Rapid Detection of Mycoplasma Contamination in Cell Cultures by Enzymatic Detection of Polymerase Chain Reaction (PCR) Products

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ABSTRACT. Enzymatic detection of polymerase chain reaction (ED-PCR) was applied for rapid and easy identification of mycoplasmas from contaminated cell culture. This method was based on the capture of amplified products via biotin-streptavidine affinity and the detection of an incorporated hapten in amplified products with enzyme-linked antibody. Primers corresponding to common sequence of *Mollicutes* in 16S ribosomal RNA dominated gene was used. Nineteen of twenty *Mollicutes* so far reported as cell contaminants appeared positive by ED-PCR, whereas remaining one, *Acholeplasma axanthum*, appeared negative. Samples from sixty-two cell culture were tested for contamination of mycoplasmas by means of ED-PCR, cultivation, and electronmicroscopy. The results of ED-PCR were the same as those of cultivating method. The time required for all the detection process in ED-PCR was about 5 hr for 20 samples. We suggest that ED-PCR can be used in the rapid detection of mycoplasmas from cell culture.—**KEY WORDS:** cell-contaminant, ED-PCR, mycoplasma.

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Mycoplasmas (the trivial name for microorganisms belonging to the class *Mollicutes*) 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. It is not always easy to find mycoplasma contamination in cell cultures because the changes (e.g., turbidity of medium, abnormal pH changes, or cytopathic effects on cell lines) are not apparent.

For the detection of mycoplasma in cell cultures, the cultivating method or deoxyribonucleic acid (DNA) fluorescent methods have most commonly been used [2, 9, 12]. Although the sensitivities of both methods are satisfactory, these methods are cumbersome and time consuming [7]. As an enzyme-linked immunosorbent assay [8] seems to be handling easy, it needs specific antibodies against cell contaminant mycoplasma species. Recently, in order to detect the contaminated mycoplasmas in cell culture more rapidly and simply, the polymerase chain reaction (PCR)-based detection methods have been developed [4, 5]. The sequence of the primers were often selected from 16S ribosomal RNA (rRNA) gene because this gene abounds with genus- and species-specific region. Dussurget and Roulland-Dussoix developed a fast and simple method to detect mycoplasma contamination in simulated samples of animal sera by using genus- and species-specific primers [4]. On the other hand, the 16S-23S rRNA spacer regions in domain cell contaminant mycoplasmas were researched and analyzed by Harasawa *et al.* and Uemori *et al.* [6, 18]. Since from these analyses, Harasawa *et al.* developed a method of detection and identification of mycoplasmas in cell cultures by the PCR and restriction analysis of the 16S-23S rRNA intergenic spacer regions [5]. These methods were possible to detect a small number of mycoplasmas [1 to 10 colony-forming units (CFU)] and to identify by the nested PCR or the restriction analysis of PCR products [4, 5]. However, the main problem of mycoplasma contamination in cell culture should be whether the cell culture is infected

mycoplasmas or not. Then we considered that the detection of mycoplasmas could be performed a time of PCR. Because five mycoplasma species, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinitis*, *M. orale*, and *Acholeplasma laidlawii*, have been known to account for more than 95% of the mycoplasma contamination in cell cultures [1, 3, 11]. Furthermore, for more shortening time, easy handling and development of a diagnostic kit, we employed a new method based on the enzymatic detection of the PCR (ED-PCR) products generated by a pair of primers labeled with either biotin or dinitrophenol on their 5' ends [10, 17]. The sequences of these primers were constructed based on the common sequence in 16S rRNA dominated gene of *Mollicutes* [15]. After PCR, the reaction mixture was applied to a microtiter well pre-coated with streptavidine. Thereafter, bound PCR products were detected colorimetrically with alkaline phosphatase-conjugated anti-dinitrophenol antibody [10, 17].

The procedure of ED-PCR was carried out as follows: 10 μ l of 3- to 7-day cultivated cell-line culture was added to 1.0 ml of phosphate-buffered saline (8 mM Na₂PO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.2) and centrifuged at 15,000 rpm for 5 min. The pellet was suspended in 40 μ l of lysis solution containing 110 mM Tris-HCl (pH 8.9), 1.5 mM MgCl₂, 80 mM KCl, 0.05% of bovine serum albumin (BSA), 0.1% sodium cholate, 0.1% Triton X-100, 200 μ g/ml of proteinase K, 0.45% Tween 20, and 0.45% Noindet P-40. Prior to subject for PCR, the mixture was incubated at 60°C for 10 min and subsequently at 94°C for 5 min. Forty μ l of the mixture was used as template for PCR. PCR amplification was performed in a 50 μ l reaction mixture containing 110 mM Tris-HCl (pH 8.9), 1.5 mM MgCl₂, 80 mM KCl, 0.05% of BSA, 0.1% Triton X-100, 200 pM each of the oligonucleotide primers, 1 mM each of the four dNTP, and 1 unit of *Tth* DNA polymerase (Toyobo Co., Ltd., Osaka). The oligonucleotide primers were designed from 16S ribosomal RNA gene and that of spacer region between 16S and 23S rRNA genes in *M. arginini* or *M. orale*. The sequence

used were as follows; 5'-CGCTGCGGTGAATACGTTCT-3', and the reverse complement of 19-mer was 5'-CCCCACGTTCTCGTAGGGA-3'. The reaction mixture was overlaid with paraffin oil and subject to 30 cycles of amplification in a programmable heating block (Hoei Science, Tokyo). The program used was as follows: 94°C, 30 sec for denaturation, 50°C, 30 sec for annealing, and 72°C, 60 sec for extension. After amplification, 10 μ l of the amplified mixture was transferred to a streptavidin-conjugated microtiter well followed by reacting with 100 μ l of alkaline phosphatase-conjugated anti-dinitrophenol antibody. The optical density (OD) of the mixture was determined at wave length of 405 nm after the mixture was left at room temperature for 30 min, or when the positive control showed an OD higher than 0.500. Microtiter wells with OD more than 0.150 were considered to be positive for mycoplasma contamination. The detection limit in this assay was at 200 CFU and 100 CFU per tube for *M. arginini* and *M. orale*, respectively.

The cultivating method was carried out as follows: Ten-fold dilution of cell culture samples were incubated on mucin agar plates [19], and in arginine broth [19] and mucin broth [19]. These media, except mucin broth, were incubated at 37°C for 2 weeks in duplicate in 5% CO₂ and air, and anaerobic condition. Mucin broth was incubated aerobically for 2 weeks. Broth media were used for propagation of cell contaminant mycoplasmas and agar medium was used for viable titer, cloning of isolate and observation of the colony morphology.

Until now, 20 species of *Mollicutes* have been reported as cell culture contaminants [1, 3]. Our preliminary examinations showed that 19 of them could be detected by

ED-PCR, the remaining one was *Acholeplasma axanthum*. The prevalence of this organism in cell culture has reported to be less than 0.1% in all isolates [3].

Sixty-two cell lines were examined for contamination by means of ED-PCR, cultivation, and electronmicroscopy [13]. The results were summarized in Table 1. Mycoplasmas were detected in 14 cultures of 62 test samples by both ED-PCR and cultivation. The contaminated mycoplasmas were identified as either *M. orale* or *M. fermentans* by metabolism-inhibition test [16]. Numbers of mycoplasmas detected were determined to be between 5×10^4 to $4 \times$

Table 1. Detection of mycoplasma contamination in cell cultures by enzymatic detection of polymerase chain reaction (ED-PCR), cultivation and electron microscopy

No. of cases	Detection by		
	ED-PCR	cultivation	electron microscopy
14	+	+ ^{a)}	ND ^{b)}
1	+ ^{c)}	—	+
1	—	+ ^{d)}	ND
1	—	—	+ ^{e)}
45	—	—	—

a) Mycoplasma species were identified to be *M. orale* and *M. fermentans*. The viable titers of these contaminants in the cell cultures ranged from 5×10^4 to 4×10^8 CFU/ml.

b) ND: Not done.

c) The contaminant could not be identified.

d) The contaminant was identified to be *M. orale* (less than 10^2 CFU/ml).

e) Unknown *Mollicutes*-like organisms were observed by electron microscopy (see Fig. 1).

Fig. 1. Electron micrograph of *Mollicutes*-like organisms in a 5-day-old cell culture. This hybridoma cell has already been broken. Bar represents 1 μ m.

10^8 CFU per ml of cell culture. Forty-five samples were proved to be negative for mycoplasma contamination by any of the methods employed. The contradictory results were obtained for remaining three samples according to the methods employed. One sample was positive by cultivation but negative by ED-PCR, from which *M. orale* was isolated with low titer of less than 100 CFU/ml of the cell culture fluid. This suggests the importance of timing of sampling, especially in case of slow growing mycoplasmas are contaminated. It may be preferable to submit for examination when test cells begins to come off the tubs. Another sample (cell name: 2D-14, IE gene of Aujeszky virus were transformed to PK-15 cell) was negative by cultivation but positive by both ED-PCR and electron microscopy, suggesting that the non-cultivable mycoplasmas were contaminated and ED-PCR could detect some of the non-cultivable mycoplasmas. The other sample (hybridoma cell) was negative by both cultivation and ED-PCR but positive by electron microscopy (Fig. 1).

In conclusion, the results of detecting mycoplasmas from cell cultures by ED-PCR were in good agreement with that of cultivation method. The time required for all the detection process in ED-PCR was about 5 hr for 20 samples. Since ED method was employed instead of agarose gel electrophoresis, much more samples could be treated in a short time. If an ELISA reader is not available, it is possible to read the results by the naked eye. From the results obtained here, we consider that ED-PCR would be useful for rapid detection of cell culture contaminating mycoplasmas.

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