Comparison of the Sensitivity of Two Methods for Isolation of *Mycoplasma pneumoniae*

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Throat swab specimens from 1,494 Marine Corps recruits were inoculated into vials containing diphasic (broth/agar) mycoplasma medium and also onto PPLO agar plates, which were subsequently overlayed with sheep erythrocytes in saline agar. Strains of *Mycoplasma pneumoniae* were isolated from 89 (6%) of the specimens by one or both of the methods. Eighty-one of the 89 (91%) positive specimens were cultured with diphasic medium, whereas only 42 (47%) were cultured with the overlay method ($\chi^2 = 7.44$, $P < 0.01$). The diphasic system for isolation of *M. pneumoniae* should be the method of choice in clinical and research application.

Two methods for isolating and identifying *Mycoplasma pneumoniae* in clinical specimens are in current use (1, 6). The diphasic medium method utilizes vials containing nutrient broth overlying a layer of agar. In this method *M. pneumoniae* growth is indicated by a color change in the medium due to acid production. In the agar overlay technique, the presence of *M. pneumoniae* colonies is determined by the appearance of surrounding areas of beta-hemolysis in the overlay containing sheep or guinea pig erythrocytes.

During a field trial of an inactivated *M. pneumoniae* vaccine (R. P. Wenzel, R. B. Craven, J. A. Davies, J. O. Hendley, B. H. Hamory, and J. M. Gwaltney, Jr., *J. Infect. Dis.*, in press), a comparison was made of the sensitivity of these two methods for isolating *M. pneumoniae* in clinical specimens.

MATERIALS AND METHODS

Specimens. Throat swab specimens were obtained from 1,494 Marine Corps recruits at Parris Island, S.C., and immediately placed in 2 ml of transport medium collecting broth. Liquid was expressed from the swab by pressing it against the inner wall of the vial. One hundred and thirty-five specimens were obtained from recruits hospitalized with pneumonia, 337 from recruits seen as out-patients with bronchitis, and 1,022 from asymptomatic recruits in training. The vials were kept at 4°C before testing.

The vials were shipped to Charlottesville, Va., and processed within 24 to 36 h from collection, a delay that reportedly will not adversely affect isolation rates (5).

Media. The collecting broth medium contained 7 parts PPLO broth base (Difco Laboratories, Detroit, Mich.), 2 parts horse serum (GIBCO, Grand Island,

N.Y.), 1 part fresh yeast extract, 2.5% thallium acetate, and 1,000 μ g of penicillin per ml (4). To this was added 0.002% phenol red, 1% D-glucose, and 1 N NaOH sufficient to adjust the pH to 7.8. Broth for the diphasic medium contained all of the above components in the same ratio plus 0.1 ml of 2% methylene blue per 100 ml. In both techniques the solid medium was PPLO agar (Difco) to which was added horse serum, yeast extract, thallium acetate, and penicillin in the same proportions as above. The overlay contained 0.5 ml of 50% whole sheep blood in Alsever solution and 2 ml of 1% agar (Difco) in 0.85% saline.

A sample of the specimen (0.1 ml) was inoculated onto a petri dish (60 by 15 mm) containing 4 ml of agar, and 0.1 ml was also inoculated into a vial containing diphasic medium. Inoculated agar plates were incubated at 36°C in a jar with 100% relative humidity. The overlay medium was applied on day 10 of aerobic incubation of the PPLO plate. The plates were read at 24 and 48 h for evidence of beta-hemolysis.

After inoculation, the diphasic vials were observed daily for color change for a period of 6 weeks. If a color change occurred, 0.1 ml of the broth layer was inoculated onto PPLO agar, on which was placed a disk impregnated with *M. pneumoniae* hyperimmune guinea pig serum (Microbiological Associates, Bethesda, Md.). The plate was incubated for 10 days, overlaid with erythrocytes as described above, and read for growth inhibition around the antiserum disk, thus confirming the isolate as *M. pneumoniae* (2).

RESULTS

M. pneumoniae was isolated from 89 (6%) of the 1,494 recruits studied by the two methods combined. The diphasic method was clearly superior to the overlay technique whether the

TABLE 1. Relative isolation rates of *M. pneumoniae* from 89 positive throat swab specimens

Clinical status of recruits	Total no. positive	No. positive (%) ^a	
		Agar overlay	Diphasic
Pneumonia	40	17 (42)	39 (98)
Bronchitis	19	11 (58)	19 (100)
No symptoms	30	14 (46)	23 (77)
Total	89	42 (47) ^a	81 (91) ^a

^a $\chi^2 = 7.44$; $P < 0.01$.

specimen was obtained from recruits with pneumonia, bronchitis, or no apparent illness (Table 1). Of all isolates, the diphasic method yielded *M. pneumoniae* in 98% of culture-proven cases of pneumonia, in 100% of culture-proven cases of bronchitis, and in 77% of asymptomatic pharyngeal carriers of the organism. This contrasts with rates for the agar overlay method of 42, 58, and 46%, respectively. Overall, the diphasic method was twice as sensitive as the overlay method, 91 versus 47% in total isolations ($\chi^2 = 7.44$, $P < 0.01$).

DISCUSSION

In the experience of Grayston et al., approximately one-half of *M. pneumoniae* isolates were detected in the direct plate, whereas the remainder were isolated through use of the diphasic medium (3). However, no data were presented by the authors in their review article.

The increased sensitivity of the diphasic method we observed may be a result of increased time of incubation with this system compared to the overlay method. The plates in the overlay method were incubated for 10 days prior to the sheep cell overlay and then observed for a maximum of 2 more days. Unless they became positive earlier, all the diphasic cultures were observed for 6 weeks, although none became positive after 4 weeks. It is possible that specimens containing a smaller number of colony-forming units may have been detected using the longer period of incubation. A second explanation may be that the observation of color change is less exacting than the proper overlaying of erythrocytes and subsequent interpretation of hemolysis. A third explanation might be that in a diphasic medium the liquid

phase acts to dilute toxic by-products of metabolism, whereas on solid media such toxic by-products might accumulate near the colony and prevent or inhibit growth.

A further question is the relative sensitivity of the cultural method to the serological method for diagnosing *M. pneumoniae* illness. Of 43 patients with pneumonia on whom we performed cultural and serological tests (R. P. Wenzel, R. B. Craven, J. A. Davies, J. O. Hendley, B. H. Hamory, and J. M. Gwaltney, Jr., *J. Infect. Dis.*, in press), 15 of 21 (71%) of those who had fourfold serum metabolic inhibition antibody rises had isolation of *M. pneumoniae*. Similarly, 15 of 21 (71%) who had a positive culture had fourfold serum metabolic inhibition antibody rises. Sixteen patients had negative tests by both methods. Thus, in our experience serological tests and the diphasic cultural tests are of equal value.

Both cultural methods require a great deal of time and offer the clinician only retrospective data. The diphasic technique is the more time consuming of the two, requiring days of observation for confirmation by the disk inhibition method. Nevertheless, the markedly increased sensitivity of the diphasic medium suggests that it should be the method of choice for clinical usage and for use as a research tool for epidemiological or vaccine studies that involve the isolation of *M. pneumoniae*.

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