

Comparison of Laboratory Diagnostic Procedures for Detection of *Mycoplasma pneumoniae* in Community Outbreaks

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Background. *Mycoplasma pneumoniae* continues to be a significant cause of community-acquired pneumonia (CAP). A more definitive methodology for reliable detection of *M. pneumoniae* is needed to identify outbreaks and to prevent potentially fatal extrapulmonary complications.

Methods. We analyzed 2 outbreaks of CAP due to *M. pneumoniae*. Nasopharyngeal and/or oropharyngeal swab specimens and serum samples were obtained from persons with clinically defined cases, household contacts, and asymptomatic individuals. Real-time polymerase chain reaction (PCR) for *M. pneumoniae* was performed on all swab specimens, and the diagnostic utility was compared with that of 2 commercially available serologic test kits.

Results. For cases, 21% yielded positive results with real-time PCR, whereas 81% and 54% yielded positive results with the immunoglobulin M and immunoglobulin G/immunoglobulin M serologic tests, respectively. For noncases, 1.8% yielded positive results with real-time PCR, whereas 63% and 79% yielded serologically positive results with the immunoglobulin M and immunoglobulin G/immunoglobulin M kits, respectively. The sensitivity of real-time PCR decreased as the duration between symptom onset and sample collection increased, with a peak sensitivity of 48% at 0–21 days. A specificity of 43% for the immunoglobulin M antibody detection assay was observed for persons aged 10–18 years, but the sensitivity increased to 82% for persons aged ≥19 years.

Discussion. Thorough data analysis indicated that no single available test was reliable for the identification of an outbreak of CAP due to *M. pneumoniae*. A combination of testing methodologies proved to be the most reliable approach for identification of outbreaks of CAP due to *M. pneumoniae*, especially in the absence of other suspected respiratory pathogens.

Mycoplasma pneumoniae is estimated to cause 15%–20% of cases of community-acquired pneumonia (CAP) [1]. Most infections are mild or asymptomatic and often self-limiting; however, 1%–5% of infections may require hospitalization and can lead to serious extrapulmonary complications, such as encephalitis [1–4]. Outbreaks of *M. pneumoniae* infection occur in such institutions as schools, prisons, and hospitals and in

the community. Reliable and rapid methods for confirming the etiology of such outbreaks are needed to enable an effective public health response.

Several diagnostic methods detect *M. pneumoniae* infection, including isolation, complement fixation, serologic testing, and molecular-based detection assays [1, 5]. Each of these methods has limitations. Isolation is tedious and time consuming, requires expertise, and yields inconsistent results [5]. Complement fixation testing lacks sensitivity and specificity [5]. Commercially available serologic test kits for detection of antibodies to *M. pneumoniae* possess inherent limitations of specificity and sensitivity and, more importantly, depend on patient compliance with the timely acquisition of acute- and convalescent-phase serum samples for accurate interpretation [6]. Molecular-based assays, such as real-time PCR, have recently been shown to aid

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in the rapid identification of outbreaks of *M. pneumoniae* infection and provide greater sensitivity than do traditional methods [7–10]. However, many published real-time PCR assays have not been thoroughly evaluated and are not available at most public health laboratories. Furthermore, the reliability of these diagnostic assays is often confounded by challenges inherent to investigations of outbreaks of unknown etiology (e.g., appropriate specimen collection and transport for testing for numerous suspected agents). These mitigating factors may impact the interpretation and reliability of the diagnostic methods used to identify an outbreak.

We evaluated a recently validated real-time PCR assay and 2 widely available serologic detection kits for their reliability in detecting *M. pneumoniae* infection during 2 recent CAP outbreaks [9]. Isolation was also attempted for all swab specimens with a positive real-time PCR result.

METHODS

During the investigation of outbreaks of *M. pneumoniae* infection at a university and in the community, oropharyngeal and/or nasopharyngeal swab specimens and serum samples were obtained on the same date for each individual in 1 of 4 groups: (1) persons with radiographically or clinically confirmed pneumonia, (2) persons who had recently sought the advice of a physician for a cough, (3) household contacts of symptomatic individuals, and (4) age-matched persons with no illness or symptoms [9, 10]. Cases were defined as fever (temperature, $\geq 38^{\circ}\text{C}$ [$\geq 100.4^{\circ}\text{F}$]) and cough or a clinical or radiologic diagnosis of pneumonia [10]. Asymptomatic contacts of cases or age-matched control subjects were considered to be non-case patients.

Swabs were placed in 2 mL of either BD Universal Viral Transport Medium (Becton-Dickinson), SP4 media, or PBS and were transported at 4°C or were frozen [11]. Serum samples were collected from a subset of these patients and were transported at 4°C or were frozen. Only individuals who provided both a swab and a serum specimen were included in this study. Total nucleic acid extraction was performed on all swab specimens, in accordance with the manufacturer's instructions, using 1 of 3 kits: the QIAamp DNA Blood BioRobot MDx kit (Qiagen), the QIAamp DNA Blood Mini kit (Qiagen), or the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Applied Science). Swab specimens were vortexed in the Viral Transport Medium prior to sample extraction. The remaining volume of each specimen was frozen at -70°C . A validated real-time PCR assay specific to *M. pneumoniae* (as well as an RNase P assay as a control for sample extraction and integrity) was performed in duplicate on all extracted specimens, as described elsewhere [9].

Serum samples were tested for the presence of antibodies to *M. pneumoniae* using the IgM-specific Mycoplasma Immuno-

Card (Meridian Bioscience; hereafter referred to as the "Meridian IgM assay") and the *Mycoplasma pneumoniae* IgG/IgM Antibody Test System (Remel; hereafter referred to as the "Remel IgG/IgM assay") in accordance with the manufacturers' instructions.

Isolation was attempted for all PCR-positive swab specimens. Two hundred μL of each supernatant was placed in 1.8 mL of SP4 media with glucose [11]. Ten-fold serial dilutions down to 10^{-6} were performed in SP4 media. A second 200 μL of each supernatant was filtered through a 0.45- μM syringe filter and was treated as above. All tubes were incubated at 37°C with 5% CO_2 along with a negative control tube containing media only. All tubes were observed regularly for change in media color, and blind passages were performed weekly for 4 weeks [11]. Tubes with media color change were extracted and tested by the described real-time PCR assay to confirm the presence of *M. pneumoniae*.

The diagnostic sensitivity, specificity, and positive predictive value for a clinically defined case and noncase were calculated for each laboratory procedure [12]. SAS software, version 9.1 (SAS Institute), was used for these calculations [13].

RESULTS

A total of 263 individuals were tested for the presence of *M. pneumoniae* by real-time PCR and by the Meridian IgM and Remel IgG/IgM assays. Of these persons, 97 had clinically defined cases, and 166 were classified as non-case patients. Table 1 summarizes the specificity and sensitivity results for all 3 diagnostic assays. Twenty case patients (21%) had positive results of real-time PCR (mean crossing-threshold value [C_t], 34.7; range, 27.4–40.2), 79 (81%) had positive results of the Meridian IgM assay, and 52 (54%) had positive results of the Remel IgG/IgM assay (table 1). Individuals with positive results of real-time PCR routinely had positive results of Meridian IgM (90%) and Remel IgG/IgM (70%) assays (data not shown). Among 77 case patients with negative real-time PCR results, 61 (79%) had positive Meridian IgM assay results, and 38 (49%) had positive Remel IgG/IgM assay results (data not shown). Interestingly, the 3 samples obtained from non-case patients that yielded positive results by real-time PCR also yielded positive results of the Meridian IgM and Remel IgG/IgM assays.

Isolates were recovered from 11 (48%) of 23 PCR-positive specimens, including samples obtained from the 3 aforementioned non-case patients. The mean C_t value for swab specimens that yielded isolates was 34.8 (range, 30–40.2). Although the Meridian IgM assay appeared to be the most sensitive method studied, it also had the lowest specificity (63%) (table 1). The Remel IgG/IgM assay was more specific (specificity, 79%) but was the least sensitive of the methods. Although the sensitivity for real-time PCR was only 21%, it had the highest rate of specificity (98%). In addition, real-time PCR had the highest positive predictive value (87%), with the Meridian IgM

Table 1. Diagnostic sensitivity and specificity of real-time PCR, IgM-specific *Mycoplasma* ImmunoCard (Meridian Bioscience; hereafter referred to as Meridian IgM assay), and the *Mycoplasma pneumoniae* IgG/IgM Antibody Test System (Remel; hereafter referred to as the Remel IgG/IgM assay) results for 97 case patients and 166 non-case patients, by age and interval from reported onset of symptoms to specimen collection.

Characteristic	Sensitivity assessment				Specificity assessment			
	No. of subjects	PCR	Meridian IgM assay	Remel IgG/IgM assay	No. of subjects	PCR	Meridian IgM assay	Remel IgG/IgM assay
All subjects	97	21 (13–29)	81 (74–89)	54 (44–64)	166	98 (96–100)	63 (55–70)	79 (73–85)
Age, years ^a								
0–9	33	9 (0–19)	97 (91–100)	67 (51–83)	23	96 (87–100)	48 (27–68)	74 (56–87)
10–18	44	20 (9–32)	89 (79–98)	52 (38–67)	58	97 (92–100)	43 (30–56)	76 (65–87)
≥19	20	40 (19–61)	40 (19–61)	35 (14–56)	83	100	82 (74–90)	82 (74–90)
Interval between symptom onset and specimen collection, days ^b								
0–21	21	48 (26–69)	76 (58–94)	33 (13–54)
22–59	17	29 (8–51)	94 (83–100)	76 (56–97)
≥60	26	12 (0–24)	100	73 (56–90)

NOTE. Data are percentage (95% CI), unless otherwise indicated.

^a Information on age was not available for 2 non-case patients.

^b Information to calculate interval was not available for 33 case patients.

and Remel IgG/IgM assays demonstrating positive predictive values of 56% and 60%, respectively. The positive predictive value of real-time PCR was significantly higher than that of the Meridian IgM assay ($P < .01$) and the Remel IgG/IgM assay ($P < .01$).

The diagnostic sensitivity and specificity of each laboratory procedure varied by age and by interval, in days, from the onset of symptoms to specimen collection (table 1). The proportion of individuals with detectable antibody was highest for children (age, <18 years) and appeared to decrease with age (figure 1). The specificity of the Meridian IgM assay was 48% among subjects aged 0–9 years and 43% among those aged 10–18 years, but it increased to 82% among persons aged ≥19 years. Although there was not a statistically significant difference in the specificity for the 0–9-year-old versus the 10–18-year-old age groups, it was significantly higher for the ≥19-year-old versus the 0–9-year-old age groups (relative specificity, 1.71; 95% CI, 1.10–2.66). No age-related differences in specificity were found for either the Remel IgG/IgM assay or real-time PCR. The sensitivity decreased with age for both antibody-based tests but increased with age for real-time PCR (table 1).

Analysis of the real-time PCR data showed that the sensitivity decreased as the interval between symptom onset and specimen collection increased. For intervals of 0–21 days, the sensitivity was 48%, but it decreased to 29% for intervals of 22–59 days and to 12% for intervals >60 days (figure 2). The relative sensitivity for real-time PCR was 4.13 (95% CI, 1.30–13.1) for intervals of 0–21 days and 2.55 (95% CI, 0.70–9.30) for intervals of 22–59 days when both were compared with intervals >60 days. In contrast to the downward trend in sensitivity of real-time PCR with increasing interval, the sensitivity of Meridian

IgM assay increased from 76% to 94% and to 100% as the interval increased (table 1). The sensitivity of the Remel IgG/IgM assay followed a similar upward trend as the interval increased (table 1).

DISCUSSION

Our findings reinforce the difficulty of achieving rapid and reliable laboratory identification of *M. pneumoniae* outbreaks. We found a wide range of sensitivities and specificities among the 3 methods tested, all of which were affected by the age of the patient and the timing of specimen collection. Our results indicate that no currently available single test can reliably confirm the etiology of outbreaks of *M. pneumoniae* infection. A combination of *M. pneumoniae*-specific testing modalities, performed in comparison with appropriate negative control samples and in the absence of other suspected agents, is the most prudent and pragmatic approach to confirming *M. pneumoniae* as the cause of an outbreak.

Laboratories that offer testing for *M. pneumoniae* largely rely on commercially available serologic test kits and, to a lesser extent, molecular assays such as PCR. Although both these approaches tend to be more sensitive than culture for detection of *M. pneumoniae* infection, results must be interpreted with care. Real-time PCR appears to be more useful during early stages of infection, when more organisms are likely to be present. However, over time, the likelihood of a positive PCR result diminishes, because the sensitivity decreases significantly as the interval from symptom onset to specimen collection increases. Our data suggest that PCR is >2.5 times more sensitive if specimens are collected during the first 21 days after the onset of

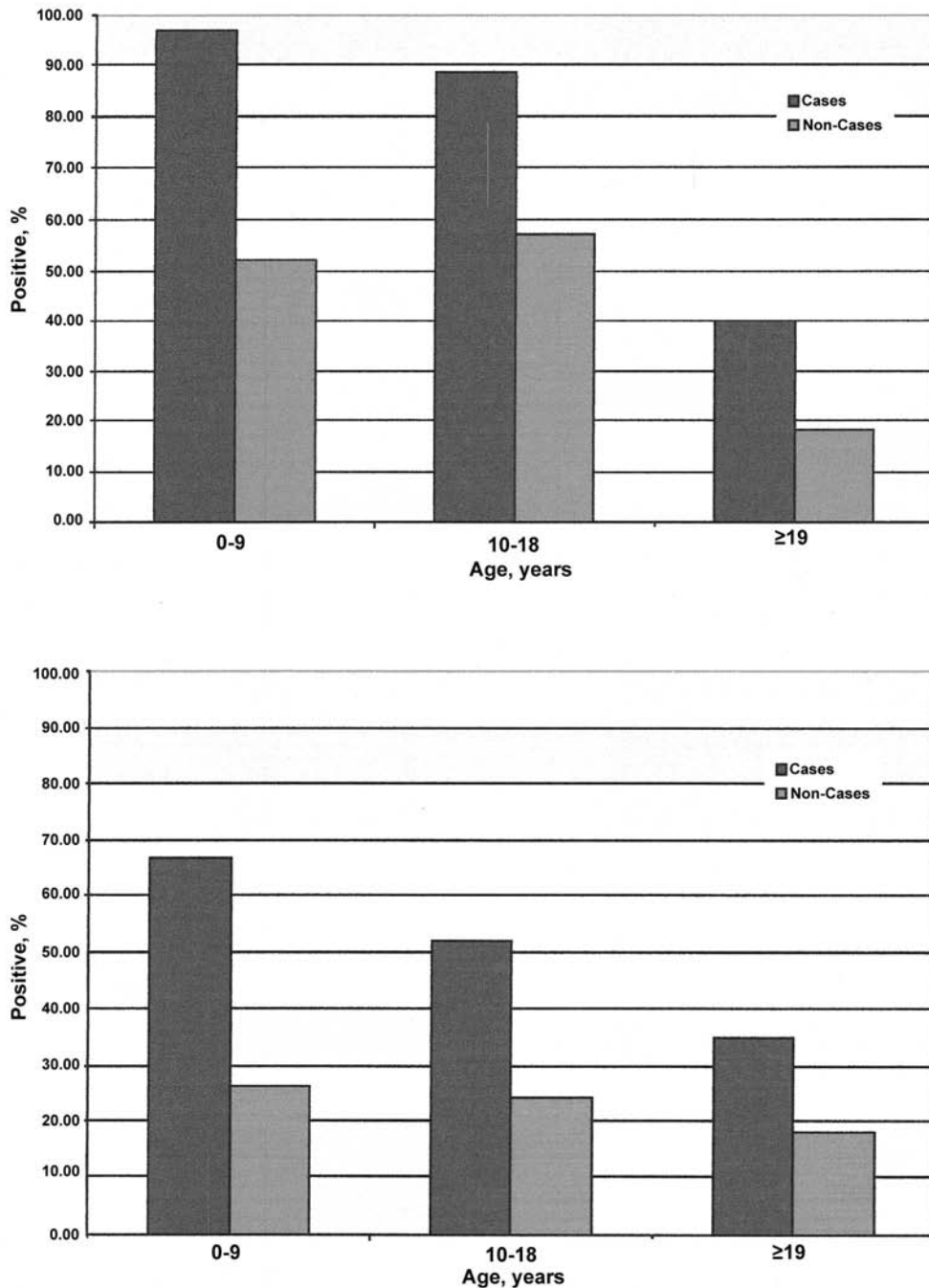


Figure 1. Percentage of serum specimens that tested positive for *Mycoplasma pneumoniae* using the IgM-specific Mycoplasma ImmunoCard (Meridian Bioscience; A) or the *Mycoplasma pneumoniae* IgG/IgM Antibody Test System (Remel; B), by age group, among case patients and non-case patients.

symptoms. This principle is further demonstrated by analyzing the 2 outbreaks described in this study separately. In one outbreak, a mean interval of 4 days elapsed between symptom onset and sample collection, and 66.7% of samples tested positive by real-time PCR (mean C_t , 30.4); in the other, a mean interval of 54 days elapsed, and 19.2% of samples tested positive

by real-time PCR (mean C_t , 37.1; data not shown). This suggests that timely collection of nasopharyngeal and/or oropharyngeal swab specimens is critical for successful nucleic acid detection. Sample quality, storage, and transportation conditions also impact PCR performance [14]. Concurrent collection of oropharyngeal and nasopharyngeal swab specimens and of serum

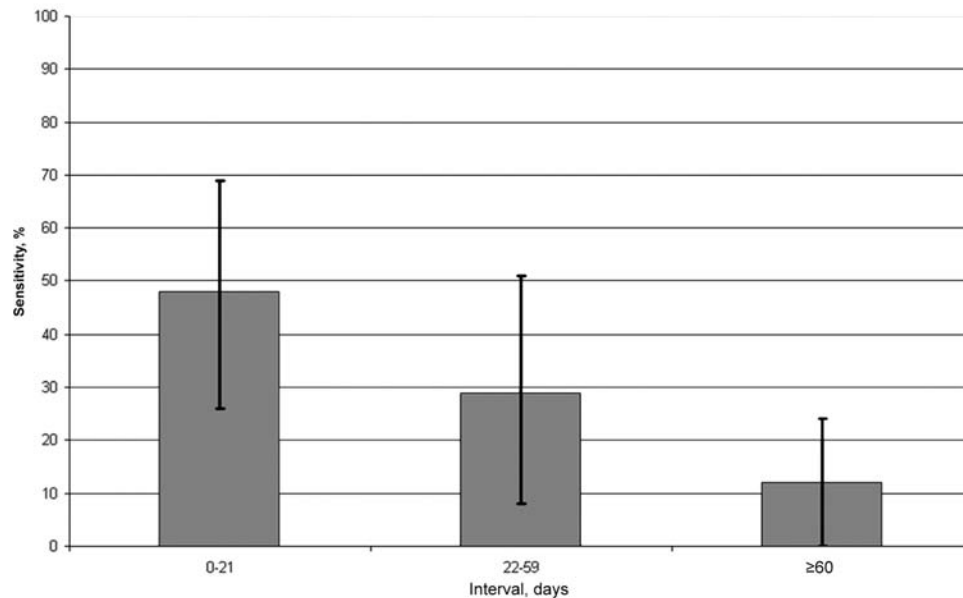


Figure 2. Real-time PCR sensitivity according to the interval from symptom onset to specimen collection. Information to calculate the interval was not available for 33 case patients. Error bars represent 95% CIs for sensitivity.

samples from both patients and negative controls may be useful adjuncts to real-time PCR data. Testing for other possible causative agents should also be performed, especially early in an investigation, because a variety of different respiratory pathogens can present with the same clinical picture.

Serologic tests, although useful, also have restrictions that must be kept in mind when analyzing results. Many require collection of both acute- and convalescent-phase samples to identify a 4-fold increase in titer suggestive of an active infection [6]. Although serologic tests may provide important retrospective information, the impracticality of obtaining the follow-up sample limits their use when rapid confirmation of etiology is needed. Furthermore, as recent studies indicate, IgM antibody can persist for years, with levels remaining elevated in asymptomatic individuals [15]. Nir-Paz et al. [15] found that healthy children (age, 6–18 years) were most likely to have positive IgM results, whereas the likelihood of a positive IgG result increased progressively throughout adulthood. The data from our study support these findings, because the specificity of the Meridian IgM assay was lower for younger populations but increased with age. We found that sizable proportions of non-case patients had detectable antibody (figure 1). Therefore, use of serologic assays as the sole test may provide misleading information, because the high baseline rate of positivity may give investigators and clinicians the false impression that *M. pneumoniae* is the etiology of the CAP outbreak. However, our data suggest the Meridian IgM assay may be more useful than the Remel IgG/IgM assay for the early identification of outbreaks of *M. pneumoniae* infection and that all serologic data

are most useful when results for symptomatic individuals versus age-matched asymptomatic individuals are compared.

One limitation of our study is that a single serum serologic test was used in each calculation, as opposed to paired serologic tests, as most studies recommend. This limitation, however, was minimized by the collection and testing of serum specimens obtained from non-case patients to establish a baseline of seropositivity among the different age groups. Many of the non-case patients had exposure to case patients (they were household contacts or attended the same college), which may have increased the likelihood of asymptomatic infection or prior immunity, leading to higher seropositivity rates. However, a previous study that evaluated seropositivity among asymptomatic individuals found rates consistent with those found for the non-case patients in our study [15]. A second limitation is that no gold standard diagnostic assay exists, making it difficult to draw conclusions for determining the most accurate and reliable method to use for identifying positive and negative specimens. Furthermore, many laboratories depend on nonstandardized and nonvalidated assays and, therefore, struggle with interpretation of results, sometimes allowing for *M. pneumoniae* infection to be misdiagnosed or for outbreaks to go undetected entirely.

The present study provides insight for establishing optimal testing strategies for the accurate and timely diagnosis of *M. pneumoniae* infection in the context of an outbreak. Early recognition, combined with proper sample collection, handling, and processing, increases the likelihood that *M. pneumoniae* will be correctly identified. Analysis of test results from 2 CAP

outbreaks suggests that a combination of *M. pneumoniae*-specific testing modalities simultaneously performed on suspected case patients and control subjects is the most reliable strategy to determine the etiology of an outbreak, especially in the absence of other agents.

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