Detection of mycoplasma contamination in cell cultures and bovine sera

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ABSTRACT: Contamination of cell cultures and sera used for animal virus propagation with mycoplasmas represents a serious problem, especially in virology. Therefore specific control measures must be used. To achieve this we introduced PCR for the detection of mycoplasma species in cell cultures and compared its results with ELISA and microbiological culture. Seven mycoplasma species which are the most common contaminants of cell lines (*Mycoplasma arginini, M. fermentans, M. hyorhinis, M. bovis, M. orale, M. hominis,* and *Acholeplasma laidlawii*) were used to verify the method. Then we assessed five selected cell lines and three bovine sera by the PCR, ELISA and culture methods and compared the results. PCR was positive for all of the mycoplasma species tested. ELISA kit used (Mycoplasma detection kit, Roche, Germany) allowed detection of only four species of contaminating mycoplasmas (*Acholeplasma laidlawii, Mycoplasma arginini, M. hyorhinis,* and *M. orale*). All the methods detected contamination of the VERO and RK13 cell lines. The agents of contamination were determined by the species-specific ELISA kit as *Mycoplasma arginini* and *M. orale*, respectively. Other cell lines and sera tested were not contaminated with mycoplasma. The results confirmed that the PCR method used in the present study is a sensitive, fast and specific detection method of mycoplasma contaminations and is suitable for routine mycoplasma detection in cell cultures and bovine sera.

Keywords: mycoplasma; cell line; PCR; ELISA; microbiological culture; contamination

Mycoplasmas are a group of bacteria which lack a cell wall; many of them are pathogenic for people, animals, insects and plants. Some mycoplasma species also contaminate cell cultures used for isolation and propagation of viruses (Tang et al., 2000). Frequencies of such contaminations generally vary between 5 and 87% (Rawadi and Dussurget, 1995; Garner et al., 2000). Since cell lines are used for culture of animal viruses, their contaminations represent a serious problem in virology. They may produce unreliable experimental results with consequent potential risk during production of biological preparations. Natural hosts of contaminating mycoplasma species are particularly humans and various animal species. Primary sources of contamination with *Mycoplasma orale, M. fermentans,* and *M. hominis* in the laboratories are infected people who handle cell cultures and suspensions of viruses. Sources of *M. arginini, M. bovis, Acholeplasma laidlawii,* and *M. hyorhinis* are usually animal donors of tissues and biological constituents used for cell culture, e.g. calf serum and trypsin (Razin and Tully, 1995). Widely practised interlaboratory exchange of cell cultures which may have been contaminated, can also lead to transmission of mycoplasma contaminations. Whereas it is easy to detect the contamination of cell cultures with other bacteria, moulds or yeasts either macroscopically or by inverse microscopy, mycoplasma may

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persist in cell cultures for a long time without being obvious; however, they induce alterations in metabolism, cell growth rate and cell morphology (Roulland-Dussoix et al., 1994).

The following methods of testing mycoplasma infections have been specified by the Anonymous (2002): culture in liquid media and detection of colony forming mycoplasmas on solid agars, and a method for agar testing with an indicator in the cell culture (fluorescent DNA staining). Nevertheless, a number of other alternative methods for mycoplasma detection have been developed: histochemical staining, electron microscopy, infrared spectroscopy (Melin et al., 2004), and immunological methods, e.g. ELISA, immunofluorescence, and immunoblotting (Razin and Tully, 1996). Commercially available diagnostic kits based on biochemical and immunological detection can also be used for that purpose. However, all mycoplasma species contaminating cell cultures cannot be detected by a single test due to their limited sensitivity or specificity (Uphoff et al., 1992). Recently, several methods of detection of mycoplasma contaminations of cell cultures based on the polymerase chain reaction (PCR) technology have been developed. Some of the PCR methods use species-specific or family-specific 16S-23S rRNA intergenic region as a target sequence (Tang et al., 2000; Kong et al., 2001). Most of the PCR methods are however based on the direct detection of the 16S rRNA genes, these are either specific for particular groups of mycoplasma (Van Kuppeveld et al., 1994; Pruckler and Ades, 1995; Nissen et al., 1996; Uphoff and Drexler, 2002) or are species-specific (Roulland-Dussoix et al., 1994). The recently developed PCR method (Uphoff and Drexler, 2002, 2004) also amplifies sequences of the 16S rRNA, and detects the presence of the most common contaminants of cell lines: *Mycoplasma arginini, M. bovis, M. fermentans, M. hominis, M. hyorhinis, M. orale,* and *Acholeplasma laidlawii.* The applied primers also recognize more than 18 other *Mycoplasma* and *Acholeplasma* species, which can be associated with primary cell cultures (Wirth et al., 1994).

The purpose of the present study was to introduce the PCR method (Uphoff and Drexler, 2002, 2004) for mycoplasma detection, to test its specificity in seven mycoplasma species and to use the PCR method for testing selected cell lines and bovine sera stored in the Collection of Animal Pathogenic Microorganisms (Veterinary Research Institute, Brno, Czech Republic). The results obtained by PCR were finally compared with the results of ELISA and microbiological culture.

MATERIAL AND METHODS

Mycoplasma strains used for testing the PCR and ELISA methods

Seven mycoplasma species which are the most common contaminants of cell lines were used for testing the PCR and ELISA methods. Before propagation, the mycoplasma strains were stored in the freeze-dried form in the Collection of Animal Pathogenic Microorganisms (Table 1). Liquid media containing the following compounds were used for propagation: PPLO Broth (Difco) (7.4 g), redistilled water for cell culture (CC) (350 ml), 1% phenol red

Table 1. Mycoplasma strains used for verification of PCR and ELISA

Species	Strain	Origin
Acholeplasma laidlawii	$T_1M_1$51; CAPM M-23	isolate, Jurmanova, Czech Republic
Mycoplasma arginini	6/III ; CAPM M-43	isolate, Jurmanova, Czech Republic
Mycoplasma bovis	Donetta; ATCC 25523	type strain, Freundt, Denmark
Mycoplasma fermentans	PG 18; ATCC 19989	type strain, Freundt, Denmark
Mycoplasma hominis	PG 21; ATCC 23114	type strain, Freundt, Denmark
Mycoplasma hyorhinis	TR 32; CAPM M-32	isolate, Gois, Czech Republic
Mycoplasma orale	CH 19299; ATCC 23714	type strain, Freundt, Denmark

CAPM = Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Czech Republic
ATCC = American Type Culture Collection, Manassas, USA

(1 ml), equine serum for CC (100 ml), 5% yeast extract (50 ml), ampicilin (500 mg) and 5% thallium acetate (3 ml), supplemented with arginin, glucose, or urea (0.5 g) according to metabolic activity of respective species (Razin and Tully, 1995). Culture medium with mycoplasma inoculum was incubated in capped tubes at 37°C for several days until the colour of the pH indicator changed.

Cell cultures and bovine sera tested

Cell lines MARC-145 (monkey kidney cell line), MDBK (Madin Darby bovine kidney cell line), VERO (African Green monkey kidney cell line), FBTy (foetal bovine thyroid gland) and line RK13 (rabbit kidney cell line) were tested for potential mycoplasma contamination. These cell lines were obtained from different laboratories. Prior the DNA isolation, the cells were cultured on antibiotic-free medium for three weeks with the aim to meet requirements for propagation of detectable amounts of potential mycoplasma contaminants.

Further, calf sera used for culture medium supplementation, i.e. precolostral calf serum (PTS, batch No. 020301 and No. 030502, distributed by SML-ZVOS Hustopece, Ltd.) and calf serum (TS, batch No. 24/2000, distributed by BOSE-TASOV) were tested.

PCR

The recently developed PCR method for mycoplasma detection (Uphoff and Drexler, 2002, 2004) was used. One millilitre of cell culture suspension, bovine serum or propagated mycoplasma culture was centrifuged for 6 min at 13 000 × g. The pellet was washed twice with 1 ml PBS and then resuspended in 100 µl PBS. After incubation for 15 min at 95°C, the Wizard DNA Extraction kit (Promega, Germany) was used for DNA isolation.

PCR was carried out in a volume of 25 µl in 1× PCR buffer (Qiagen, Germany) containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.33 µM of each oligonucleotide, 10 ng internal control DNA, 100 ng extracted DNA and 1 U Taq DNA polymerase. Primers (Table 2) were complementary to regions of the 16S rRNA genes of seven mycoplasma species which are most commonly found in cell cultures (*Acholeplasma laidlawii, Mycoplasma arginini, M. bovis, M. hyorhinis, M. hominis, M. fermentans, M. orale*). The resulting PCR product was separated by electrophoresis in 1.3% agarose gel, stained with ethidium bromide, visualized under UV light and documented by photography. The length of the amplification product was 502 to 520 bp depending on mycoplasma species.

Table 2. Primers used in the PCR according to Uphoff and Drexler (2002)

Forward primers	Reverse primers
cgc ctg agt agt acg ttc gc	gcg gtg tgt aca aga ccc ga
cgc ctg agt agt acg tac gc	gcg gtg tgt aca aaa ccc ga
tgc ctg ggt agt aca ttc gc	gcg gtg tgt aca aac ccc ga
tgc ctg agt agt aca ttc gc	
cgc ctg agt agt atg ctc gc	
cgc ctg ggt agt aca ttc gc	

Internal control DNA was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and its preparation was described previously (Uphoff et al., 1994). It is a DNA fragment with the length 986 bp, which is competitively amplified in the PCR reaction when *Acholeplasma laidlawii* is the contaminating mycoplasma species and noncompetitively amplified when other mycoplasma species are present in the cell cultures (Uphoff et al., 1994).

ELISA

Mycoplasma species identification by ELISA test (enzyme-linked immunosorbent assay) was performed with commercial diagnostic Mycoplasma detection kit (Roche, Germany) following the manufacturer's instructions. The kit contains four species-specific polyclonal antibodies to *Acholeplasma laidlawii, Mycoplasma arginini, M. hyorhinis* and *M. orale*.

Microbiological culture

Culture examination of cell cultures and bovine sera for mycoplasmas was performed by an internationally accepted method (Anonymous, 2002) under both anaerobic and microaerobic conditions. Selected types of liquid media with the above mentioned composition and a solid agar were used:

PPLO agar (Difco) (8 g), redistilled water for CC (210 ml), equine serum for CC (60 ml), 5% yeast extract (30 ml), ampicilin (200 mg) and 5% thallium acetate (1.5 ml).

Restriction fragment length polymorphism analysis of PCR products

A revision of the positive reaction of the strain M-32 (*Mycoplasma hyorhinis*) with antibodies to *Acholeplasma laidlawii* in the ELISA test was carried out by digestion of the PCR product with restriction enzymes (Uphoff and Drexler, 2004). To distinguish between *Mycoplasma hyorhinis* and *Acholeplasma laidlawii*, the PCR amplicon was digested with the *Asp I* and *Sfu I* restriction enzymes. *Mycoplasma hyorhinis* is digestible with the restriction enzyme *Asp I*, the resulting fragment sizes are 303 and 213 bp. *Acholeplasma laidlawii* is digestible with *Sfu I*, with the fragments of 436 and 81 bp (Uphoff and Drexler, 2004). One microliter of the appropriate 10 × buffer and 1 µl of the respective restriction enzyme were added to 8 µl of the PCR reaction mix containing the amplicon and incubated at 37°C for one hour. The restriction fragment patterns were determined by electrophoresis in a 1.5% agarose gel and visualised by ethidium bromide staining.

RESULTS

Seven mycoplasma species were used in the present study for the PCR and ELISA method verification. Although the strains were propagated after a long-time storage (10 to14 years), all remained vital. The PCR was positive for all of the species tested. Electrophoresis of the PCR products of two selected mycoplasma species (*Mycoplasma arginini, M. fermentans*) is shown in Figure 1. The PCR products of the size 502 to 520 bp were mycoplasma-specific and products of the size 986 bp resulted from the amplification of the internal control DNA. The results of the ELISA test confirmed that the kit used (Mycoplasma detection kit, Roche, Germany) is species-specific and only four species of contaminating mycoplasmas could be detected (Table 3). The culture of *Mycoplasma hyorhinis* (strain M-32) propagated in our laboratory was also positive in the reaction with antibodies to *Acholeplasma laidlawii* (Table 3). However, the digestion of its PCR amplicon with the restriction enzymes *Asp I* and *Sfu I* showed only the fragment pattern typical for *M. hyorhinis* (Figure 2).

In the cell lines (MARC 145, MDBK, VERO, FBTy, RK13) and bovine sera (PTS 02 03 01, PTS 03 05 02 and TS 24/2000), mycoplasma contamination was tested by the three methods (PCR, ELISA and culture). The contamination with mycoplasma was de-

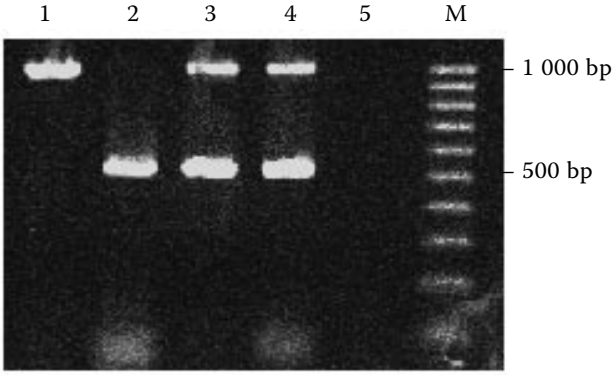

Figure 1. Ethidium bromide-stained gel containing the PCR products of two selected cell lines, two mycoplasma species and water: (1) FBTy + internal control, (2) RK13 + internal control, (3) *Mycoplasma arginini* (CAPM M-43) + internal control, (4) *M. fermentans* (ATCC 19989) + internal control, (5) water, (M) 100 bp ladder

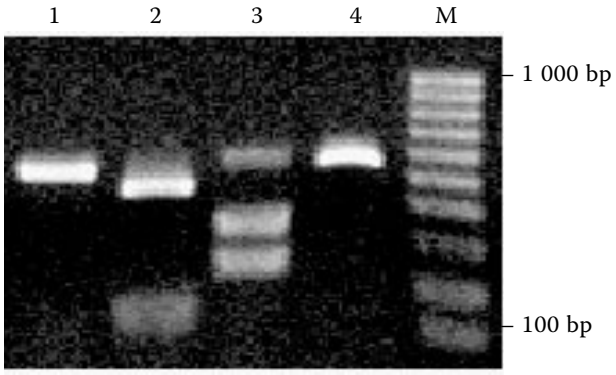

Figure 2. Identification of mycoplasma species by restriction fragment length polymorphism analysis of PCR products: (1) *Acholeplasma laidlawii* (CAPM M-23) digested with *Asp I*, (2) *Acholeplasma laidlawii* (CAPM M-23) digested with *Sfu I*, (3) *Mycoplasma hyorhinis* (CAPM M-32) digested with *Asp I*, (4) *Mycoplasma hyorhinis* (CAPM M-32) digested with *Sfu I*, (M) 100 bp ladder

Table 3. Verification of the PCR and ELISA methods

	A. laidlawii (CAPM M-23)	*M. arginini* (CAPM M-43)	*M. bovis* (ATCC 25523)	*M. fermentans* (ATCC 19989)	*M. hominis* (ATCC 23114)	*M. hyorhinis* (CAPM M-32)	*M. orale* (ATCC 23714)
PCR	+	+	+	+	+	+	+
ELISA	+ (*A. laidlawii*)	+ (*M. arginini*)	–	–	–	+ (*M. hyorhinis, A. laidlawii*)	+ (*M. orale*)

+ positive result of examination; – negative result of examination

Table 4. Examination of cell lines and sera

Method	MARC-145	MDBK	VERO	FBTy	RK13	PTS 020301	PTS 030502	TS 23
PCR	–	–	+	–	+	–	–	–
Microbiological culture	–	–	+	–	+	–	–	–
ELISA	–	–	+ (*M arginini*)	–	+ (*M. orale*)	–	–	–

– negative result of examination; + positive result of examination; PTS = precolostral calf serum; TS = calf serum

tected by the PCR method in two cell lines, i.e. in VERO and RK13 lines (Figure 1), the results for the other cell lines were negative. In the cell line RK13, amplification of internal control DNA was suppressed, which was caused by massive mycoplasma contamination of that line (Figure 1). All sera tested by PCR were mycoplasma-negative. The results of testing of cell lines and sera by PCR were further compared with the results of ELISA and culture examination (Table 4). The ELISA method confirmed contamination of the VERO and RK13 lines, and due to its species-specificity, it was detected that the VERO and RK13 lines were contaminated with the *Mycoplasma arginini* and *M. orale* species, respectively (Table 4). Contamination of the cell lines VERO and RK13 was likewise confirmed by the culture method because typical mycoplasma colonies were grown on the solid agar. The negative result in the other cell lines and sera was also confirmed by culture.

DISCUSSION

The purpose of the present study was to test and compare sensitivity and applicability of three commonly used methods (culture, PCR and ELISA) for the detection of mycoplasma contaminations of cell cultures and to reveal potential contamination of five selected cell lines and three batches of bovine sera.

Through testing the PCR method (Uphoff and Drexler, 2002, 2004), our results confirmed that primers detected presence of all seven tested species which are the causal agents of 98% mycoplasma contaminations of cell lines (Rawadi and Dussurget, 1995). The results obtained with the ELISA kit proved that the test allows a detection of four species only and it is species-specific. The positive reaction of the strain M-32 (*Mycoplasma hyorhinis*) with antibodies to *Acholeplasma laidlawii* was questionable. Since a contamination of the strain was not verified by the restriction fragment length polymorphism analysis of the PCR product, this result was probably caused by an unspecific cross reactivity of this strain with the ELISA test.

Among five cell lines examined (by PCR, ELISA and culture), mycoplasma contamination was detected in two cell lines, i.e. in 40% of them. Contamination rates given by literature sources vary between 5 and 87% (Rawadi and Dussurget, 1995; Garner et al., 2000), hence the contamination rate detected in the present study is close to the average value. Although serum used for culture is a common primary source of contamination with the species *M. arginini* (Garner et al., 2000) and

contamination of the VERO line with that species was recorded, all types of sera presently used in our laboratory, warranted by the manufacturers as mycoplasma-tested, were found to be mycoplasma-negative.

The conventional method of culture is sensitive; however, it is not suitable for a routine assessment of cell line contaminations, particularly because it is time-consuming. The culture is carried out for up to 4 weeks, because four subcultures must follow before a negative conclusion can be drawn (Anonymous, 2002). It is also necessary to use both solid and several types of liquid agars, as requirements for biochemical composition of media, and metabolic activity of various mycoplasma species are different (Razin and Tully, 1995, 1996).

The PCR method is very fast and suitable for assessment of more samples in comparison with the culture method. Internal control included in the reaction ensured the integrity control of PCR used (Uphoff and Drexler, 2002) and detection of potential inhibitory agents. The level of sensitivity of the reaction is also satisfactory. In the present study, 100% congruency between PCR, ELISA and microbiological culture was observed. Also in the previously published data, congruency of the results of the PCR and culture method was described to be 100% (Spaepen et al., 1992; Hopert et al., 1993), or higher than 90% (Van Kuppeveld et al., 1994). The examination of 598 samples from 377 cell lines by the PCR by Uphoff and Drexler (2002) revealed only 1 false-negative result. Fourteen false-positive results detected by Uphoff and Drexler (2002) were most probably caused by residual mycoplasma DNA on the cell line which resisted the decontaminating process.

The sensitivity of the ELISA kit is not very high and is different for different species (variation declared by the manufacturer was 10^4 to 10^7 CFU per ml). However, by its species-specificity, the kit is useful for investigation of contamination sources. Using the kit, we detected that the VERO and RK13 lines were contaminated with *Mycoplasma arginini* and *Mycoplasma orale*, respectively. The primary sources of contamination with *M. arginini* and *M. orale* are sera used for the culture and laboratory personnel, respectively (Garner et al., 2000). A transfer of mycoplasmas from other cultures by using the same media and laboratory equipment is also possible (Drexler and Uphoff, 2000).

The PCR method used in the present study is a fast, sensitive, and specific detection method of mycoplasma contaminations. We recommend it for routine mycoplasma detection in cell cultures and bovine sera.

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