Detection of multiple mycoplasma infection in cell cultures by PCR

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Abstract

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A total of 301 cell cultures from 15 laboratories were monitored for mycoplasma (*Mollicutes*) using PCR and culture methodology. The infection was detected in the cell culture collection of 12 laboratories. PCR for *Mollicutes* detected these bacteria in 93 (30.9%) samples. Although the infection was confirmed by culture for 69 (22.9%) samples, PCR with generic primers did not detect the infection in five (5.4%). Mycoplasma species were identified with specific primers in 91 (30.2%) of the 98 samples (32.6%) considered to be infected. *Mycoplasma hyorhinis* was detected in 63.3% of the infected samples, *M. arginini* in 59.2 %, *Acholeplasma laidlawii* in 20.4%, *M. fermentans* in 14.3%, *M. orale* in 11.2%, and *M. salivarium* in 8.2%. Sixty (61.2%) samples were co-infected with more than one mycoplasma species. *M. hyorhinis* and *M. arginini* were the microorganisms most frequently found in combination, having been detected in 30 (30.6%) samples and other associations including up to four species were detected in 30 other samples. Failure of the treatments used to eliminate mycoplasmas from cell cultures might be explained by the occurrence of these multiple infections. The present results indicate that the sharing of non-certified cells among laboratories may disseminate mycoplasma in cell cultures.

Introduction

Mycoplasmas (*Mollicutes*) inhabit plants, insects, animals, and humans. Most belong to the normal flora of their hosts; however, some are primary pathogens and many possess an opportunistic character. There is some controversy about other species as the cause of syndromes of unknown etiology or of diseases in immunosuppressed hosts. These bacteria can also seriously interfere with biomedical research by infecting laboratory animals or cell cultures (1,2).

The infection of cell cultures with mycoplasma can have different cytogenetic effects (3). Mycoplasmas usually adhere to cells but, depending on the species, may fuse with the host cell or even invade it (4-6). These bacteria deplete the nutrients of cell cultures and interfere with the response of these cells when challenged experimentally (7,8). Interruption of cell metabolism (9), modulation of the immune response (10,11), modification of cellular morphology, inter-

ference with viral replication, chromosome modifications, or cell transformation may occur (12). The identification of these phenomena in accidental or experimental infections may contribute to the understanding of the relationship between mycoplasmas and the host cell (13).

The first isolation of mycoplasma from a cell culture was reported in 1956 (14). At present, about 20 species isolated from this system have been reported. *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. orale*, *M. salivarium*, *M. fermentans*, and *M. hyorhinis* are detected in about 95% of cell cultures accidentally infected with this microorganism. Species such as *Ureaplasma urealyticum*, *M. pneumoniae* and *M. pirum* are rarely present in cell cultures and some were isolated only once (15-18).

In most cases, mycoplasma infection originates from contaminated animal serum, but contaminated aerosols in the laboratory contribute to its dissemination. The incidence of mycoplasmas in cell cultures depends on sampling, institution and duration of the evaluation period (19,20). *A. laidlawii* and *M. arginini* are of bovine origin and *M. hyorhinis* originates from swine (21).

The presence of mycoplasmas of swine origin in bovine serum is justified by the contamination of this product in mixed slaughterhouses (22). Trypsin, a compound of swine origin, is used in cell cultures and mycoplasmas have never been isolated from cultures containing it because of the mycoplasmacidal activity of this compound (22), but their DNA has been detected in our laboratory (Timenetsky J, personal communication). *M. orale* and *M. salivarium* inhabit the human oropharynx and usually initially infect cell cultures through the aerosol generated by mouth pipetting (3,12).

Mycoplasmas usually infect specific hosts, but in the case of animals some species may be found in different animal hosts. The human host barrier is rarely crossed by mycoplasmas of animal origin. However,

cell cultures might be infected with species of human or animal origin (23). The literature proposes that the sharing of non-certified cells between laboratories might be the main route of dissemination of mycoplasma (21). The frequency of infection of cell cultures with mycoplasma is approximately 30% (21), but the distribution of species causing multiple infections is still unknown. The aim of the present study was to determine the most frequent association of mycoplasmas in cell cultures.

Material and Methods

Microorganisms

The following mycoplasma ATCC strains were used as control: *U. urealyticum* T960, *A. laidlawii* P8, *M. pneumoniae*-FH, *M. genitalium* G37, *M. hominis* PG-21, *M. hyorhinis* BTS7, *M. orale* CH19299, *M. salivarium* PG-20, *M. buccale* CH202247, *M. fermentans* PG-18, *M. pulmonis* PG-34, *M. arginini* G230, and *M. arthritidis* PG-6.

Sampling

A total of 301 cell culture samples were studied, including 219 lineage cells, 47 hybridomas, 31 human blood cultures infected with *Plasmodium falciparum*, and 4 cultures containing the trypomastigote forms of *Trypanosoma cruzi*. The samples were received from 15 laboratories in the State of São Paulo, Brazil. The cells presented elongation, intracellular inclusions, vacuolization, dark granulations, acidification of the culture medium, growth reduction, or rupture of the monolayer. Some cell cultures showed no morphological alterations and were monitored for the first time.

Culture

Each cell sample was diluted 10^{-1} to 10^{-3} in 1.8 mL SP4 broth and 100 µL of each

dilution was inoculated onto solid medium. The cultures were incubated for 15 days at 30ºC under aerobic conditions. A subculture of each culture was performed if the initial culture showed no mycoplasma. The microorganism was presumptively identified based on alterations in the pH of the broth in the absence of turbidity, production of "fried egg" colonies on agar plates and positive subculture after filtration of the initial culture through 0.22-µm membranes (24).

The cell culture samples were also inoculated into thioglycolate broth (Difco, Detroit, MI, USA) and BHI agar (Difco) containing 5% sheep blood and incubated for 5 days at 37ºC under aerobic and anaerobic conditions.

Polymerase chain reaction

The technique described by Fan et al. (25) was used. Target DNA was extracted from the cell culture samples and from cultures of the reference strains by boiling 1.0 mL of each sample.

Nucleotide sequence of primers and amplified products (26)

Primers for the generic detection of Mollicutes. GPO3 5' GGGAGCAAACACGAT AGATACCCT 3', MGSO 5' TGCACCATC TGTCACTCTGTTAACCTC 3 (270 bp).

Specific primers for the detection of: i) *M. salivarium*: MSALF 5' ATGGATTG TAAAGTGCTGTTGCTAG 3', MSALR 5' GCGTCAACAGTTCTCTGCCG 3' (434 bp); ii) *M. arginini*: MAGF 5' GCATGGAA TCGCATGATTCCT 3', GP4R 5' GGTG TTCTTCCTTATATCTACGC 3' (545 bp); iii) *M. hyorhinis*: MHRHF 5' GAACGGGA TGTAGCAATACATTC 3', MHRHR 5' AGCGGACTGAAGTTGAGCTTCAG 3' (604 bp); iv) *M. fermentans*: UNI-5' TAAT CCTGTTTGCTCCCCAC 3', FER+ 5' AAGAAGCGTTTCTTCGCTGG 3' (579 bp); v) *M. orale*: UNI- 5' TAATCCTG

TTTGCTCCCCAC 3', ORA5 5' GGAGCG TTTCGTCCGCTAAG 3' (583 bp): vi) *A. laidlawii*: UNI- 5' TAATCCTGTTTGC TCCCCAC 3', ACH3 5' AGCCGGACTGA GAGGTCTAC 3' (505 bp).

For the detection of *Mollicutes* by polymerase chain reaction (PCR), 50 pmol of each *GPO3/MGSO* primer, 2 U *Taq* DNA polymerase (Biotools, São Paulo, SP, Brazil), 2.0 mM MgCl$_2$, 100 µM of each deoxyribonucleotide triphosphate (dNTP), 1 µL DNA, and ultrapure water were added to a final volume of 50 µL. The thermocycler (model PTC-100, MJ Research, Inc., Watertown, MA, USA) was programmed for one cycle at 94ºC for 5 min, 35 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s, and a final step at 72ºC for 10 min.

Twenty-five picomoles of each primer, 1 U *Taq* DNA polymerase (Biotools), 1.4, 1.8, and 1.6 mM MgCl$_2$ for *M. salivarium*, *M. arginini* and *M. hyorhinis*, respectively, 200 µM of each dNTP, 1 µL DNA extracted from the cell culture sample, and ultrapure water to a final volume of 50 µL were used for the detection of *M. salivarium*, *M. arginini* and *M. hyorhinis* by PCR. The thermocycler was programmed for 40 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 60 s and a final step at 72ºC for 5 min.

For the detection of *M. fermentans*, *M. orale* and *A. laidlawii*, the reaction mixture contained 40 pmol of each primer, 1 U *Taq* DNA polymerase (Biotools), 1.2 mM MgCl$_2$, 200 µM of each dNTP, 1 µL DNA extracted from the cell culture sample, and ultrapure water to a final volume of 50 µL. The thermocycler was programmed for one cycle at 95ºC for 15 min, 30 cycles at 95ºC for 30 s, 64ºC for 90 s, and 72ºC for 90 s, and a final step at 72ºC for 10 min.

A positive control (DNA of the reference strains) and a negative control (ultrapure water) were added to each amplification.

The sensitivity of the PCR for the detection of mycoplasmal DNA was determined by culture of 20 mL of each species studied.

The DNA concentration of each solution was measured by absorbance at 260 nm (GIM GENE QUANT 2, Pharmacia®, Bjork-gatar, Sweden). The solutions were then diluted up to the 10^{-9} in decimal steps and submitted to PCR as described above.

Statistical analysis (27)

The culture results were used as reference for comparison with the PCR results. The sensitivity parameters were calculated as follows: true-positive/true-negative + true-positive results. Specificity was calculated as follows: true-negative/true-positive + true-negative results. Accuracy was obtained as follows: true-positive + true-negative/total number of samples.

Results

The mycoplasma reference strains grew on solid and liquid medium and were confirmed at the species level by PCR. Table 1 shows the general results obtained for the samples evaluated by culture and PCR. Mycoplasmas were detected by culture in 69/301 (22.9%) of the cell culture samples. No other bacterial genera were identified. The cultured mycoplasmas presented "fried egg" colonies, shifted the pH of the broth without turbidity and were subcultured after filtration. PCR using primers to detect *Mollicutes* revealed the presence of targeted DNA in 93/301 (30.9%) samples. Thus, these bacteria were present in 24/301 (8.0%) additional samples. Five (5.4%) samples were positive

Table 1. Detection of Mollicutes in cell culture samples by PCR and culture methodologies.

Culture	PCR		
	Positive	Negative	Total
Positive	64	5	69
Negative	29	203	232
Total	93	208	301

by culture and negative by PCR using the generic primers. *Mollicutes* were detected by PCR in 86/219 (39.7%) cell lineage samples, 5/47 (10.6%) hybridoma samples and 2/31 (6.5%) *P. falciparum* culture samples.

The 98 samples (32.6%) positive by PCR with generic primers or by culture were again submitted to PCR using species-specific primers and the sequences used were able to detect the target *Mollicutes* (Figure 1). Most 60/98 (61.2%) of the infected cell cultures presented at least two mycoplasma species. Some samples were infected with three or four species. *M. hyorhinis* was detected in 62/98 (63.3%) of these samples, *M. arginini* in 58/98 (59.2%), *A. laidlawii* in 20/98 (20.4%), *M. fermentans* in 14/98 (14.3%), *M. orale* in 11/98 (11.2%), and *M. salivarium* in 8/98 (8.2%). PCR was able to identify the mycoplasma species analyzed in 91/98 (92.9%) samples, while none of these species were identified in seven (7.1%) samples. *M. hyorhinis* and *M. arginini* were present as the single infecting agent in 12/98 (12.4%) and 10/98 (10.2%) samples, respectively. These species, however, were associated
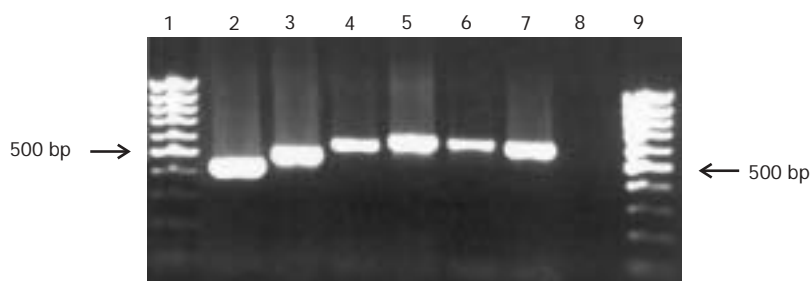

Figure 1. Agarose gel electrophoresis of the PCR assay performed as positive control to detect 16SrRNA of the searched mycoplasmas in pure culture. Lanes 1 and 9, size markers DNA-1 kb plus (Invitrogen); lane 2, Mycoplasma salivarum - 434 bp; lane 3, Acholeplasma laidlawii - 505 bp; lane 4, M. arginini - 545 bp; lane 5, M. fermentans - 579 bp; lane 6, M. orale - 583 bp; lane 7, M. hyorhinis - 604 bp; lane 8, negative control (water).

in 30/98 (30.6%) positive samples. *M. fermentans*, *A. laidlawii* and *M. orale* were also detected as single agents in 2/98 (2.0%), 3/98 (3.1%) and 4/98 (4.1%) samples, respectively, but they were associated with other species in 12/98 (12.3%), 17/98 (17.4%) and 7/98 (7.1%) positive samples, respectively. *M. salivarium* was only detected in association with other species. Mycoplasmas were identified infecting cell cultures in 12 laboratories and were present in association in four. Table 2 also shows mycoplasma associations other than *M. hyorhinis* and *M. arginini* detected in cell cultures.

The sensitivity of culture compared to PCR using primers for the detection of *Mollicutes* was 68%, specificity was 97%, and accuracy was 89%. The respective primer concentrations were 1 ng/μL for the detection of *M. arginini*, 1 pg/μL for *M. salivarium* and *M. orale*, and 100 fg/μL for *M. hyorhinis*, *M. fermentans* and *A. laidlawii*. The primers used for the detection of *Mollicutes* showed a sensitivity of 100 fg/μL. Figure 2 illustrates the sensitivity of the primers in detecting the DNA target of *M. hyorhinis*.

Discussion

Accidental infection of cell cultures with mycoplasma (*Mollicutes*), especially when unnoticed, usually invalidates the results of biomedical research. The cell cultures may not die but remain altered and inadequate for experimentation (28).

The frequency of mycoplasma infection

Table 2. Cell cultures in which more than one species of mycoplasma other than Mycoplasma hyorhinis and Mycoplasma arginini was detected by PCR.

N	Mh	Ma	Mf	Ms	Mo	Al
1	nd	+	+	nd	nd	nd
2	+	+	+	nd	nd	nd
3	+	+	+	nd	nd	nd
4	nd	+	+	nd	nd	nd
5	nd	+	+	nd	nd	nd
6	+	nd	nd	+	nd	+
7	nd	nd	+	nd	nd	+
8	+	nd	nd	+	+	+
9	+	+	nd	nd	+	+
10	+	+	nd	nd	+	+
11	+	+	nd	nd	nd	+
12	+	+	nd	nd	nd	+
13	+	+	nd	nd	nd	+
14	+	+	nd	nd	nd	+
15	+	+	nd	+	nd	+
16	+	+	+	nd	nd	nd
17	+	nd	nd	nd	+	nd
18	nd	nd	nd	+	nd	+
19*	nd	+	+	nd	nd	nd
20	nd	+	nd	nd	nd	+
21	+	+	nd	+	nd	nd
22	nd	nd	nd	nd	+	+
23*	+	+	+	nd	nd	nd
24	+	nd	+	+	nd	+
25*	nd	+	nd	+	nd	+
26	+	+	nd	nd	nd	+
27	nd	nd	nd	nd	+	+
28	+	nd	nd	+	+	nd
29	+	nd	+	nd	nd	nd
30	+	+	nd	nd	nd	nd

Thirty cultures of 98 tested (30.6%) were positive. N = number of the cell culture sample: 1-5: L929, 6: CTY, 7: TCTY, 8-11: HeLa, 12-15: PLF, 16: AT, 17: CHO, 18: BHK, 19: OX3, 20: MDBK, 21: NHI3T3, 22: HT29, 23: C15.6, 24: T end, 25: N418, 26: HEK, 27: VERO, 28: PK136, 29 and 30: WEH. Mh = M. hominis; Ma = M. arginini; Mf = M. fermentans; Ms = M. salivarium; Mo = M. orale; Al = Acholeplasma laidlawii; nd = non-detected mycoplasma species. +Detected species specific mycoplasma. *Hybridomas.

Figure 2. Agarose gel electrophoretic analysis of the PCR assay performed with MHRHF/MHRHR and different concentrations of DNA/μL obtained by dilution from a culture of Mycoplasma hyorhinis. Lane 1, 1000 ng; lane 2, 100 ng; lane 3, 10 ng; lane 4, 1 ng; lane 5, 100 pg; lane 6, 10 pg; lane 7, 1 pg; lane 8, 100 fg; lane 9, negative control (water); lane 10, 10 fg; lane 11, 1 fg; lane 12, 100 atg; lane 13, size markers DNA-1 kb plus (Invitrogen).
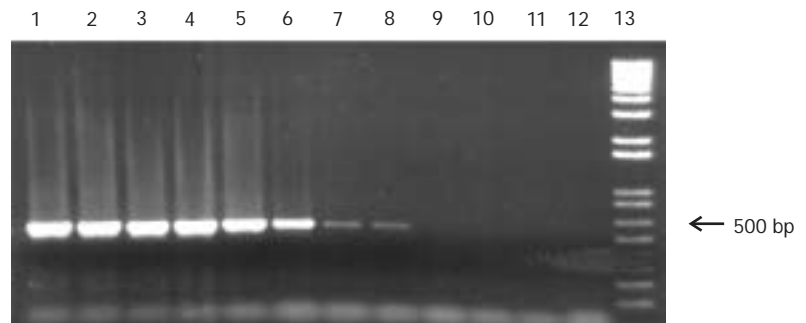
in cell cultures can reach 100% (29), but the outcome depends on the sampling variables. The 32.6% rate of cell culture infection observed in the present study agrees with the literature regarding the frequency of tested samples but not regarding infected laboratories. Twelve of the 15 laboratories studied were found to be infected. This finding is important because all are research laboratories, except for one which only commercializes cells. This type of information has not been available in Brazil before this report and it is hoped that these data may contribute to the control of this infection.

The laboratory that commercializes cells takes specific care and possesses a rigid quality control system, including the monitoring for mycoplasmas. Most laboratories surveyed in the present study maintain cells for research or laboratory diagnosis. Some of these cells were labeled as originating from the institution that commercializes cells. However, these cells were probably infected with mycoplasmas over time due to their continuous manipulation. This situation, if not analyzed, may compromise the reputation of the institution that provides the cell culture.

Primary infection of cell line cultures with one mycoplasma species is the most widely reported situation in the literature. The presence of various species is related to primary cell cultures; however, this possibility was not specifically examined in the present study.

The species detected in the present study derived from animals or humans or both. The diversity of mycoplasma species in the same cell culture indicates the occurrence of different initial infection sources. The subculturing of a cell culture among laboratories over time due to successive sharing may explain the detection of multiple mycoplasma species. Mycoplasmal diversity accumulates over time mainly due to failure to control the infection.

Several methods for the detection of mycoplasmas in cell cultures have been reported. The use of two methods has been the most recommended strategy to minimize false results. PCR in combination with culture is the most widely recommended procedure (30). In the present study, PCR detected more infected samples than culture. Five positive cultures were not confirmed by PCR for *Mollicutes* but their identification was possible by specific PCR. The low amount of mycoplasmas or their DNA in the cell culture sample, the presence of inhibitors or failure of the diagnostic method may explain the contradictory nature of the results obtained by culture and PCR (23).

PCR permits the detection of femtogram amounts of mycoplasma DNA, corresponding to one bacterial cell. However, the sensitivity of specific PCR varies from species to species.

The isolation of more than one mycoplasma species is time consuming even for a specialized laboratory. In this respect, PCR permits not only the diagnosis of mycoplasmas in cell cultures but also allows the determination of the distribution of species (21).

M. orale, *M. hyorhinis* and *A. laidlawii* are the species most frequently found in cell cultures (29). They were also detected in the present study but at different frequencies. *M. hyorhinis* was the most frequent, followed by *M. arginini*, findings that are in contrast to the literature (23,31).

M. fermentans is considered to be a normal inhabitant of the human urogenital tract. It is a fastidious species, a fact that impaired its isolation in the past. In 1986, *M. fermentans* was associated with the development of AIDS in HIV-positive individuals, a fact that, in turn, attracted the interest of the scientific community. Subsequently, *M. fermentans* has been detected in or associated most frequently with tissues and blood of individuals with poorly studied diseases or diseases of unknown etiology (1,32). The increase in the frequency of *M. fermentans* in cell cultures can be explained by the grow-

ing use of human blood cells or tissues for primary culture (23).

M. hyorhinis and *M. fermentans* were detected co-infecting a cell culture inoculated with *Coxiella burnetii*, without evidence of morphological alterations caused by this association (data not shown). Mycoplasma infections in cell cultures that do not cause cell death or alterations in cellular organization, on the other hand, may represent models for the study of the biology of these bacteria and of the cell cultures. In fact, in the last decade the number of experiments in this field, especially on mycoplasmas of human or animal interest, has been increasing (13).

A cell culture infected with mycoplasmas must be promptly eliminated and the non-infected cells should be carefully preserved. An attempt can be made to treat cell cultures of special value for research if infected with mycoplasmas. Antibiotics or antibodies are the most widely used methods; however, it should be remembered that these compounds are not necessary mycoplasma-eliminating agents when they infect cells and that the biology of mycoplasmas varies among species (33). In this context, analysis

of the toxicity of the antimicrobial agent as well as the sensitivity of mycoplasma to the drug should be performed (34,35). An attempt to eliminate a multiple mycoplasma infection in cell culture is not considered, a fact that might explain the failures of treatment of mycoplasma-infected cell cultures. The sensitivity of mycoplasma to antibiotics is variable and the treatment is directed at a cell culture infected with a single mycoplasma species (21). On the other hand, the use of antibiotics to prevent the growth of microorganisms in cell cultures may hide the agent for years and impair its characterization.

Mycoplasmas are sensitive to the habitual processes of decontamination used in a laboratory. Some persistent laboratory negligence such as the production of aerosols or other inadequate techniques contributes to the dissemination of mycoplasmas among cell cultures (21). Comments by laboratory staff confirm failures in the control of cell cultures, as well as disregard about mycoplasma infection in this system. This context compromises the efforts made to improve the quality of biomedical research.

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