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SPREAD AND CONTROL OF MYCOPLASMAL INFECTION OF CELL CULTURES

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SUMMARY

Environmental sampling was performed during trypsinization and passage of 3T-6 cell cultures that contained a mean of 4.3×10^7 colony forming units (CFU) per ml supernatant of *A. laidlawii*. The lip of the culture flask and the outside of the used pipet were always heavily contaminated. The outside of the culture flask (3/7), the work surface (8/12) and the outside of a pan of disinfectant (4/5) were regularly contaminated with mycoplasmas. Airborne mycoplasmas were detected eight of 32 times (25%) by settling plates; simultaneous forced-air samplers by two different methods were always negative. The technician's hands were contaminated two of 15 samples. When hands were contaminated, more contamination was detected in the environment. Droplets of *A. laidlawii* and *M. orale* inoculated onto work surfaces survived drying for a minimum of 3 days, even in laminar airflow cabinets. Twenty-five of 31 (80.6%) cell culture technicians carried *M. salivarium* in their throats; only two carried *M. orale*. It is concluded that mycoplasma-infected cultures are the most common source of further infection. Recommendations for prevention and control of mycoplasmal infection are listed.

Key words: mycoplasma; contamination; environmental sampling.

INTRODUCTION

Despite many advances in media, procedures and supplies, mycoplasmal infection of cell cultures remains a major problem. In this laboratory, 290 of 3,872 cultures assayed (7.4%) contained mycoplasma during a 5-year period (*unpublished data*). Barile and co-workers reported that 99% of 1,374 mycoplasmas isolated from cell cultures were either human, bovine or swine species (1). The modes of transmission of mycoplasmal infection of cell cultures are unclear although bovine serum (2), trypsin (3) and technicians (1) are proven or suggested sources. These primary sources of infection cannot always explain the appearance of mycoplasmas in laboratories where changes in supplies, techniques or personnel have not occurred.

In our assays it has been noted that if one cell culture in a laboratory contains mycoplasma, most, perhaps all, cultures in that laboratory are infected with the same mycoplasmal species. Retrospective analysis has shown that several laboratories had their first mycoplasmal isolation after receiving cultures from laboratories with a history of mycoplasmal infection. The relative importance of primary source of infection (serum, trypsin, technicians, etc.) versus secondary source (other infected cultures) is unclear. O'Connell and co-workers failed to recover mycoplasmas from the air during work with infected cultures, but believed mycoplasmal infection spread via aerosols (4). The purpose of this communication is to document the relative importance of other infected cultures and personnel and offer effective preventive and control measures.

MATERIALS AND METHODS

Media. Mycoplasma media consisted of 75 parts brain-heart infusion, 20 parts select horse serum, and 5 parts yeast extract. Supplements included 0.5% glucose, 0.1% l-arginine hydrochloride, 0.002% DNA and 0.002% phenol red. Noble agar, 0.9%, was added for solid media. The final pH was 7.2 to 7.4. Incubation was at 37°C under aerobic and anaerobic atmospheres (GasPak, Bioquest, Cockeysville, Md.) (5).

Environmental samples. Samples of the air, the technician's hands and the environment were obtained before, during and after work with 3T-6 mouse fibroblast cell cultures inoculated with approximately 10^5 colony forming units (CFU) of *A. laidlawii*. The cell culture was passed twice

weekly in Hanks'-Eagle's media with 10% fetal bovine serum (FBS) without antibiotics. The samples were obtained during trypsinization and passage of the cell culture during a 15-week study period to determine if mycoplasmal contamination of the environment occurred by aerosols and/or droplets generated during these procedures. Samples of hands and flat surfaces were obtained with Rodac plates (Bioquest, Inc., Cockeysville, Md.) containing 16.5 ml mycoplasma agar per plate. Air samples were obtained with both agar sedimentation plates and forced-air samplers. Sedimentation plates (100 by 15 mm) were placed in the immediate area where the infected cell cultures were being trypsinized and passed. Simultaneous air samples were obtained with either Andersen samplers (Anderson 2000 Inc., Salt Lake City, Utah) which monitor 1 ft^3 air per min onto a series of six agar plates, or all glass impingers (AGI-4, Ace Glass Co., Vineland, N.J.) which monitor 0.44 ft^3 air per min into 22 ml mycoplasma broth containing 0.05 ml Antifoam Emulsion (Dow Corning Co., Midland, Mich.). Mycoplasma media used in environmental studies contained penicillin (1,000 units per ml) and streptomycin (100 μg per ml).

Throat swabs. Throat swabs of cell culture personnel were inoculated onto mycoplasma agar containing penicillin (1,000 units per ml), streptomycin (100 μg per ml) and thallium acetate (0.1%). This medium was also used to sample oral droplets generated by personnel during talking and sneezing. Mycoplasmal isolates were identified by the growth inhibition test (6).

RESULTS

Environmental sampling. 3T-6 cultures containing a mean of 4.3×10^7 CFU per ml of *A. laidlawii* of cell culture fluid (ā of 10 tests) were used to study dissemination of mycoplasma in a laboratory. Results of relevant environmental samples are summarized in Table 1.

The lip of the opened plastic culture flask was heavily contaminated immediately after the cap was removed in all 20 samples. The tip and bottom outside surface of the used pipet were heavily contaminated. Most other areas sampled were only occasionally positive. The hands of the technician were contaminated after work two of 15 times. When the hands were contaminated, more contamination was detected in the environment; on these occasions mycoplasmas were also detected on the propipet, the cap of the flask, and the outside of the flask. Mycoplasma was never detected on the technician's hands before work.

TABLE 1

ENVIRONMENTAL SAMPLING FOR MYCOPLASMA[a]

Sample Site	No. Positive/ No. Tested	ā CFU[b] (range)
Lip of culture flask	20/20	TN[c]
Used pipet	10/10	TN
Outside of flask	3/7	250 (4-TN)
Cap of flask	1/1	6
Hands	2/15	TN
Droplet on work surface	2/2	TN
Work surface	8/12	7 (1-22)
Propipet	1/7	150
Used disinfectant solution	0/10	0
Outside of disinfectant pan	4/4	157 (7-TN)
Outside of used pipet	1/1	110
Glassware submerged in disinfectant	1/11	75
Glassware partially submerged in disinfectant	2/2	185 (70-TN)
Trypan blue + infected cells	2/2	TN
Used hemacytometer	2/2	TN
Tube containing culture	4/4	65 (10-200)
Settling plates	8/32	85 (1-TN)
Air samples[d]	0/11	0

[a]Samples were taken before, during and after trypsinization and passage of 3T-6 mouse fibroblast cell cultures infected with *A. laidlawii*, 4.3×10^7 CFU/ml (ā of 10 tests). Samples obtained with Rodac and settling plates over a 15-week study period.

[b]Average CFU/plate of positive samples.

[c]Greater than 200 CFU/Rodac plate, 300 CFU/settling plate.

[d]Obtained with Anderson and all glass impinger (AGI-4) samplers.

The disinfectant solution used for discard of used glassware was always negative, but the lip of the disinfectant pan was frequently contaminated. Observations demonstrated that used, contaminated pipets were generally slid across the lip of the pan and into the disinfectant solution. Contamination of the lip probably occurred during this contact. Glassware that was submerged in disinfectant yielded mycoplasma on only one occasion—from the inside of a 1.0-ml pipet. Glassware that was only partially submerged yielded large numbers of organisms from unsubmerged surfaces. Rodac samples of the dried work surface taken at random sites were frequently positive. On two occasions, droplets were observed on the work surfaces; samples of these yielded confluent mycoplasmal growth.

A laidlawii was never recovered from the air by either Andersen samplers or AGI-4 impingers. Shortening the sampling time with the Andersen samplers to minimize drying of the agar also

failed to detect airborne mycoplasma. Inoculation of *A. laidlawii* into the sampling media used in the impingers showed that the media had no detrimental effect on the organism. The concentration of antifoam compound used in this medium had no effect on broth grown *A. laidlawii*.

Settling plates in the immediate work area detected mycoplasma 25% of the time, 8/32. The number of CFU on these plates varied from one to confluent growth.

Limited samplings were performed on cell culture procedures other than trypsinization and passage. A hemacytometer used to count mycoplasma-infected cultures yielded heavy mycoplasmal contamination on the cover slip and on the hemacytometer slide. The lip of the tube containing the cell culture in trypan blue was also contaminated. Specific tests showed that trypan blue was also contaminated. Specific tests showed that trypan blue did not affect the viability of *A. laidlawii*. Mycoplasma was detected on the hands after handling the contaminated hemacytometer.

A 3T-6 culture free of detectable mycoplasma by two different assay methods was trypsinized and passed weekly in the same area immediately after work with the mycoplasma-infected cells. No disinfection or other control measures were performed. *A. laidlawii* was detected in this "clean" culture 6 weeks after the beginning of this experiment. Many cell cultures, including 3T-6, have been maintained free of mycoplasma in this laboratory by appropriate control measures.

To determine the survival of mycoplasmas on surfaces, 0.05-ml aliquots of supernatants from cell cultures infected with either *A. laidlawii* or *M. orale* were separately inoculated onto the work surface of a laminar flow biohazard cabinet. Results are listed in Table 2. *A. laidlawii* was recovered with Rodac plates as long as 4 days later, whether or not the air unit was in operation. *M. orale* was recovered after 4 to 6 days with the unit not in operation, and 3 to 4 days when the laminar flow unit was in operation. The relative humidity was not regulated nor measured during these studies.

The role of the laboratory technician as a possible source of mycoplasmal contamination was investigated. Throats of technicians were swabbed and inoculated onto mycoplasma agar and broth media containing antibiotics. Data on mycoplasmal carriage are presented in Table 3. The majority of technicians tested carried mycoplasmas in their throats. The number of CFU per plate was frequently too numerous to count. *M. salivarium* was isolated from every positive plate. Included in these samplings were three technicians from laboratories with a history of extensive *M. orale* infection. *M. orale* was not recovered from any of these technicians. Intensive efforts to isolate other species resulted in two isolates of *M. orale* type 1 from plates containing several hundred CFU of *M. salivarium*. On several other occasions, mycoplasma colonies that differed morphologically from the predominant *M. salivarium* were observed, but subsequent transfers into broth and onto agar yielded *M. salivarium*, indicating either failure to successfully isolate and transfer the proper colony or an atypical *M. salivarium* colony. When personnel known to carry mycoplasma in their throats read a book passage aloud 6 inches from mycoplasma plates, one isolate of *M. salivarium* was recovered. With more violent oral activity (sneezing), *M. salivarium* was detected on three of eight sneeze plates held inches from the mouth.

TABLE 2

SURVIVAL OF MYCOPLASMA ON FLAT SURFACES[a]

Organism	Days Survival	
	LAF[b] On	LAF Off
M. orale (6)[c]	3-4	4-6
A. laidlawii (4)	3-4	3-4

[a]Aliquot of 0.05 ml of infected cell culture, 10^7 to 10^8 CFU/ml, inoculated onto work surface.
[b]Laminar airflow.
[c]Number of determinations.

TABLE 3

RECOVERY OF MYCOPLASMA FROM CELL CULTURE PERSONNEL

Source	No. Pos./No. Tested	%
Throat swab[a]	25/31	80.6
Talking[b]	1/16	6.2
Sneezing[b]	3/8	37.5

[a]Anaerobic broth and agar culture.
[b]Anaerobic agar culture.

DISCUSSION

Mycoplasma-infected cell cultures are unacceptable in standardized, controlled experiments. Concentrations of mycoplasmas in cell cultures have ranged from 10^7 to 10^9 ml of cell culture fluid (*these studies*), 10^5 to 10^8 CFU per ml (1) and less (*unpublished data*). Assuming a mycoplasma concentration of 1×10^7 CFU per ml, a 75-cm^2 cell

culture flask using 20 ml of media will have a minimum of 20×10^7 or 2×10^8 CFU mycoplasma. There are, on the average, 2 to 3 logs more mycoplasmas than cells in infected cell cultures. A significant number of additional organisms are attached to the cell membranes. Mycoplasma infection will have profound, often unknown, effects on cellular nucleic acid metabolism, amino-acid metabolism and other pathways (7). Mycoplasma "infection," not "contamination," more accurately describes the parasitism, metabolism and reproduction of mycoplasmas in cell cultures.

The high concentrations of mycoplasmas in cell cultures partially explain the ease with which the organisms spread in a laboratory. The relatively gentle disturbance of the cell culture supernatant during removal of the flask from the incubator was sufficient to heavily contaminate the lip of the flask in these studies. A glass flask could be heated to disinfect the lip, but the effect of this procedure on environmental dissemination is unclear.

Any manipulation of cell cultures (pipetting, refeeding, centrifugation, etc.) can generate droplets. These droplets are relatively large, sedimenting within seconds in the immediate area. This explains the failure of O'Connell and co-workers to isolate mycoplasmas from the air during transfer of infected cultures (4). The present study confirms this although our conclusions are different. We contend that infection is spread by droplets, not aerosols, which contaminate hands, equipment, supplies, and work surfaces. The organisms are inoculated into cell cultures by direct or indirect contact with contaminated sources.

In infected cultures, a droplet (0.1 ml) may contain 10^6 or more mycoplasmal CFU. These remain viable on flat surfaces for days. Undisturbed, they can readily infect cultures transferred in the same laboratory. This was demonstrated in this study by infection of a cell culture handled immediately after one infected with *A. laidlawii* when no control practices are employed. Conversely, many cell cultures have been maintained free of mycoplasmas in this laboratory by practices listed at the end of this section, even though many cultures in the same laboratory are deliberately infected with mycoplasmas for experimental studies.

Survival of *M. orale* and *A. laidlawii* on flat surfaces was not significantly influenced by the use of laminar airflow. Kundsin showed that mycoplasmas could persist in aerosol form despite the lack of a cell wall (8). Wright and co-workers demonstrated that aerosols of *A. laidlawii* were very stable at humidity levels of 25% or lower and at 75% or higher (9).

Environmental sampling demonstrated the effectiveness of careful aseptic technique. A common practice of sliding pipets across the lip of the pan into the disinfectant solution transfers organisms from the outside of the pipet to the lip. The hands can become contaminated when handling the pan.

Although a majority of personnel tested carried mycoplasmas in their throats, it was difficult to isolate *M. orale* type 1, the human species most frequently encountered in cell cultures. This species was isolated from only two of 31 throat swabs, and then it was present in much lower concentration than *M. salivarium*. There is a possibility that other *M. orale* colonies were overgrown by *M. salivarium*. Use of immunofluorescent detection methods would clarify this.

If personnel are a major source of cell culture mycoplasmas, *M. salivarium* would be expected to constitute a major percentage of cell culture infections. Yet Barile and co-workers have not detected this species among 1,374 isolates from cell cultures (1). Our laboratory has isolated *M. salivarium* from 47 cultures; all were lymphocyte cultures and 46 were from one laboratory. The optimum pH for growth of *M. salivarium* is 5.5 to 6.5 (10), below the range of most cell cultures but closer to that obtained in lymphocyte cultures. The strain of *M. salivarium* isolated from the 46 lymphocytic cultures grew over a wide pH range (5.0 to 9.0) (*unpublished data*). The prevalence of *M. orale*, not *M. salivarium*, as the major human species isolated from cell cultures requires further study.

The failure to isolate *M. orale* from three technicians from a laboratory with widespread *M. orale* infection indicates that personnel are not the major source of infection. When *M. orale* is present in the throat, it is in low concentrations, based on results of these limited studies. When present in bovine serum and perhaps other biologicals, other mycoplasma species are evidently in low concentrations due to the large volume of sample required for detection (2). These are small compared to the 10^6 to 10^8 CFU per ml and higher found in infected cultures. Consideration of these results and the results of the environmental sampling leads us to conclude that although the ultimate source of mycoplasmas in cell cultures may

be personnel and serum, most mycoplasma infection comes from other infected cultures.

Effective practices to prevent and control mycoplasma and other microorganisms are known. Some of these have been partially described in earlier reports (11, 12), and are summarized here:

1. Acquisition of cell cultures only from reliable sources that certify them mycoplasma-free by standard test methods.

2. Regular assay of cell cultures for mycoplasma by standard methods.

3. Use of antibiotic-free media.

4. Use of hand pipets and prohibition of mouth pipetting.

5. Use of quarantine/isolation facilities and techniques for handling cultures whose mycoplasmal status is unknown. If separate laboratory facilities are not available, questionable cultures should be handled after clean cultures.

6. Disinfection of work surfaces between handling of different cell cultures.

7. Prompt autoclaving of infected cultures.

8. Liquid-nitrogen storage of clean cell cultures in early passage. These serve as a reservoir in the event cultures eventually become infected or mutate.

9. Use of high efficiency particulate air (HEPA) filters in laminar airflow or mass airflow cabinets or rooms to prevent airborne microbiological contamination during cell culture procedures, media preparation and sterility testing. These units should be checked at least yearly to insure proper filtration, airflow and air balance.

10. Effective quality-control testing to detect contamination in serum, distilled water and media before they are used to grow cell cultures.

11. Monitoring the effectiveness of ovens and autoclaves.

12. Packaging of sterile supplies in volumes that will be used quickly. The probability of opened, partially used packages remaining sterile decreases with time.

13. Strict enforcement of effective housekeeping measures. Particular attention should be paid to chemical disinfection of floors, the general laboratory area, sinks and faucets.

14. Use of protective clothing and head covering to minimize contamination by microorganisms shed by the technician.

15. No unnecessary activity in the immediate work area during sterile procedures. This includes general traffic and talking.

16. Use of serum-free media if possible.

17. Careful aseptic technique by all personnel who handle cell cultures.

18. Education and periodic review of aseptic procedures. All new equipment and procedures must be examined to determine if they may contribute to potential of contamination.

19. Proper design of facilities for cell culture work.

These measures are prudent and effective. They will significantly reduce the incidence of mycoplasmal and other microbiological infection of cell cultures and result in improved experimental control.

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