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Received November 23, 1964. P.S.E.B.M., 1965, v118.

A Selective Medium and Color Test for *Mycoplasma pneumoniae*.* (30018)

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Isolation and propagation of *Mycoplasma pneumoniae* depends upon a heart infusion-peptone base medium enriched with horse serum and yeast extract(1). While this formula permits luxuriant growth of *M. pneumoniae,* other mycoplasma (PPLO) are also supported. Once isolated the identity of *M. pneumoniae* is now established by a hemolytic plaque test(2,3) or serologically with specific rabbit antisera(4-9). This report will demonstrate that methylene blue chloride (MB) is an inhibitor for all mycoplasma species of human origin except *M. pneumoniae.* It will be shown that 2,3,5-triphenyltetrazolium chloride (TTC) can be used in a color test which is easily performed and specific for *M. pneumoniae.* Results from a study which applied MB inhibitor medium and TTC color tests will also be summarized.

Materials and methods. Mycoplasma, media, and cultural techniques. Six mycoplasma species of human origin and one saprophytic species were studied. Human species included *M. fermentans, M. salivarium, M. hominis* type I, *M. hominis* type II, *M. pneumoniae,* and *M. pharyngis*(10), while a type B strain of *M. laidlawii* was the saprophyte.† All were maintained at 36°C in fluid medium with serial transfers every 3-4 days. Growth in liquid cultures, including those containing MB‡ and TTC,§ was initiated from a 10% inoculum derived from 3-4-day-old stock cultures. To insure that inocula were viable when liquid to liquid transfers were made, the inoculum was plated, and then after 3, 6, or 7 days the subculture was plated to estimate growth. Plates for aerobic incubation were sealed with paraffin while anaerobic conditions in glass jars were created with 95% N_2, 5% CO_2 gas as recommended by Barile(11). The medium used was that of Chanock *et al*(1,8) with 3 modifications: horse serum was gamma globulin-free,‖ 2,000 U/ml penicillin were used, and amphotericin B was omitted from both cultures. The MB and TTC were prepared in 1.0% aqueous stock solutions sterilized by autoclave before adding to the medium.

MB procedures. Growth inhibition by MB was measured for each species in agar and broth. In agar the concentration tested ranged from 0.00001% to 0.02%. In broth the range was 0.00002% to 0.1% because resistance of PPLO to the dye was somewhat greater in broth. Plates of MB agar were

* From Research Project MR 005.09-1305, Bureau of Medicine & Surgery, Navy Dept., Washington, D. C. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

† *M. fermentans, M. salivarium, M. hominis* type I, *M. hominis type* II, and the FH strain of *M. pneumoniae* were provided by Drs. N. L. Somerson and R. M. Chanock, Nat. Inst. Health, Bethesda, Md. *M. pharyngis* was received from Dr. W. A. Clyde, Jr., Univ. of North Carolina, Chapel Hill, who recently described this species. Dr. Ruth G. Wittler, Walter Reed Army Inst. of Research, Washington, D. C. furnished *M. laidlawii* type B.

‡ National Aniline Division, Allied Chemical and Dye Corp., New York.

§ Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.

‖ Agamma horse serum—Hyland Laboratories, Los Angeles, Calif.

TABLE I. Inhibition of Mycoplasma by Various Concentrations of Methylene Blue Chloride (MB) in Agar.

Mycoplasma species	% MB					
	.00001	.0001	.001	.002	.01	.02
	(% of colonies inhibited*)					
M. pneumoniae	ND†	ND	ND	0	0	0
M. fermentans	0	0	100	100	100	100
M. salivarium	0	80	100	100	100	100
M. hominis I	0	99	100	100	100	100
M. hominis II	0	0	80	100	100	100
M. pharyngis (new species)	99	100	100	ND	ND	ND

* Determined by reference to control plate lacking MB.
† ND = not done.

incubated 38 days before negative readings were accepted. The percent of colonies inhibited was determined from colony counts of test and control plates, identically inoculated, using the formula $P = C\text{-}T/C \times 100$, where P = percent inhibited, C = number on control plate, and T = number on test or MB plate. Inocula showing less than 500 colonies on a control plate were not accepted. Broth cultures of each species grown with and without MB were plated after 3 days' incubation while the number of colonies developing from these inocula were recorded after 30 days' incubation. In case a strain might adapt to MB, tubes containing 0.001%, 0.0002% and 0.1% MB, after incubating until the 6th or 7th day, were plated again to test for increasing numbers.

TTC procedures. Color phenomena arising from TTC reduction were studied in colonies and surrounding agar under aerobic and anaerobic conditions while varying the TTC concentration. Because the physical state of the inoculum was considered another variable, agar plates were seeded with fluid cultures as well as agar block inocula. Agar block inocula were taken from 6-day-old agar cultures. Broth inocula were diluted in PPLO broth with added thallium acetate. All TTC agar plates were held at 36°C for 27-48 days while observations were made every 1-3 days. Whether or not TTC would undergo color change in fluid PPLO cultures under anaerobic conditions was also determined in tubes containing 0.02% TTC.

MB and TTC applied to recovery and identification of M. pneumoniae. To evaluate MB inhibitor plates in conjunction with TTC color tests, mycoplasma isolations were attempted on pneumonia wards of the U. S. Naval Hospital, Great Lakes, Ill. Throat swabs from a total of 235 patients were inoculated onto conventional PPLO agar, while duplicates were streaked onto medium similar in composition but containing 0.002% MB. Plates were incubated aerobically and examined on the 14th, 21st, and 30th day using 40× or 120× magnification. Isolates were transferred serially by push-block, usually twice, until the colonies numbered at least 200/cm^2 of agar surface. Blocks taken from these areas were then transferred to 0.02% TTC agar for color tests. A broth culture was also seeded for the PPLO serodentification test of Huijmans-Evers and Ruys(5,9) to confirm further the identification.

Results. MB activity in agar. The experimental data in Table I, which illustrate the inhibitory property of MB for PPLO of human origin, typify the findings to date. The resistance of *M. pneumoniae* is in contrast with the susceptibility of other species to the dye. It may be observed that *M. pneumoniae* was totally resistant to 0.02% MB, while growth of *M. hominis* type II, the next most resistant species, was blocked by one-tenth this amount. During attempts to prepare a selective medium, the 0.02% quantity was found insufficiently translucent for microscopic study of agar cultures. The level of dye that permitted good microscopic viewing, still effectively inhibitory, and selective in action, was 0.002%.

MB activity in broth. The effect of varying amounts of MB on growth in broth is presented in Table II. That more MB was necessary for inhibition in broth than on agar is evident when Table II data are compared with Table I. *M. pneumoniae* tolerated 0.1% MB, whereas the others were completely inhibited by one-fifth this amount. Methylene blue reduction was not observed although colony counts suggested that adaptive processes in *M. fermentans* were occurring. The 0.001% and 0.0002% levels of dye lowered the count more than 90% during the first 3 days of incubation. With incubation continued to the 6th or 7th day,

TABLE II. Inhibition of Mycoplasma by Various Concentrations of MB in Broth.

Mycoplasma species	% MB							
	3 days‡					4 days	6 days	7 days
	.00002	.0002	.001	.002	.02	.1	.001	.0002
	(% of viable count inhibited*)							
M. pneumoniae	ND†	ND	ND	ND	0	0	ND	ND
M. fermentans	0	93	99	100	100	ND	0	80
M. salivarium	0	88	98	99	100	ND	99	99
M. hominis I	0	0	80	99	100	ND	99	99
M. hominis II	0	0	0	0	100	ND	99	100

* Determined by reference to plate counts of broth lacking MB.
† ND = not done.
‡ Days incubation of broth cultures.

M. fermentans tended to overcome the inhibitor, whereas *M. salivarium, M. hominis* I and II colony counts showed decreasing numbers.

TTC reactivity in agar cultures. Results with mycoplasma in agar have emphasized that atmospheric conditions and kind of inoculum are factors of prime importance for obtaining TTC reduction reactions. If a liquid inoculum was used, and incubation was anaerobic, all species produced reddish hues that were usually confined to the colony, but not to the agar. Another factor was whether or not the colonies were scattered or crowded. If crowded, the colonies rarely produced coloration. Contrasting with anaerobic conditions, colonies grown aerobically on agar were rarely colored even though well spaced. One species, *M. pneumoniae,* showed pinkish centered colonies over a narrow range of diluted inoculum, but such color was only briefly retained.

When TTC plates were inoculated by agar push-block, the reaction appeared in a more convenient and colorful form. In these cultures a pinkish hue plainly visible to the unaided eye spread throughout the resting blocks (Fig. 1). Further study indicated that the agar block method coupled with aerobic incubation had practical application in the recognition of *M. pneumoniae.* Table III presents results of an experiment which stressed the point that only *M. pneumoniae* produced pink blocks when incubation was aerobic. Under anaerobic conditions nonspecific coloration of agar blocks by *M. fermentans, M. salivarium* and *M. pharyngis,* as well as *M. pneumoniae,* were usual. The effect of TTC concentration can also be studied from Table III. It appeared that 0.02% TTC is near optimum since the depth of color obtained was uniformly satisfactory while persisting for a prolonged period. A total of 8 authentic *M. pneumoniae* strains have been tested and all reacted with TTC in the same way. Testifying to the specificity of this reaction (Fig. 1), the following 18 strains of human origin have not produced pink blocks

TABLE III. Effect of 2,3,5-Triphenyltetrazolium Chloride (TTC) Concentration and Atmospheric Conditions on Duration and Specificity of Color Reaction by Mycoplasma Species.

Mycoplasma	% TTC							
	.005	.02	.05	.10†	.005	.02	.05	.10
	Aerobic				Anaerobic			
M. pneumoniae	4–6*	4–22	4–27>	11–13	19	6–48>	4–48>	4–41
M. fermentans	0 ‡	0	0	0	4–7	4–48>	4–48>	11–48>
M. salivarium	0	0	0	0	0	0	19–27	0
M. hominis I	0	0	0	0	0	0	0	0
M. hominis II	0	0	0	0	0	0	0	0
M. pharyngis	ND§	0	0	0	ND	0	2–4	ND

* 1st number = days incubation for color change in block; 2nd number = number days before positive reaction becomes negative or number days the color was observed to be retained.
† Aerobically and anaerobically incubated agar containing 0.10% TTC irregularly inhibited some of the species.
‡ 0 = no reaction.
§ ND = not done.

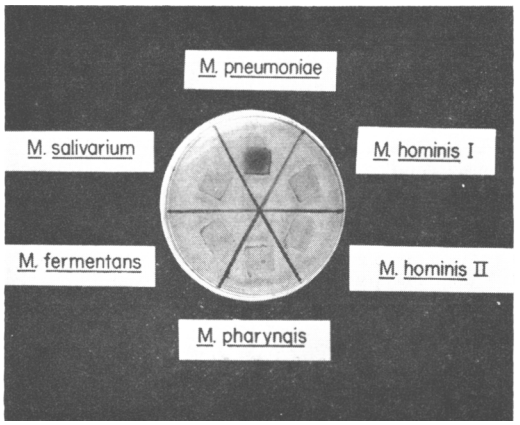

FIG. 1. Agar block color test for *M. pneumoniae* showing specificity of reaction.

in repeated trials under aerobic conditions: 2 *M. fermentans*; 6 *M. salivarium*; 4 *M. hominis* I; 3 *M. hominis* II; and 3 of *M. pharyngis*.

From experience gained, the following method of performing the TTC color test was found optimum for identification of *M. pneumoniae*. A section of TTC-free agar bearing 6-day-old colonies was placed on freshly prepared 0.02% TTC agar. The plate was incubated aerobically at 36°C after sealing the lid with paraffin. The enveloping pink color usually permeated the inoculating block in 3 to 6 days. A dense growth of colonies was necessary to initiate the reaction. Freshly isolated, slowly growing stains most often were transferred twice until 200 or more colonies could be counted on 1 cm^2 of agar surface. Failures of *M. pneumoniae* to reduce TTC under aerobic conditions usually could be traced to an insufficient number of colonies. Economy of time and material was achieved by placing as many as 8 blocks on 1 plate.

TTC in broth. When broth containing 0.02% TTC was inoculated with fluid cultures of test species of human origin, only *M. pneumoniae* generated response. The broth became red in 3 to 5 days followed by precipitation of dark red particles as the supernatant layer slowly decolorized.

MB and TTC responses of M. laidlawii. The non-parasitic PPLO, *M. laidlawii,* was tested for reactivity with TTC and MB as were the other species. Growth response on MB plates indicated that this organism was totally inhibited by 0.002% of MB in agar. Experiments with TTC agar and broth revealed that response of *M. laidlawii,* the free living saprophyte, were identical to those of *M. pneumoniae,* the respiratory tract pathogen.

MB inhibitor plates and TTC color tests in primary isolation of PPLO from pneumonia patients. In the first trial throat swabs from 50 of the 235 pneumonia patients were streaked on MB and conventional agar plates. Of these, 20 patients yielded typical *M. pneumoniae* colonies on 29 plates when both media were scored. Nine isolations were made on both media, 4 on MB plates only, and 7 on conventional medium only (Table IV). An additional 185 patients have been studied subsequently by identical methods. *M. pneumoniae* was recovered from 11 of these patients. Seven isolations were made on both media, 3 on MB plates only, and 1 on conventional medium only. The effect of MB on large colony PPLO belonging to other species was carefully observed. From the total of 235 patients, 47 of these isolations were made, and all grew out on only conventional agar indicating that MB had totally suppressed these species. In 5 examples, *M. pneumoniae* and another species were found as a mixed culture on a conventional plate. The MB test plate on the same patient showed only *M. pneumoniae* colonies. It was mandatory to test the *M. pneumoniae* colonies from MB plates for TTC responses. Of the 23 isolated, all reacted positively, without exception, in the aerobic TTC agar block test indicating that resistance to MB and ability to reduce TTC under these conditions are markers of equivalent significance occurring in all strains. Serologic evidence was obtained which confirmed the *M. pneumoniae* identity of 12 of 23 TTC reactive MB isolates described above. By the paper disk growth inhibition tests against known specific rabbit anti-serum, 12 cultures were positive in their reaction to *M. pneumoniae* anti-serum. Two isolates were not then available and the remaining 9 failed to adapt to broth. In addition, of 24 TTC reactive isolates from conventional agar, the 9 which adapted immediately to broth were

TABLE IV. Selective Action of MB for *M. pneumoniae* Recovery from Throat Swabs.

Cultural status without MB	Cultural status with MB: Negative	*M. pneumoniae* only	*M. pneumoniae* + other mycoplasma	Other mycoplasma only	Total patients cultured without MB
Negative	10	1*	0	0	11
M. pneumoniae only	7‡	5†	0	0	12
M. pneumoniae + other mycoplasma	0	4†	0	0	4§
Other mycoplasma only	20	3*	0	0	23§
Total patients cultured with MB	37	13	0	0	50

* *M. pneumoniae* isolated on MB only. † *M. pneumoniae* isolated on both agars. ‡ *M. pneumoniae* isolated only on agar without MB. § Large colony mycoplasma.

positive in their reaction to *M. pneumoniae* anti-serum.

Discussion. The growth of contaminating bacteria and fungi is commonly prevented in PPLO media with penicillin, thallium acetate, and amphotericin B. While chemical inhibitors against mycoplasma have not been applied in cultures, their existence has been noted by earlier workers. Smith, Morton, and Leberman reported mycoplasma inhibitory activity with Nile Blue A, thionin, and sodium azide(12). Crystal violet was found toxic for PPLO under certain conditions by Morton and Lecce(13). The observation that MB will rule out all human strain mycoplasma and allow *M. pneumoniae* to grow is a new finding with an obvious potential. The PPLO agar plate with MB inhibitor should prove useful in epidemiological surveys of respiratory diseases. Other uses such as purifying mixed cultures or even to identify may be envisioned. In this study the number of species tested was limited to those of human origin. Information of interest and diagnostic importance may evolve from studies of MB inhibition in the mycoplasma species of animals and poultry.

In the first 50 patients cultured for *M. pneumoniae,* an excess of 3 recoveries was noted on conventional agar (16 conventional *vs* 13 MB). Conceivably, MB may have contributed to the difference. If due to MB and not to chance variation, then fewer recoveries on MB agar should have been obtained in the 2nd series of 185 cultures. Instead, the trend was opposite in direction (8 conventional *vs* 10 MB) indicating that MB was not contributing significantly to the error.

The study of TTC was here undertaken because the literature did not include observations on *M. pneumoniae* or species authentically identified. Furthermore, the factor of aerobiosis needed to be considered. Switzer used 0.005% TTC to determine growth in cultures of swine strain mycoplasma(14). Somerson and Morton reported on 6 human strains of PPLO(15). They stated that all 6 reduced TTC under anaerobic conditions. By comparing TTC reactions of identified species when grown under aerobic and anaerobic conditions, our results agree with and extend the Somerson and Morton findings.

The observation that *M. laidlawii* type B was aerobically reactive to TTC under the same conditions as *M. pneumoniae* stresses an important point; namely, that when agar block TTC color tests are performed for diagnostic purposes, the source of the culture must be known. Conceivably, the Laidlaw PPLO could interfere with *M. pneumoniae* identification. However *M. laidlawii* can be distinguished by habitat and growth characteristics, while ordinarily they are not found in the respiratory tract of human subjects. The TTC color test should assist those wishing a reliable easily performed test of identification. Among the major advantages are: TTC is inexpensive, readily available, and can be sterilized in aqueous solution by autoclave or after being added to the medium; the technic entails simple procedures, macroscopic observations, and excludes the need of animals; the time and work expended is less than needed for identifying technics currently in use.

Summary. Two common laboratory compounds, methylene blue chloride (MB) and 2,3,5-triphenyltetrazolium chloride (TTC), were studied for their reactivity with or

against human strain mycoplasma. Addition of 0.002% of MB to PPLO agar converts it to a medium which is highly selective for *Mycoplasma pneumoniae*. This modification is recommended for epidemiological surveys, for purification of cultures, and even for identification of *M. pneumoniae*. A test based on a color reaction between *M. pneumoniae* and TTC has been described. This test is recommended for confirming or establishing the identity of this species from other mycoplasma of the human respiratory tract.

The authors are indebted to Dr. Irvin S. Snyder, Univ. of Iowa Coll. of Med., for helpful suggestions in the tetrazolium study.

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Received November 30, 1964. P.S.E.B.M., 1965. v118.

Enhancement of Ethanol Toxicity by Ethacrynic Acid. (30019)

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Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl) phenoxy] acetic acid is a new potent saluretic-diuretic agent that was developed through a search for biologically active compounds that react selectively with sulfhydryl groups(1). Although ethacrynic acid probably most closely resembles the mercurial diuretics(2), its mechanism of action is thought to be qualitatively different from diuretics employed commonly.

During studies in our laboratory on the effect of ethacrynic acid on thiol groups it was noticed that mice treated with ethacrynic acid became much more susceptible to the toxic effects of ethanol. Doses of alcohol, non-lethal normally, were lethal to a large percentage of mice after treatment with this diuretic. This problem was further investigated because of the importance of sulfhydryl containing enzymes in the metabolism of ethanol, and the possible clinical implications of ethanol toxicity.

This paper presents data concerning the increased lethality of ethanol after ethacrynic acid treatment and preliminary studies on the mechanism of this action.

Methods. Male and female general purpose mice weighing 20-30 g were used in these experiments. Mice were generally treated subcutaneously (s.c.) with the drug prior to administration intraperitoneally (i.p.) of alcohol. Alcohol was administered as a volume/volume solution prepared such that the dose/kg of alcohol was brought to a volume of 10 ml with distilled water or saline. One-tenth ml of this solution was administered per 10 g of body weight.

Blood alcohol was determined by the alcohol dehydrogenase (AlDH) method of Bucher and Redetzki as described(3). Enzymes and cofactor were obtained from Calbiochem. Yeast AlDH was used as received. Equine liver AlDH was used after dialysis for 48 hours to remove residual ethanol.