2.A1 Testing for the Presence of Mycoplasma in Stock Cell Cultures Appendix 1

K. McMenamin-Snekvik
C. Teitzel

Washington Animal Disease Diagnostic Laboratory
College of Veterinary Medicine
Washington State University
Pullman, WA 99164
509/335-9696

A. Introduction

The contamination of cell cultures by mycoplasma organisms remains a major problem for laboratories using cell culture assays for virus isolation. *Mycoplasma* spp. can produce a virtually unlimited variety of effects in the cell cultures they infect including changes in cell proliferation, metabolism, decreased viability and adhesion properties. The severity of these effects depend on the mycoplasma species, the infected cell line and the culture conditions. Mycoplasma organisms are small, 0.3-0.8μm in diameter, and cannot be detected by visual inspection or with routine light microscopy and conventional staining methods (i.e. Gram stain). In addition, the small size and flexibility of their cell membrane allow mycoplasma organisms to pass through commonly used anti-bacteriological filters with diameters of 0.45um. Unfortunately, due to the aforementioned reasons, mycoplasma-infected cell cultures may remain unnoticed for long periods interfering with accurate interpretation of biological results.

Treatment of infected cell cultures is difficult as mycoplasma organisms are often resistant to most antibiotics commonly employed in cell cultures. Although there are treatment options for contaminated cell cultures, many are complex, time consuming or labor intensive. Thus, if possible, contaminated cell cultures should be discarded.

Sources of mycoplasma contamination include infected cell cultures brought into the laboratory, contaminated media and reagents used to maintain cell stocks, and personnel handling the cell cultures. All cell cultures should be mycoplasma screened prior to routine use in the laboratory and at approximately 3 month intervals to monitor for possible mycoplasma introduction.

To reduce the possibility of mycoplasma contamination, culture procedures should be performed in a laminar flow biosafety cabinet or clean room reserved for sterile cell culture work. All work surfaces should be disinfected before initiating cell culture procedures, between cell lines and after completion of cell culture work. Supplies, reagents, and equipment used for cell culture should be sourced from reputable and

reliable vendors and kept separate from other laboratory operations. Personnel working with cell cultures should employ strict aseptic techniques.

There are various detection methods used to identify mycoplasma in cell cultures, with the two most common being microbiological culture and detection by PCR. PCR can be sensitive, specific, fast, and reliable, although it is expensive compared to other methods. PCR protocols utilizing primer sequences for mycoplasma detection have been published, or several complete commercial test kits are also available (e.g. Sigma-Aldrich, ATCC). Although PCR offers a fast and simple technique, the high sensitivity of this method makes it susceptible to contaminants and can produce false positive as well as false negative results if performed incorrectly. The microbiological culture method is sensitive and detects viable mycoplasma contaminants, although it is more time consuming. Here we will include the microbiological culture technique commonly used in the laboratory.

B. Principle/Purpose:

To detect possible mycoplasma infection of all cell lines. Mycoplasma infection may go undetected for many passages, causing a variety of unpredictable effects and leading to disruption of the host cell function. Mycoplasma infection may also influence the sensitivity of host cells for growth of viruses.

C. Specimen:

All cell lines currently in use for certification and diagnostic purposes.

D. Materials:

1 blood agar plate
1 mycoplasma agar plate
1 mycoplasma broth tube
Sterile Dacron swabs with plastic shafts (wood or aluminum may inhibit growth)
Plastic loops
Plastic transfer pipettes
CO_2 incubator
Dissecting microscope
Dienes stained coverslips
Digitonin discs

E. Procedure:

1. Over two consecutive weeks, split one $25cm^2$ flask of each cell line to be tested. Follow cell maintenance and amplification procedures in all steps except for when preparing media—omit all antibiotics from media formulations. When performing this test, use the flasks with the most recent split date in media without antibiotics.
2. Swab the inside of the first flask with a Dacron swab, making sure to pick up cells as well as media.
3. Swab approximately one-third of the mycoplasma plate. Using a second swab, streak for isolation/growth.

4. Using a plastic transfer pipette, draw up just over 0.5ml of the contents of the flask. Dispense a drop of this suspension onto the blood agar plate, and dispense the remaining suspension into the mycoplasma broth tube.
5. Streak for isolation on the blood agar plate using a plastic loop.
6. Repeat steps 1- 5 for all cell lines to be tested.
7. Incubate the tubes and plates in a 35°C CO_2 incubator.
8. After 72 hours of incubation read mycoplasma plates using dissecting microscope for typical colonies, usually small (50-500μm diameter), fried-egg-like with an opaque, granular central zone embedded in the agar and a flat translucent peripheral zone on the agar surface. Colony appearance is dependent on many factors related to the media and incubation conditions and some mycoplasma isolates do not form the typical colony morphology. Also read blood agar plates at this time, and if no colonies grow along the streak lines consider the samples to be negative for other bacterial pathogens and discard the blood agar plates.
9. After 72 hours if the mycoplasma plate does not show mycoplasma growth, perform a subculture by saturating a Dacron swab with the mycoplasma broth and restreaking the original mycoplasma plate along the original streak lines. Re-incubate plate and broth in the same 35°C CO_2 incubator. Read mycoplasma plates for 14 days. If no mycoplasma colonies are visible by 14 days, discard plates and broth tubes and consider cell line to be negative for mycoplasma infection. Record negative results of the mycoplasma screening.
10. If colonies with typical morphology are present subculture to a new plate and place a digitonin disc on the plate with sterile forceps. Incubate 24-48 hours and read for a zone of inhibition.
11. *Mycoplasma* spp. show a zone of inhibition of 10 mm or greater. Zone sizes of less than 10 mm are consistent with *Acholeplasma* spp. rather than *Mycoplasma* spp. Bacterial isolates show no zone of inhibition.
12. Suspect colonies should be tested with Dienes stain. Drop a coverslip coated with a layer of Dienes stain onto the colonies on the plate. Mycoplasma colonies stain blue and retain the stain for more than 10 minutes, while bacterial colonies appear colorless or lose the stain quickly because of rapid reduction of the methylene blue.
13. Mycoplasma organisms may also be identified using 16s rDNA PCR and sequencing, or may be sent to a reference laboratory for identification.

Additional References

Cumitech 19. 1984. Laboratory Diagnosis of Chlamidial & Mycoplasmal Infections. ASM. Washington, D.C. Aug.

Drexler, H.G. and C.C. Uphoff. 2002. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology 39:75-90.

Hargreaves, F.D. and L.H. Leach. 1970. The Influence of Mycoplasma Infection on the Sensitivity of HeLa Cells for Growth of Viruses. J Med Microbiol. May; 3:259-65.

Koneman, E.W., S.D. Allen, W.M. Janda, P.C. Schreckenberger, and W.C. Winn. 1992. Color Atlas & Textbook of Diagnostic Microbiology, 4th Edition. J.B. Lippincott & Company. pp. 675-697.

Nikfarjam, L. and P. Farzaneh. 2012. Prevention and Detection of Mycoplasma Contamination in Cell Culture. Cell Journal 13:203-212.

Razin, S. and J. Tully. 1983. Methods in Mycoplasma, Vol.I. Academic Press. pp. 103-107, 83-87, 356-357.