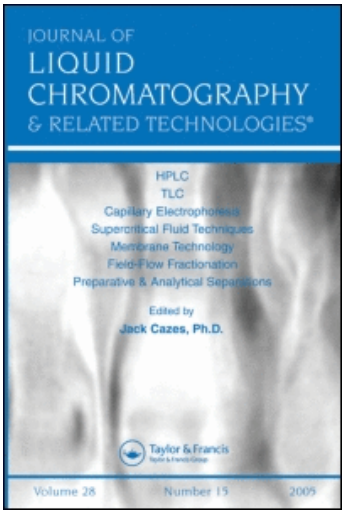

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DETECTION OF MYCOPLASMA CONTAMINATION IN LYMPHOBLASTOID CULTURES BY A SIMPLE, HPLC METHOD

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ABSTRACT

A simple method for detecting mycoplasma contamination of lymphoid cell cultures is discussed. This method is based on high pressure liquid chromatographic (HPLC) detection of citrulline by reversed-phase separation of its ortho-phthaldialdehyde (OPA) derivative. Formation of citrulline via conversion of arginine using the enzyme arginine deiminase present in most of the commonly encountered strains of mycoplasma is measured by this sensitive technique. The use of HPLC techniques offers an extremely sensitive indication of mycoplasma contamination in cells having large nuclei which make detection by staining with Hoechst dyes difficult to interpret.

INTRODUCTION

The presence of mycoplasma in animal cell culture is of constant concern in most tissue culture laboratories. Several methods which enable the investigator to detect low level contamination are in use and have been reviewed recently (1). A culturing technique based on use of an indicator cell line known to be

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mycoplasma-free has been developed by del Giudice and Hopps (2) and consists of inoculation of this indicator line with culture supernatant, media or serum, or cells to be tested. After incubation for 3-5 days, the cells are stained with Hoechst 33258 and examined microscopically. Over an eleven-year period, 17,666 specimens were tested, and 13.8% were found to be mycoplasma-infected. Although the frequency of appearance of each strain varied from year-to-year, suggesting continued reinfection with environmentally present organisms, the most common strains were M. orale, M. arginini, M. hyorhinis, and A. laidlawii, accounting for 85% of all species identified. The indicator cell culture method is not necessary for direct examination of cellular components of culture. In these instances, staining with Hoechst dyes alone is sufficient. Convenient staining methods have been described by Russel et al (3) and Chen (4). All of these methods are based on the binding of DNA by Hoechst fluorochrome and the ability to detect densely stained mycoplasma in the cytoplasm. In lymphoblastoid cell lines having extensive nuclear areas, this method requires experienced technical personnel unless contamination is very advanced. Other methods, including conventional culture, electron microscopy, and uridine incorporation, have been reviewed and compared recently by Hessling et al (1).

An alternate method, first used in 1963 (5), detected the citrulline resulting from arginine deiminase modification of arginine. All of the contaminating mycoplasma encountered in this study were of the species which contained the arginine deiminase activity. This colorimetric assay has not been used extensively in recent literature because of its lack of sensitivity and inability to recognize low level contamination as conveniently as the fluorescent staining method. The technique which we report utilizes the speed and resolving power of HPLC in combination with the fluorescent staining method to screen cultures, such as lymphoblasts, having large nuclei, which are difficult to analyze by the staining technique alone when infected at a low level.

This extra screening sensitivity allows either confirming data in negative cultures or detecting as positive those cultures which may be questionable by other techniques. It also enables one to screen cell culture supernatants for the presence of citrulline levels, indicating the culture that produced them was mycoplasma-infected. We have found this method to be of value in the production of lymphokines from lymphoblastoid cell cultures.

MATERIALS AND METHODS

Cell Culture

The human lymphoblastoid cell line RPMI 1788 was used for the studies described. The cells were grown as previously described (6) in RPMI 1640 (Gibco, Grand Island, NY) containing 2% heat-inactivated pooled human serum. Cells were seeded at an initial concentration of 1 x 10^6/ml and were allowed to grow for 24 hours at 37°C, during which time the cell number doubled. No antibiotics were used for culture. Sterility was monitored by inoculation of trypticase soy broth and thioglycollate.

Fluorescent Staining Methods

Fluorescent staining of RPMI 1788 lymphoblasts was carried out according to the methods of Chen (4). One ml samples of RPMI 1788 were removed from spinner flasks and placed into a sterile 15 ml centrifuge tube. The cells were fixed in 2 ml of Carnoy's fixative (3:1 100% methanol:glacial acetic acid) added directly to the sample without removing the growth media. After fixation for 2 minutes the cells were centrifuged at 300 X g for 5 minutes, the supernatant decanted, and the cells were washed in 2 ml Carnoy's fixative for 10 minutes and again centrifuged. The pelleted fixed cells were resuspended in 1.0 ml of Hoechst 33258 (2-[2-(4 hydroxyphenyl)-2-benzimidazolyl]-6-[methyl-4-piperazyl] benzimidazol)-trihydrochloride in phosphate-buffered saline at

0.05 µg/ml. An aliquot of the stained cells was placed onto a microscope slide and examined at 390 X on a Leitz Dialux 20 at 390-490 nm excitation, >515 nm emission. Cultures were judged to be positive, negative, or questionable for mycoplasma contamination based on appearance of intensely staining extranuclear areas of DNA.

High Performance Liquid Chromatography

HPLC identification of citrulline was performed by modification of the procedure of Turnell and Cooper (7) for separation of OPA derivatives of amino acids.

1. Reagents. Citrulline, homoserine, norvaline, OPA, 2-mercaptoethanol, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Iodoacetic acid was purchased from Eastman (Rochester, NY). Solvents were purchased from Fisher (St. Louis, MO). All reagents for sample processing and derivatization were prepared as described by Turnell and Cooper (7).

a. OPA/2-ME Reagent. Five hundred mg of OPA was dissolved in 10 ml of methanol and diluted to 100 ml with a 400 mM sodium borate buffer, pH 9.5. Four hundred µl 2-ME was added to this solution followed by an additional 40 µl every three days. This reagent was stored for about three weeks at room temperature.

b. Iodoacetic Acid Reagent. Iodoacetic acid (0.74 g) and boric acid (0.62 g) were dissolved in HPLC grade water. The pH was adjusted to 9.5 with 2M NaOH solution and the mixture was brought to 100 ml with water.

c. Acetonitrile Precipitation Reagent. Fifty microliters of 2-ME was added to 25 ml of acetonitrile. This reagent was prepared daily.

d. Stock Sodium Propionate Solution. This solution was prepared by addition of 250 mmoles propionic acid (relative density = 0.990-0.995 kg/l) and 350 mmoles of anhydrous Na_2HPO_4. The solution was adjusted to pH 6.50 at room temperature with a 4M NaOH solution.

e. Internal Standard Solution. In 1.0 l of water, 1.0 mmole each of homoserine and norvaline was dissolved. Aliquots of this solution were stored at -20°C until used.

f. Solvent A. Water/stock sodium propionate solution/acetonitrile (72/20/8 by volume).

g. Solvent B. Water/acetonitrile/methanol/dimethyl sulfoxide (42/30/25/3 by volume). All HPLC solvents were filtered and degassed prior to use.

2. Sample Processing and Derivatization. Samples for citrulline analysis were processed and derivatized as described by Turnell and Cooper (7) for amino acid analysis. Twenty µl of internal standard solution and 200 µl of acetonitrile precipitation reagent were added to a 20 µl sample in a polypropylene tube. The contents of all tubes were vortexed and centrifuged at 13,000 X g for 2 minutes. Twenty µl of the resulting supernatant solution was placed into another polypropylene tube, and 100 µl of the iodoacetic acid reagent was added. The contents of the tube were mixed by vortexing, 100 µl of the OPA/2-ME reagent was added, and exactly 30 seconds later 20 µl was injected onto the column.

3. Instrumentation. The HPLC system used includes two Waters model 6000A pumps, a model U6K injector, a model 660 solvent programmer (Waters Assoc., Milford, MA), and LDC fluoro-monitor III model 1311 (LDC, Riviera Beach, FL) equipped with an excitation band width of 340 to 380 nm and an emission wavelength filter of 418-700 nm. Data were acquired via a Smartface a/d converter (P.J. Cobert, Assoc., St. Louis, MO) and analyzed using custom software and a DEC LSI 11/23 computer (Digital Equipment Co., Maynard, MA), a DSD 880 disc drive (Data Systems Designs, Dallas, TX), and a Tektronix 4662 digital plotter (Tektronix, Beaverton, OR). The column used was a 3.9 mm ID x 30 cm analytical column prepacked with Waters µBondapak C18 (particle diameter 10 microns). The analytical column was fitted with a 30 mm x 2 mm ID Waters guard column packed with Waters Bondapak C18/Corasil (particle diameter 37-50 microns).

4. Chromatographic Conditions. The procedure used was based on the method for amino acid analysis described previously by Turnell and Cooper (7). We have modified their procedure to obtain conditions suitable for detection of citrulline in cell culture medium and culture supernatants. Samples or standards were processed and derivatized as described above, and 20 µl portions were injected onto the HPLC column. Elution was carried out at room temperature with a concave gradient (gradient curve #10 on the Waters model 660 solvent programmer), ranging from 100% solvent A at the time of injection to 100% solvent B 20 minutes after injection. The flow rate was 2.0 ml/min. Under these conditions, the citrulline derivative eluted near the end of the gradient run with a retention time of approximately 19.2 minutes and clearly was separated from derivatives of the amino acids found in the culture medium and supernatants. Derivatives of the remaining amino acids in the sample were eluted over the next 10 minutes with 100% solvent B. The column was equilibrated then with 100% solvent A prior to the next sample injection.

5. Identification and Quantitation. Citrulline peaks were identified by their retention times relative to the reference peaks produced by homoserine and norvaline. Quantitation of citrulline in samples was accomplished by measuring the citrulline peak area and comparing with the peak area produced by a known amount of the internal standard homoserine. The ratio of the peak areas obtained, along with the relative molar fluorescence response of citrulline with respect to homoserine under identical derivatization and chromatographic conditions, could be used to calculate the concentration of citrulline in a sample. A standard curve of citrulline in RPMI 1640 medium was prepared so that the concentration range of added citrulline in the medium ranged from 0.05 mM to 2.0 mM. Twenty µl aliquots from each preparation were then derivatized, processed, chromatographed, and quantitated as described above.

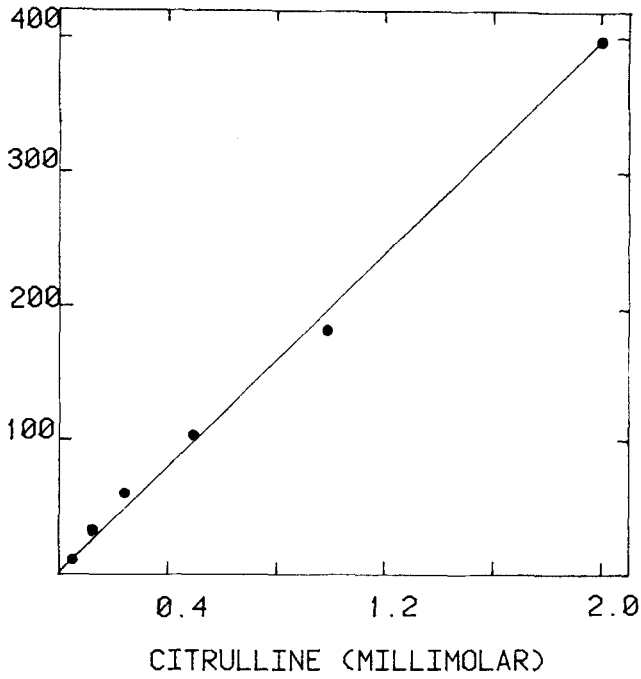

FIGURE 1. Standard curve relating citrulline concentration to peak area. Samples of tissue culture medium containing 2% human serum were spiked with citrulline and derivatized as described in Methods. Peak areas of citrulline were determined by computer analysis with respect to internal standard peaks and are expressed in arbitrary units. Citrulline is expressed as mM concentration of sample, r^2 = 0.9961; y = 192.2x + 6.7.

RESULTS AND DISCUSSION

Figure 1 is a standard curve for citrulline concentration versus peak area using the conditions described. A linear response of peak area to citrulline concentration was obtained. Using a range of 5.0 fluorescent units full scale, one can easily detect citrulline at the lowest concentration (0.05 mM) used, which corresponds to 84 picomoles of citrulline actually injected.

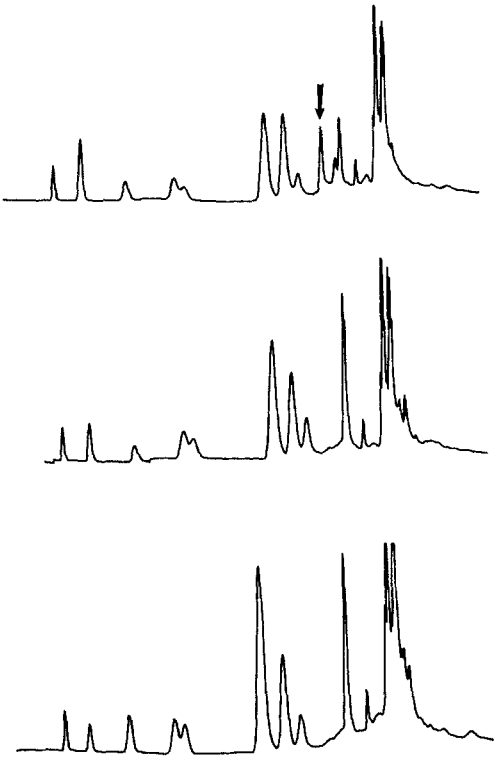

FIGURE 2. Detection of mycoplasma contamination in tissue culture. RPMI 1788 lymphoblastoid cell cultures were examined by staining with Hoechst dye as described in Methods and then supernatants were subjected to citrulline determination. Lower profile is negative control preparation of medium and serum only, middle profile is of supernatant of culture which was shown not to contain mycoplasma by staining, and upper figure is supernatant of culture which was questionable by staining techniques. Arrow indicates the citrulline peak which is present only in the contaminated culture.

Figure 2 shows the results obtained when this method is applied to detection of mycoplasma in tissue culture. The lower profile is tissue culture medium containing 2% heat-inactivated human serum. This sample has no citrulline detectable at the

sensitivity employed. The middle profile is supernatant after growth of lymphoblastoid cells which were shown, by staining with Hoechst 33258, to be negative for mycoplasma contamination. It also has no detectable citrulline present. The upper figure is an example of the usefulness of this technique. An aliquot was obtained from a culture which gave questionable findings when stained, yet was shown by subsequent staining to be mycoplasma-contaminated. As indicated by the arrow, citrulline is present in this preparation.

We estimate that a concentration of citrulline as low as 0.01 mM (15-20 picomoles of citrulline injected) could be detected with this method at the sensitivity employed currently. If necessary, for other applications or media sources, one could increase sensitivity easily by relative increases in the injection volume or ranging of the detector. However, for our purposes, those cultures which are negative or questionable by staining and which, later, can be shown to be positive by staining have a detectable citrulline peak at the sensitivity range described. We routinely culture cells in RPMI 1640 medium containing 2% human serum. Human serum contains approximately 25 μmoles of citrulline per liter, which corresponds to a citrulline concentration in medium supplemented with 2% human serum of only 0.5 pM, well below the detection limit of our assay. Accordingly, detection of a peak corresponding to citrulline in our assay represents a level of citrulline in the sample which cannot be attributed to citrulline in the serum added to the medium for cell culture. We calculate that addition of 10% fetal calf serum to culture medium also would not interfere with our assay unless the citrulline content of the added serum were unusually high.

This assay is simple, quick, and easy to interpret. It is important, however, to control the time from derivatization to injection as closely as possible because of the relatively short half-lives of the OPA/2-ME derivatives of the amino acids. In a study of stability of such derivatives, Turnell and Cooper (7) reported that the citrulline derivative in OPA/2-ME has a half-life

of only 3.5 minutes while the half-life of the same derivative diluted in solvent A is 79.3 minutes. Thus, the citrulline derivative is relatively stable once injected onto the column and delay in injection should be avoided. This instability causes no problems because the derivatization procedure is very uncomplicated technically and can be carried out easily during a 30-second period.

This technique is not intended to be a substitute method for the widely used staining techniques. It is, however, extremely useful in instances where fluorochrome staining of cells may yield questionable results, such as in those instances where cells with large nuclei make interpretation of results difficult in low level contamination. Even though the possibility of mycoplasma contamination in these questionable instances could be determined by inoculation of indicator cell lines which are easier to interpret by staining, this would require an additional week, during which time the disposition of supernatants and continued cultivation of cells in question would be hampered. In a laboratory such as ours, where 20-30 l/day of lymphoblastoid culture is processed, the storage of supernatants for such long periods is not acceptable nor is the expense of continued culture of cells while such testing is being performed. The use of a combination of initial fluorochrome staining with additional HPLC determination of citrulline in questionable cultures allows us a degree of certainty which enables us to pool and process these supernatants immediately.

This technique is limited to detection of strains of mycoplasma which contain the enzyme arginine deiminase. However, it is our experience that the presence of arginine deiminase activity correlates highly with total mycoplasma detectable by staining techniques. This observation confirms the findings of Barile and Schimke (5) in which 73 cultures submitted by 22 laboratories were examined colorimetrically for arginine deiminase activity and were shown to have 100% correlation with isolatable mycoplasma by growth in culture. According to del Guidice and Hopps (2), the

most commonly encountered mycoplasma species over an 11-year period included a significant portion which would not have been detectable by our assay. However, they point out that the species encountered vary greatly from year-to-year and among laboratories.

An additional advantage of our technique is that it does not require intentional growth of mycoplasma-infected cultures in the laboratory. With the indicator cell line technique, one must culture mycoplasma for use as a positive control and also culture potential mycoplasma-infected cells, supernatants, sera, or other medium components in order to establish them in indicator cell lines. Although these techniques are extremely reliable, most laboratories are neither equipped with separate tissue culture areas for intentional growth of mycoplasma nor are the investigators comfortable with the presence of mycoplasma-contaminated cell lines.

We have described a convenient, sensitive assay system based on the detection of derivatized citrulline by reversed-phase HPLC for the detection of mycoplasma contamination of cell cultures. This technique is based on the presence of arginine deiminase activity, an enzyme not found in mammalian systems, and will detect most commonly encountered mycoplasma cell culture contaminants. It is especially useful as a confirming technique when conventional detection by staining is questionable or in instances requiring rapid disposition of cell cultures or supernatants.

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