Detection of mycoplasma contamination in cell cultures

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Abstract

Mycoplasma contamination is one of the most common contaminants that can cause serious problems in cell cultures. Mycoplasma is the smallest self-replicating prokaryote characterized by lack of cell wall. Due to their small size and lack of cell wall, mycoplasma can pass through the filters regularly used in the laboratory and cannot be seen under conventional microscope. Cultures contaminated with mycoplasma do not usually show obvious signs of contamination such as medium turbidity. Therefore, mycoplasma may persist in cultured cells indefinitely and spread to other cultures. Mycoplasma contamination can cause various effects on cultured cells such as change in growth rate and morphology, alteration of gene expression, and modulation of cytokine production. For these reasons, mycoplasma contamination should be routinely monitored in the laboratory. Currently, there are many detection methods available such as microbiological culture, DNA staining, PCR, and biochemical assays. All detection methods have their advantages and disadvantages in terms of reliability, specificity, sensitivity, simplicity, time, and cost. It is recommended that combination of at least 2 methods should be performed to detect mycoplasma contamination.

***Keywords**: cell culture; detection method; mycoplasma contamination*

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Introduction

Microbial contamination is one of the major problems in cell culture. The consequences of contamination vary from minor disturbances to serious issues such as loss of valuable data and undermining validity of research. Among the major causative agents, bacteria, fungi, and mycoplasmas are the most common contaminants.[1, 2] The bacterial and fungal contaminations can be readily detected by direct visual or microscopic observation. Those contaminations may cause turbidity and/or pH shift of cultured medium, as well as cell destruction. Unlike bacteria and fungi, mycoplasmas may not cause any obvious signs of contamination. Mycoplasma-contaminated cultures do not usually show major cell damage. Therefore, mycoplasma contamination may persist in the cultured cells over a long period of time and be transferred to other cultures, causing spread of contamination. In chronic contamination, cultured cells may demonstrate alterations in cell behaviors such as slow proliferation rate and reduced saturation density. Previous studies have reported that 1% of primary cell cultures are contaminated with mycoplasmas, whereas continuous cell lines are more frequently contaminated in the range of 15-35%.[1] These contaminations can pose a significant problem on cell culture-related research.

Mycoplasma biology

Mycoplasmas belong to the class *Mollicutes* (*mollis*, soft; *cutis*, skin), a group of specialized bacteria characterized by absence of rigid cell wall. The terms mycoplasmas and mollicutes have been used interchangeably. The class *Mollicutes* is classified into several orders including *Mycoplasmatales*, *Entomoplasmatales*, and *Acholeplasmatales*. Mycoplasma-contaminated culture was first reported in 1956.[3] To date, more than 190 species of mycoplasmas have been identified.[4] Most of the recognized species belong to the family *Mycoplasmataceae*, which comprises the genus *Mycoplasma* and *Ureaplasma.* [5] Among the existing species, more than 20 species have been found contaminated in the cell lines. Theses wall-less bacteria are ubiquitously found in human, mammals, reptiles, fish, and plants. They have small genome size (0.58-2.20 Mb), rendering limited metabolic activities.[6] As a consequence, most mycoplasmas are parasites that typically display host and tissue specificity.[6] Mycoplasma is the smallest self-replicating prokaryote with diameter ranging from 0.3-0.8 μm, which is bounded by a triple-layered membrane.[4, 7] They have a wide variety of shapes, e.g. spherical, pear-shaped, flask-shaped cells, as well as filaments.[4] Their minute size and flexibility enable mycoplasma to pass through 0.22- and 0.45-μm filters regularly used in cell culture laboratories. Since these microorganisms have no cell walls, mycoplasmas are resistant to penicillin, which targets cell-wall synthesis. Furthermore, it has been reported that some strains of mycoplasmas are also resistant to streptomycin, the antibiotic commonly used in cell cultures.[7] Mycoplasmas usually have long generation times ranging from 1-9 h and relatively long lag phase.[1] Therefore, mycoplasma culture on agar plate may take longer than one week to obtain visible colonies. Once contaminated, the mycoplasma can grow up to 10^8 CFU/ml without medium turbidity.[8] It was estimated that each infected cell contains 100-1000 mycoplasmas adhered to the cell.[1] Due to their small size and lack of cell wall, mycoplasmas cannot be observed under conventional microscope. The cryptic contamination can cause subtle but significant impact on cell behaviors.

Source of mycoplasma contamination comes from individuals or culture medium components such as animal sera. Most of mycoplasma contaminations in cell cultures are caused by various strains found in human, bovine, and swine; those are *M. orale, M. fermantans, M. hominis, M. arginini, Acholeplasma laidlawii, M. hyorhinis.* [9-12] *M. orale,* usually found in human oropharyngeal tract, accounted for 20-40% of all mycoplasma contaminations, while culture contaminated with *M. arginini* was in the range of 20-30%.[1] *M. orale, M. fermantans, M. hominis* contaminations are primarily found associated with the person who handles the cell cultures, whereas the main sources of contamination with *M. arginini, M. bovis, A. laidlawii, M. hyorhinis* are usually found in animal tissues in primary cell culture and animal-derived components such as fetal calf serum and trypsin.[13] Mycoplasma-infected cells are also another major source of contamination. The route of spreading contamination includes droplet dispersion during handling cell cultures. In addition, the prolonged survival of dried mycoplasmas enables spreading contamination to other cultures easily. One of the major virulence factors of mycoplasmas is their adherence to host tissues for colonization and infection. Many mycoplasmas attach to the surface of host cells, whereas several strains such as *M. penetrans* and *M. hominis* can enter the cells.[5] Intracellular localization provides protection against the immune system and the action of antibiotics.[4] Proposed mechanisms of damage to host cells include competition for precursors in the biosynthesis of macromolecules, interference with membrane receptor, alteration of membrane transport mechanisms.[6] It has been reported that mycoplasmas can cause various effects on cells such as change in growth rate and morphology, alteration in membrane composition, chromosomal aberrations, disturbance in the amino acid and nucleic acid metabolism, change of gene expression, and modulation of cytokine production.[14-16] Due to the hidden contamination and the insidious effects on cultured cells, mycoplasma testing is recommended to regularly perform to maintain mycoplasma-free cultures.[17]

Mycoplasma detection methods

There have been various methods developed to detect mycoplasma contamination in cell cultures. Those methods include microbiological culture, microscopy observation, nucleic acid assay (e.g. PCR-based methods, DNA/RNA hybridization), immunological assays such as Enzyme-Linked Immunosorbent Assay (ELISA) and immunofluorescence, and biochemical assays. The microbiological culture was traditionally referred to as the direct method, whereas other methods that measure gene product or metabolic activities were regarded as indirect methods. Each method has different strengths and limitations (Table 1). It is recommended that the sample should be tested by at least two methods to validate the result.[8] In addition, cultured cells should be grown in antibiotic-free medium prior to mycoplasma testing for at least 3 subcultures or 2 weeks.[8]

Table 1 Advantages and disadvantages of mycoplasma detection methods

Method	Advantages	Disadvantages
Microbiological culture	Highly sensitive	Time consuming, require expertise
DNA staining	Fast, simple	Low sensitivity, difficult to interpret if low-level contamination, more time consuming if culture with indicator cell line
PCR	Fast, highly sensitive, can discriminate between viable and non-viable mycoplasma	Requires optimization
DNA/RNA hybridization	Moderate sensitivity	Requires optimization
ELISA	Fast, moderate sensitivity	Narrow range of species detected

Microbiological culture

Microbiological culture is one of the most conventional assays, yet still officially recommended by several international institutes such as United States Pharmacopeia (USP) and European Pharmacopeia (EP).[15] The cultured cells are inoculated into broth for 4-7 days and then plated onto a special nutrient agar (e.g. pleuropneumonia-like organisms; PPLO) in an aerobic and anaerobic condition. Anaerobic incubation is more preferable due to its higher detection rate. The agar plate is subsequently incubated for at least 14 days. After incubated, the colonies will appear as characteristic fried-egg morphology with diameter of 100-400 μm.

The advantage of this technique is its high sensitivity. However, this technique requires special growth conditions and can take approximately 28 days to obtain the result. In addition, it is essential to include culture of live mycoplasma as a positive control. Therefore, this technique should be performed by experienced personnel in an isolated facility. It should be noted that there are some strains of mycoplasmas (e.g. fastidious strain of *M. hyorhinis)* that are non-cultivable on standard agar or broth-culture media, thereby remaining undetected by this method.[1, 18] Due to its long testing time, this method is unfit when results are needed rapidly. Other disadvantage of this method is the batch-to-batch variation of the media components and media preparation, which can significantly affect the growth of mycoplasma.[19]

Fluorescent DNA staining

The principle of this method is based on the specific binding of fluorescent dye to DNA. The cultured cells can be fixed and directly stained by fluorescent dye such as Hoechst 33258 and DAPI (4', 6-diamidino-2-phenylindole). Upon staining, mycoplasma DNA appears as fine particulate or filaments of fluorescence on the cell surface and/or nearby the cells in case of heavy contamination (Figure 1). Bacterial and fungal contamination can be detected as well. An indicator cell free of mycoplasma (e.g. mouse

embryo fibroblast 3T6, Vero cell) is recommended to include in the protocol to support the adherence and growth of mycoplasma. Basically, the test sample is added to the indicator cells grown on cover slip and incubated for 3-5 days. The culture is then fixed, stained, and examined by fluorescence microscope. Co-culture with indicator cell line enhances the mycoplasma proliferation, thus increasing the sensitivity. The indicator cell assay, also referred to as the indirect fluorescent staining, is recommended by EP and USP for screening of mycoplasma contamination. In combination with the microbiological culture, these 2 assays are considered as the gold standard approach for detection of mycoplasma contamination.[19] This technique alone is simple and rapid, giving results within 1-3 days. It requires mycoplasma at a minimum of 10^6 CFU/ml to produce obvious positive result. [14] Due to its low specificity, the fluorescent DNA staining should be done in conjunction with other mycoplasma-specific techniques.

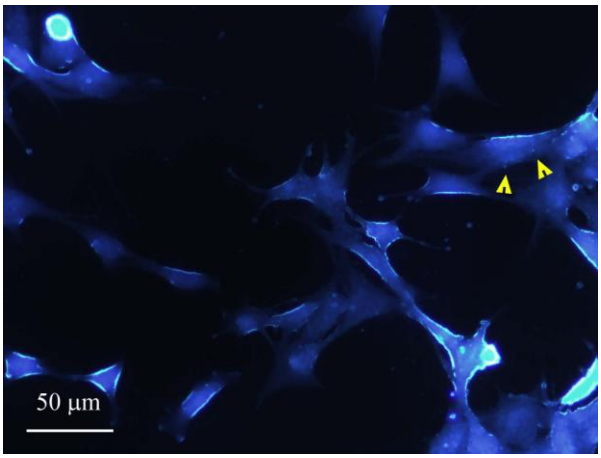

Figure 1 Photomicrograph of 3T6 cells stained with Hoechst 33258. Arrowheads indicate small dots corresponding to mycoplasmas. Scale bar = 50 µm.

PCR-based methods

The polymerase chain reaction (PCR) assay is a powerful tool widely used in many areas of molecular biology. The PCR-based methods commonly used to detect mycoplasmas include endpoint, nested, and real-time PCR. The PCR reaction can either perform with mycoplasmal DNA or cDNA of rRNA synthesized by enzyme reverse transcriptase. These methods are highly specific, highly sensitive, rapid, and reliable. [9, 18, 20, 21] It has been shown that these methods can detect as low as single organism[22] and one copy per µl of sample.[18] Currently, PCR is considered as the most sensitive detection method. [1] Reverse transcription-polymerase chain reaction (RT-PCR) was shown to have comparable sensitivity with the conventional microbiological culture method. [23] However, RT-PCR is more time-consuming compared to other PCR that can readily detect the mycoplasmal DNA. RT-PCR is more suitable when the detection is aimed at live mycoplasma cells after eradication treatment. The conserved regions of 16S rDNA or rRNA are frequently used as the target sequences. In addition, the spacer

region between 16S and 23S rDNA has been reported to be a highly specific sequence to identify mycoplasmas.[24, 25] Primer design is one of the important parameters in the procedure. To detect a wide range of mycoplasma species, the highly conserved sequences are usually targeted to amplify. Identification of mycoplasma species is also feasible using this method. [26] This is useful when source of contamination is needed to determine. It should be noted that there are sequence homologies between *Mollicutes* spp. and *Chlamydia* spp., which can lead to false-positive results. [27]

DNA/RNA hybridization

DNA/RNA hybridization is based on molecular hybridization of complementary oligonucleotide probes to mycoplasmal DNA/RNA. The labeled oligonucleotide probes are usually targeted to the 16S rRNA or 23S rRNA sequences. Positive signals are developed in the proportion to the amount of mycoplasma rRNA and are monitored using a colorimetric plate reader. This method was shown to be very sensitive with the detection limit at 1 ng of mycoplasmal DNA, which is equivalent to approximately 10^5 CFU of mycoplasmas.[28]

ELISA

ELISA is based on binding of the mycoplasma-specific monoclonal or polyclonal antibodies to specific antigen. The secondary antibody is usually conjugated to system that can produce colorimetric readout such as horseradish peroxidase enzyme. The species-specific identification can also be performed by this method to distinguish different strains of mycoplasmas. Broad-range and species-specific mycoplasma antibodies are commercially available. The sensitivity of the ELISA kit declared by the manufacturer was in the range of 10^4-10^7 CFU/ml. [13] Although ELISA was previously reported to have high sensitivity, several studies demonstrated that PCR is far more sensitive than ELISA. [18, 20-22]

Biochemical methods

Biochemical methods are based on detection the metabolic activities such as the enzyme activities (e.g. arginine deiminase, thymidine, uridine, adenosine or pyrimidine nucleoside phosphorylase, hypoxanthine or uracil phosphoribosyl transferase). The enzyme adenosine phosphorylase is commonly found in mycoplasmas but is undetectable in mammalian cells.[29] The adenosine phosphorylase assay has been shown to have low specificity because other bacteria such as *Bacillus subtilis* and *Escherichia coli* can produce adenosine phosphorylase as well. Furthermore, some strains of mycoplasmas such as *M. pneumoniae* do not possess adenosine phosphorylase, thereby being undetected by this assay. [30] Other modified method is based on cytotoxicity generated by adenosine phosphorylase and 6-methylpurine deoxyriboside (6-MPDR), an analog of adenosine. Degradation of 6-MPDR by adenosine phosphorylase produces 6-methylpurine and 6-methylpurine riboside, which are toxic to mammalian cells. However, this method was reported to yield false-negative results.[31] In low level contamination, it is difficult to detect mycoplasma by these methods since it depends on arbitrary values to obtain the positive results. Recently, a test based on degradation of the *Gaussia*

luciferase reporter in the conditioned medium has been reported to be more sensitive to monitor the mycoplasma contamination.[32]

In summary, mycoplasma contamination is commonly found in cell cultures and can cause serious problems in biomedical research. It is recommended that cell cultures should be routinely screened for mycoplasma contamination. There are many detection methods including microbiological culture, DNA staining, and PCR. Combination of microbiological culture and indicator cell assay is regarded as the gold standard approach for detection of mycoplasma contamination. Other alternatives include PCR-based methods and biochemical assays. All detection methods have their advantages and disadvantages in terms of reliability, specificity, sensitivity, simplicity, time, and cost. Given that all available detection methods have some limitations, testing of mycoplasma contamination should not be performed by a single technique.[31, 33]

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การตรวจสอบการปนเปื้อนของเชื้อไมโคพลาสมาในเซลล์เพาะเลี้ยง

ศิษฎา ตันนุกิจ สุปรียา วาณิชย์ปกรณ์**

บทคัดย่อ

การปนเปื้อนไมโคพลาสมาเป็นปัญหาหลักที่พบบ่อยในเซลล์เพาะเลี้ยง เชื้อไมโคพลาสมาเป็นโปรคารีโอตที่มีขนาดเล็กที่สุดที่สามารถเพิ่มจำนวนได้เองโดยไม่ต้องพึ่งพาโฮสต์ มีลักษณะเฉพาะคือไม่มีผนังเซลล์ ไมโคพลาสมาสามารถถูกกรองผ่านแผ่นกรองที่ใช้ในการเพาะเลี้ยงเซลล์ในการทำให้ปราศจากเชื้อและไม่สามารถมองเห็นได้ผ่านกล้องจุลทรรศน์ทั่วไป เนื่องจากไมโคพลาสมามีขนาดเล็กมากและไม่มีผนังเซลล์ โดยทั่วไปเซลล์เพาะเลี้ยงที่ปนเปื้อนเชื้อไมโคพลาสมาจะไม่แสดงลักษณะให้เห็นเด่นชัดว่ามีการปนเปื้อน เช่น อาหารเลี้ยงเซลล์ขุ่น ดังนั้น เชื้อไมโคพลาสมาที่ปนเปื้อนในเซลล์เพาะเลี้ยงจะสามารถคงอยู่ได้เป็นระยะเวลานานและแพร่กระจายไปยังเซลล์เพาะเลี้ยงอื่นๆ การปนเปื้อนเชื้อไมโคพลาสมาส่งผลต่อเซลล์เพาะเลี้ยงได้หลากหลาย เช่น มีการเปลี่ยนแปลงของอัตราการเจริญเติบโตและรูปร่างของเซลล์ การเปลี่ยนแปลงการแสดงออกของยีนและการผลิตไซโตไคน์ เป็นต้น ด้วยเหตุที่กล่าวมาข้างต้นการตรวจสอบการปนเปื้อนไมโคพลาสมาในเซลล์เพาะเลี้ยงจึงควรทำเป็นประจำในห้องปฏิบัติการ ปัจจุบันมีวิธีการตรวจสอบหลายวิธี เช่น การเพาะเลี้ยงทางจุลชีววิทยา การย้อมดีเอ็นเอ เทคนิคปฏิกิริยาลูกโซ่โพลีเมอเรส และวิธีทางชีวเคมี แต่ละวิธีมีข้อดีข้อเสียแตกต่างกันไปในด้านของความเชื่อมั่น ความจำเพาะ ความไว ความยากง่าย ระยะเวลาที่ใช้และค่าใช้จ่าย ในการตรวจสอบการปนเปื้อนเชื้อไมโคพลาสมาจึงควรเลือกใช้วิธีการตรวจสอบอย่างน้อย 2 วิธีควบคู่กันเพื่อให้ได้ผลที่ถูกต้องและแม่นยำ

คำสำคัญ: *การเพาะเลี้ยงเซลล์; วิธีการตรวจสอบ; การปนเปื้อนไมโคพลาสมา*

**ภาควิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่*