Tetrazolium Agar Overlay in Test for *Mycoplasma pneumoniae*

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In this rapid presumptive test for *Mycoplasma pneumoniae*, subculture is not required, and tetrazolium reduction is evident in 1 hr or less.

Tetrazolium reduction (3), as a method of presumptively identifying *Mycoplasma pneumoniae*, has been suggested for laboratories lacking more refined techniques (4). The tetrazolium agar test of Kraybill and Crawford (7) established the specificity of tetrazolium reduction for *M. pneumoniae* among human mycoplasma species but required a 3- to 6-day subculture period for interpretation of results. In addition, formazan production depends on the density of mycoplasma growth on agar and on whether a broth or agar block inoculum is used for transfer. Repeated subculture may also be necessary to achieve proper colony density for tetrazolium reduction.

We report a specific, rapid, color test by a tetrazolium agar overlay technique for presumptive identification of *M. pneumoniae*, which yields results often in 15 min and is independent of inoculum type and colony density.

Four human mycoplasma species were tested in our procedure: 17 strains of *M. pneumoniae* (recent clinical isolates and the FH strain of L. Hayflick, Stanford Medical School), 7 strains of *Mycoplasma salivarium*, 7 strains of *Mycoplasma orale* type I, and 2 strains of *Mycoplasma hominis*, the latter 16 strains all being clinical isolates. All clinical isolates tested were primary cultures with the exception of the *M. hominis* strains which had been passed once in broth. Definitive identification of all species was by specific growth inhibition by antiserum-impregnated discs (2, 6).

For broth cultures and agar plates, we used the medium formulated by Chanock et al. (1) with penicillin (1,000 units/ml), thallium acetate (1:2,000), and amphotericin B (5 μg/ml).

Agar plates of all mycoplasma specimens were incubated aerobically at 37 C prior to overlay with tetrazolium agar. Equal volumes of 0.21% sterile aqueous 2,3,5-triphenyltetrazolium chloride (Aldrich Chemical Co., Inc.) and 1.3% Ionagar no. 2 (Colab Lab., Inc., Chicago Heights, Ill.) were equilibrated at 45 C, and 2 ml of the mixture was added to each plate. All plates were incubated aerobically (7, 8) and examined periodically at ×100 magnification.

With the tetrazolium overlay technique, the first evidence of reduction and color change specific for *M. pneumoniae* isolates (7) could be detected within 15 min after overlay application, although small colonies frequently required 1 hr. The colonies were initially pink and progressed to purple-black after 3 to 4 hr. Previous hemadsorption tests, whether positive or negative, had no effect on formazan production and did not hinder evaluation of the reducing activity of the colonies.

Inadequacies intrinsic to the tetrazolium reduction test requiring subculture, reported by Kraybill and Crawford (7), do not affect the results of the overlay method described here. *M. pneumoniae* colonies initiated by either agar block or fluid inoculum reduced tetrazolium with equal permanence and rapidity. Similarly, variation of colony density from 1 colony per plate to greater than 200 colonies/cm² did not influence the results.

The feasibility of presumptive tests for *M. pneumoniae* requiring subculture is questionable since large numbers of primary isolates normally cannot be serially transferred (5). Identification by growth inhibition with specific antisera also requires subculture. Consequently, it is important that a battery of presumptive tests be available which can be performed on the initial agar plate.

If only one agar plate is used for primary isolation, an agar block for serial transfer can be removed before the tetrazolium agar is added. Alternatively, tetrazolium overlay
could be used with other tests in plastic 100-mm Y plates (BioQuest) with standard agar (1) in two sections and selective methylene blue agar in a third. One section of standard agar could be used for the hemolytic plaque test; the second section of standard agar could be used for hemadsorption followed by tetrazolium overlay. Appearance of colonies on the methylene blue section would provide a fourth presumptive test.

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