (Hu *et al.*, 1995; Harasawa and Kanamoto, 1999; Kong *et al.*, 2001; Loens *et al.*, 2003; Khanna *et al.*, 2005). However, the degree of sensitivity of these techniques tends to be somewhat low, as the nucleo-

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tide sequences of primers tend not to perfectly match the target DNA from different species. Primers have, however, been specially designed to target the conserved region of the 16S ribosomal RNA (rRNA) gene (van Kuppeveld *et al.*, 1992; Rawadi and Dussurget, 1995; Quirt *et al.*, 2001; Jurstrand *et al.*, 2005) or the 16S-23S rRNA regions encountered in *Mycoplasma* species (Harasawa *et al.*, 1993; Harasawa, 1995; Tang *et al.*, 2000). These procedures have been associated with a sensitivity of between 5 and 100 organisms.

The nucleotide sequences of the rRNA genes from various *Mycoplasma* species have been determined, and used to construct the basis for a systematic phylogenetic analysis of these organisms. Computer-aided rRNA sequence alignments have revealed the existence of highly-conserved regions between many of the relevant species, enabling the selection of common PCR primers. This report describes a two-stage nested PCR assay technique, which can be used to detect one copy of target DNA from 13 species and to identify mycoplasmic contaminants at the species level.

Materials and Methods

Bacterial strains, growth conditions, and culture media

The 13 strains of mycoplasma employed in this study were obtained directly from the American Type Culture Collection (ATCC) and grown at 37°C in PPLO medium (DIFCO). They include: *M. arthritidis* ATCC 13988, *M. bovis* ATCC 25025, *M. fermentans* ATCC 19989, *M. genitalium* ATCC 33530, *M. hominis* ATCC 23114, *M. hyorhinis* ATCC 17981, *M. neurolyticum* ATCC 15049, *M. orale* ATCC 23714, *M. pirum* ATCC 25960, *M. pneumoniae* ATCC 15531, *M. pulmonis* ATCC 23115, *M. salivarium* ATCC 23064, *U. urealyticum* ATCC 27618. To grow the O_2-sensitive strains, either an anaerobic jar or CO_2 incubator was used.

Preparation of genomic DNA

Genomic DNA from these 13 mycoplasma species was prepared via the standard protocols, as previously described (Tang *et al.*, 2000). In brief, mycoplasma cultures growing at the late log phase stage were centrifuged for 30 min at 12,000 × g. The resultant pellets were resuspended in STE buffer (10 mM NaCl, 20 mM Tris HCl [pH 8.0], 1 mM EDTA) and incubated for 2 h at 37°C with 1% SDS and 50 μg of protease K (Promega, USA) per ml. Nucleic acids were extracted three times with an equal volume of phenol saturated with TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA), once with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), and once with chloroform-isoamyl alcohol (24:1). The total nucleic acids were then precipitated via the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 96% ethanol. After washing and drying, the pellets were then resuspended in sterile distilled water containing 1 μg of RNase A. Genomic DNA from the non-mycoplasmal bacteria was prepared via the lysozyme treatment technique, as previously described (Song and Park, 1997). In the case of the human and murine cell lines, the cells were lysed with 0.5% NP-40 and treated for 1 h with 20 μg protease K at 55°C. Total genomic DNA was precipitated for 30 min with 0.6 volumes of isopropanol at -20°C followed by centrifugation and washing in cold 75% ethanol. The pellet was then dried and resuspended in 50 μl of sterile distilled water.

Primers

A total of 6 primers (listed in Table 1) were employed in the amplification of a conserved spacer region encompassing the 16S and 23S rRNA gene from 13 mycoplasma strains. In addition to the three previously recommended primer sequences (Nakagawa *et al.*, 1992; Harasawa *et al*, 1993), three additional primers were selected in this study, according to the acquired sequence alignment data. During the first-stage PCR, one primer pair, F1 and R1, was utilized to amplify the outer spacer regions from the 13 mycoplasmal species. During the second-stage PCR, two primer pairs targeting the inner regions of the spacer DNA (FN2 and R2, FN3 and RN3) were used, ac-

Table 1. PCR primers for detection of mycoplasmal rRNA genes

Name	Sequences	Reference
F1	ACACCATGGGAG(C/T)TGGTAAT	Harasawa *et al.*, 1993
R1	CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT	Nakagawa *et al.*, 1992
FN2	ACCTCCTTTCTACGGAGTACAA	This study
R2	GCATCCACCA(A/T)A(A/T)AC(C/T)CTT	Harasawa *et al.*, 1993
FN3	TATTTGCTATTCAGTTTTCAAAGAAC	This study
RN3	GGGGTGAAGTCGTAACAAGGTAT	This study

cording to the mycoplasma species.

PCR conditions

PCR was conducted in a total volume of 25 μl, containing 1× PCR buffer, 50 μM each of dNTP, 10 pmol of each primer, and 0.5 U Taq DNA polymerase, in accordance with the manufacturer's recommendations (Solgent, Korea). 0.1 μg of genomic DNA from the mycoplasma, 1 μg of purified chromosomal DNA of the cell lines, or defined copy numbers of the cloned rRNA genes were employed as templates. The PCR reactions were conducted in a PCR machine with a heated lid. Each of the cycles was conducted as follows: 30 sec at 94°C, 30 sec at 55°C, and 60 sec per Kb of amplification products at 72°C, followed by 5 min of incubation at 72°C. The number of cycles was less than 45. Prior to cloning, DNA sequencing, or restriction enzyme digestion, the PCR products were treated with phenol/chloroform and precipitated with ethanol. Second stage PCR was conducted via the addition of 0.5 μl of the first PCR product directly to 19.5 μl of the reaction mixture containing the primer pair for the nested PCR. During the PCR analysis, decontamination processes were conducted periodically, and each of the PCR components were optimized in order to ensure a higher degree of sensitivity, as was previously described (Furrer *et al.*, 1990; Sarkar and Sommer, 1991). Aliquots of the final PCR products were analyzed on 2% agarose gel. DNA bands were visualized with a UV transilluminator after ethidium bromide staining, and then photographed.

Analysis of the amplified DNA with restriction enzyme digestion

In order to identify and differentiate between mycoplasma species, the sizes of the second-stage PCR products were determined via electrophoresis on MetaPhore agarose or Nusieve 3:1 agarose. Further differentiation of species was accomplished via the *Sau*3AI digestion of the purified PCR products, after which the digested fragments were subjected to agarose gel electrophoresis (Ausubel *et al.*, 1995). Portions (10 μl) of the PCR products were analyzed on 2% agarose gel (SeaKem ME; FMC, USA), via electrophoresis in Tris-borate-EDTA buffer. Electrophoresis was conducted for 1 h at 100 V, and the gels were stained for 30 min with a solution containing 0.5 μg of ethidium bromide per ml. The DNA fragments were visualized via UV illumination at 312 nm.

Sensitivity and specificity determination

In order to evaluate the specificity of the PCR developed in this study, the genomic DNA from a murine fetal thymus and human cell line were analyzed under the same PCR reaction conditions. Chromosomal DNA from *Escherichia coli, Mycobacterium avium, Staphylococcus aureus,* and *Saccharomyces cerevisiae* was also tested, under the same reaction conditions. In order to evaluate the sensitivity, clones of the first-stage PCR products were serially diluted and two-stage PCR was conducted in the presence of 1 μg of salmon sperm DNA as a background competitor. The PCR amplicons were then analyzed on 2% agarose gel.

DNA manipulation and sequencing of PCR products

In order to clone the PCR products into plasmid vectors and prepare the plasmid DNA, *E. coli* strain DH5α was grown in Luria broth (LB) and stored at -80°C in LB containing 15% glycerol (v/v). The PCR products amplified from the 13 mycoplasmal species were then ligated using the pGEM-T cloning system (Promega, USA), and the resultant mixtures were transformed into DH5α. Transformants harboring plasmid clones were selected on LB media containing 100 μg/ml of carbenicillin. Each of the plasmid clones containing the rDNA region from the 13 mycoplasmas were then purified, and sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems, USA). Each reaction volume included 4 μl of BigDye, 100 ng of the purified plasmid DNA, and 3.2 pmol of the sequencing primer. The sequencing reaction was run in a total volume of 10 μl. The thermal profile included 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and a final extension at 60°C for 4 min. The samples were then analyzed with a 96-lane capillary DNA analyzer (ABI PRISM 3700; PE Biosystems, USA).

Results

Design of mycoplasma-specific primers

In this study, we attempted to devise a highly sensitive two-stage PCR technique for the detection of 13 commonly encountered mycoplasmas, including *M. arthritidis, M. bovis, M. fermentans, M. genitalium, M. hominis, M. hyorhinis, M. neurolyticum, M. orale, M. pirum, M. pneumoniae, M. pulmonis, M. salivarium,* and *U. urealyticum*. The spacer DNA region between the 16S and 23S rRNA genes of 13 *Mycoplasma* species was then selected for the development of a new diagnostic procedure. The nucleotide sequences in the spacer region were acquired from three different nucleotide sequence libraries, namely the Genbank, EMBL, and RDP databases. Alignment studies were conducted using Clustal W sequence analysis software. The previously described general mycoplasmic primers, F1 and R1 (Table 1), evidenced considerable similarity with the spacer regions of the 13 species (data not shown). Therefore, we adopted

these general primers for our first-stage PCR procedure. To select the primers for the second-stage PCR, we conducted extensive analyses of the sequence data from the 13 species. Careful observation showed that these *Mycoplasma* species could be divided into two groups, in accordance with sequence similarity. As the two-consecutive amplification procedure resulted in an enhancement of the detection limit, we developed a two-stage PCR method. After first-stage PCR, conducted with the general mycoplasmal primers F1 and R1, nested PCR was conducted with specific primers targeting the inner rDNA region. In addition to the outer similar region used for binding to the first PCR primer, the inner homologous region was chosen, as well as the FN2 and R2 primer pairs, which were selected for the second PCR. Fig. 1 shows the alignment data obtained from 6 among 11 major mycoplasmal species. Indeed, this similarity group was comprised of 11 species, including *M. arthritidis, M. bovis, M. fermentans, M. genitalium, M. hominis, M. orale, M. pirum, M. pneumoniae, M. pulmonis, M. salivarium, U. urealyticum.* Two species, *M. hyorhinis* and *M. neurolyticum,* evidenced a lesser degree of similarity to the major group, and thus comprised another similarity group. As is shown in Fig. 1B, a different set of the second PCR primers,

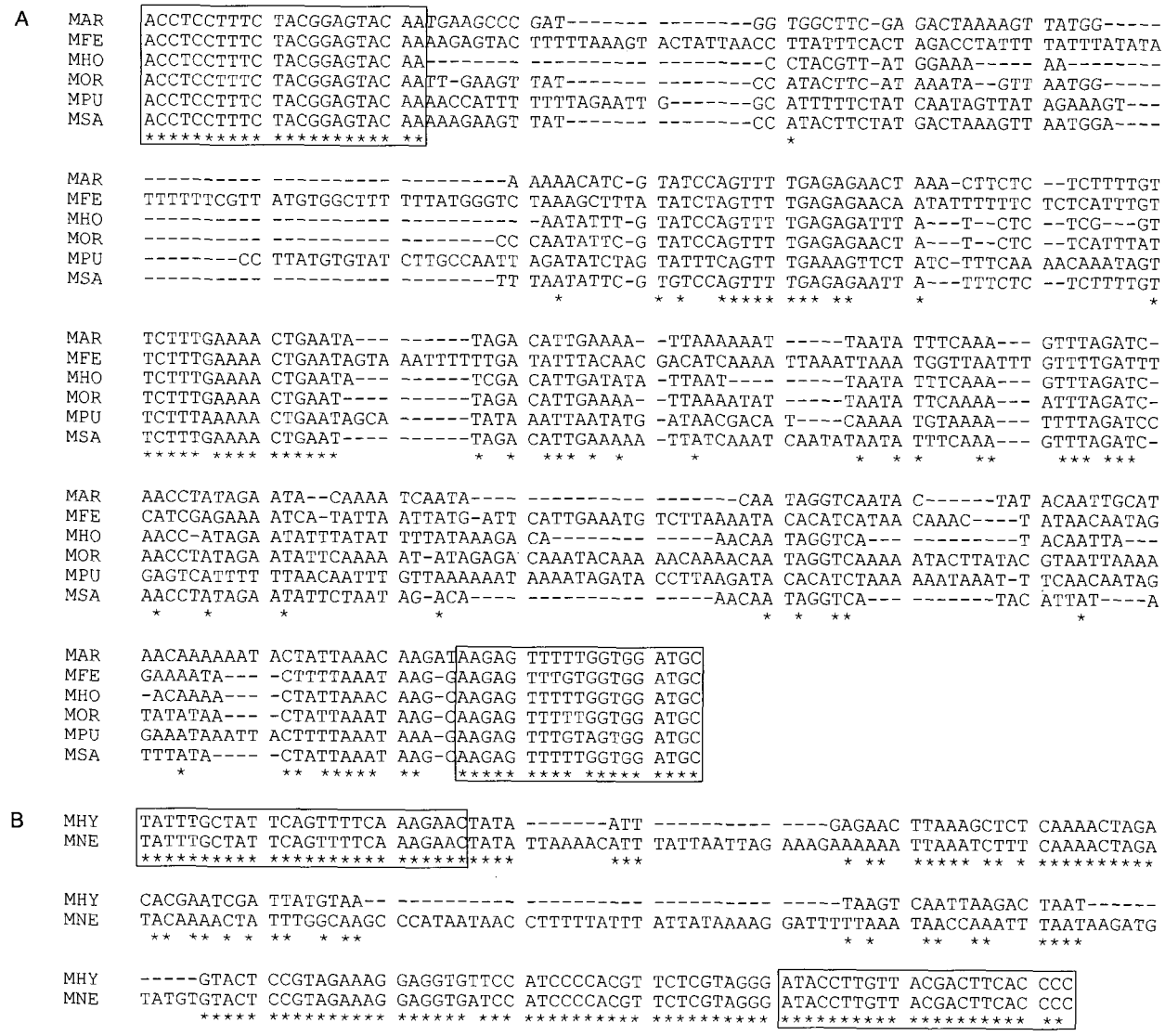

Fig. 1. Multiple alignments of the spacer regions spanning the 16S and 23S rRNA genes from eight *Mycoplasma* species. The nucleotide sequences of the spacer regions between the 16S and 23S rRNA genes from six major species (A) or two species (B) were aligned using the Clustal W program. The boxed sequences represent a region exhibiting perfect homology, and were selected as primers (FN2 and R2 in A, FN3 and RN3 in B) for the second-stage nested PCR. Symbol: dashes, deletion; asterisk, conserved nucleotide; MAR, *M. arthritidis*; MFE, *M. fermentans*; MHO, *M. hominis*; MHY, *M. hyorhinis*; MNE, *M. neurolyticum*; MOR, *M. orale*; MPU, *M. pulmonis*; MSA, *M. salivarium.*

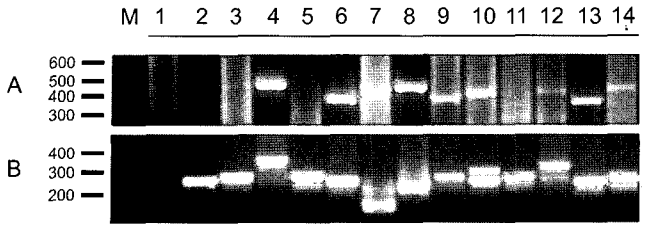

Fig. 2. Analysis of PCR products using two-stage PCR amplification. First-stage PCR was conducted using the general mycoplasma primers F1 and R1 and 30 ng of genomic DNA from 13 mycoplasma species (A). 0.5 μl of the resulting reactants was then mixed with PCR reaction mixture and subjected to second-stage PCR amplification using two different nested PCR primer sets; one primer pair, FN3 and RN3, for *M. hyorhinis* and *M. neurolyticum*, or the FN2 and R2 pair for the remaining 11 species (B). M, size marker; lane 1, no DNA; lane 2, *M. arthritidis*; lane 3, *M. bovis*; lane 4, *M. fermentans*; lane 5, *M. genitalium*; lane 6, *M. hominis*; lane 7, *M. hyorhinis*; lane 8, *M. neurolyticum*; lane 9, *M. orale*; lane 10, *M. pirum*, lane 11, *M. pneumoniae*; lane 12, *M. pulmonis*; lane 13, *M. salivarium*; lane 14, *U. urealyticum*.

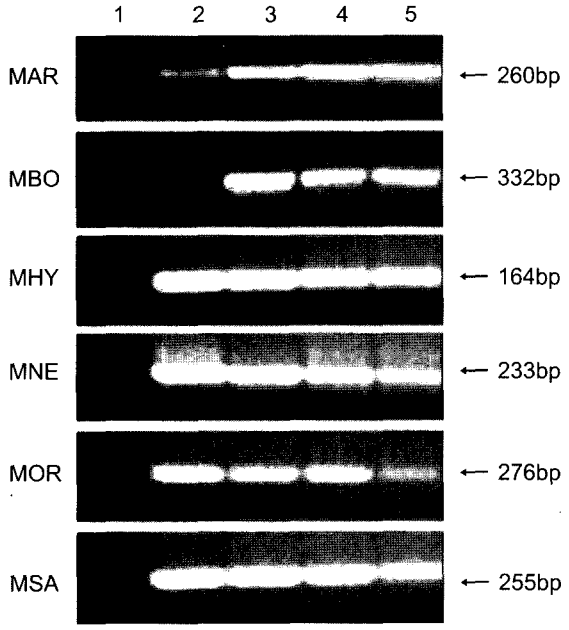

Fig. 3. Detection sensitivity of the two-stage PCR assay. Each clone of the rRNA genes from 6 species was serially diluted and subjected to two-stage PCR analysis with the primer sets, as was described in Fig. 2. lane 1, no template; lane 2, 1 copy; lane 3, 10 copies; lane 4, 10^2 copies; lane 5, 10^3 copies. MAR, *M. arthritidis*; MBO, *M. bovis*; MHY, *M. hyorhinis*; MNE, *M. neurolyticum*; MOR, *M. orale*; MSA, *M. salivarium*.

namely FN3 and RN3, were selected for the detection of both *M. hyohinis* and *M. neurolyticum* (Table 1).

Two-stage PCR amplification of rDNA genes

In order to test the primers designed for the nested PCR in our study, we initially amplified the target region with the general primers. First-stage PCR was then conducted using 30 ng of the genomic DNA purified from the 13 mycoplasma species. As is shown in Fig. 2A, the general PCR primers generated amplicons from 370 bp to 500 bp. However, the PCR products from four species, namely *M. arthritidis, M. bovis, M. genitalium,* and *M. pneumoniae,* were not visible when analyzed via agarose gel electrophoresis. Second-stage PCR was then conducted with the two different sets of selected primers, in accordance with the sequence similarity between species. The major group, which included 11 species, was readily detected using the FN2 and R2 primer pair, and the remaining 2 species, *M. hyorhinis* and *M. neurolyticum*, were efficiently diagnosed with the FN3 and RN3 specific primers (Fig. 2B). In conclusion, the second-stage PCR produced PCR products from all 13 of the tested species, which is indicative of enhanced amplification of the first-stage PCR products.

Sensitivity of the two-stage PCR

In an attempt at species confirmation, we cloned the amplified rDNA regions of each of the mycoplasmal species from the first-stage PCR into the pGEM-T plasmid vector. 13 purified rDNA clones were then subjected to the sequencing program. The nucleotide sequences of the amplicons were determined via dideoxy chain-termination, as is described in the Materials and Methods section. The mycoplasma species were then confirmed via alignment of the GeneBank sequence data (data not shown). In order to characterize the sensitivity of the two-stage nested PCR, the clones of each of the mycoplasmal rRNA genes from the 13 mycoplasmas were diluted 10-fold. To simulate samples prepared from cultured cells, 1 μg of salmon sperm DNA was added to each dilute as a background DNA, then subjected to PCR analysis. Distinct bands were observed in ethidium bromide-stained agarose gels, and the last visible DNA fragments were generated from 1 copy dilute of template DNA. This indicates that the detection limit of this protocol was 1 copy. This, in turn, suggests that a single microorganism can be detected with the method used in this study. In order to determine the sensitivity of the present method when genomic DNA was used as a template, we conducted the two-stage PCR reaction using the purified genomic DNA from four different species: *M. orale*, *M. pneumoniae*, *M. hyorhinis*, and *M. neurolyticum*. The last visible fragments were generated from 2 fg of purified chromosomal DNA (data not shown), equivalent to approximately 2 genome copies. In conclusion, the two-stage PCR reaction used in the present study was sufficient for the detection of 1-2 copies of rDNA from 13 mycoplasma species.

Specificity of the two-stage PCR

Under our experimental conditions, the target DNA regions from 13 species were detected readily, via two amplification steps. Cross-reactions can be observed with some microorganisms, which are common contaminants in cultured cells. In order to evaluate this possibility, we conducted an investigation into the specificity of this two-stage PCR technique. The same PCR procedure was applied to chromosomal DNA as a template, which had been purified from 6 different species, namely *E. coli, M. hyorhinis*, *M. orale*, *S. cerevisiae,* mouse fetal thymus, and the human THP1 cell line. As is shown in Fig. 4, the two mycoplasma species were detected efficiently. However, these primers produced no distinct DNA fragments from either the primary or secondary PCR procedures, in cases in which the genomic DNA from other species was used as a template. This data shows that the primers for the two-stage PCR are sufficiently specific for the diagnosis of mycoplasmic contamination.

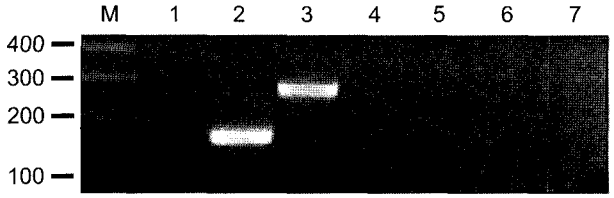

Fig. 4. Specificity of the two-stage PCR amplification. 100 ng of purified DNA from the mycoplasmas and other species was subjected to primary PCR using the general mycoplasma primers, F1 and R1. A 1/20 volume of the reaction products was analyzed in the nested PCR reaction with a primer mixture consisting of FN2, R2, FN3, and RN3. One-third of the total reaction volume was analyzed via electrophoresis. M, size marker; lane 1, no DNA; lane 2, *M. hyorhinis*; lane 3, *M. orale*; lane 4, human THP1 cell line; lane 5; fetal mouse thymus cell; lane 6, *E. coli*; lane 7, *S. cerevisiae.*

Table 2. Size variations of restriction fragments in the 16S-23S intergenic region

Species	Size of second-stage PCR products	Size of *Sau*3AI digests
M. arthritidis	261	170, 90
M. bovis	332	332
M. fermentans	353	353
M. genitalium	280	160, 120
M. hominis	223	160, 63
M. hyorhinis	165	120, 45
M. neurolyticum	233	190, 43
M. orale	275	155, 120
M. pirum	315	315
M. pneumoniae	280	160, 120
M. pulmonis	329	210, 119
M. salivarium	266	180, 86
U. urealyticum	332	182, 150

Identification of *Mycoplasma* species

Although the sizes of the products of the second-stage PCR varied depending on species, these size differences were not readily observable by normal agarose gel electrophoresis, and the PCR products of some species were of very similar sizes. As different *Mycoplasma* species tend to be encountered in different hosts, species identification is particularly useful with regard to the tracing of the source of contamination of a given cell line. In order to determine the origins of the PCR products, we conducted RFLP assays. The PCR products from the second-stage PCR were digested using the *Sau*3AI 4 bp-recognizing restriction enzyme. Electrophoretic analysis with *Sau*3AI-digested DNA generated distinct bands and allowed for the species-level differentiation of *Mycoplasma* (Table 2). However, the amplicons from *M. bovis*, *M. fermentans*, and *M. pirum* were not digested with this restriction enzyme.

Discussion

Mycoplasmas, which are invisible to light microscopy, are frequent contaminants of *in vitro* animal cell cultures. These cryptic contaminations can be recognized via several specific procedures, including the recognition of color changes in a dye applied to the culture media, ELISA for the detection of specific proteins, or PCR methods (Clyde *et al.*, 1984; Hopert *et al.*, 1993; Razin, 1994; Harasawa, 1995). As mycoplasma infections in cultured cells tend to induce both biochemical and genetic changes, experimental results can often be misinterpreted. Therefore, contamination surveys should be periodically conducted in laboratories working with susceptible cultures.

Our primary objective was to develop a sensitive and reliable method for the detection of mycoplasmas in cell culture, via the application of PCR technology. We selected the evolutionary conserved region of the rRNA genes as an amplification target, due both to the abundance of usable nucleotide sequences and the size difference of this factor among many species. Generally, the PCR analysis of mycoplasma contamination requires primers that are specific to the selected species, and allows for the discrimination of mycoplasma from non-mycoplasmal contaminants. Moreover, the procedure must be free of cross-re-

action with the DNA from the cell lines themselves. Our technique, which involved two amplification steps, was able to detect 1-2 copies of the target DNA molecule, and evidenced no cross-reactivity with the genomic DNA of other organisms, including human, mouse, *E. coli*, and *S. cerevisiae*, thereby indicating that the second PCR primers, including FN2, R2, FN3, and RN3 are highly specific under the PCR conditions adopted in this study.

Classical diagnosis methods do not encompass a wide range of mycoplasmas, and have some limitations, due primarily to the fact that they require experience and skill, as well as a series of time-consuming and laborious steps (Chen, 1977). For example, immunological detection by ELISA (Roche, Germany) and biochemical detection using mycoplasma-specific adenosine phosphorylase (MycoTect; BRL, USA) are both limited by cross-reactivity and sensitivity, and therefore require large numbers of mycoplasma cells, and still exhibit a tendency toward false-positive results. Also, the detection of mycoplasma contamination via fluorochrome staining, using agents such as Hoechst 33258 and DAPI, is complicated and hindered by the existence of a nonspecific background comprised of cell debris, media components, or mitochondrial DNA (Chen, 1977). Despite its many limitations, then, the time-consuming practice of microbiological cultivation has traditionally constituted the method of choice, in cases in which the other methods do not yield conclusive results. However, certain mycoplasmas, such as non-culturable *M. hyorhinis* strains, have proven difficult to cultivate, and may elude such diagnostic tests.

In order to circumvent the problems posed by the aforementioned classical methods, a host of other methods have been developed, some employing nucleic acid technology. In the present study, we have devised a two-stage PCR protocol, which is superior to the traditional mycoplasma cultivation and serological techniques, in terms of its ability to detect mycoplasma infections. This method is very effective, as it is able to simultaneously detect 13 species, and cultured cells are often eventually contaminated by more than one mycoplasma species.

The results presented in this report indicate that one or two copies of rDNA from 13 mycoplasma species could be detected by our two-stage PCR, using highly specific primers in the second stage of PCR. The unique nucleotide sequence features harbored within the spacer region between the 16S and 23S rRNA genes enabled us to select highly specific primers for the nested PCR. Among the 13 species tested in this study, 11 species, which evidenced a high degree of sequence similarity, were detected readily with the FN2 and R2 primers. Another group, including only two species, *M. hyorhinis* and *M. neurolyticum*, evidenced a lesser degree of similarity with the other major mycoplasmas, and generated distinct amplicons when the FN3 and RN3 primers were used. Moreover, the differences in the lengths of the spacer regions resulted in the production of differently sized amplicons between species, thereby allowing for the identification of the different tested mycoplasmal species. Further identification was accomplished via the analysis of the digested DNA fragments with *Sau*3AI.

Reliability and sensitivity are both prerequisites for a PCR-based microorganism detection method. Previous studies have shown that several species can be detected via two-stage nested PCR. However, these procedures remain controversial with regard to their detection sensitivity characteristics. The optimization of primer sequences and the reaction conditions presented here enhanced the sensitivity and ensured the high degree of specificity of the two-stage nested PCR. It was revealed that the specificity of the method presented here was sufficient for the discrimination of mycoplasma contamination from other probable contaminants, including *E. coli*, *S. aureus*, and budding yeasts.

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