Acute-Phase, Indirect Fluorescent Antibody Procedure for Diagnosis of *Mycoplasma pneumoniae* Infection

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ABSTRACT

An indirect fluorescent antibody (IFA) technique was developed to detect IgG and IgM-specific antibodies to *Mycoplasma pneumoniae*. The presence of IgM-specific mycoplasma antibody was interpreted as reflecting active infection in patients with atypical pneumonia or other clinically compatible illness. The procedure is suitable for use in routine clinical laboratories, correlated well with complement fixation test results and did not show cross reaction with *Legionella pneumophila* antibody. The ready availability of an acute-phase procedure for diagnosis of *Mycoplasma pneumoniae* infection permits therapeutic judgments based on testing of the acute serum sample.

Introduction

Laboratory diagnosis of *Mycoplasma pneumoniae* infection has been limited to non-specific procedures such as detection of cold agglutinins and streptococcus MG antibody, or to complement fixation (CF) techniques and cultural isolation procedures. The latter procedures are available only in larger or reference laboratories and often involve a delay of 10 days to two weeks prior to receipt of test results. Recent reviews of mycoplasma infection refer to the need for a rapid and readily available diagnostic procedure to assist in clinical evaluation of mycoplasmal diseases. This communication presents a rapid, indirect fluorescent antibody (IFA) procedure specific for *Mycoplasma pneumoniae* that is suitable for use in the routine medical laboratory.

Materials and Methods

Equipment

The procedure requires only routinely available immunology laboratory equipment. A good quality fluorescent microscope is important, particularly fluorescein isothiocyanate (FITC)-specificity of the excitor and barrier filters. While transmission fluorescent illumination
may be acceptable, the IFA Mycoplasma procedure is significantly easier in performance and interpretation using epi-illuminated fluorescent microscopy.

**Reagents**

**Antigen:** A commercially available, freeze-dried, whole-organism *Mycoplasma pneumoniae* preparation (#VA-10)* serves as the antigen substrate.

**Fluorescein Labeled Antiserum:** Fluorescein isothiocyanate (FITC) anti-human IgM (sheep) (#MF04)* and anti-human IgG (sheep) (#MF03).* These are reconstituted according to manufacturer's instructions. Purity of the conjugate is confirmed according to the procedure of Nakamura and associates.9

**FA Rhodamine Counterstain:** This is reconstituted according to manufacturer’s instructions. It is diluted 1:20 in PBS with working concentration of FITC-conjugate (e.g., 1.0 ml FITC-conjugate of stock concentration, 8.5 ml phosphate buffered saline (PBS) 0.5 ml reconstituted rhodamine).

**Three Percent Normal Yolk Sac Suspension:** This is available from the Center for Disease Control. |

**Phosphate Buffered Saline:** The pH is 7.2 to 7.4.

**Glycerol Mounting Medium:** Glycerol/PBS—9/1.

**Multiwell Slides:** These are commercially available.§

**Cover-Slips:** #1 thickness, 24 x 50 mm.

**Controls**

Commercially available positive control sera for *Mycoplasma pneumoniae* are positive for the IgG-specific antibody. The IgM-specific control serum is obtained from confirmed active cases of *Mycoplasma pneumoniae* infection, pooled and stored at −20°C in 0.5 ml aliquots. Negative control serum may be obtained commercially or prepared as a pool of known negative patients. Known positive and negative control sera should be run with each test batch.

**Sample**

The patient sample consists of a single, routinely collected and separated serum specimen. Samples are usually fasting owing to routine laboratory collection procedures. However, non-fasting samples have not posed a problem. Separated serum is stable for testing purposes at ambient temperatures for a brief period of storage or transport and is stored at 4°C for periods greater than 24 hours. Minor hemolysis has not affected test results.

**Substrate Slide Preparation**

1. Commercial *Mycoplasma pneumoniae* antigen is rehydrated according to manufacturer’s instructions.

2. Using a calibrated pipet, 3 μl of antigen suspension are placed on each well of a multi-well fluorescent microscopic slide. The slides are dried for 30 minutes at room temperature.

3. Slides are fixed in acetone at room temperature for 15 minutes.

4. Slides are dried at room temperature and stored at −20°C until used. Slides are stable at this temperature for at least six months.

**Testing Procedure**

1. A single routinely drawn and separated patient serum sample constitutes the testing specimen in most circumstances. All serum samples are retained at 4°C for a minimum of two weeks in the event of need for follow-up testing, at which time initial and follow-up sera are tested in parallel.
2. Previously prepared substrate slides are removed from the freezer and equilibrated at room temperature for five to 10 minutes.

3. Using a calibrated pipet, the patient’s serum is serially diluted. The initial dilution (1:8 or 1:10) is made in three percent normal yolk sac suspension; subsequent dilutions are made in PBS.

4. Using a 25 μl calibrated pipet, 25 μl of appropriately titered serum are added to each well or reaction site.

5. Slides are incubated in a moist chamber at 37°C for 60 minutes.

6. Slides are incubated in at least two changes of PBS for at least 10 minutes.

7. Slides are blotted carefully with smooth absorbent paper to avoid disruption of the antigen substrate.

8. Twenty-five μl of appropriate IgG- or IgM-specific anti-human FITC are added with rhodamine counterstain to each reaction site.

9. Slides are incubated in a moist chamber at 37°C for 30 minutes.

10. Slides are rinsed and dried, as in steps 6 and 7.

11. Using PBS buffered glycerol as the mounting medium, coverslips are placed on slides.

12. Slides are read using incident light fluorescence at 200 × magnification with an FITC-specific filter combination.

13. A positive result appears as an apple-green, fluorescent slurry of particulate matter consistent with the almost submicroscopic morphology of the Mycoplasma pneumoniae organism (figure 1). Incorporation of the rhodamine counterstain suppresses non-specific background fluorescence. A negative result appears as a muted, reddish-orange granular field. Strict comparison with known positive and negative sera of both antibody classes and blind reading between observers to establish reproducible visual thresholds will provide consistent and reproducible results.

14. Titers or dilutions considered “positive” should be individualized for each laboratory emphasizing clinical correlation and reproducibility between observers. Borderline or weak results should be interpreted with caution and repeated with follow-up samples if clinical suspicion persists.

Results

Development of the IFA mycoplasma procedure included correlation of test results with the patient’s clinical status and parallel testing with reference laboratory CF results in an initial series of 128 cases presenting as febrile pneumonitis with “normal flora” bacterial cultures (table I). Results of this comparison show the IFA test to be an adequate screening test as it produced no “false-negatives” relative to CF results. All CF-positive cases were also positive with the IFA procedure.

Four cases which were borderline positive, using IFA criteria, involved patients with febrile illness of unknown etiology; two of these responded to erythromycin therapy, and two recovered spontaneously.

An additional 165 healthy, asymptomatic persons were tested using the IFA mycoplasma procedure to determine the incidence of community antibody frequency for Mycoplasma pneumoniae. All 165 sera were negative for mycoplasma-IgM; 48 percent were positive for low

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td>Comparison of Mycoplasma Complement Fixation and Indirect Fluorescent Antibody* Results</td>
</tr>
<tr>
<td>CF Result</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
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<td>Positive</td>
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*Consecutive samples submitted for mycoplasma testing.
levels of mycoplasma-IgG (titers of 8 to 32) suggesting previous infection or antigen exposure in these patients. To date, a total in excess of 300 patients has been tested and clinical correlation has remained excellent.²

Clinical interpretation of the significance of class-specific antibody titers for *Mycoplasma pneumoniae* is outlined in table II, according to criteria established in our own laboratory. Initial dilution titers of mycoplasma-IgM (8 or 10) are interpreted with caution and with correlation with the total clinical picture. Follow-up studies are suggested if the clinical situation warrants.

Concurrent testing of the original series of 128 patients for *Legionella pneumophila* disclosed nine serologically positive cases of which five were confirmed using direct fluorescent antibody (DFA) studies on pulmonary tissue or culture isolation of the organism.¹ No patient had a duel positive result for both legionella and mycoplasma antibodies.

**Discussion**

The IFA mycoplasma procedure is presented as a rapid diagnostic test for *Mycoplasma pneumoniae* infection and is recommended for testing febrile patients with pneumonitis with normal sputum bacterial studies (“atypical pneumonia”). Interpretation of a positive mycoplasma-IgM titer as indicative of active infection is analogous to current testing interpretation for hepatitis A virus infection.⁶ An initially negative or borderline result in a clinically suspect patient should be followed with serial studies which may disclose seroconversion to positive titers.

In our experience, approximately 90 percent of test results are clearly positive or negative on testing of the initial, acute serum sample. Only two initially nega-

## Table II

<table>
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<tr>
<th>Interpretation</th>
<th>IgM titer</th>
<th>IgG titer</th>
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<tbody>
<tr>
<td>Negative result</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Previous infection</td>
<td>neg</td>
<td>&gt; 8</td>
</tr>
<tr>
<td>Active or recent infection*</td>
<td>&gt; 16</td>
<td>&gt; 16</td>
</tr>
<tr>
<td>Equivocal result†</td>
<td>8 - 16</td>
<td>neg - 8</td>
</tr>
</tbody>
</table>

*Correlate with clinical picture.
†Suggest follow-up study if clinically indicated.
tive patients tested thus far have shown conversion to mycoplasma-IgM antibody positivity, illustrating the usefulness of testing for IgM-specific mycoplasma antibody in the acute serum sample.

All patients having positive titers of mycoplasma-IgM antibody were tested for rheumatoid factor (RF) with no evidence of dual positivity or cross reaction encountered thus far. A mycoplasma-IgM positive serum also positive for rheumatoid factor would be interpreted cautiously with close correlation with the patient's clinical presentation and other laboratory data. The low incidence of rheumatoid factor positivity in the general population should not preclude routine use of an IgM-specific IFA mycoplasma procedure, particularly if the IFA procedure is restricted to testing febrile patients with "bacterial negative" pneumonitis.

Most patients presumptively diagnosed as having mycoplasma pneumonia using IFA test results presented with the expected clinical findings of low-grade fever and mild pneumonitis and responded rapidly to erythromycin therapy. One patient presented with acute pericarditis, demonstrated seroconversion from negative to positive mycoplasma IFA titers, and responded promptly to antibiotic therapy. Another patient presented with acute polyarthritis, positive mycoplasma IFA titers, and responded promptly to appropriate therapy.

Subsequent to presentation of the procedure abstract, communication with the director of the Mycoplasma Reference Laboratory in Norwich, England indicated that a similar, IgM-specific IFA procedure for Mycoplasma pneumoniae infection had been found, in their experience, equally sensitive to cultural isolation and identification of the organism.

The use of class-specific antibody detection in the immunologic diagnosis of Mycoplasma pneumoniae infection reflects a personal bias and experience with fluorescent microscopy. The basic concept should readily translate to enzyme labeled immunosorbant assay (ELISA) and/or radioimmunoassay procedures and thus prove susceptible to automation.

**Conclusion**

In response to a need for a rapid and specific testing procedure for Mycoplasma pneumoniae infection, an IgM and IgG-specific indirect fluorescent antibody technique was established using whole-organism Mycoplasma pneumoniae as the antigen substrate for detection of class-specific antibodies in the acute-phase serum sample. The technique has proven to be a successful on-site screening test in the analysis of more than 300 patients presenting with clinical "atypical pneumonitis" and offers rapidly available results of comparable sensitivity and specificity to traditional complement fixation procedures. Increased case finding resulting from use of the IFA mycoplasma procedure has led to an increased appreciation of the spectrum of the clinical disease.

**Acknowledgments**

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**References**


