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Rapid, Sensitive PCR-Based Detection of Mycoplasmas in Simulated Samples of Animal Sera

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A fast and simple method to detect mycoplasmal contamination in simulated samples of animal sera by using a PCR was developed. The following five mycoplasma species that are major cell culture contaminants belonging to the class *Mollicutes* were investigated: *Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, and *Mycoplasma fermentans*. After a concentration step involving seeded sera, genus-specific primers were used to amplify a 717-bp DNA fragment within the 16S rRNA gene of mycoplasmas. In a second step, the universal PCR was followed by amplification of variable regions of the 16S rRNA gene by using species-specific primers, which allowed identification of contaminant mycoplasmas. With this method, 10 fg of purified DNA and 1 to 10 color-changing units of mycoplasmas could be detected. Since the sensitivity of the assay was increased 10-fold when the amplification products were hybridized with an internal mycoplasma-specific ^{32}P-labelled oligonucleotide probe, a detection limit of 1 to 10 genome copies per PCR sample was obtained. This highly sensitive, specific, and simple assay may be a useful alternative to methods currently used to detect mycoplasmas in animal sera.

Mycoplasmas (the trivial name for microorganisms belonging to the class *Mollicutes*) are the smallest self-replicating bacteria. They are common and serious contaminants of cell cultures, and this remains one of the major problems encountered in biological research, in biological diagnosis, and in biotechnological production with cell cultures. This problem is actually widespread, and the incidence of contamination varies from 5 to 87% of cell cultures (3, 28, 31, 42) depending on the cell line, the test used, and the quality control practices used. The class *Mollicutes* is subdivided into more than 120 species (41), but only 20 species have been isolated from cell cultures (1, 2, 40). Of these 20 species, which have been isolated from bovine, human, and porcine sources, 5 (*Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, and *Mycoplasma fermentans*) account for 95% of all contaminations (1, 3, 25, 26). It has been shown that mycoplasmas produce a variety of effects on cultured cells. Besides affecting cellular growth and morphology, mycoplasmas are known to be able to alter amino acid and nucleic acid metabolism and immunological and biochemical properties and to induce chromosome aberrations, leading to experiments whose results are often unreliable (1, 3, 12, 24, 25). The main sources of contamination of clean cultures are mycoplasma-infected cultures (1). The origins of the contaminants (i.e., the direct sources) are often laboratory personnel (*M. orale* and *Mycoplasma salivarium* have been isolated from oropharynxes of 25 to 80% of healthy individuals; *M. fermentans* has been isolated more rarely) (38) and commercial animal sera used in culture media (*M. arginini*, *A. laidlawii*, *M. hyorhinis*) (1, 2).

Mycoplasma cells are pleomorphic, have diameters of 300 to 800 nm, and lack cell walls (41). Because of their small size and pliability, mycoplasmas can pass through the 450- and 220-nm-pore-size membrane filters usually used for cell culturing (17). When high-pressure filtration is used some mycoplasmas can occasionally penetrate 100-nm-pore-size final filters (5, 41). Like most cell cultures infected by mycoplasmas, serum infections are rarely detected by visual inspection or light microscopy in the absence of obvious signs of infection (e.g., turbidity of sera or media, pH changes, or cytopathic effects on cell lines) (42). Moreover, serum is not very propitious for mycoplasma growth since it lacks host cells and some essential nutrients and also contains toxic and inhibitory components (27). Low contamination rates and reduction of contamination rates by filtration for a long time made detection and isolation of mycoplasmas from commercial sera impossible (2, 5). Three methods are currently sensitive enough to detect mycoplasmas: microbiological culturing, adenosine phosphorylase (AdoP) screening, and the indicator cell culture technique. However, each method has certain disadvantages. The method based on specific broth or agar medium culturing is time-consuming (several weeks), some strains (especially *M. hyorhinis* strains) are difficult to grow (3, 15, 24, 42), and experience is needed to perform the technique and interpret the results (42). The biochemical method lacks specificity since some bacteria (e.g., *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*) produce nucleoside phosphorylase (4, 5, 16), while some mycoplasma species (e.g., *Mycoplasma pneumoniae* FH, *Mycoplasma pirum*, *Mycoplasma lipophilum*) produce practically none (16). Moreover, AdoP is a soluble enzyme that is active in the absence of live mycoplasmas (i.e., contaminants) (24). The method based on inoculation into mycoplasma-free indicator cell cultures is time-consuming because three passages and a final detection technique (e.g., DNA fluorescent staining with 4′,6-diamidino-2-phenylindole dihydrochloride [34] or bisbenzimidazole fluorochrome Hoechst 33258 [8] or an enzyme-linked immunosorbent assay [ELISA] with specific antibodies) are needed (19). Except for the ELISA, these methods cannot be used for direct identification of the contaminants.

In this paper we describe the development of a PCR-based method to detect mycoplasmas in simulated samples of animal sera. Our data show that this rapid and reproducible method possesses not only the high sensitivity necessary for detecting mycoplasmas in sera and the specificity necessary for identifying contaminant mycoplasmas (thus indicating possible

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sources), but also the simplicity necessary for easy, unambiguous interpretation of results.

MATERIALS AND METHODS

Growth media. The broth and agar media used for growth and titration were BDA, BDG, and GD, as described by Roulland-Dussoix et al. (33). The base medium contained 21 g of PPLO broth (Difco Laboratories, Detroit, Mich.), 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and enough distilled water to bring the volume to 1 liter. For BDG, 78 ml of autoclaved base medium was supplemented with 20 ml of horse serum, 0.2 ml of 1% phenol red, 66 mg of ampicillin per ml, and 1 ml of 50% glucose under sterile conditions, and the pH was adjusted to 7.8 with 1 M sodium hydroxide. For BDA, the base medium was supplemented as described above except that the glucose was replaced with 5 ml of 5% arginine hydrochloride and the pH was adjusted to 7.2. For GD, 1 g of agar was added to 80 ml of base medium and the preparation was autoclaved; the medium was completed by adding 20 ml of horse serum, 1 ml of 50% glucose, and 1 ml of ampicillin (66 mg/ml) under sterile conditions.

Strains and growth conditions. The strains used included *M. hyorhinis* BTS7^T (= ATCC 17981^T) (T = type strain), *M. orale* CH19299^T (= ATCC 23714^T), *M. fermentans* PG18^T (= ATCC 19989^T), and *A. laidlawii* PG8^T (= ATCC 23206^T). *M. arginini* G230^T was obtained from the Collection of the Institut Pasteur. Portions (0.2 ml) of late-exponential-phase mycoplasmas were grown on 2-ml portions of BDA and BDG at 37°C under microaerophilic conditions. Arginine-hydrolyzing mycoplasmas alkalinize BDA, and the yellow color of BDA changes to purple. Glucose-fermenting mycoplasmas acidify BDG, and the red color of BDG changes to yellow.

In addition, the following microorganisms were investigated to evaluate the specificity of the primers used for the PCR: *M. pirum*, *M. salivarium*, *M. pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Mycoplasma pulmonis*, *Ureaplasma urealyticum*, *Staphylococcus pasteurii*, *B. subtilis*, *Enterococcus faecalis*, *Streptococcus* sp., *Clostridium difficile*, *Clostridium perfringens*, *Clostridium ramosum*, *Clostridium innocuum*, *Rhodococcus* sp., *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus flavus*.

DNA extraction. Genomic DNAs were extracted from mycoplasmas by the method described by Carle et al. (7) to evaluate PCR sensitivity. Following extraction, DNAs were purified by equilibrium centrifugation in CsCl-ethidium bromide continuous gradients as described by Sambrook et al. (36).

Sera and infection. Commercial mycoplasma-free tested sera from horses, newborn calves, and fetal calves were obtained from GIBCO-BRL, Cergy Pontoise, France. These sera were diluted five times in a physiological salt solution and infected with known amounts of broth-grown mycoplasmas, defined by determining the numbers of CFU and color-changing units (CCU) as described by Rodwell and Whitcomb (32). The CCU titration was based on the correlation between the growth of mycoplasmas and the color changes of broth media supplemented with appropriate pH indicators.

Treatment of seeded samples. Simulated samples (10 ml) were centrifuged in 13.2-ml Ultra-Clear tubes (Beckman Instruments, Palo Alto, Calif.) by using a type SW41 Ti rotor (Beckman Instruments) at 4°C for 30 min at 20,000 × *g*. The resulting pellets were resuspended in washing buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), and the resulting preparations were mixed well and centrifuged with an Eppendorf model 5414 instrument at 4°C for 15 min at the maximum speed. DNAs were released from the samples by adding 25 μl of solution A (10 mM Tris-HCl [pH 8.3], 100 mM KCl, 2.5 mM $MgCl_2$) and 25 μl of solution B (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 1% Tween 20, 1% Triton X-100, 120 μg of proteinase K [Appligene, Inc., Pleasanton, Calif.] per ml; the proteinase K was from a 20-mg/ml stock solution in 5 mM Tris-HCl [pH 7.8]–50% glycerol kept at −20°C) to the washed pellets, incubating the preparation for 1 h at 60°C, boiling it for 10 min, and then chilling it on ice.

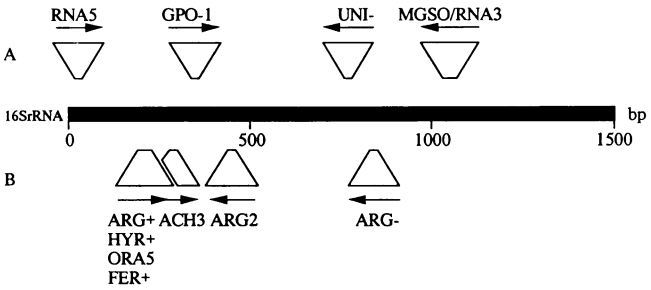

FIG. 1. Positions of the oligonucleotide primers used for detection of mycoplasmas at both the genus (A) and species (B) levels on a schematic physical map of the mycoplasmal 16S rRNA gene.

Oligonucleotide primers for the PCR and oligonucleotide probe. The existence of regions which exhibit sequence variability at the genus and species levels in mycoplasmal 16S rRNA genes allowed us to select genus- and species-specific primers for the PCR (Fig. 1 and Tables 1 and 2). A 40-pmol portion of radioactive DNA probe GPO-1 (43) was labelled in 25 μl of a solution containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 200 μCi of [γ-^{32}P]ATP (Amersham International, Amersham, England), and 30 U of T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, Ohio) by incubating the preparation for 45 min at 37°C. The enzyme was inactivated by heating the preparation for 5 min at 65°C. The 5′-end-labelled oligonucleotide was purified on a Nick column (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions.

DNA amplification. To avoid contamination by naturally occurring DNA or by PCR product carryover, we used separated areas for PCR preparations and products. We routinely autoclaved and used UV to irradiate reagents that could be autoclaved and irradiated without affecting their performance.

TABLE 1. Sequences of the primers used in this study

Primer[a]	Sequence	Location (positions)
RNA5	5′-AGAGTTTGATCCTGGCTCAGGA-3′	10-31
RNA3	5′-ACGAGCTGACGACAACCATGCAC-3′	1043-1065
GPO-1	5′-ACTCCTACGGGAGGCAGCAGTA-3′	338-359
MGSO	5′-TGCACCATCTGTCACTCTGTTAACCTC-3′	1029-1055
UNI−	5′-TAATCCTGTTTGCTCCCCAC-3′	763-782
ARG+	5′-GTGAAAGGAGCCCTTAAAGC-3′	193-212
ARG2	5′-TCAACCAGGTGTTCTTTCCC-3′	440-459
ARG−	5′-CTGCGTCAGTGAACTCTCCA-3′	829-848
HYR+	5′-GAAAGGAGCTTCACAGCTTC-3′	198-217
ORA5	5′-GGAGCGTTTCGTCCGCTAAG-3′	199-218
FER+	5′-AAGAAGCGTTTCTTCGCTGG-3′	203-222
ACH3	5′-AGCCGGACTGAGAGGTCTAC-3′	277-296

[a] RNA5, RNA3, GPO-1 (43), MGSO (43), and UNI− are genus-specific primers. ARG+, ARG2, and ARG− (*M. arginini*), HYR+ (*M. hyorhinis*), ORA5 (*M. orale*), FER+ (*M. fermentans*), and ACH3 (*A. laidlawii*) are species-specific primers.

TABLE 2. Primer use and lengths of amplified fragment

Organism	Primer 1	Primer 2	Length of amplified fragment (bp)
Mycoplasmas	GPO-1	MGSO	717
M. arginini	RNA5	ARG2	449
A. laidlawii	ACH3	UNI−	505
M. hyorhinis	HYR+	UNI−	584
M. orale	ORA5	UNI−	583
M. fermentans	FER+	UNI−	579

The 1,000-μJ irradiation treatment was performed in a UV box (model 1800 Stratalinker UV crosslinker; Stratagene, La Jolla, Calif.) as described by Ou et al. (30). UV irradiation was also used for microcentrifuge tubes, racks, surfaces of laboratory benches, and instruments by using UV lamps on the benchtop. We included negative controls, which contained all of the reagents except template DNA, with each set used for amplification. Careful laboratory procedures (aliquoting reagents, using tips with aerosol barriers, changing gloves frequently, premixing reagents, and adding DNA last) were used in order to minimize the risk of contamination (10, 22).

Oligonucleotide primers were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, Calif.) by the methoxyphosphoramidite method at the Unité de Chimie Organique of the Institut Pasteur, Paris, France. Sequences were amplified with a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using the method described by Saiki et al. (35). Each PCR assay was performed in 100 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) autoclaved gelatin, 25 μM tetramethylammonium chloride (Aldrich, Milwaukee, Wis.), 80 pmol of primer 1, 80 pmol of primer 2, each deoxynucleoside triphosphate at a concentration of 200 μM, 2 U of *Taq* polymerase (Amersham International), and 10 μl of DNA sample. Each reaction mixture was overlaid with 2 drops of mineral oil to prevent evaporation. The DNA samples were added last, through the oil. The samples were subjected to an initial denaturation cycle for 15 min at 95°C and then to 30 cycles consisting of 95°C for 30 s for denaturation, 58°C for 1.5 min (genus-specific PCR) or 64°C for 1.5 min (species-specific PCR) for annealing, and 72°C for 1.5 min or more (increased 1 s per cycle) for extension. Finally, the mixtures were subjected to an additional extension cycle at 72°C for 10 min.

Analysis of the amplified DNA. Portions (20 μl) of the PCR products were analyzed on a 2% agarose gel (SeaKem ME; FMC, Rockland, Maine) by electrophoresis in Tris-borate-EDTA buffer. Electrophoresis was performed at 100 V for 1 to 1.5 h, and then the gel was stained with a solution containing 0.5 μg of ethidium bromide per ml for 30 min. DNA fragments were visualized by UV illumination at 312 nm. For Southern blotting, the gel was denatured in 1.5 M NaCl–0.5 N NaOH, neutralized in 0.5 M Tris-HCl (pH 7.4)–3.0 M NaCl, and transferred to a nylon membrane (Hybond-N^+; Amersham International) by vacuum blotting (VacuGene XL apparatus; Pharmacia) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). The DNA was covalently linked to membranes by exposure to 1,200 μJ of UV irradiation (254 nm) in a UV box. The nylon membranes were prehybridized for 2 h at 25°C in 10 ml of a solution containing 4× SSC, 1× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.02 M Tris-HCl (pH 7.4), 40% (vol/vol) formamide, 0.1 g of dextran sulfate per ml, and 20 μg of denatured fragmented salmon sperm DNA per ml of prehybridization mixture. Hybridization was performed for 16 h at 25°C in the solution described above containing 6×10^5 cpm of ^{32}P-5′-end-labelled oligonucleotide probe per ml of hybridization mixture. The blots were washed once for 5 min and three times for 20 min at 30°C in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and then twice for 20 min at 30°C in 0.1× SSC–0.1% SDS. The blots were autoradiographed for 3 h at −80°C on Hyperfilm-MP (Amersham International) film.

RESULTS

Evaluation of sensitivity. In preliminary studies, the sensitivity of the PCR method was determined by examining serial dilutions of purified DNAs from *M. arginini*, *A. laidlawii*, *M. hyorhinis*, *M. orale*, and *M. fermentans*. The genus-specific PCR produced a visible fragment of the expected size (717 bp) for all of the mycoplasma species tested (Fig. 2). Distinct bands were observed in ethidium bromide-stained agarose gels, and the detection limit was 10 fg, which is equivalent to 5 to 15 genome copies depending on the species tested. The species-specific PCR produced 449-, 505-, 584-, 583-, and 579-bp fragments for *M. arginini*, *A. laidlawii*, *M. hyorhinis*, *M. orale*, and *M. fermentans*, respectively (Table 2). The last visible fragments were produced from 10 fg of template DNA, indicating that 5 to 15 microorganisms could be detected, depending on the species tested. When the amplified segments of DNA were hybridized with the complementary ^{32}P-labelled oligonucleotide probe, GPO-1, the detection limit was raised 10-fold under both genus-specific and species-specific conditions (Fig. 2). It was then possible to see bands on autoradiograms that were not visible on the stained gel, even after a short exposure time (3 h). Aliquots from PCR mixtures containing 1 fg of template DNA (approximately one genome copy) gave a hybridization signal. Eventual contamination of animal sera by more than one species of mycoplasma led us to determine the limit of detection of our assay by using a combination of mycoplasmas having different origins. Thus, we used various DNA mixtures (DNAs from two to four different species) that had human and/or serum origins. For each combination, under genus-specific conditions, the limit of detection was still 10 fg. On the other hand, under species-specific conditions, the limit of detection varied from 10 to 25 fg, depending on the association tested.

However, these results were obtained when purified chromosomal DNA was used as the template in the PCR. The sensitivity achieved with purified DNA may not be achieved with DNAs extracted from simulated samples or commercial samples of animal sera. Therefore, the sensitivity of the PCR method was assessed with DNAs extracted from simulated samples by performing a limiting dilution experiment; 10 CCU per PCR sample was consistently detected, and 1 CCU was detected in only 50% of the reactions. A Southern blot analysis with GPO-1 increased the sensitivity 10-fold. Thus, the limit of detection was estimated to range from 1 to 10 CCU/ml of PCR sample (i.e., 0.25 to 2.5 CCU/ml of serum sample). The results obtained by determining the number of CCU were confirmed by determining the number of CFU.

Evaluation of specificity. Under our conditions, the genus-specific primer set consisting of MGSO and GPO-1 was not absolutely specific to mollicutes depending on the concentration of DNA studied. Cross-reactions not mentioned by Van Kuppeveld et al. (43) were observed with some bacteria that are phylogenetically closely related to mollicutes (e.g., *Enterococcus faecalis*, *C. innocuum*, *B. subtilis*), with bacteria that arose from branches that are more distant on the mycoplasma phylogenetic tree (e.g., *Streptococcus* sp., *Staphylococcus pas-*

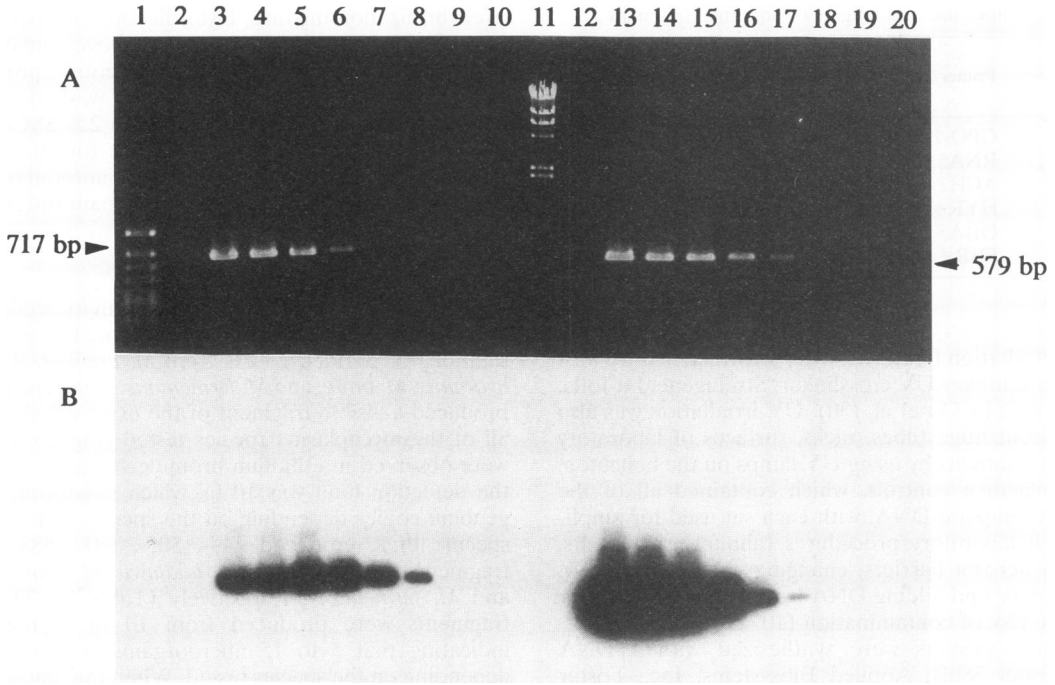

FIG. 2. Sensitivity of the PCR detection assay. Purified DNA from *M. fermentans* was serially diluted and subjected to a PCR with genus-specific primers (lanes 2 to 10) or with species-specific primers (lanes 12 to 20). (A) Ethidium bromide staining of DNA on the agarose gel. (B) Southern blot analysis of the gel shown in panel A, using [γ-^{32}P]ATP-labelled probe GPO-1. Lanes 1 and 11, DNA markers (lane 1, ϕX174 replicative-form DNA *Hinc*II digest [Pharmacia]; lane 11, λ DNA *Hin*dIII digest); lanes 2 and 12, negative controls (sterile distilled water); lanes 3 and 13, 100 pg; lanes 4 and 14, 10 pg; lanes 5 and 15, 1 pg; lanes 6 and 16, 100 fg; lanes 7 and 17, 10 fg; lanes 8 and 18, 1 fg; lanes 9 and 19, 100 ag; lanes 10 and 20, 10 ag.

teuri, *Escherichia coli*, *Rhodococcus* sp., *C. perfringens*), and even with yeasts (e.g., *Saccharomyces cerevisiae*). The specificity of the species-specific primer sets was also investigated. At an annealing temperature of 64°C, no amplification product was detected when mycoplasmal DNA from a species other than the species tested or nonmycoplasma bacterial DNA was examined (see Materials and Methods for the species tested). Use of primer sets specific for *M. arginini*, *A. laidlawii*, *M. hyorhinis*, *M. orale*, and *M. fermentans* resulted in amplification of DNA fragments having the expected sizes (449, 505, 584, 583, and 579 bp, respectively). Tests with purified DNA were then completed by performing specificity studies with simulated samples. The primer sets exhibited the same specificity with all five type strains mentioned in Materials and Methods and with cell culture contaminant strains identified in our laboratory (13 *M. arginini* strains, 3 *A. laidlawii* strains, 46 *M. hyorhinis* strains, 13 *M. orale* strains, and 12 *M. fermentans* strains).

In order to enhance the specificity of our method, we used the three-primer PCR described by Kai et al. (20). We used RNA3, RNA5, and inner primer UNI− for the genus-specific PCR and ARG+, ARG−, and inner primer ARG2 for the species-specific PCR for *M. arginini* (Table 1). The reaction produced an additional band having the predicted size (772 bp in the genus-specific PCR and 266 bp in the PCR specific for *M. arginini*), which confirmed the identity of the initial PCR product (1,055 bp in the genus-specific PCR and 655 bp in the PCR specific for *M. arginini*). The detection limit with this technique was also 10 fg (Fig. 3).

DISCUSSION

Contamination of animal sera by mollicutes is a widespread problem that has biological and economic importance for cell culturists and serum processors. Since there is at present no reliable, reproducible, fast, sensitive, specific assay for detecting mycoplasma infections in animal sera, our aim was to develop a procedure which is superior to classical microbiological culturing, AdoP activity screening, and indicator cell culture techniques. In this paper we describe the use of the PCR in a mycoplasma infection detection system for animal sera that is useful in laboratory and industrial applications.

The exquisite sensitivity of our PCR-based detection tech-

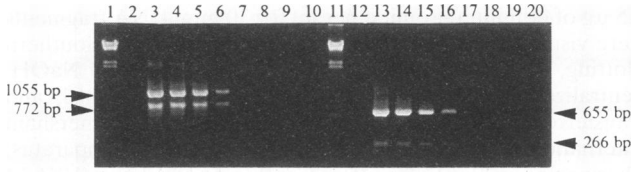

FIG. 3. Sensitivity of the three-primer PCR detection assay. Purified DNA from *M. arginini* was serially diluted and subjected to the universal PCR by using three genus-specific primers (lanes 2 to 10) or to the specific PCR by using three species-specific primers (lanes 12 to 20). Lanes 1 and 11, DNA markers (*Hin*dIII-digested λ DNA); lanes 2 and 12, negative controls (sterile distilled water); lanes 3 and 13, 100 pg; lanes 4 and 14, 10 pg; lanes 5 and 15, 1 pg; lanes 6 and 16, 100 fg; lanes 7 and 17, 10 fg; lanes 8 and 18, 1 fg; lanes 9 and 19, 100 ag; lanes 10 and 20, 10 ag.

nique presented two major limits that we had to control. First, inhibition of the amplification reaction because of the presence of contaminants in samples has been described previously as a source of false-negative results (9). No internal positive controls were included in the PCR, since the sera were intentionally infected. Tests to determine the detection limit with seeded animal sera revealed amplification around the threshold of mycoplasma concentration (10 organisms). Moreover, the PCR was not inhibited when undiluted sera were used (data not shown). Therefore, the possibility that main inhibitors were present under the conditions of our assay was eliminated. Second, since the PCR can generate millions of DNA copies in a 100-μl reaction volume, product carryover and exogenous DNA have been described in many cases as sources of contamination and, thus, false-positive results (9, 11, 22, 30). Strict laboratory procedures (see Materials and Methods) were used to minimize the risk of contamination. Moreover, negative controls from concentration to extraction and the PCR steps were included to monitor contamination.

The first requirement of the assay was high sensitivity in order to detect the presence of rare mycoplasmas in large volumes. Initially, we concentrated the samples and optimized each technical step of the method. The detection limit ranged from 5 to 15 mycoplasmas and was increased to 1 mycoplasma by using radioactive labelling. The results obtained with purified DNA were in agreement with the results of previous studies. Recently published detection limits have varied from 1 to 100 mycoplasmas, depending on the species tested, the PCR target, the primer design, and the PCR conditions used (6, 14, 21, 23, 29, 33, 37, 39). Since between 10 and 100 mycoplasmas (1 to 10 mycoplasmas after hybridization) could be detected in PCR samples, fourfold concentration by centrifugation allowed the detection of less than 10 organisms per ml of original serum sample. Obviously, the concentration factor could be increased. Apart from the potential presence of inhibitors in sera, the loss of sensitivity of the PCR assay compared with purified DNA could be explained mainly by the yields of resuspension of the cells after centrifugation (estimated to be near 80%) and also by the DNA extraction yields. No other investigation has explored the use of the PCR technique for detecting mycoplasmas in animal sera. Nevertheless, data evaluating the use of the PCR technique for diagnosis of mycoplasma infections by detection in clinical samples are available. Jensen et al. (18) observed a detection limit of 40 *M. pneumoniae* cells in simulated clinical samples (throat swabs from healthy individuals) after 70 cycles of amplification of a 153-bp segment of the gene for the P1 virulence protein and a detection limit of 4 organisms after hybridization with a ^{32}P-labelled oligonucleotide probe. Skakni et al. (37) were able to detect 10 *M. pneumoniae* cells (1 organism in 25% of the reactions) in clinical samples (nasopharyngeal aspirations or bronchoalveolar lavages) obtained from children. Using 35 cycles of amplification of a 375-bp segment of the gene coding for the P1 cytadhesin protein, Buck et al. (6) were able to detect between 1 and 10 *M. pneumoniae* cells in simulated specimens prepared from throat swabs. Dot blot hybridization with a biotin-labelled DNA probe did not increase the sensitivity. Our results obtained with simulated animal sera were comparable to the results of these previous studies. Since the rRNA copy number is high (up to 10,000 copies per cell [43]) and is much more than the copy number of the rRNA gene (1 or 2 copies per cell [13]), the reverse transcription PCR (43) was used as a detection technique in our method. The results obtained under the conditions described by Van Kuppeveld et al. (43) could not be reproduced in our assay. We failed to increase the sensitivity, and nonspecific amplification was observed. This was due in part to stable RNA secondary structures that hampered reverse transcription and thus cDNA synthesis. The use of a nested PCR (29) resulted in an increase in sensitivity (data not shown), but this time-consuming technique was a source of contamination and nonspecific amplification. Therefore, we did not routinely use these two PCR-derived techniques.

The second requirement of our assay was specificity to detect only mycoplasmas and not other microorganisms constantly present in unprocessed serum sublots and possibly in final lots of manufactured sera (2). Because of cross-reactions observed with nonmycoplasma bacteria and even yeasts, the results obtained under genus-specific conditions alone lacked specificity. These cross-reactions were not mentioned by Van Kuppeveld et al. (43), although we used more specific PCR conditions (a higher annealing temperature) than Van Kuppeveld et al. did. Since specificity experiments are dependent on DNA concentrations, the divergence could be explained by the use of higher concentrations in our assay. However, the combination of a universal (i.e., genus-specific) PCR followed by a specific (i.e., species-specific) PCR and Southern blot hybridization proved to be a highly specific approach. In fact, it enabled us not only to confirm that the amplification fragment was the fragment expected, but also to identify the contaminant mycoplasmas at the species level. With the exception of serological tests, including the immunoenzymatic detection kit (Boehringer Mannheim Biochemica, Mannheim, Germany), which at present is limited to four species, the specific PCR approach is the only detection assay that allows direct identification and thus determination of the source of contamination. Actually, a contamination course and eventually a hypothesis concerning the origin of the contaminant can be determined from the species identified. The combination of a universal three-primer PCR and a specific three-primer PCR is an attractive alternative to the protocol described above, because this technique allows rapid and simple identification of contaminant mycoplasmas. Unlike Southern blot hybridization, the three-primer PCR does not increase sensitivity, but it is faster and much simpler and avoids the disadvantages of handling radioactive materials. For these reasons, this approach can be used in every routine diagnostic laboratory or research laboratory in which the PCR is used.

When the detection methods used at this time are compared, only our PCR-based assay combines specificity that allows direct contaminant identification and the high level of sensitivity required to detect small contamination rates in commercial filtered sera. Our assay is also faster than culture-based methods. The results are available within 1 day, in contrast to the 2 to 6 weeks required by the microbiological culture techniques and the 1 to 3 weeks required by the cell culture-based techniques. Moreover, DNA staining (e.g., staining with 4′,6-diamidino-2-phenylindole dihydrochloride) after cell culturing and microbiological culturing relies on subjective reading of the results. These detection assays require training and experience. On the other hand, interpretation of the PCR results is easy because it relies on an objective reading. Moreover, the PCR method is very repeatable, with no variation between replicate results of an experiment, and reproducible, with only slight variations between the results of replicate experiments. However, unlike culture-based methods, our assay does not differentiate dead mycoplasmas from viable mycoplasmas. Therefore, it could give a diagnosis of excess contamination and lead to elimination of sera containing dead mycoplasmas.

Our results suggest that the PCR has significant potential as a rapid, sensitive method for detecting and identifying myco-

plasmas in animal sera. Compared with the time-consuming and fastidious indicator cell culture and broth-agar culture detection methods and the enzymatic detection method, which is not very specific, the PCR assay is a promising method, for the diagnosis of animal serum contamination by mycoplasmas. The method described above could provide an interesting alternative to the currently used detection methods. The viability and rapidity of the assay could be useful for cell culturists. Moreover, in the serum supply industry, this assay could be combined with a confirmation test to provide quality control procedures for animal serum producers, to improve the validity of results, and to reduce the time of quarantine. Although the results obtained with simulated samples described above are encouraging, the suitability of the assay for detection and identification of mycoplasmas in commercial animal sera remains to be established. Therefore, we are currently performing additional studies with a large number of commercial samples in order to validate the PCR assay.

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REFERENCES

1. **Barile, M. F.** 1979. Mycoplasma-tissue cell culture interactions, p. 425–474. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. 2. Academic Press, New York.
2. **Barile, M. F., and J. Kern.** 1971. Isolation of *Mycoplasma arginini* from commercial bovine sera and its implication in contaminated cell cultures. Proc. Soc. Exp. Biol. Med. **138:**432–437.
3. **Bölske, G.** 1988. Survey of mycoplasma infections in cell cultures and comparison of detection methods. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **269:**331–340.
4. **Bonissol, C., and B. Stoïljkovic.** 1989. AdoP assay detection of mycoplasmal contamination in biological media. Res. Virol. **140:** 241–251.
5. **Bonissol, C., F. Traincard, B. Stoïljkovic, and P. Hosli.** 1984. Adenosine phosphorylase activity as a technique for detection of mycoplasmas in biological media. Ann. Inst. Pasteur Microbiol. **135A:**63–72.
6. **Buck, G. E., L. C. O'Hara, and J. T. Summersgill.** 1992. Rapid, sensitive detection of *Mycoplasma pneumoniae* in simulated clinical specimens by DNA amplification. J. Clin. Microbiol. **30:**3280–3283.
7. **Carle, P., C. Saillard, and J. M. Bové.** 1983. DNA extraction and purification. Methods Mycoplasmol. **1:**295–299.
8. **Chen, T. R.** 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. **104:**255–263.
9. **Clewley, J. P.** 1989. The polymerase chain reaction, a review of the practical limitations for human immunodeficiency virus diagnosis. J. Virol. Methods **25:**179–188.
10. **Erlich, H. A., D. Gelfand, and J. J. Sninsky.** 1991. Recent advances in the polymerase chain reaction. Science **252:**1643–1651.
11. **Fox, J. C., M. Ait-Khaled, A. Webster, and V. C. Emery.** 1991. Eliminating PCR contamination: is UV irradiation the answer? J. Virol. Methods **33:**375–382.
12. **Gabridge, M. G., and D. J. Lundin.** 1989. Cell culture user's guide to mycoplasma detection and control. Bionique Laboratories, New York.
13. **Glaser, G., H. C. Hyman, and S. Razin.** 1992. Ribosomes, p. 169–177. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas. Molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
14. **Grau, O., R. Kovacic, R. Griffais, and L. Montagnier.** 1993. Development of a selective and sensitive polymerase chain reaction assay for the detection of *Mycoplasma pirum*. FEMS Microbiol. Lett. **106:**327–334.
15. **Harasawa, R., H. Mizusawa, and K. Koshimizu.** 1986. A reliable and sensitive method for detecting mycoplasmas in cell cultures. Microbiol. Immunol. **30:**919–921.
16. **Hatanaka, M., R. Del Giudice, and C. Long.** 1975. Adenine formation from adenosine by mycoplasmas: adenosine phosphorylase activity. Proc. Natl. Acad. Sci. USA **72:**1401–1405.
17. **Hay, R. J., M. L. Macy, and T. R. Chen.** 1989. Mycoplasma infection of cultured cells. Nature (London) **339:**487–488.
18. **Jensen, J. S., J. Sondergard-Andersen, S. A. Uldum, and K. Lind.** 1989. Detection of *Mycoplasma pneumoniae* in simulated clinical samples by polymerase chain reaction. APMIS **97:**1046–1048.
19. **Jurmanova, K., M. Hajkova, and O. Fischer.** 1990. Detection of mycoplasmas in cell cultures. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Suppl. **20:**947–948.
20. **Kai, M., S. Kamiya, S. Sawamura, T. Yamamoto, and A. Ozawa.** 1991. Simplified method for confirmation of PCR products. Nucleic Acids Res. **19:**4562.
21. **Kai, M., S. Kamiya, H. Yabe, I. Takakura, K. Shiozawa, and A. Ozawa.** 1993. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by the polymerase chain reaction. J. Med. Microbiol. **38:**166–170.
22. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
23. **Lüneberg, E., J. S. Jensen, and M. Frosch.** 1993. Detection of *Mycoplasma pneumoniae* by polymerase chain reaction and nonradioactive hybridization in microtiter plates. J. Clin. Microbiol. **31:**1088–1094.
24. **McGarrity, G. J., and H. Kotani.** 1985. Cell culture mycoplasmas, p. 353–390. *In* S. Razin and M. F. Barile (ed.), The mycoplasmas, vol. 4. Academic Press, New York.
25. **McGarrity, G. J., H. Kotani, and G. H. Butler.** 1992. Mycoplasmas and tissue culture cells, p. 445–454. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas. Molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
26. **McGarrity, G. J., H. Kotani, and D. Carson.** 1986. Comparative studies to determine the efficiency of 6-methylpurine deoxyriboside to detect cell culture mycoplasmas. In Vitro Cell. Dev. Biol. **22:**301–304.
27. **Miles, R. J.** 1992. Cell nutrition and growth, p. 23–40. *In* J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas. Molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.
28. **Mowles, J. M.** 1988. The use of ciprofloxacine for the elimination of mycoplasma from naturally infected cell lines. Cytotechnology **1:**355–358.
29. **Narita, M., Y. Matsuzono, T. Togashi, and N. Kajii.** 1992. DNA diagnosis of central nervous system infection by *Mycoplasma pneumoniae*. Pediatrics **90:**250–253.
30. **Ou, C.-Y., J. L. Moore, and G. Schochetman.** 1991. Use of UV irradiation to reduce false positivity in polymerase chain reaction. BioTechniques **10:**442–446.
31. **Polak-Vogelzang, A. A., J. Brugman, and R. Reijgers.** 1987. Comparison of two methods for detection of Mollicutes (Mycoplasmatales and Acholeplasmatales) in cell cultures in the Netherlands. Antonie Leeuwenhoek **53:**107–118.
32. **Rodwell, A. W., and R. F. Whitcomb.** 1983. Methods for direct and indirect measurement of mycoplasma growth. Methods Mycoplasmol. **1:**185–196.
33. **Roulland-Dussoix, D., A. Henry, and B. Lemercier.** Detection of mycoplasmas in cell cultures by PCR: a one year study. J. Microbiol. Methods, in press.
34. **Russell, W. C., C. Newman, and D. H. Williamson.** 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature (London) **253:**461–462.
35. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

37. **Skakni, L., A. Sardet, J. Just, J. Landman-Parker, J. Costil, N. Moniot-Ville, F. Bricout, and A. Garbarg-Chenon.** 1992. Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. J. Clin. Microbiol. **30:**2638–2643.
38. **Somerson, N. L., and B. C. Cole.** 1979. The mycoplasma flora of human and nonhuman primates, p. 191–216. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. 2. Academic Press, New York.
39. **Spaepen, M., A. F. Angulo, P. Marynen, and J. J. Cassiman.** 1992. Detection of bacterial and mycoplasma contamination in cell cultures by polymerase chain reaction. FEMS Microbiol. Lett. **99:**89–94.
40. **Spierenburg, G. T., A. A. Polak-Vogelzang, and B. J. E. G. Bast.** 1988. Indicator cell lines for the detection of hidden mycoplasma contamination, using an adenosine phosphorylase screening test. J. Immunol. Methods **114:**115–119.
41. **Tully, J. G.** 1992. Mollicutes, p. 181–191. *In* J. Lederberg (ed.), Encyclopedia of microbiology, vol. 3. Academic Press, New York.
42. **Uphoff, C. C., S. Brauer, D. Grunicke, S. M. Gignac, R. A. F. MacLeod, H. Quentmeier, K. Steube, M. Tümmler, M. Voges, B. Wagner, and H. G. Drexler.** 1992. Sensitivity and specificity of five different mycoplasma detection assays. Leukemia **6:**335–341.
43. **Van Kuppeveld, F. J. M., J. T. M. Van der Logt, A. F. Angulo, M. J. Van Zoest, W. G. V. Quint, H. G. M. Niesters, J. M. D. Galama, and W. J. G. Melchers.** 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl. Environ. Microbiol. **58:**2606–2615.