A Rapid Chemical Method for Detecting PPLO Contamination of Tissue Cell Cultures. (28767)

MICHAEL F. BARILE AND ROBERT T. SCHIMKE (Introduced by M. Pittman)
Division of Biologics Standards and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

Schimke and Barile(1,2) have shown that extensive conversion of arginine to ornithine by the HeLa-S_3 cell culture was an artifact of pleuropneumonia-like organisms (PPLO-Mycoplasma) contamination. In PPLO contaminated cell cultures and in PPLO grown in broth culture, conversion of arginine to ornithine was rapid and extensive and occurred by the 3-enzyme arginine dihydrolase pathway involving arginine deiminase, ornithine transcarbamylase and carbamyl phosphokinase. This pathway was previously reported to occur in a few species of bacteria (3) and in a human urethal PPLO strain(4, 5).

The presence of arginine deiminase activity in PPLO has been exploited for development of a rapid chemical method for detection of PPLO contamination. This enzyme is not present in animal tissue. It will be shown that the results obtained with the enzyme assay method based on the presence of arginine deiminase activity in cell culture extracts agree entirely with the most sensitive cultural procedures for PPLO contamination.

Materials and methods. Cull cultures. On invitation, 22 laboratories in the Bethesda area submitted 73 actively proliferating primary and continuous cell culture lines for PPLO examination. Cells were derived from human, rhesus monkey, vervet monkey, rabbit, dog, mouse, chick and quail tissue. On delivery, 10 millimolar L-arginine was added to the cell culture media fluid since these culture media usually contain only small amounts of L-arginine. The cell culture was incubated for 2 or 3 days. An aliquot was removed for PPLO detection by cultural procedures and the remainder assayed for arginine deiminase activity. The two assays were carried out in different laboratories, and the results were not compared until both tests were completed.

PPLO cultivation. Three types of PPLO-media were used, a human blood, and a horse serum media described by Barile *et al*(6) and the Hayflick media(7). Duplicate agar plates were inoculated; one was incubated aerobically and the other anaerobically in a 95% N_2 and a 5% CO_2 atmosphere(6,8) at 37°C for 14 days.

Preparation of cells. Twenty ml of cell culture grown in suspension or 30 square centimeters of monolayer cells were used. Cells were scraped free from the glass (monolayer) with a rubber policeman, and concentrated by centrifugation at 2000 $\times$ *g* for 4 minutes in 12 ml thick-walled centrifuge tubes. The supernatant was decanted and the cell button (1-2 mg protein) was suspended in 0.5 ml of distilled water and frozen and thawed twice to disrupt the cells.

Enzyme assay. Arginine deiminase in cell extracts was detected by the formation of citrulline from arginine at pH 6.5. Citrulline was determined colorimetrically by the method of Archibald(9) as modified by Ratner (10).

Reagents—(a) 0.1 M L-arginine in 0.05 M potassium phosphate pH 6.5.

(b) Urease (Sigma Type V, Sigma Chemical Co.), 10 mg/ml in potassium phosphate 0.05 M pH 6.5.

(c) Acid mixture containing 90 ml of concentrated sulfuric acid, 250 ml of concentrated phosphoric acid, 600 ml of distilled water.

(d) Perchloric acid 0.5 M.

(e) Aqueous diacetyl monoxime, 0.75% (2,3-Butanedione 2-Oxime, Eastman Organic Chemicals).

Assay procedure. A 3-tube assay was used, the test material and 2 control blanks. For the test assay tube, 0.2 ml of the cell culture lysate was added to 0.4 ml of reagent (a) and 0.01 ml of reagent (b). The control (1) tube contained 0.2 ml of cell culture lysate, 0.4 ml of reagent (a) and 0.01 ml of reagent

(b) plus 1.0 ml of reagent (d) which stopped the reaction at zero time. Control (2) tube contained 0.4 ml reagent (a) and 0.01 ml of reagent (b) but no cell culture lysate. The test assay and control (2) tubes were incubated at 37°C for 1 to 3 hours. Control (1) tube was not incubated. After incubation, 1.0 ml of reagent (d) was added to precipitate the protein. The tubes were than centrifuged at 5000 × *g* for 5 minutes. One ml of the resulting clear supernatant fluid from each of the 3 tubes was transferred to clean tubes and 2 ml of reagent (c) were added, followed by 0.1 ml of reagent (e). The tubes were then boiled for 15 minutes in a covered pan, cooled in the dark and the color was measured at 490 mμ in a suitable spectrophotometer. The presence of color in the complete assay tube in excess to that occurring in both control blanks constituted evidence of PPLO contamination.

Notes on the method. Citrulline produced a characteristic peach, yellow-orange color under the conditions described above with an adsorption maximum at 490 mμ. If the protein was not removed completely from the supernatant fluid following addition of the perchloric acid, a yellow color developed. This was prevented by careful pipetting and centrifugation. This interfering protein chromogen was differentiated readily from the color of citrulline by its absorption peak at 480 mμ, and by the longer time of heating required to obtain maximal color development. Light was excluded during the boiling and cooling steps because of the light sensitivity of the citrulline chromogen.

The amount of chromogenic material present in the control (2) tube (cell-free blank) increased with time of incubation at 37°C. If the arginine deiminase assay was carried out for only one hour, the optical density of control (2) tube was lower than that of control (1) tube (cell preparation stopped at 0 time). However, when the incubation period was increased to 3 hours, the control (2) tube was greater than that of the control (1) tube.

The lower limit of color development considered indicative of PPLO contamination was set at twice the combined optical density at 490 mμ of the 2 control blanks. The total number of PPLO required for a positive reaction for arginine deiminase activity as described in the assay was between 100,000 and one million PPLO cells, as determined both in PPLO contaminated cell culture lysates and in PPLO broth culture. Contaminated cell cultures generally contain 10 times or more PPLO in the sample specified for the test. Samples that are smaller or contain fewer PPLO would be satisfactory if the incubation period of the test were extended up to 6 hours.

TABLE I. Detection of PPLO in Tissue-Cell Culture.

Procedure	No. pos/No. examined	Positive %
Arginine deiminase activity	27/73	37
Agar culture	27/73	37
Agreement	73/73	100

Although the optimum pH of the arginase reaction, *i.e.*, arginine ⟶ ornithine + urea, in cell cultures occurred at a pH of 9-10, several cell lines were tested with extremely high levels of arginase, resulting in a significant amount of urea formation at pH 6.5, the pH used for the arginine deiminase assay. Since urea produced a yellow color with diacetyl monoxime under the conditions described above, the urease was added to prevent false positive reactions due to urea formation.

Results. There was complete agreement in the results obtained with the cultural and chemical procedures for detection of PPLO in cell cultures (Table I).

Typical optical density measurements of arginine deiminase activity are given in Table II for the 2 control blanks and for lysates from cell cultures grown with and without additional arginine. Although additional arginine was not required for the test procedure, it should be noted that arginine supplementation increases the content of arginine deiminase 3-fold.

The human blood agar and the Hayflick media were comparable in promoting growth of contaminating PPLO and the maximum number of positive isolations was obtained

TABLE II. Typical Determinations for Arginine Deiminase Activity with PPLO-Contaminated Tissue Cell Cultures.

Tissue cell culture No.	Type	Colorimetric assay (O.D. 490) — Blank controls: Zero time	Blank controls: No cells	Arginine* added: —	Arginine* added: +	Arginine deiminase activity
10	M.K.S.	.025	.02	.595	1.50	+
20	P.R.K.	.057	.02	.061	.053	—

* Tissue-cell cultures grown in accustomed medium with and without additional arginine to final concentration of 10.0 mM.

TABLE III. Primary Isolation of Tissue-Cell Culture PPLO Strains on Artificial Media.

Growth atmosphere		PPLO agar media: Human blood*	Horse sera*	Hayflick media†
Aerobic	No. pos/No. tested	22/73	8/73	18/73
	Percent	30	11	25
Anaerobic* 95% N_2 – 5% CO_2	No. pos/No. tested	27/73	25/73	27/73
	Percent	37	34	37

* Barile, Yaguchi and Eveland, *Am. J. Clin. Path.*, 1958, v30, 176. (BYE Media, BBL, Inc., Baltimore.)

† Chanock, Hayflick and Barile, *Proc. Nat. Acad. Sci.*, 1962, v48, 41-49.

when a 95% N_2 and a 5% CO_2 atmosphere was used (Table III).

In addition to PPLO contamination, low-order bacterial contamination (100-1000/ml tissue culture media fluid) was seen in 20 of the 73 cell cultures tested (27%). The bacteria were identified as members of *Corynebacterium, Micrococcus* and *Bacillus* and gram negative enteric rods. These bacterial contaminations in no way interfered with detection of PPLO in cell culture lysates by the enzyme assay procedure. When tested in broth culture, none of the bacterial isolates produced arginine deiminase activity.

Discussion. The presence of arginine deiminase activity in PPLO allows for an effective method for detection of PPLO contamination of tissue-cell cultures. The method compares favorably with detection by the best cultural procedures and has the advantage of giving rapid results. It can be used in laboratories not equipped for PPLO cultivation, or by investigators who prefer not to maintain PPLO in their laboratory because of the increased risk of PPLO contamination involved.

PPLO contamination of tissue cell cultures has been shown to affect the metabolic characteristics(1,2,11), and the morphology of the cells(12) and ability of cell cultures to support viral plaque formation(13). The high frequency of PPLO contamination in tissue-cell culture(8), the inapparent signs of PPLO contamination and the effects of such contamination on the normal functions of tissue-cell culture require that each investigator establish whether his cell lines are contaminated with PPLO before interpretation of his results can be properly made, particularly in investigations dealing with the arginine metabolism of cell cultures, since PPLO infection is associated with the rapid breakdown of arginine(1,2,11,12,13). Continuous surveillance of cell cultures for PPLO is necessary since in the authors' experience cell cultures can become contaminated at any time. We suggest that information concerning the presence or absence of PPLO contamination be included in each report of studies in which tissue-cell cultures were used; further that cell cultures free of PPLO should be preserved in case the working cell cultures become contaminated.

The results on detection of PPLO obtained with the chemical procedure were in complete agreement when the best cultural procedures were used, a rich media and appropriate atmospheric condition, namely an atmosphere

of 5% CO_2 and 95% N_2. Barile *et al*(6) have previously emphasized the advantages of the human blood PPLO agar and a nitrogen-carbon dioxide atmosphere for isolation of PPLO from human tissue.

The need for adequate culture media for detection of adventitial agents in cell cultures is further indicated by the finding of contamination of certain cell lines by bacteria. There was neither turbidity of the cell culture media fluids nor apparent signs of cytopathologic effect of cells and further, none of the investigators was aware that his cell culture was contaminated with bacteria. These cell lines generally had been checked for bacterial contamination only with thioglycollate broth media, without regard to the inhibitory activity of the antibiotic or the need for special nutrients after being subjected to antibiotic insult. An enriched agar medium such as the human blood agar medium used in these studies for PPLO cultivation is preferable. It will support growth of fastidious organisms as well as detect the presence of minimal bacterial contamination.

Certain potential false results using the arginine deiminase method for PPLO contamination should be mentioned. Cell cultures contaminated with bacteria which have arginine deiminase activity(3) would produce a positive test for PPLO. However, in our experience a positive test has been found only when PPLO were present and not when bacteria were present. In any case, the presence of arginine deiminase activity in cell cultures would indicate microbial contamination. Furthermore, since no survey can be inclusive, the possibility that a PPLO contaminant may not contain arginine deiminase can not be excluded.

Summary. A rapid, simple and routine chemical method for detection of PPLO contamination in cell cultures is reported. The method is based on the presence of arginine deiminase activity in all tissue culture PPLO strains, but absence from all mammalian tissue and PPLO-free cell cultures. In a total of 73 cell cultures examined, the detection of PPLO determined by presence of arginine deiminase activity agreed entirely with the most sensitive cultural procedure which requires 14 days.

The authors would like to acknowledge the excellent technical assistance of M. W. Grabowski, D. B. Riggs and G. Pla in carrying out these studies.

1. Schimke, R. T., Barile, M. F., *Exp. Cell Res.,* 1963, v30, 593.

2. ———, *J. Bact.,* 1963, v86, 195.

3. Cohen, P. P., Brown, G. W., Jr., *Comparative Biochemistry,* vII, ed., M. Florkin, H. S. Mason, Academic Press, Inc., New York, 1960, p161.

4. Smith, P. F., *J. Bact.,* 1955, v70, 552.

5. ———, *ibid.,* 1957, v74, 801.

6. Barile, M. F., Yaguchi, R., Evelad, W. C., *Am. J. Clin. Path.,* 1958, v30, 171.

7. Chanock, R. M., Hayflick, L., Barile, M. F., *Proc. Nat. Acad. Sci.,* 1962, v48, 41.

8. Barile, M. F., Malizia, W. F., Riggs, D. B., *J. Bact.,* 1962, v84, 130.

9. Archibald, R. M., *J. Biol. Chem.,* 1944, v156, 121.

10. Ratner, S., *Methods in Enzymology,* vII, S. P. Colowick, N. O. Kaplan, Eds., Academic Press, Inc., New York, 1955, p356.

11. Powelson, D. H., *J. Bact.,* 1961, v82, 288.

12. Kenny, G. E., Pollock, M. E., *J. Inf. Dis.,* 1963, v112, 7.

13. Rouse, H. C., Bonifas, V. H., Schlesinger, R. W., *Virol.,* 1963, v20, 357.

Received September 3, 1963. P.S.E.B.M., 1963, v114.