

New Laboratory Techniques for Isolation of *Mycoplasma pneumoniae*

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Received January 4, 1983

The SP-4 culture medium, developed originally for newly isolated plant and insect mycoplasmas (spiroplasmas), has markedly improved the recovery of *Mycoplasma pneumoniae* from human clinical materials. This medium, in combination with a direct fluorescent antibody test, can enhance the recovery and identification of the organism by 30-40 percent over conventional culture procedures. Although these modifications are a clear improvement in diagnostic techniques for *M. pneumoniae*, the time required for growth and identification of the agent is still a major disadvantage for rapid clinical diagnosis. Thus, there remains a critical need for techniques that can specifically identify the major antigens (or other components) of the organism within the first week of the infection.

INTRODUCTION

The laboratory diagnosis of *Mycoplasma pneumoniae* infections is an important and necessary adjunct to clinical impressions in establishing the role of the agent in acute human respiratory disease. In addition, clinical experiences reported over the past ten years have emphasized the involvement of this organism in a wide spectrum of other pulmonary manifestations and in some important and serious extrapulmonary syndromes [1]. In these latter situations, the association with *M. pneumoniae* may occur with, or in the absence of, overt respiratory disease symptoms. Thus, it is important that both clinical and laboratory personnel encourage performance of proper laboratory diagnostic techniques for this organism on all patients in early stages of respiratory disease and in those individuals with symptoms known to be associated with extrapulmonary *M. pneumoniae* infections (e.g., pericarditis, hemolytic anemia, encephalitis, and so on). Adequate and early diagnosis of *M. pneumoniae* infections also has important clinical significance, since effective antibiotic therapy is available in the form of tetracycline or erythromycin drugs.

The laboratory diagnosis of *M. pneumoniae* infections rests primarily on an adequate serological analysis of the patient's serum (which is discussed elsewhere in this symposium) and on the isolation and identification of the organism from secretions of the respiratory tract or other appropriate sites [2,3].

Few technical improvements in cultivation techniques have been proposed for this organism since the initial discovery of the agent [4]. The basic mycoplasma medium employed for most work has centered around a formulation developed by Derrick Edward [5], consisting of a base medium of seven parts beef heart infusion and sup-

plements of two parts sterile horse serum, one part fresh yeast extract, glucose (0.5 percent final), and phenol red indicator (0.002 percent final) [4]. Broth cultures showing a decline in pH (from glucose fermentation) or increased turbidity are plated to a solid medium (of the above formulation, but excluding glucose and phenol red). Agar colonies of *M. pneumoniae* are identified on the basis of hemadsorption [6], hemolysis [7], or by a plate immunofluorescence test with specific *M. pneumoniae* antiserum [8].

MATERIALS AND METHODS

Culture Medium

The formulation of the SP-4 medium is given in Table 1. A diphasic medium is prepared in 4 ml vials by placing 1 ml of melted SP-4 agar medium (prepared with 0.8 percent Noble agar) in the bottom of a sterile vial. The agar is allowed to solidify and 2 ml of SP-4 broth is overlaid on to the agar. Broth medium for throat cultures should contain penicillin G (final 1,000 units/ml) and thallium acetate (final 0.5 mg/ml). For some throat specimens with heavy bacterial loads (such as in pediatric patients), thallium levels should be increased (to final 1 mg/ml) and polymyxin B (final 500 units/ml) added to the broth overlay. Mycoplasma agar plates are prepared from either the SP-4 formula or from conventional mycoplasma broth containing 20 percent horse serum. It is important to use only washed or purified agar (Noble, agarose) and bacterial inhibitors (thallium acetate, penicillin G) in any solid medium.

Quality control of medium components is a critical factor in cultivation of the organism, since unsatisfactory lots of base medium, fresh yeast extract, or fetal bovine serum are encountered (see [2] or [9] for detailed procedures).

Specimen Collection and Transport

Throat and nasopharyngeal swabs, tissues from lung or brain, and spinal, pleural, or pericardial fluids should be placed immediately in diphasic SP-4 medium and

TABLE 1
SP-4 medium for recovery of *Mycoplasma pneumoniae*

Basal Medium	Amounts for 1 liter
Mycoplasma Broth Base	3.5 g
Bacto Peptone	5.3 g
Bacto Tryptone	10.0 g
Glucose	5.0 g
De-ionized water	560 ml
Adjust pH to 7.5-7.6. Sterilize at 121°C for 15 minutes.	
Sterile supplements	Amounts for 1 liter
CMRL 1066 tissue culture supplement— (10×) (with glutamine)	50 ml
Fresh yeast extract (25% solution)	35 ml
Yeastolate (2% solution) (Difco)	100 ml
Fetal bovine serum (heated 56°C—1 hour)	170 ml
Penicillin G (100,000 units/ml)	10 ml
Thallium acetate (1:50 solution)	50 ml
Polymyxin B (100,000 units/ml) (optional)	5 ml
Phenol red (0.1% aqueous)	20 ml
Final pH, 7.4-7.5	Osmolality, 332 mOsm

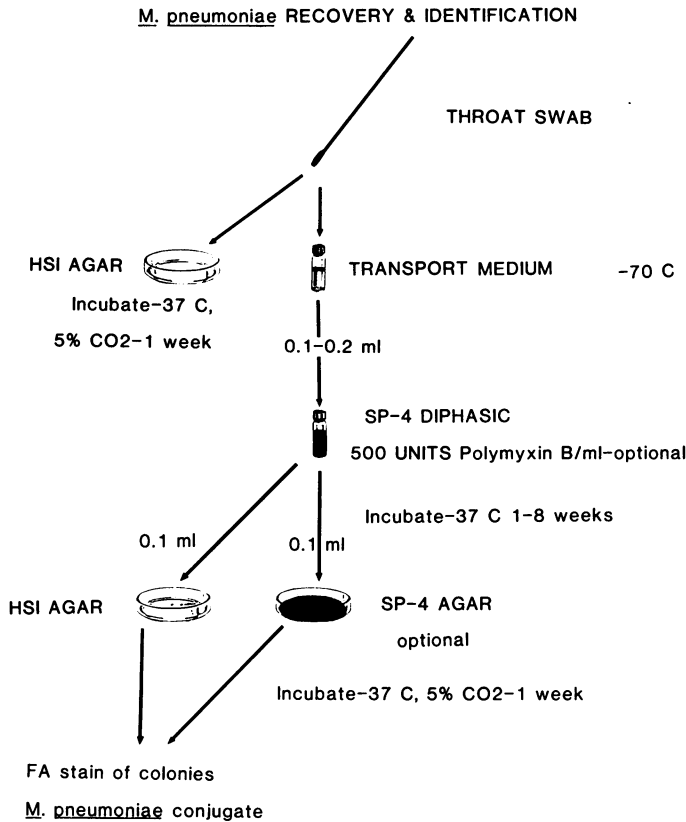


FIG. 1.

onto solid mycoplasma media [5], or into a suitable transport medium (such as conventional mycoplasma broth containing 20 percent horse serum) (Fig. 1). If the specimen cannot be cultured within 24 hours of collection, it should be frozen (in transport medium) at -70°C until appropriate materials are available.

Cultivation Procedures

A recommended scheme for cultivation of *M. pneumoniae* is given in Fig. 1. After addition of 0.1 to 0.2 ml of the transport specimen to SP-4 diphasic medium, the transport specimen should be refrozen at -70°C in case repeat tests are required. Tissues should be minced coarsely and then added to about 5 ml of SP-4 broth. At least two tenfold dilutions of the initial suspension should be made in SP-4 broth to reduce possible inhibitory substances from tissues (antibiotics, antibodies, hemoglobin, lysolecithin, and so on). During incubation, the SP-4 diphasic vials should be observed every 3-5 days for decreases in the pH indicator or for increased turbidity in the broth phase. When this occurs, small volumes (0.1 to 0.2 ml) are plated to conventional mycoplasma agar plates and subcultures made to fresh SP-4 diphasic medium. Plates are incubated (37°C) aerobically (but sealed to prevent dehydration), or are placed in an environment of 95 percent nitrogen-5 percent carbon dioxide. Mycoplasma colonies usually appear after incubation periods of 5-14 days.

Identification of Isolates

The most rapid and specific identification of *M. pneumoniae* colonies growing on agar plates is accomplished through a direct plate immunofluorescence antibody test

[8]. The plates are flooded with about 1–2 ml of phosphate-buffered saline and then allowed to soak for 20–30 minutes at room temperature. The wash fluid is discarded in a beaker (for eventual disinfection) and about 1 ml of an appropriate dilution of a fluorescein-conjugated antiserum specific for *M. pneumoniae* is added to the plate. The dilution of conjugate selected will depend upon the potency of the antiserum, but a conjugate dilution of 1:20 to 1:40 will usually provide strong fluorescence of *M. pneumoniae* colonies. After a 20- to 30-minute incubation of agar colonies and conjugate, the conjugate is poured off (in beaker for disinfection) and the plates again washed two or three times with about 2–3 ml of saline. After final wash, the plates are inverted and dried for 20 minutes. The plates are examined with a fluorescence microscope equipped with incident illumination. Colonies are scanned under magnification of about $\times 160$, using incandescent light for transmitted illumination and a quartz-halogen or high-pressure mercury vapor lamp source for incident illumination. Recommended filter systems include the following: BP 450/490, FT 510, and LP 520, for exciter, beam splitter, and barrier filters, respectively.

Confirmation of *M. pneumoniae* colonies on agar can be accomplished by preparing duplicate plates of the throat culture specimen and selecting colonies of the size and shape of those staining with specific conjugate. These colonies are then transferred to fresh broth or agar and the resulting growth eventually obtained on agar plates identified by conventional growth inhibition tests [10]. Conversely, duplicate agar plates of the primary culture can be flooded with a suspension of 5 percent guinea pig erythrocytes in saline, and the colonies examined microscopically for adsorption of erythrocytes to specific colonies [6], or an overlay of guinea pig erythrocytes in agar added to the plate and plates re-incubated for observations on the development of hemolytic zones around *M. pneumoniae* colonies [7].

RESULTS AND DISCUSSION

The SP-4 medium formulation was initially developed for the growth of helical mycoplasmas [11]. The value of the medium to support the growth of *M. pneumoniae* from human throat culture specimens was shown in a study [12] where SP-4 medium was compared to recovery rates of the organism in the standard mycoplasma medium containing 20 percent horse serum. An increased isolation rate of 30–40 percent was found with SP-4 medium. In addition, that study also indicated that the incorporation of methylene blue [13], at least when added to SP-4 medium, significantly inhibited the growth of *M. pneumoniae*.

In those individuals who are shedding significant amounts of the organism (such as in acute respiratory disease or early convalescence), the diphasic medium will show changes in pH indicator (to yellow) within 8–15 days. When these SP-4 broth cultures are transferred to agar plates, a pure culture of the organism is usually apparent by immunofluorescent tests. In those individuals in late convalescence phases of *M. pneumoniae* infection (or even in some asymptomatic individuals), few *M. pneumoniae* colonies may be apparent on agar plates stained with specific conjugates. Frequently, these colonies may be mixed with colonies of other mycoplasmas common to the normal flora of the mouth or throat (*M. orale* and *M. salivarium*). In these situations, it is important to examine the plate carefully by the fluorescent technique to search out the one or two *M. pneumoniae* colonies that might be present.

Finally, we have frequently observed an alkaline shift in the SP-4 broth, presumably due to the growth of such arginine-hydrolyzing mycoplasmas as *M.*

orale or *M. salivarium*. When these broth cultures were plated to agar and resulting colonies examined by fluorescence with *M. pneumoniae* conjugates, as much as 50 percent of the colony population was found to be *M. pneumoniae*. We believe this is perhaps the result of more vigorous metabolism of arginine-hydrolyzing mycoplasmas in competition with glucose-fermenting *M. pneumoniae*. This observation suggests that when any change occurs in the indicator of the SP-4 broth, either to the acid or alkaline side, appropriate platings of the culture to solid media should be done as soon as possible.

The modifications described here generally enhance the recovery of *M. pneumoniae* and provide laboratory confirmation of the role of the organism in infection. However, even in the best of circumstances, growth of the organism in liquid medium, then growth on the agar medium, and final identification by direct immunofluorescent antibody test may require 2-3 weeks. This is not an ideal situation, particularly from a clinical standpoint when decisions on appropriate therapy may rest on more adequate information as to the nature of the etiologic agent. Thus, there is still a critical need for a rapid diagnostic test that will specifically identify the major antigens (or other important components) associated with this organism. Logically, this test should be performed on sputum or throat washings at an early stage of the infection and when the patient is shedding the largest numbers of *M. pneumoniae*. The recent identification of *M. pneumoniae* proteins associated with hemadsorption and cytdesorption (such as the P1 protein) [14-16] might eventually offer a suitable detection system for rapid diagnosis of *M. pneumoniae* infections.

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