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A polymerase chain reaction based method for detecting *Mycoplasma*/*Acholeplasma* contaminants in cell culture

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Abstract

A detection system that utilizes a primer mixture in a nested polymerase chain reaction for detecting *Mycoplasma* contaminants in cell cultures is described. Primers were designed to amplify the spacer regions between the 16S and 23S ribosomal RNA genes of *Mycoplasma* and *Acholeplasma*. This detection system was able to detect 20–180 colony forming units per milliliter of sample. Eight commonly encountered *Mycoplasma* and *Acholeplasma* contaminants, which include *Mycoplasma* (*M.*) *arginini*, *M. fermentans*, *M. hominis*, *M. hyorhinis*, *M. orale*, *M. pirum*, *M. salivarium*, and *Acholeplasma laidlawii*, were consistently amplified. *Mycoplasma* contaminants generated a single DNA band of 236–365 base pairs (bp), whereas *A. laidlawii* produced a characteristic two-band pattern of 426 and 219 bp amplicons. Species identification could be achieved by size determination and restriction enzyme digestion. Minor cross-reactions were noted with a few closely related gram positive bacteria and DNA from rat cell lines. A *Mycoplasma* Detection Kit for detecting *Mycoplasma* contaminants in cell cultures has been developed based on this approach.

Keywords: *Acholeplasma laidlawii*; Cell culture contaminants, *Mycoplasma* detection; Polymerase chain reaction (PCR)

1. Introduction

Mycoplasmas, which belong to the class *Mollicutes*, are one of the most frequent contaminants found in cell cultures (Rawadi and Dussurget, 1995). Among 100 *Mollicute* species that have been classified, at least 20 *Mycoplasma* and *Acholeplasma* species have been found as cell culture contaminants. Over 95% of these were identified as either *Mycoplasma* (*M.*) *arginini*, *M. fermentans*, *M. orale*, *M. hyorhinis*, *M. hominis*, *M. salivarium*, *M. pirum*, or *Acholeplasma laidlawii* (Boolske, 1988; McGarrity et al., 1992; Arai et al., 1994). *Mycoplasma* contamination affects many different aspects of the infected cell culture, leading to unreliable experimental results and potential harmful biological products (McGarrity and Kotani, 1985a,b; Harasawa et al., 1993). Therefore, routine testing for *Mycoplasma* contamination is necessary to ensure reliable research results and quality biotechnological products.

Available methods for *Mycoplasma* detection include microbiological culture, DNA fluorochrome

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staining, enzyme-linked immunosorbent assay, immunofluorescence, biochemical assays, and DNA probe assays (McGarrity and Kotani, 1985a,b; Hay et al., 1989; Uphoff et al., 1992; Barile and Rottem, 1993). While each of these methods has its own advantages for detecting *Mycoplasma* contamination, the procedures are generally time-consuming, insensitive, difficult to interpret, or may not detect all the *Mycoplasma* contaminants that are commonly found in cell cultures. Polymerase chain reaction (PCR)-based methods that have been developed for detection and identification of *Mycoplasma* are fast and sensitive (Rawadi and Dussurget, 1995; Wirth et al., 1994; Dussurget and Roulland-Dussoix, 1994; Razin, 1994). Primers have been designed from conserved regions of the 16S ribosomal RNA (rRNA) gene and used to identify *Mycoplasma* and *Acholeplasma* (Rawadi and Dussurget, 1995; Wirth et al., 1994; Dussurget and Roulland-Dussoix, 1994). However, the processes involved using genus-specific primers followed by species-specific primers for each organism, thus requiring many sets of primers for each identification. Others have utilized primers based on the 16S–23S rRNA region of the genus *Mycoplasma* (Harasawa et al., 1993; Harasawa, 1995). The heterogeneous intergenic spacer sequences among different bacteria allowed identifying *Mycoplasma* contaminants to the species level. However, the sensitivity for detecting *A. laidlawii* was low (10^4 colony forming units (CFU)/ml) with these primers (Hu et al., 1995). Since *A. laidlawii* is one of the predominant contaminants in cell cultures, it is important to design primers which are able to detect *A. laidlawii* as well as *Mycoplasma* with good sensitivity and reliability.

This study describes a two-stage PCR reaction, which amplifies the 16S and 23S rRNA spacer region sequences from *Mycoplasma* and *Acholeplasma*. This approach allows detection and identification of *Mycoplasma* contaminants in a single nested PCR reaction, which reduces the number of primers needed in the process. Furthermore, a two-banded amplicon is generated from *A. laidlawii*. This distinct feature allows one to differentiate *Acholeplasma* from other *Mycoplasma* species. Thus this detection method is highly sensitive, specific, and reproducible.

2. Materials and methods

2.1. Organisms and cell cultures

Bacteria used in this study were provided by the Bacteriology Collection at the American Type Culture Collection (ATCC). These include species of *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Mesoplasma*, and *Entomoplasma*, six strains of *A. laidlawii*, and other closely related bacteria. DNA from mammalian cells was also obtained from the ATCC (Table 1).

2.2. Sample preparation

Pellets from 500 μl of reconstituted bacterial culture or frozen cell culture were resuspended in 100 μl of lysis buffer containing 1 × PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin (w/v)], 0.5% NP-40, 0.5% Tween 20 and 1 μl proteinase K (17.3 mg/ml, Boerhinger Mannheim). The cell suspension was lysed by incubating at 60°C for 1 h. Proteinase K was inactivated by heating at 95°C for 10 min and 500 μl of dH_2O was then added. For experiments involving bacterial extracts, 5 μl of the resulting solution was used for PCR tests. In the case of cell lines, nucleic acid was precipitated with 600 μl of isopropanol and 1 μl of glycogen (20 mg/ml, Gibco) at −20°C for at least 30 min followed by DNA pelleting and washing with cold 75% alcohol. The pellet was then dried and resuspended in 50 μl sterile dH_2O, and 200–500 ng of nucleic acid was used for the PCR reaction.

2.3. Primers

A total of seven primers were present in the two-stage PCR. In addition to the four primer sequences recommended by Harasawa (Harasawa et al., 1993; Harasawa, 1995), three additional primers (M78, M89 and R34) specifically selected from the 16S and 23S rRNA region of *A. laidlawii* were incorporated (Fig. 1, Table 2). Two outer primer pairs (F1 and R1, M78 and M89) were used in the first-stage PCR. Inner primers F2, R2, and R34 were used in the second-stage PCR.

Table 1
List of *Mycoplasma*, wall-related bacteria and cellular DNAs used for this study

Species	ATCC No.	Results	Species	ATCC No.	Results
Genus *Mycoplasma*:			Genus *Acholeplasma*:		
M. fermentans	19 989	+	*A. laidlawii* (type strain)	23 206	+
M. orale	23 714	+	*A. laidlawii*	14 089	+
M. pirum	25 960	+	*A. laidlawii*	31 166	+
M. hyorhinis	17 981	+	*A. laidlawii*	25 937	+
M. hominis	23 114	+	*A. laidlawii*	29 804	+
M. salivarium	23 064	+	*A. laidlawii*	27 085	+
M. arginini	23 838	+			
Wall-related bacteria:			Cellular DNA:		
Bacillus subtilis	6 051	±[a]	Human	CRL 1611	−
Lactobacillus casei	393	+	Human	CCL 119	−
Lactobacillus catenaformis	7 469	−	Human	CCL 86	−
Lactobacillus rhamnosus	25 536	−	Human	CCL 187	−
Clostridium ramosum	25 582	−	Mouse	HB 72	−
Clostridium innocuum	14 501	±[a]	Mouse	HB 96	−
Clostridium pasteurianum	6 013	−	Mouse	HB 144	−
Enterococcus faecalis	19 433	−	Mouse	HB 167	−
Escherichia coli	11 775	−	Mouse	HB 194	−
			Rat	CCL 107	+
			Rat	CRL 1570	+
			Mouse/rat	HB 221	−
			Mouse/hamster	HB 226	−
			Mouse/hamster	HB 218	−
			Monkey	CRL 1587	−
			Monkey	CCL 81	−

[a] Amplicon band seen when 100 ng extracted DNA from the corresponding organism was used for the two-stage PCR.

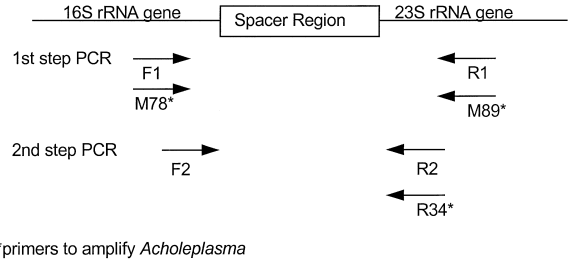

Fig. 1. Positions of primers at the 16S and 23S RNA genes.

2.4. Two-stage PCR reaction

PCR was carried out in a total volume of 50 μl containing 1× PCR buffer as mentioned above, 50 μM each of deoxynucloeside triphosphate, 20 pmol of each primer, and 1 U Taq DNA polymerase. A 200–500 ng mass of extracted nucleic acid (cell lines) or 5 μl reconstituted cells of each organism was used as template. The thermal profile included initial denaturation at 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 2 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 5 min.

For first-stage PCR, 5 μl of sample were added to 45 μl of reaction mixture with two outer primer-pairs. Second-stage PCR was carried out by adding 1 μl of the first-stage PCR product to 49 μl of reaction mixture with three inner primers (Table 2). Aliquots of the final amplification products were electrophoresed on a 1.2% agarose gel; DNA bands were

Table 2
Primer locations and nucleotide sequences: (the first four primers comprise the first-stage primer mixture; the last three primers comprise the second-stage primer mixture)

Primer	Ref.
ACACCATGGGAG(C/T)TGGTAAT	F1 (Harasawa et al., 1993)
CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT	R1 (Nakagawa et al., 1992)
AAAGTGGGCAATACCCAACGC	ATCC primer M78[a]
TCACGCTTAGATGCTTTCAGCG	ATCC primer M89[a]
GTG(C/G)GG(A/C)TGGATCACCTCCT	F2 (Harasawa et al., 1993)
GCATCCACCA(A/T)A(A/T)AC(C/T)CTT	R2 (Harasawa et. al.,1993)
CCACTGTGTGCCCTTTGTTCCT	ATCC primer R34[a]

[a] Patented sequences (US Patent No. 5 693 467).

visualized by UV fluorescence after ethidium bromide staining.

2.5. PCR product identification

Sizes of the second-stage PCR amplicons were estimated by electrophoresis on MetaPhor agarose (FMC) or Nusieve 3:1 agarose (FMC). Further differentiation of *Mycoplasma* species was accomplished by digestion with *Vsp*I, *Cla*I and/or *Hind*III, and the digest patterns were determined by agarose gel electrophoresis.

2.6. Sensitivity determination

Mycoplasma and *Acholeplasma* cultures were grown to exponential phase in *Spiroplasma* medium SP-4 (Pienta and Tang, 1996). Tenfold serial dilutions were performed. Fifty μl of each dilution was plated on SP-4 medium for CFU and 5 μl was subjected to PCR assays. Sensitivity was determined for each organism by correlating the cell concentration with the PCR results.

3. Results and discussion

Using the primer mixture described here, we could detect all eight common *Mycoplasma* contaminants of cell cultures (Table 1, Fig. 2). The sensitivity of this assay ranged from 20–180 CFU/ml between different species of *Mycoplasma* and *Acholeplasma*. The sensitivity for detecting *A. laidlawii* was greatly improved. With this system we could detect 20 CFU/ml of *A. laidlawii*, whereas the sensitivity of other primers was 10^4 CFU/ml. Thus, a single nested PCR test allowed the detection of both *Mycoplasma* and *Acholeplasma* contaminants present in cell culture with high sensitivity without the addition of species-specific inner primers. This highly sensitive method is especially useful for monitoring the success of eliminating *Mycoplasma* from valuable cell lines.

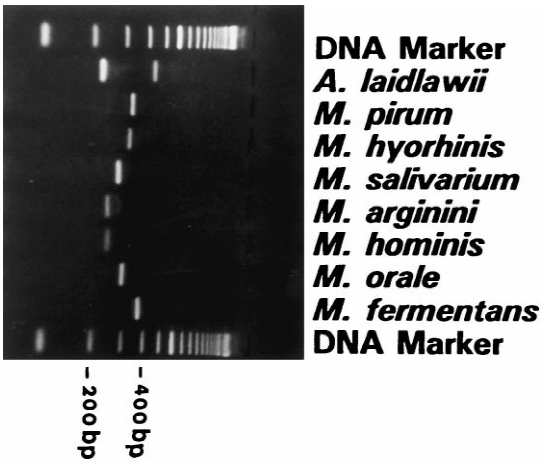

Fig. 2. Agarose gel electrophoresis of the second-step PCR products from eight commonly encountered *Mycoplasma* and *A. laidlawii*. Amplified DNA products were electrophoresed on 2% MetaPhor agarose (FMC) gel and 100 bp DNA ladder (Gibco BRL) was used as a DNA size marker.

The lengths and sequences of the spacer region in the rRNA operon differ among *Mycoplasma* species, and this heterogeneity can be used for species differentiation (Harasawa et al., 1993; Harasawa, 1995). A single DNA amplicon ranging in 236–365 base pair (bp) was produced from all commonly encountered *Mycoplasma* contaminants (Fig. 2). In contrast, since *A. laidlawii* has at least two rRNA

operons with tRNA genes inserted in the 16S–23S spacer region (Nakagasawa et al., 1992), a double banded PCR product with the size of 426 bp and 219 bp was consistently observed. The 426 bp and 219 bp DNA amplicons were also produced from five additional strains of *A. laidlawii* (Fig. 3). This feature provides a practical way to differentiate *Mycoplasma* species and *A. laidlawii*. Further analysis of the second-stage PCR products with restriction enzyme digestions allowed differentiation of *Mycoplasma* to the species level (Table 3). Species identification is particularly useful for tracing the source of contamination of a cell line, since *Mycoplasma* species come from different origins.

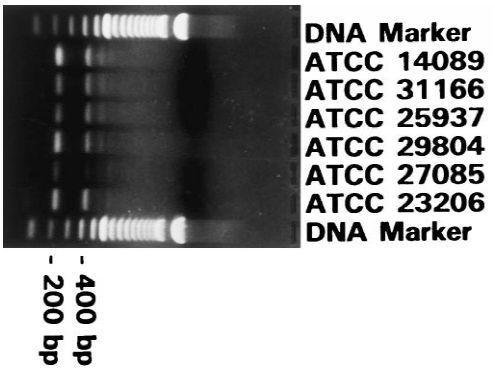

Fig. 3. Agarose gel electrophoresis of the second-step PCR products for six strains of *A. laidlawii*. Amplified DNA products were electrophoresed on 1.2% agarose (Gibco, BRL). ATCC numbers were listed for each strain of *A. laidlawii*, and 100 bp DNA ladder (Gibco, BRL) was used as a DNA size marker.

Table 3
Restriction fragment size variations in the 16S–23S rRNA intergenic regions of the commonly encountered *Mycoplasma* species

Mycoplasma species	Size of second-stage PCR product (bp)[a]	Size of restriction fragments (bp)		
		*Vsp*I	*Cla*I	*Hind*III
M. arginini	236	134,102	–[b]	–
M. fermentans	365	270,95	241,124	–
M. hominis	236	123,113	–	–
M. hyorhinis	315	–	–	253,62
M. orale	290	151,139	–	–
M. pirum	323	169,154	285,38	–
M. salivarium	269	–	–	–
A. laidlawii	426,219	219,189, 148,89	–	–

[a] Size was deduced from published sequences and confirmed with high resolution agarose gel.
[b] No restriction site.

A number of wall-related bacteria were included in the test for specificity, and the results indicated that the primer mixture provided good specificity for detecting *Mycoplasmas* in cell cultures (Table 1). A few cross-reactions were observed with closely related gram positive organisms. Amplification was observed with *Lactobacillus casei* DNA at concentration of 1 ng, where a band of approximately 280 bp was generated. The system also amplified DNAs from *Clostridium innocuum* (approximately 315 bp) and *Bacillus subtilis* (approximately 220 bp) when high DNA concentration of 100 ng was used as templates. Since these gram positive organisms tested are closely related to *Mollicutes* phylogenetically, it is not surprising that some cross-reaction was observed. It should be pointed out that most of these organisms are unlikely contaminants in cell cultures. Contamination by *B. subtilis* and *C. inocuum* at cell densities yielding 100 ng of DNA would most likely cause the cell culture media to become turbid and easily noticeable.

The enhanced primer mixture also successfully amplified members of *Spiroplasma*, *Entomoplasma* and *Mesoplasma*, the genera to which most plant or insect *Mycoplasmas* belong. A single amplicon ranging between 325–340 bp was observed from these organisms. These results demonstrate the broad range feature of our primer mixture, which is valuable for detecting plant and insect Mycoplasmas. Efforts are being made to further explore this potential application.

The primer mixture described has been tested with cell cultures and virus stock samples from a variety of sources. No specific amplification was observed with DNA from human, mouse or monkey cell lines (Table 1). An exception was seen in two rat cell lines where an approximately 340 bp amplicon was present. Based on the limited number of rat cell lines tested, reasons for detecting this band were unclear. More testing needs to be done when additional rat cell lines are available.

This detection system has been used in a variety of contaminated cell lines, and the results agreed with microbiological culturing and Hoechst-staining techniques (data not shown). In order to obtain reproduc-

ible and unambiguous results, we caution that DNA extraction is necessary for lyophilized or frozen cultures, since the presence of possible inhibitors seem to interfere with the amplification process.

In conclusion, we have described a two-stage PCR reaction that amplifies the 16S–23S rRNA spacer region sequences from *Mycoplasma* and *Acholeplasma*. This approach allows detection and identification of *Mycoplasma* contaminants in a single nested PCR reaction with high sensitivity. Furthermore, a two-banded amplicon is generated from *A. laidlawii*. This distinct feature allows the differentiation between *Acholeplasma* and *Mycoplasma* species. Further identification of *Mycoplasma* to the species level is possible by performing restriction enzyme digestions. Thus, we present a *Mycoplasma* detection method that is highly sensitive, specific, and reproducible.

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