Isolation of Acholeplasma laidlawii from Commercial, Serum-Free Tissue Culture Medium and Studies on Its Survival and Detection

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This report documents the first isolation of Acholeplasma laidlawii from a commercial lot of serum-free Dulbecco basal medium. Experimental studies demonstrated survival of the organism for at least 1 year depending on the concentration of the contaminating organism as well as pH and temperature of storage of the serum-free medium. A comparison of isolation methods showed that concentration by filtration through 220-nm membrane filters and testing the filters for mycoplasma recovery, especially when using phenol red-diphasic Hayflick medium, was both sensitive and practical for the average laboratory.

In recent years, Acholeplasma laidlawii has become one of the most prevalent mycoplasma contaminants of tissue cultures, partly due to the presence of this organism as well as Mycoplasma arginini and other bovine mycoplasmas in commercially available bovine sera (2, 3).

This report documents the first isolation of A. laidlawii from a commercial lot of serum-free basal tissue culture medium and describes possible conditions for the organism's survival as well as methods for detection of suspected mycoplasma contamination in tissue culture materials.

MATERIALS AND METHODS

Mycoplasma isolation. Mycoplasma testing of tissue cultures and biological materials used in animal cell cultures is performed routinely in this laboratory by utilizing primarily Hayflick medium (8) for isolation and growth of the contaminating microorganisms. The following cultures are set up: duplicate Hayflick agar medium plates to include aerobic and anaerobic incubation, at least one diphasic tube with 1% suitable substrate and 0.002% phenol red as indicator, one plate with Shepard's A-2 formulation (14) to detect any thallium acetate-sensitive or acid pH-prefering organisms, plus a blood agar plate as a check on the possibility of L-form isolation as well as to monitor other microbial contamination. The phenol red-diphasic tube has been one of the most useful media for mycoplasma isolation, and both 1% glucose, to detect primarily fermenting organisms by their acid pH shift, and 1% arginine diphasic tubes, to detect arginine utilizing mycoplasma by an increased pH, have been used. However, as a practical point, the 1% glucose diphasic tube has been consistently efficient in the isolation of both fermenting and nonfermenting mycoplasma tissue culture contaminants.

Although a shift in pH usually is considered presumptive evidence for mycoplasma contamination, all diphasic tubes are subcultured at least once to agar plates for identification. False positive pH shifts can occur with low grade bacterial and yeast contaminations, and acid pH changes can be observed with actively metabolizing tissue culture cells; false negatives can be reported if the mycoplasma's metabolism causes no significant pH changes or if the organisms do not grow to sufficient titer to insure detectable pH changes.

All cultures are incubated at 36°C and examined at 2- to 3-day intervals, with appropriate subcultures, for several weeks before discarding as negative. The precaution of avoiding both desiccation and inadequate incubation was essential, especially in allowing detection of low levels of mycoplasma contamination.

Identification of mycoplasmas was by the growth inhibition paper disk method with appropriate antisera (5). Every mycoplasma isolation throughout the experimental period was re-identified using the disk growth inhibition test.

"Reconstruction" experiments. The principal serum-free tissue culture solution used was Dulbecco modified Eagle medium with and without glutamine or glucose; other basal medium solutions such as minimal essential medium and 199 were tested to check that A. laidlawii survival was not limited to any one formulation. Dulbecco medium was prepared
from instant tissue culture powder (Grand Island Biological Co.), although some experiments were performed by using commercial liquid preparations. Adequate mycoplasma testing was performed on all media used; all solutions were sterilized by double filtration through 200- or 220-nm membrane filters, avoiding high pressures as recommended by Lemcke (11). Although residual mycoplasmas have been reported after 220-nm filtration (1) and a second filtration using 100 rather than 220-nm filters would be preferable, especially with high-pressure filtration, our laboratory has used more practical methodology has proven quite effective. The following filtration units were used interchangeably: disposable units from Nalgene (200 nm) and Falcon Plastics (220 nm) as well as standard membrane filters (220 nm; Millipore Corp.).

For the "reconstruction" experiments, 100-ml duplicate quantities of serum-free tissue culture solutions were inoculated with varying concentrations of A. laidlawii and stored under variable conditions of time, temperature, and pH as described in Results. At various time intervals, the infected Dulbecco medium was tested for recovery of A. laidlawii, (i) directly, without further treatment, (ii) concentrated approximately 100-fold by centrifugation at 10,000 rpm for 45 min, or (iii) filtered through 200- to 220-nm membrane filters. The filters were tested for mycoplasma growth on both solid and diphasic medium. The fluid samples in 0.1- to 0.2-ml volumes were tested initially on duplicate aerobic and anaerobic Hayflick agar plates, diphasic phenol red-glucose tubes, A-2 plates, blood agar plates, and BHK-21 tissue cultures. Subsequently A-2 plates, anaerobic cultures, duplicate plates, and BHK-21 tissue cultures were omitted because these cultures offered little additional data for A. laidlawii recovery experiments.

Standard quantitative methods were performed for A. laidlawii, either by colony counts on agar by appropriate dilutions and expressed as colony-forming units per milliliter (CFU/ml) or by titration in complete Hayflick medium by using phenol red as indicator with 1% glucose as substrate and expressing the resulting metabolic acid pH shift in color-changing units per milliliter.

RESULTS

Isolation of A. laidlawii from serum-free, commercial Dulbecco medium. The isolation of a mycoplasma from several previously mycoplasma-negative cell lines in a tissue culture laboratory with a good mycoplasma surveillance program pointed to the possibility of either a new unsuspected source of contamination or a previously undetected, perhaps low-level, mycoplasma infection. A very intensive retesting of all cell lines and serum lots, as well as rechecking of techniques in both tissue culture and mycoplasma laboratories, revealed neither a break in sterile methods, a source of contamination from materials under suspicion at that time, nor deficiencies in mycoplasma testing. Yet the following facts seemed to imply a new tissue culture medium component as the source of the contamination: (i) the isolate was a glucose-positive, hemolytic, preferentially aerobic organism during a period when most of the contaminating mycoplasmas were anaerobic nonfermenters; (ii) all of the originally contaminated cell lines had been fed a particular tissue culture medium; and (iii) when clean stock cultures or other previously negative cell lines were fed with that medium, they also became mycoplasma positive. At that time, mycoplasmas were not thought to replicate or survive any length of time in serum-free tissue culture solutions. However, when the different basic formulations with the same serum were tested by using mycoplasma-free cell lines, it became obvious that the contaminating mycoplasma came from one lot of Dulbecco medium. When medium in individual, plastic-wrapped sterile bottles was concentrated by centrifugation at 10,000 rpm for 45 min and cultured both in our laboratory and in a mycoplasma-free laboratory to avoid any possible charge of cross-contamination, the same mycoplasma was isolated. On one testing even an unconcentrated sample became positive with a few CFU per plate; a very rough estimate suggested a level of contamination of 1 to 2 CFU/ml, but the tendency to grow in clumps, noted in subsequent experiments, may have caused an underestimation of the actual number of contaminating mycoplasmas.

The organism was identified through the courtesy of L. Hayflick's laboratory as well as by our laboratory as A. laidlawii. Eventually the commercial company corroborated the isolation for that particular lot of serum-free Dulbecco medium; although no explanation for the occurrence of this single mycoplasma contamination was obtained, aerosol exposure, perhaps to contaminated bovine serum, was suspected.

Because "secondary contamination" could occur in any tissue culture laboratory during the preparation or use of media in close proximity to mycoplasma-positive materials, reconstruction experiments were undertaken with the same resilient A. laidlawii organism to examine the effect of concentration of the organism, temperature, pH, and duration of storage as well as to study methods of detection feasible for the smaller laboratory.

Reconstruction experiments. Duplicate 100-ml bottles of Dulbecco medium were inocu-
lated with three concentrations of *A. laidlawii* representing a "low" level of contamination with 50 CFU per bottle, an arbitrary "medium" level of 5,000 CFU, and a "high" level of contamination with $5 \times 10^4$ CFU per bottle. These represent final concentration levels of 0.5, 50, and 50,000 CFU/ml, respectively. The Dulbecco medium was stored at room temperature and at 4 C, and was frozen at -20 C for periods of up to a year, simulating the most likely storage conditions in a tissue culture laboratory. At intervals of 1 day, 1 week, and 1 month or more, in some instances 1 year, the infected Dulbecco medium was (i) tested directly, without further treatment, (ii) concentrated approximately 100-fold by centrifugation at 10,000 rpm for 45 min or (iii) filtered through 200- to 220-nm membrane filters. The filters and the fluids were tested for mycoplasma growth as described in Materials and Methods.

The results from one such experiment are summarized in Table 1. The isolation and re-identification of *A. laidlawii* from one or more of the inoculated cultures was considered a positive result (+). It was predictable that, with more organisms, the period of positive isolations would be longer, but the survival of *A. laidlawii* at 4 and -20 C for a period of 1 year was unexpected even for an organism originally isolated from sewage and capable of replication on chemically defined medium in the laboratory (16). For the shorter testing periods of 1 month or less, it was the higher temperature of storage that gave consistently positive results for all levels of contamination, and quantitative studies showed that multiplication did occur at room and, occasionally, at refrigeration temperatures.

Table 1 also clearly indicates the inadequacies of small volume testing of uncentrated material for detecting low levels of mycoplasma contamination. Concentration by centrifugation was consistently more efficient and more practical for serum-free tissue culture materials than was concentration by centrifugation.

Similar results were obtained using minimal essential medium and the more complex 199 basal medium in place of Dulbecco medium. However, the addition of glutamine and glucose at concentration in themselves not mycoplasminedical decreased the number of positive recoveries from media with low levels of mycoplasma contamination; whether inhibitory levels of metabolic products were formed with availability of the above substrate(s) was not checked. The addition of sodium bicarbonate affected recovery of *A. laidlawii* only in correlation with any change in pH of the media.

**Effect of pH.** Most of the experiments were performed in the range of pH 7.5 ± 0.1 preferred by most tissue culturists. However, the facts that variations exist in the pH of commercial

| Time       | Test condition            | Effect on survival | Effect on survival | Effect on survival |
|------------|---------------------------|--------------------|--------------------|--------------------|
|            |                           | Room temp*         | Room temp*         | Room temp*         |
|            |                           | 4 C                | -20 C              | 4 C                | -20 C              |
| 1 day      | Not concn                 |                    |                    |                    |
|            | Concen by centrifugation  |                    |                    |                    |
|            | Concen by filtration      |                    |                    |                    |
| 1 week     | Not concn                 |                    |                    |                    |
|            | Concen by centrifugation  |                    |                    |                    |
|            | Concen by filtration      |                    |                    |                    |
| 1 month    | Not concn                 |                    |                    |                    |
|            | Concen by centrifugation  |                    |                    |                    |
|            | Concen by filtration      |                    |                    |                    |
| 1 year     | Not concn                 |                    |                    |                    |
|            | Concen by centrifugation  |                    |                    |                    |
|            | Concen by filtration      |                    |                    |                    |

* Contamination level.

* Temperatures tested; room temperature is about 25 C.

+/- , Results from duplicate or repeat experiments.
tissue culture products (7.0 to 7.4) and that prolonged storage, especially at room temperatures, caused occasional pH drifts above 8.0, prompted a closer look at the effect of pH on survival of *A. laidlawii*. Table 2 shows one such quantitated experiment after 1 week of exposure at the indicated pH values. Again, contrary to expectation, *A. laidlawii* survived at rather high pH values but was quite sensitive to neutral and slightly acidic conditions that even *M. pneumoniae* can survive. The random survival of small numbers of *A. laidlawii* on subculture even at pH 6.0 and the variation in pH sensitivity of possibly other contaminating mycoplasma prevent consideration of acidification of basal media as a safe method for eradication of low levels of mycoplasma contamination.

**Sensitivity of testing procedure including the use of the BHK-21 cell line.** Table 3 details the effectiveness of the various testing maneuvers at pH 7.0 and 8.0 at "low" levels of contamination. Because anaerobic Hayflick plates, A-2 medium, and blood agar plates were only positive with overwhelming *A. laidlawii* contamination, these media are omitted from Table 3. The data reemphasize the need for concentration, point to filtration as the method of choice for serum-free solutions, and also indicate that, of all the culturing systems used, the phenol red-diphasic medium was the most sensitive. At 1 year, for a high concentration of organisms, it was the diphasic medium inoculated with the filter that gave a clear indication of mycoplasma survival. The Hayflick plates inoculated with filter were also positive, but only two atypical colonies were found after staining of Hayflick plates inoculated with concentrated centrifuged material. These plates could easily have been interpreted as negative under routine examination.

The question as to whether the negative culture results at low *A. laidlawii* concentrations actually reflected absence of mycoplasmas led to a search for more sensitive detection methods. Tissue cultures provide a naturally occurring, albeit undesirable, amplification of even small numbers of infecting mycoplasmas. This amplification as well as the occurrence of noncultivable mycoplasma contaminants in tissue culture (10, 16), primarily the porcine strain *M. hyorhinis*, emphasized the possible need for a sensitive in vitro animal cell system. BSC and Vero cell lines were employed originally to detect the source of the *A. laidlawii* contamination, but the previously published work of Zgorniak-Nowosielska et al. (18) led to the use of BHK-21 cells.

**Table 2. Effect of pH at room temperature**

| pH | Conc of *A. laidlawii* | 1* | 2 | 3 | 4 | 5 |
|----|------------------------|----|---|---|---|---|
| 8.5| High                   | +  | + | + | + | + |
|    | Medium                 | +  | + | - | - | - |
|    | Low                    | -  | - | - | - | - |
| 8.0| High                   | +  | + | + | - | - |
|    | Medium                 | -  | - | - | - | - |
|    | Low                    | -  | - | - | - | - |
| 7.5| High                   | +  | + | + | - | - |
|    | Medium                 | +  | - | - | - | - |
|    | Low                    | -  | - | - | - | - |
| 7.0| High                   | -  | - | - | - | - |
|    | Medium                 | +  | - | - | - | - |
|    | Low                    | -  | - | - | - | - |
| 6.5| High                   | -  | - | - | - | - |
|    | Medium                 | +  | - | - | - | - |
|    | Low                    | -  | - | - | - | - |
| 6.0| High                   | -  | - | - | - | - |
|    | Medium                 | -  | - | - | - | - |
|    | Low                    | -  | - | - | - | - |

* Incubation was for 7 days at indicated pH values.
* Log of color-changing units per milliliter.
* Symbols: +, color change; -, no color change; ±, partial color change, positive subculture.
* One colony per plate on subcultures.

Preliminary experiments showed that *A. laidlawii* grew to identical titers in BHK-21 cells and in Hayflick medium; BHK-21 cell-free extracts had no mycoplasmacidal activity, and neither medium components nor agarose used in the overlay method (18) showed any inhibitory activity. Because the mycoplasma-free BHK-21 cell line possessed a very active metabolism causing considerable and rapid acid pH shifts, a more rigorously buffered minimal essential medium + 10% fetal calf serum medium with Good buffers (7) was eventually used. As tricine had been reported (15) to have eliminated mycoplasma tissue culture contamination, the *N*-*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (10 mM), *N*-tris(hydroxy-methyl)methyl-2-aminomethanesulfonic acid (10 mM), and *N*-2-hydroxyethyl-piperazine-*N*-2'-ethanesulfonic acid (15 mM) combination was subjected to rigorous testing with negative effect on *A. laidlawii* viability.

Extensive testing by using monolayers with overlay and sandwich techniques (18) or direct inoculation into tissue culture tubes with subsequent plate testing revealed that only the most heavily contaminated materials became positive in the BHK-21 systems (Table 3). It is possible that the pinpoint mycoplasma colonies formed in agarose overlay may have been
Table 3. Effectiveness of testing procedures at low concentrations of A. laidlawii

| Test procedure* | 1 Day | 1 Week | 1 Month |
|-----------------|-------|--------|---------|
|                 | Room temp | Room temp | Room temp |
|                 | 4 C | -20 C | 4 C | -20 C | 4 C | -20 C |
| **pH 8.0**      |       |        |       |        |       |        |
| Not concn       |       |        |       |        |       |        |
| HM              | -   | -      | +     | -      | +     | -      |
| D-PR            | -   | -      | +     | -      | +     | -      |
| BHK-21          | -   | -      | -     | -      | -     | -      |
| Conc by centrifugation |       |        |       |        |       |        |
| HM              | ±   | -      | +     | -      | +     | -      |
| D-PR            | +   | -      | +     | -      | +     | -      |
| BHK-21          | -   | -      | -     | -      | -     | -      |
| Conc by filtration |     |        |       |        |       |        |
| HM              | ±   | -      | +     | -      | +     | -      |
| D-PR            | +   | +      | ±     | +      | +     | +      |
| **pH 7.0**      |       |        |       |        |       |        |
| Not concn       |       |        |       |        |       |        |
| HM              | -   | -      | -     | -      | +     | -      |
| D-PR            | -   | -      | -     | -      | +     | -      |
| BHK-21          | -   | -      | -     | -      | -     | -      |
| Conc by centrifugation |       |        |       |        |       |        |
| HM              | -   | -      | -     | -      | +     | -      |
| D-PR            | -   | -      | +     | -      | +     | -      |
| BHK-21          | -   | -      | -     | -      | -     | -      |
| Conc by filtration |     |        |       |        |       |        |
| HM              | ±   | ±      | ±     | ±      | -     | -      |
| D-PR            | +   | +      | +     | +      | +     | +      |

* Abbreviations: D-PR, diphasic phenol red tube; HM, Hayflick medium plate.

| Temperature tested; room temperature is about 25 C. |
| ±, A few colonies per plate (not overtly positive). |

misplaced from time to time, but the cumbersome-ness of the technique of searching for mycoplasmas together with the lack of any overt cytopathology or consistently positive autoradiography (unpublished data) created doubt as to the acceptability of this method for the average laboratory. The practical difficulties in obtaining and maintaining mycoplasma-free tissue cultures for some laboratories postponed any evaluation of other cell lines for detecting low levels of mycoplasma contamination of tissue culture products.

Although BHK-21 and other cell lines have been useful in detecting M. hyorhinis tissue culture contamination and may be important in detecting other noncultivable mycoplasmas, the results with A. laidlawii emphasize that reliance on any one technique can be misleading.

**DISCUSSION**

Since the first reported mycoplasma isolation from tissue cultures in 1956 (13), a vast literature has accumulated on the problems that these filterable organisms lacking cell walls pose for the cell biologist and virologist. Some of these effects exerted by mycoplasmas on macromolecular synthesis, cytology, stability of genetic material, susceptibility to viruses, and other exogenous materials as well as the misinterpretations of experimental results due to the sheer mass of their numbers and/or competition with cells for medium constituents or presence of mycoplasmal enzymes have been reviewed by Stanbridge (16). New reports on the effect of mycoplasmas in cell culture, such as the influence on microcytotoxicity tests possibly involving membrane alterations (4), will continue to appear.

There have been distinctive patterns of mycoplasma infections: initially, predominantly human strains were isolated and, although droplet infection was implicated, this was never directly proven. Also suspected but never proven was the contamination by the porcine...
strain, *M. hyorhinis* (GDL isolate), prevalent in the mid-1960s; implicated were trypsin or bovine sera contaminated in slaughter houses that handle pigs (1). However, the present epidemic of bovine mycoplasma strains, predominantly *A. laidlawii* and *M. arginiini* (2, 3), has been definitely linked to the use of contaminated bovine serum products by Barile and Kern (3). Because prevention is the best protection against mycoplasma contamination, recognition and adequate testing of potential sources of contamination is of paramount importance. Aerosolization has been shown to cause cross-contamination in the tissue culture laboratory (9, 10) and has been suspected in the case of contamination of horse serum by bovine mycoplasmas (1) (L. Hayflick, personal communication) as well as in this incidence resulting in the isolation of *A. laidlawii* from one lot of commercial serum-free basal medium. At the present time, no further isolations from commercial serum-free tissue culture media have been reported; however, this isolation of *A. laidlawii* and demonstration of prolonged survival for even 1 year in serum-free tissue culture media, depending on concentration of the contaminating organism as well as temperature, pH, and duration of storage, indicate possible new hazards for any tissue culture laboratory that could be difficult to trace unless careful and sensitive detection maneuvers are instituted.

The possibility of aerosol contamination from tissue culture to tissue culture is well recognized, but less awareness exists that aerosols from tissue cultures, sera, and other biological products can infect such seemingly nonsupportive solutions as the Dulbecco basal medium for any length of time. That such contaminated material can then initiate a round of infection either by direct use in tissue culture medium or more indirectly by aerosolization was demonstrated by "mini-experiments" done in our laboratory. Hayflick agar plates were placed at varying distances from the working center where the contaminated Dulbecco basal medium was handled; irrespective of whether pipetting, with or without blowing, or pouring was employed, a majority of plates within a 1-ft (30 cm) radius, although not 2 ft (60 cm), became contaminated by droplet infection. The percentage of positive cultures increased in direct relation to the casualness of laboratory techniques employed. The suspicion has grown that this kind of secondary infection may be occurring in some cases of *A. laidlawii* and *M. arginiini* contamination despite reasonable information that no bovine or horse sera or tissue cultures using such commercial products have been introduced into the laboratory (unpublished data).

In addition to awareness of potential sources of contamination, adequate detection systems for mycoplasma contamination must be worked out for each tissue culture laboratory; many of these guidelines have been published in the past (2, 3, 16).

Although commercial companies have been trying to improve their quality control standards under FDA guidelines, including double filtration through 220-nm filters, the use of high pressure allows occasional low levels of contamination to occur (1, 11). Thus, testing of tissue culture materials as well as using double filtration, if feasible, by individual laboratories is important. Furthermore, heat inactivation at 56°C of serum components might be considered for those cultures not affected nutritionally by such a maneuver. Other means of inactivating mycoplasma, such as irradiation, face the problem of adequately penetrating and killing of mycoplasma aggregates. For the larger tissue culture laboratory it is not too difficult to incorporate additional safeguards by testing large lots of sera or tissue culture media by using the large volume method suggested by Barile and Kern (3). However, for the smaller laboratory this may not be feasible; the use of concentrating techniques such as the centrifugation and filtration methods used in the reconstruction experiments offer considerable advantage for their simplicity in any testing procedure. Centrifugation proved to be less effective for serum-free solutions but was useful for serum where particulate matter allowed a concentrated pellet to be formed. Filtration through 200- to 220-nm membrane filters with use of the filters for culture proved to be optimal for detection of mycoplasma contamination in serum-free media. It has also the advantage that a second filtration, if possible by using a 100-nm filter, would make the media mycoplasma-free. The disadvantages of filtration are that any solution with a high concentration of serum necessitates the use of high pressure (although filters can still be tested) and also that the use of filters may seriously affect experimental results as reported, for example, by Fowles et al. in a paper on the effect of activated lymphocyte products on macrophage bacteriostasis (6).

The effectiveness of using a phenol red-dichloroquinone medium has been discussed; however, it is urged that reliance should not be placed on one medium or on any single method for mycoplasma testing; other mycoplasma contaminants may appear, variability of medium components as well as toxic factors may occur from
time to time affecting mycoplasma isolations, and noncultivable mycoplasmas should be taken into account (10, 18). This concern for the sensitivity of the present testing methods led to the use of BHK-21 cells, and, although it was not useful for A. laidlawii in this instance, it seems important to urge the use of more than one method of detection (for example, use of radioactive nucleic acid precursors for mycoplasma labeling, autoradiography, and electron microscopy) in addition to carefully controlled isolation techniques. It will be equally important to continue to investigate other and newer approaches to achieve an optimal surveillance program.

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