Detection of Mycoplasma Contamination in Cell Cultures by a Mycoplasma Group-Specific PCR

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The suitability of a 16S rRNA-based mycoplasma group-specific PCR for the detection of mycoplasma contamination in cell cultures was investigated. A total of 104 cell cultures were tested by using microbiological culture, DNA fluorochrome staining, DNA-rRNA hybridization, and PCR techniques. A comparison of the results obtained with these techniques revealed agreement for 95 cell cultures. Discrepant results, which were interpreted as false negative or false positive on the basis of a comparison with the results obtained with other methods, were observed with nine cell cultures. The microbiological culture technique produced false-negative results for four cell cultures. The hybridization technique produced false-negative results for two cell cultures, and for one of these cell cultures the DNA staining technique also produced a false-negative result. The PCR may have produced false-positive results for one cell culture. Ambiguous results were obtained with the remaining two cell cultures. Furthermore, the presence of contaminating bacteria interfered with the interpretation of the DNA staining results for 16 cell cultures. For the same reason the hybridization signals of nine cell cultures could not be interpreted. Our results demonstrate the drawbacks of each of the detection methods and the suitability of the PCR for the detection of mycoplasmas in cell cultures.

Cell cultures are widely used in both biomedical and biotechnological research centers and industry, as well as for diagnostic tests in hospitals. These cell cultures can be contaminated with species belonging to the genera *Mycoplasma* and *Acholeplasma* (which are collectively referred to as mycoplasmas). Contaminating mycoplasmas may alter host cellular characteristics. For instance, alterations in growth characteristics, enzyme patterns, cell membrane composition, chromosomal abnormalities, and the induction of cytopathogenic changes have been described (1, 11, 15). Such effects can greatly influence the experimental results obtained with cell lines and, therefore, result in misleading conclusions. In addition to scientific drawbacks, contamination can also result in economic setbacks because of the loss of time and, since mycoplasmas are hard to eradicate, the loss of precious cell lines. These drawbacks emphasize the need to regularly screen cell cultures for mycoplasmal contamination. Routine screening is essential since contaminated cell cultures are the most important source of cross-contamination.

Current screening methods rely on microbiological culture techniques, microscopy, biochemical methods, and the use of DNA probes. Culturing mycoplasmas can take 2 to 4 weeks and can be difficult because of a requirement for special growth conditions (3, 7). Therefore, routine culturing is restricted to specialized laboratories. The results obtained by microscopy, which are based on DNA staining with a fluorescent dye, are hampered by difficulties in interpretation. Biochemical detection methods (6, 12), which are based on measuring the uptake of uridine or thymidine into cells, analyzing mycoplasma-induced cytotoxicity, or detecting various enzymatic activities, are time consuming, and their

results are difficult to interpret. The use of DNA probes to detect either mycoplasmal DNA or mycoplasmal rRNA has been shown to be a rapid screening method, with a detection limit of approximately 10^4 organisms (5, 8, 13). Although this level of sensitivity is sufficient in most cases, it may be too low for newly contaminated cell lines and cell lines treated with antibiotics or grown in the presence of low serum concentrations. In addition, probes are not always mycoplasma specific since they can cross-hybridize with gram-positive bacteria (5, 8). On the other hand, oligonucleotide ribosomal DNA probes that can be used to detect mycoplasmas which do not cross-hybridize have been described (10).

Recently, we developed a 16S rRNA-based PCR assay to detect mycoplasmas at both the genus level and the species level (16). The genus-specific primers reacted with all of the mycoplasmal species investigated and with members of the genera *Ureaplasma*, *Spiroplasma*, and *Acholeplasma*, but not with any of the walled relatives of these organisms. In this study we investigated the applicability of the mycoplasma group-specific PCR assay for the detection of mycoplasma contamination in cell cultures by comparing the results obtained with microbiological culture, DNA fluorochrome staining, and DNA-rRNA hybridization methods.

MATERIALS AND METHODS

Cell cultures. The cell cultures used in this study came from research laboratories, clinical centers, and biomedical industries. The cell cultures were sent to the Mycoplasma Laboratory at the National Veterinary Institute in Uppsala, Sweden, for screening of mycoplasmal contamination, either for routine monitoring or because it was suspected that the cell lines were contaminated.

The broad variety of cell lines tested included porcine kidney epithelial (LLC-PK1) cells, canine kidney (MDCK) cells, mouse macrophages, mouse hybridoma cells, mouse neuroblastocytoma (PC 12D) cells, monkey small-cell carci-

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noma lung (U1568) cells, human glioma (343 MG) cells, human colon carcinoma (HT29) cells, hamster kidney fibroblasts (BHK cells), mink lung cells, and rainbow trout gonad (RTG) cells.

Mycoplasma detection methods. At the National Veterinary Institute, the cell cultures were screened for mycoplasmas by using three methods: (i) microbiological culture, (ii) DNA fluorochrome staining, and (iii) DNA-rRNA hybridization. Suspension cell cultures were used directly for the tests after careful mixing. Monolayer cells were first scraped off and suspended in their media. Aliquots from each cell culture were stored at $-70°C$.

(i) Microbiological culture. The microbiological culture technique was performed by using the media and culture techniques described by Bölske (2). Three different mycoplasma broth media were inoculated with 0.3 ml of sample. A 10-fold dilution for each broth was also cultured. Two different agar media were inoculated with 0.01 ml of sample. Mycoplasmas detected by the microbiological culture technique were identified by indirect immunofluorescence as described previously (2).

(ii) Fluorochrome DNA staining. For DNA staining, 0.15 ml of sample was inoculated onto indicator cells (Vero cells), and the preparation was stained after 4 to 6 days with Hoechst 33258 stain as described by Del Giudice and Hopps (4).

(iii) DNA-rRNA hybridization. A 0.25-ml portion of each sample was tested by DNA-rRNA hybridization with the H900 probe, which is based on a restriction fragment from the 23S rRNA gene of *Mycoplasma hyorhinis*, as described by Johansson et al. (8).

Estimating numbers of mycoplasmas. The numbers of mycoplasmas per milliliter of cell culture sample were estimated by the microbiological culture technique. Generally, growth on agar plates revealed moderate to high levels of mycoplasma contamination. No growth on the agar plates was an indication of low-level contamination (<100 mycoplasmas per ml). Growth in both broth dilutions indicated that ≥ 30 mycoplasmas per ml were present, while growth only in the first broth dilution indicated that 3 to 30 mycoplasmas per ml were present.

Selection of cell cultures for PCR analysis. A total of 104 cell cultures tested by the three methods described above were selected from material kept at $-70°C$. Two criteria were used for selection: the numbers of mycoplasma-positive and -negative cell cultures had to be about equal, and the collection had to include cell cultures which contained low numbers of mycoplasmas to evaluate the sensitivity of the PCR. For the latter purpose, cell cultures 523/89, 524/89, 525/89, and 135/89, which contained only small numbers of *M. hyorhinis* (<100 cells per ml), were selected. The identities of the mycoplasma strains isolated from the mycoplasma-positive cell cultures were not used as a criterion in the selection.

A 1-ml portion of each of the cell cultures was centrifuged at 12,000 × g for 20 min, washed with 500 μl of phosphate-buffered saline, and suspended in 200 μl of water. The samples were then immediately frozen and lyophilized. The lyophilized material was coded and sent to the Department of Medical Microbiology at the University of Nijmegen for PCR analysis.

Nucleic acid isolation and PCR analysis. Lyophilized cell cultures were suspended in 1 ml of water. The nucleic acid content obtained from 50 μl of cell culture was used as a template for PCR analysis. Isolation of nucleic acid, ampli-

TABLE 1. Comparison of the results of microbiological culturing, DNA fluorochrome staining, DNA-rRNA hybridization with probe H900, and PCR analysis for the detection of mycoplasmas in 104 cell cultures

No. of cell cultures	Cell culture(s)	Results[a] of:			
		Micro-biological culturing	DNA staining	Probe H900 analysis	PCR analysis
49		+	+	+	+
3		+ (bact)	NJ	NJ	+
35		−	−	−	−
6		− (bact)	NJ	−	−
2		− (bact)	NJ	NJ	−
1	525/89	+	+	−	+
1	524/89	+	−	−	+
4	22/89, 23/89, 24/89, 408/89	− (bact)	NJ	NJ	+
1	523/89	+	+	−	−
1	135/89	+ (bact)	NJ	−	−
1	526/89	−	−	−	+

[a] +, mycoplasma positive; −, mycoplasma negative; (bact), bacteria present; NJ, results could not be judged because of bacterial contamination.

fication by the PCR, and analysis of the amplified samples were performed as described previously (16).

Briefly, nucleic acids were isolated from 250 μl of a cell culture with sodium dodecyl sulfate and proteinase K, extracted with phenol and chloroform, precipitated, and resuspended in 50 μl of distilled water. PCR amplification was performed with 10 μl of nucleic acid in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 0.2 U of Super *Taq* polymerase (HT Biotechnology, Cambridge, England). The thermal profile consisted of 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. The mycoplasma group-specific primer set, which amplifies a 280-bp fragment, consists of the forward primer GPO-3 (5'-GGGAGCAAACAGGATTAGATACC CT-3') and the reverse primer MGSO (5'-TGCACCATCTG TCACTCTGTTAACCTC-3') (16). Aliquots (20 μl) were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Southern blot hybridization was performed with [γ-^{32}P]ATP-end-labeled internal oligonucleotide GPO-4 (5'-CTTAAAGGAATTGACGGGAACCCG-3') (16). To prevent DNA carryover contamination, strict partial partitioning of the different steps of the PCR and the recommendations of Kwok and Higuchi (9) were used.

RESULTS

The results obtained with the different detection methods are shown in Table 1. Mycoplasmas were isolated from 56 cell cultures by the microbiological culture technique. Because some cell cultures were contaminated with more than one mycoplasma, a total of 67 mycoplasmas were isolated from these cell cultures. A total of 25 of these isolates were *M. hyorhinis* isolates, 22 were *Mycoplasma arginini* isolates, 12 were *Mycoplasma orale* isolates, 5 were *Mycoplasma fermentans* isolates, 2 were *Acholeplasma laidlawii* isolates, and 1 was a *Mycoplasma pirum* isolate. Table 1 also shows that 16 cell cultures, both mycoplasma-positive and mycoplasma-negative cultures, contained bacteria as determined by the microbiological culture technique. When the DNA

staining technique was used, these contaminating bacteria produced extranuclear fluorescent signals which hindered the interpretation of the results. Thus, for these 16 cell cultures, the DNA staining results could not be judged. With 9 of these 16 cell cultures, positive hybridization signals were observed. Since these hybridization signals may have been caused by cross-hybridizing bacteria, the hybridization results obtained with these cell cultures were also considered results that could not be judged.

Apart from the interpretation difficulties described above, the results of the microbiological culture, DNA staining, hybridization, and PCR techniques agreed for 95 cell cultures (52 mycoplasma-positive and 43 mycoplasma-negative cell cultures). Moderate to high numbers of mycoplasmas (10^4 to 10^8 cells per ml) were isolated from the 52 mycoplasma-positive cell cultures. Conflicting results were obtained with nine cell cultures. The microbiological culture results for mycoplasmas were negative for four cell cultures (cultures 22/89, 23/89, 24/89, 408/89) that were positive as determined by the PCR. Because these cell cultures contained bacteria, the results of the DNA staining and hybridization techniques could not be interpreted. Discrepant results were also obtained with the four cell cultures from which only small numbers of *M. hyorhinis* cells were isolated (cell cultures 135/89, 523/89, and 524/89 contained 3 to 30 mycoplasma cells per ml, and cell culture 525/89 contained 30 to 100 mycoplasma cells per ml). These four cultures were all negative as determined by hybridization, and cultures 135/89 and 523/89 were also negative as determined by the PCR. In addition, cell culture 524/89 was also negative as determined by the DNA staining technique. Furthermore, the PCR technique produced positive results for one cell culture (526/89) that was negative as determined by the microbiological culture, DNA staining, and hybridization techniques.

Cell cultures 22/89, 23/89, and 24/89, which were negative as determined by the microbiological culture technique but positive as determined by the PCR, originated from one laboratory. Also, cell cultures 523/89, 524/89, and 525/89, which contained small numbers of *M. hyorhinis* cells as determined by the microbiological culture technique but which were negative as determined by the hybridization or PCR technique, and cell culture 526/89, which was positive as determined by the PCR but negative as determined by all other methods, originated from one laboratory. Cell cultures 135/89 and 408/89 originated from other laboratories.

DISCUSSION

There is a need for a rapid and sensitive detection method for efficient determination of mycoplasma contamination of cell cultures. Ideally, this method should recognize not only the five typical contaminating species (i.e., *M. hyorhinis, M. arginini, M. orale, M. fermentans,* and *A. laidlawii*) that cause 98% of contaminations (2), but also all other mollicute species belonging to the genera *Mycoplasma, Acholeplasma, Ureaplasma,* and *Spiroplasma,* which cause the remaining 2% of contaminations. Recently, we have developed a 16S rRNA-based mycoplasma group-specific PCR assay (16) which fulfills these requirements. It was possible to select a mycoplasma-specific primer set from systematic computer alignments of the 40 known mycoplasmal 16S rRNA sequences with sequences of other procaryotes (16). In vitro amplification by the PCR with this primer set resulted in amplification of the sequences of the members of four mollicute genera mentioned above, but not in the

amplification of sequences of any other prokaryote. The suitability of this mycoplasma group-specific PCR assay for the detection of mycoplasmal contaminants in cell cultures was investigated in this study.

A comparison of the PCR technique with the microbiological culture, DNA fluorochrome staining, and hybridization techniques indicated that the PCR is a rapid, sensitive, and efficient method. Microbiological culture takes 1 to 4 weeks. Furthermore, it is known that it is difficult to isolate certain mycoplasma strains, especially *M. hyorhinis* strains (2, 4, 11). In this study, the microbiological culture technique produced negative results for four cell cultures that were positive as determined by the PCR technique. For these cultures, interpretation of both the DNA staining and hybridization results was impossible because of contaminating bacteria. Retesting of the original four cell cultures revealed again positive amplification by the PCR, which indicates that DNA carryover contamination may be excluded. The microbiological culture technique was repeated only for cell culture 23/89, because at this time point only material from this cell culture was left. Again, culture on specialized cell-free media did not produce any mycoplasma growth directly from the sample. However, growth of mycoplasmas was observed after inoculation of Vero indicator cells. The presence of mycoplasmas in this indicator cell line was confirmed by DNA staining, hybridization, and the PCR (data not shown). The results of a sequence analysis of variable regions V6, V7, and V8 demonstrated that this mycoplasma was a *M. hyorhinis* strain (B. Pettersson, K.-E. Johansson, and M. Uhlén, unpublished data). Later, after several passages on the indicator cells, a poorly growing mycoplasma was isolated on broth and agar media. This isolate was also identified as *M. hyorhinis* by indirect immunofluorescence. Two other cell cultures that originated from the same laboratory (cell cultures 22/89 and 24/89) were also negative as determined by the microbiological culture technique but positive as determined by the PCR. These cell cultures probably were also contaminated with the same poorly growing *M. hyorhinis* strain, since cell cultures handled together in the same laboratory frequently have the same mycoplasma contaminants. Whether cell culture 408/89 also contained such a poorly growing *M. hyorhinis* strain is not known.

The main disadvantage of the DNA fluorochrome staining technique is the difficulty in interpreting the results. This difficulty is due to the presence of contaminating bacteria or broken nuclei, which produce extranuclear fluorescence signals that can mask mycoplasmas, and was observed in about 10% of the cell cultures tested in a large-scale study (2). In our study, interpretation difficulties due to the presence of bacteria were observed in 16 of the 104 cell cultures. One cell culture (cell culture 524/89) was false negative as determined by DNA staining. As this was one of the cell cultures that contained few mycoplasmas (3 to 30 cells per ml), the reason for the false-negative results may be that the small volume (0.15 ml) used for inoculation of the indicator cells did not contain any mycoplasmas.

The hybridization procedure is based on the same principle as the PCR (i.e., the specific detection of nucleic acids). Although both methods are relatively rapid, there are several differences. The results of the hybridization procedure can sometimes be difficult to interpret. For instance, it is difficult to distinguish between specific signals and aspecific signals because of the presence of cross-reacting gram-positive bacteria (5, 8). Therefore, positive hybridization results can be judged only when microbiological culturing reveals no

gram-positive bacteria. Furthermore, it can also be difficult to interpret the results for individual samples when they are applied to the filter close to samples heavily contaminated with mycoplasmas. For this reason, several samples had to be retested before they were considered positive or negative in this study. Another advantage of the PCR is its greater sensitivity. In this study, two cell cultures (cell cultures 524/89 and 525/89) which contained only a few mycoplasmas (3 to 30 and 30 to 100 cells per ml, respectively) were negative as determined by the hybridization technique but positive as determined by the PCR. The sensitivity of the DNA-rRNA hybridization method, which has a detection limit of 10^3 to 10^4 organisms, was not sufficient to detect the mycoplasmas in these two cell cultures. These results demonstrate that for the detection of low-level infections (e.g., just after the onset of the infection, after an attempt to eliminate mycoplasmas, or in the presence of low serum concentrations), the PCR is required since the hybridization procedure is not sensitive enough.

Both negative hybridization results and negative PCR results were obtained with two other cell cultures (cell cultures 135/89 and 523/89) that contained only a few mycoplasmas (3 to 30 cells per ml) as determined by the microbiological culture technique. As observed with cell cultures 524/89 and 525/89, the failure to detect mycoplasmas by hybridization was probably due to the small number of mycoplasmas. For cell cultures 135/89 and 523/89, however, the PCR was also negative. When we retested these samples by PCR with larger sample volumes (500 instead of 50 μl), we could not confirm the presence of mycoplasmas. After these findings were obtained, microbiological culturing of cell culture 135/89, for which remaining material was present, was repeated at the National Veterinary Institute. However, no mycoplasmas were isolated. A possible explanation for the negative PCR results and the failure to grow *M. hyorhinis* on the second occasion is that the cell culture initially contained a very small number of *M. hyorhinis* cells per milliliter, but that freezing of the sample resulted in the loss of these few mycoplasmas. Another possible explanation is that there was an uneven distribution of mycoplasmas, which adhere to cells within the sample. When the sample was first divided, mycoplasmas may not have been distributed to all of the vials.

These results demonstrate that the PCR is a very useful technique for detecting mycoplasma contamination in cell cultures when the necessary measures are taken to avoid DNA carryover. Cell culture 526/89 was positive as determined by the PCR, but negative as determined by the three other detection methods. The PCR assay was repeated twice for this cell culture, and on both occasions the results were negative. Therefore, although precautions were taken to avoid DNA carryover contamination, it is possible that this cell culture produced a false-positive result in the first PCR. However, as discussed above concerning uneven distribution of mycoplasmas in samples with low levels of contamination, this might also be true for sample 526/89, which originated from the same laboratory as samples 523/89, 524/89, and 525/89. One other PCR procedure for the detection of contaminating mycoplasmas in cell cultures has been described. Spaepen et al. (14) used a nested PCR system with several 16S rRNA selected primer pairs to detect all contaminants. The use of a second amplification cycle, however, strongly increases the risk of DNA carryover contamination, which makes this procedure less suitable for routine screening of large numbers of cell culture samples.

Our results clearly indicate that the mycoplasma group-specific PCR assay is a valuable tool for the detection of mycoplasmas in cell cultures. As demonstrated in this paper, PCR analyses can be performed with lyophilized cell cultures, which facilitates the transport of these samples to reference laboratories. Further research should now concentrate on optimizing the sample preparation procedure so that large numbers of samples can be processed more rapidly. Furthermore, replacement of the radiolabel by a nonradioactive label (e.g., biotin) would facilitate the introduction of this PCR procedure in many diagnostic laboratories.

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