Evaluation of a Microscopy Method for Rapid Detection and Identification of *Mycoplasma pneumoniae*

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A microscopy test that used the typical shape of *Mycoplasma pneumoniae* cells growing on glass was investigated for its value for diagnostic purposes. Suspensions from 108 throat swabs were infected artificially with 10^2 , 10^3 , and 10^4 colony-forming units of three *M. pneumoniae* strains per ml. Agar medium, a diphasic medium, and the microscopy method with liquid medium in cover slip chambers were compared for isolation of the mycoplasmas. The mycoplasmas were detected first by the microscopy method in nearly all concentrations tested. Typical *M. pneumoniae* cells could often be detected after 48 h. No differences were found between a laboratory strain and two low-passage strains. The experimental results suggest that under special circumstances the microscopy method could be a useful tool for isolation and identification of *M. pneumoniae*.

Methods using different properties of *Mycoplasma pneumoniae* can be applied for isolation and identification of these organisms from clinical material. Most commonly, the mycoplasmas are grown on agar and the colonies are tentatively identified by morphology (8), hemolysis of erythrocytes in an agar overlay (9), or by more elaborate methods like growth inhibition (10). Growth of *M. pneumoniae* can also be detected in diphasic medium (1, 8), by glucose fermentation, and reduction of methylene blue (5). More rapid methods consist of using antigenic markers by applying immunofluorescence to clinical material (14), or the formation of characteristic rosettes in the sputum of patients, as described in a preliminary report (11). So far, neither method has found widespread application.

Another property of *M. pneumoniae* not yet used for diagnostic purposes is the typical shape of its cells. Microscopy studies have revealed a typical morphology of glass-grown cells of *M. pneumoniae* (2, 6). The applicability of morphological markers for diagnostic purposes has been shown in a preliminary study for *M. hominis* and to a lesser extent for *M. pneumoniae* (7). Early detection of typical *M. pneumoniae* cells in cover slip chambers permitted identification under favorable conditions after 48 h. A study on clinical specimens obtained over several months yielded only three cases of *M. pneumoniae* infection (W. Lam, M.D. thesis, Johannes Gutenberg-Univ., Mainz, West Germany, 1974). Therefore, an experimental

study was carried out to investigate the sensitivity and reliability of the method. The results indicate that microscopy detection and identification of *M. pneumoniae* cells might provide a fast and reliable method for diagnosis of *M. pneumoniae* infection.

MATERIAL AND METHODS

Mycoplasmas. Three strains of *M. pneumoniae* were used in this study. The laboratory strain FH and the strains R6 and R54 were recently isolated and identified in our laboratory from clinical material. Strains R6 and R54 were used after four passages on artificial media. The mycoplasmas were grown in 100-ml flasks in liquid medium until the indicator changed to orange. Then they were filtered through 0.4- μ m membrane filters (Nucleopore, General Electric Co.) to break up clusters, distributed in small amounts, and stored at -70°C . After 24 h of freezing, one vial of each strain was thawed, diluted, and counted by spreading on agar plates. The concentrations were 4×10^7 , 1.5×10^5 , and 4×10^5 colony-forming units (CFU) per ml for FH, R6, and R54, respectively.

Media. Liquid medium was prepared according to the formula of Hayflick (13), with addition of 0.002% phenol red, 0.05% thallium acetate, and 1,000 U of penicillin per ml. It was distributed in 2-ml portions in screw-capped vials and stored at -20°C until use. Diphasic medium was prepared as described by Smith et al. (18). Agar medium with and without 0.1% glucose was prepared according to the method of Hayflick (13). The agar medium was poured in plastic petri dishes (60 by 15 mm) (Greiner, Nürtingen).

Cover slip chambers. Small cover slip chambers,

consisting of a glass ring and two cover slips, were prepared as described previously (2).

Preparation of artificially infected throat swabs. Throat swabs were taken from a healthy person proved to be free of other mycoplasmas and immediately immersed in 2 ml of liquid medium. The swabs were squeezed out completely and then removed from the medium. The resulting suspension was inoculated with 10 to 200 μ l of medium containing the amount of CFU of *M. pneumoniae* per milliliter required for the experiment. After thorough mixing, the suspension was used for inoculation of diphasic medium (0.1 ml), two agar plates (0.05 ml each), and for filling of three cover slip chambers (0.2 to 0.25 ml each). The remaining liquid was left in the vial and incubated together with the other media at 37 C. Three concentrations of each *M. pneumoniae* strain were tested: 10^2 , 10^3 , and 10^4 CFU per ml. Twelve throat swabs were infected and examined simultaneously for each concentration of each strain tested.

Examination of cultures. The cover slip chambers were examined daily up to 6 days after inoculation with a Zeiss phase-contrast microscope with a condensor for long working distance (7 mm). The magnification was $\times 1,250$. A chamber was declared negative if no *M. pneumoniae* cells were found within 3 min. A preparation was considered positive after one or more typical *M. pneumoniae* cells was detected. The diphasic medium was examined daily for a color change from blue to yellow. The agar media were examined beginning with day 4 after inoculation with a stereomicroscope ($\times 40$). The preparation was considered positive after detection of one or more mycoplasma colonies.

Statistical methods. The results are presented graphically (see Fig. 2). Time differences in detection of growth were checked by the test of Wilcoxon

and Wilcoxon (17) in connection with the Friedman test (17) or by the Wilcoxon test (19) for pair differences. Comparisons between the influence of different inocula or, with a constant inoculum, between different strains were checked by the Dwas and Steele test (16).

RESULTS

The mycoplasmas were easily detected in the cover slip chambers despite debris, epithelial cells, and dead or inhibited bacteria. The first *M. pneumoniae* cells that could be detected often had a typical stretched shape (Fig. 1a). Later, small colonies with cells growing out from the edges were observed most frequently (Fig. 1b).

Colonies were detected nearly simultaneously on agar with and without glucose. Therefore, only the results obtained from agar without glucose were used for statistical purposes. Colony counts were done only on a few plates. They indicated a recovery of 50 to 100% of the inoculated CFUs.

Altogether, nine separate experiments with 12 swabs each were carried out. Of the 108 swabs examined, 2 showed no growth with all methods ($R6$, 10^3 CFU/ml), suggesting an error. The microscopy method failed with two swabs (FH, 10^3 and 10^4 CFU/ml), likewise the diphasic medium (FH, 10^3 CFU/ml; $R6$, 10^2 CFU/ml). The agar medium with glucose failed once (FH, 10^2 CFU/ml).

The results of the experiments are presented in Fig. 2. They suggest in most cases a faster detection of *M. pneumoniae* by the microscopy method. This was confirmed by statistical analysis (Table 1). The microscopy test was always significantly faster than the diphasic medium. There were not always such significant differences if the microscopy test was compared with growth on agar. However, the data in Fig. 2 suggest a certain advantage of the microscopy method in these cases too, and a separate analysis (Wilcoxon test for pair differences) showed significant differences in the five ex-

TABLE 1. Comparison of isolation methods^a

Strain tested	Inoculum (CFU per ml)	Microscopy method vs. diphasic medium	Microscopy method vs. agar medium	Agar medium vs. diphasic medium
FH	10^4	0.01 ^b	0.05 ^c	NS ^d
	10^3	0.01	NS	0.05
	10^2	0.01	NS	0.05
R6	10^4	0.01	0.01	NS
	10^3	0.01	NS	0.05
	10^2	0.01	0.01	NS
R54	10^4	0.01	0.01	NS
	10^3	0.01	NS	NS
	10^2	0.01	NS	0.05

^a Friedman test and consecutive comparison by Wilcoxon and Wilcoxon.

^b If number indicates significant difference, the method mentioned first in the heading gave earlier results. Significance $P \leq 0.01$.

^c Significance $P \leq 0.05$.

^d NS, No significance $P > 0.05$.

FIG. 1. *M. pneumoniae* cells after 48 h. Phase contrast. Bar represents 5 μ m.

FIG. 2. Time of detection of *M. pneumoniae* growth (three strains, three different concentrations of each strain) by different methods. M, Microscopy method; D, diphasic medium; A, agar medium. (a) Nine tubes were positive on day 10 or later.

periments with no significance in Table 1 (microscopy versus agar).

The time of the first detection of mycoplasmas in most cases depended on the number of CFUs inoculated. A higher number of organisms per milliliter was detected significantly earlier than a lower number of cells by microscopy and diphasic medium, whereas the number of mycoplasmas was not so important for the time of detection on agar. The laboratory strain FH and the newly isolated strains (R6, R54) did not differ significantly in their reactions on agar and in the microscopy method. The FH strain was detected slightly later in the diphasic medium.

DISCUSSION

Artificially infected specimens cannot replace clinical material in an evaluation of a new diagnostic method. However, the results obtained from a critically conducted study with such specimens should permit some conclusions about the reliability and sensitivity of the method investigated.

The throat swabs in this study were taken from an individual who was not carrying in his throat any known mycoplasmas. Therefore, the experiments do not give data about the probability of diagnostic errors caused by other *Mycoplasma* species from the human respiratory tract. However, the possibility of such an error seems negligible for two reasons. First, the mycoplasmas colonizing the human respiratory and genitourinary tract have a cellular morphology distinctly different from *M. pneu-*

moniae. *M. orale* forms a thin filament with extensive branching (4) not resembling the typical shape of cells and clusters of *M. pneumoniae*. *M. salivarium* and *M. buccale* (the former *M. orale* type 2) are rather diplococoid (4) or form small disk-shaped cells and short filaments (W. Bredt, unpublished observations). Cells of *M. hominis* are much thicker and form distinctly different figures (5). *Ureaplasma urealyticum* (the former T strains) grow mostly as coccoid cells (W. Bredt, unpublished observations). Therefore, recognition of the characteristic morphology of *M. pneumoniae* (2, 6), with its stretched cells strongly adhering to the glass surface, is not difficult for somebody who has seen these cells in culture. Second, our own preliminary studies with clinical material (7; W. Lam, M.D. thesis, Johannes Gutenberg-Univ., Mainz, West Germany, 1974) did not show any interference in the approximately 150 swabs investigated. Therefore, we do not consider confusion of cells of different *Mycoplasma* species a serious problem once a certain experience has been acquired.

The detection of the mycoplasmas was successful in all experiments. The occasional recovery of less than 100% of the inoculated CFUs on agar suggests a loss, either by some mycoplasma-inhibiting activity of the pharyngeal material or by adherence to epithelial cells, resulting in aggregation and thus in reduction of CFUs.

Despite this slight reduction in CFUs, the growth on agar proved to be as sensitive as the two other methods tested. The failures of all three methods (1 to 2%) seem to be at a

tolerable level. In each case of a failure of one method, the organisms have been detected by one of the other procedures. On the other hand, this aspect of the results supports previous reports, which indicate the advantage of two separate methods of isolation (12). The results of the study suggest that even low numbers of organisms (10^2 per ml) in a specimen can be detected.

Comparing the time needed for tentative diagnosis with each method, the microscopy method clearly seems to have considerable advantages. In nearly all concentrations, microscopy allowed earlier detection of the organisms. This was evident especially with higher concentrations of organisms, but even at a level of 10^2 CFU per ml at least half of the swabs were diagnosed first in the cover slip chambers. The relation is even more in favor of the microscopy method if the detection on agar from a real clinical material is considered. There the colonies are usually observed only after 7 days (1, 8). Also, in our study the freshly isolated strains in their fourth passage may already have adapted to growth on artificial medium. This, and our additional experience, probably resulted in detection on agar as early as day 4. Considering the advantages and disadvantages of the microscopy method in comparison with other procedures, it should be noted that detection of typical *M. pneumoniae* cells allows an immediate diagnosis, whereas a color change in diphasic medium or detection of colonies on agar have to be followed by time-consuming identification procedures.

There are several circumstances under which the use of microscopy detection and identification of *M. pneumoniae* cells seems to offer some advantages. One application is the direct examination of clinical material. Here positive results can be obtained as early as 2 days after inoculation. The few clinical cases studied so far (7; W. Lam, M.D. thesis, Johannes Gutenberg-Univ., Mainz, West Germany, 1974) were promising, but are not sufficient for a final statement. Furthermore, the microscopy examination of *M. pneumoniae* cells can be used for rapid identification of suspected organisms primarily grown in diphasic medium or on agar. In heavily inoculated broth cultures, the typical cells appear on the cover slip after a few hours, and the diagnosis can be confirmed by serological methods, at the latest after overnight incubation, either by direct immunofluorescence or the rounding test (3, 5). If no phase contrast is available, the organisms

can be examined by dark field if the cover slip is removed from the chambers and mounted on a microslide.

The method requires some experience and also, to some extent, special optical equipment. This will certainly limit the number of laboratories in which microscopic examination can be used as a diagnostic tool for detection of *M. pneumoniae*. However, in places where clinicians as well as microbiologists are interested in *M. pneumoniae*, the procedure could perhaps provide a possibility for rapid diagnosis of the disease. Further studies with clinical material are needed to confirm or disprove the diagnostic value of the method.

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