Evaluation of the illumigene Mycoplasma Direct DNA amplification assay

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Abstract

*Mycoplasma pneumoniae* (MP) is a common cause of community-acquired pneumonia. The illumigene Mycoplasma Direct (iMD) DNA amplification assay is a qualitative *in vitro* test utilizing LAMP technology for the direct detection of MP DNA in respiratory specimens. The iMD assay does not require the pre-extraction of nucleic acid from specimens, which is a prerequisite step for the previously approved illumigene Mycoplasma (iM) assay. The aim of this prospective multicenter study was to evaluate the performance characteristics of the newly developed iMD assay with those of the iM assay. Subjects with symptoms of upper respiratory illness suggestive of MP were enrolled across three sites in the United States. Respiratory specimens were obtained using a dual throat swab. One swab was tested at each enrollment site by the iMD assay. Reference testing with iM assay was performed by the manufacturer. Among 456 specimens tested, the iM reference method detected MP in 25 (5.5%) specimens, while the iMD assay detected 34 (7.5%) specimens as MP positive. There were 10 false positive and one false negative detections with the iMD assay. The overall positive and negative percent agreement was 96.0% (95% CI 80.5-99.3%) and 97.7% (95% CI 95.8-98.7%), respectively. The overall percent agreement was determined to be 97.6% (95% CI 95.7-98.6%). We conclude that the iMD test results were comparable with the iM assay. The removal of DNA extraction step in the iMD assay simplifies testing, saves time, and reduces the expense of detecting MP from throat swabs when compared to the iM assay.
Introduction

*Mycoplasma pneumoniae* (MP) is a common cause of respiratory infections, especially in young adults and school-age children. Diagnosis of MP infection based on a patient’s clinical presentation alone is difficult and unreliable (1). Prompt diagnosis is essential to initiate appropriate antibiotic therapy and infection control.

There are several methods utilized for MP detection. Culture-based methods are highly specific and sensitive but are time-consuming and their sensitivity may vary depending upon laboratory skills (2, 3). Serology-based assays (complement fixation assay, enzyme-linked immunoassay, enzyme-linked immunosorbent assay, and micro particle agglutination assay) are available. The limitations of serology assay–based diagnosis include lack of sensitivity due to delayed antibody response to MP infection, lack of response in older patients, highly prevalent background antibodies in healthy individuals, and also cross-reactivity with other species of *Mycoplasma* (2, 4, 5). Rapid antigen kits targeting specific proteins are also being utilized to diagnose MP. The sensitivity of rapid antigen assay has been reported to vary from 60% to 90% (2, 6, 7).

Nucleic acid amplification assays have gained popularity due to increased assay sensitivity (1, 2), which enables detection at an early course of infection. Loop-mediated isothermal amplification (LAMP) assay is a nucleic acid amplification assay that is simple, easy to use (no thermocycler needed), and highly sensitive with a quick turnaround time (2). The *illumigene* Mycoplasma (iM) (Meridian Bioscience, Inc., Cincinnati, OH) assay utilizes LAMP technology for the detection of MP from throat and nasopharyngeal swab specimens. The primers of this diagnostic kit target a 208-bp DNA sequence found in the intracellular protease-like protein gene of the MP genome (1). A by-product of the continuous isothermal amplification reaction is magnesium pyrophosphate. This white precipitate leads to turbidity in the reaction.
mixture. The illumipro-10 incubator/reader monitors the changes in the absorbance characteristics. Significant changes in the absorbance of the reaction mixture indicate the presence of the target gene. A study found the sensitivity and specificity of the iM assay to be 100% and 99%, respectively, as compared with the culture method (1). Another recent study found both sensitivity and specificity of iM assay to be 100% as compared with the FilmArray® Respiratory Panel (Biomerieux, France) (8).

The company manufacturing the iM assay recently developed an improved version; illumigene Mycoplasma Direct (iMD) assay which does not require a Qiagen DNA extraction step. The gene target and assay chemistry is identical for both the iM and iMD assay. This multicenter clinical trial compared the clinical performance of the iMD assay with reference to the iM assay that requires nucleic acid extraction. This is the first study evaluating the performance of the iMD assay.

Materials and Methods

Study Design: This prospective multicenter clinical trial was conducted at three different sites (Florida, Texas, and Missouri) across the United States. Patients with upper respiratory infections that may be attributed to MP were enrolled after obtaining informed consent. Fresh throat swab specimens from both male and female patients were collected from August 2015 through January 2016. This study was designed to evaluate the performance of the newly improved iMD assay as compared with the iM assay. The study protocols were approved by each hospital’s Institutional Review Board.
Specimens: Specimen inclusion criteria included (a) Specimens collected from patients with symptoms of upper respiratory infections which may be attributed to MP or patients suspected of having MP infection. (b) Two throat swabs from each subject enrolled; one transported in the non-nutrient transport media and other in the M4 media. (c) Written informed consent provided by the subject. Specimen exclusion criteria included (a) Specimens received in the laboratory in unsatisfactory containers or conditions. (b) Multiple sets of specimens from the same patients at different office visits. (c) Specimen received in the laboratory with less than two throat swabs per subject. (d) Specimens stored in unapproved transport media types or combinations.

Demographic data collected at enrollment included age, gender, date of symptom onset, antibiotic use in last 4 weeks and other medication use. No other clinical or radiological findings were recorded.

A total of 456 subjects at the three different sites were included in the study. A summary of the subjects enrolled and MP prevalence across the three sites is provided in Table 1. Patients from ages 3 weeks to 97 years with upper respiratory illness that may be attributable to MP were included. A dual rayon-tip swab collection device was used to collect one specimen per patient. One swab was stored in liquid Amies medium (Becton, Dickinson and Company, NJ, USA; Catalogue No. 220105) and the second in M4 transport medium (Thermo Fisher Scientific, Remel products, KS, USA; catalogue No. 12500). Specimens were stored at 2-8°C prior to testing. At each study site, specimens were de-identified and were assigned a unique, site-specific study identifier. Testing was performed with the iMD assay at each study site with one throat swab. Operators at each study site tested external positive and negative control every day prior to running iMD assay on the study samples. The second swab in M4 medium was shipped
to the manufacturer for performing the iM assay. The results from the two assays were compared to determine the performance of the iMD assay.

\textit{illumi}gene Mycoplasma Direct assay: The swab stored in red-cap non-nutrient liquid Amies transport media was utilized to perform the iMD assay within 72 hours of sample collection at the clinical site. Briefly, the swab was inserted by breaking the swab handle in the SMP PREP tube provided with the kit. The SMP PREP tube was vortexed for 10 seconds. Five to 10 drops from the SMP PREP tube were squeezed into a 1.5 ml heat treatment tube. The heat treatment tube was heated at 95°C for 10 minutes followed by a 10 second vortex. Fifty microliters of this heat-treated sample were transferred to test chamber as well as the control chamber of the \textit{illumi}gene test device. The device was closed and all air bubbles were removed by gently tapping the test device. The \textit{illumi}gene test device was inserted into the \textit{illumi}pro-10\textsuperscript{TM} incubator/reader for direct detection of MP. Results were displayed on the instrument as positive or negative at the conclusion of the run in less than an hour.

\textit{illumi}gene Mycoplasma (iM) assay: Specimens stored in M4 media were shipped (transport temperature 2-8°C) to the company within 4 days of sample collection and tested within 14 days. Qiagen QIAamp\textsuperscript{®} DSP DNA Mini Kit was utilized to extract DNA from 150µl of the specimen. The comparator iM assay was performed by the company according to the assay package insert.

**Statistical Analysis**

**Descriptive statistics:** Overall characteristics of the subjects enrolled and the MP prevalence at each individual site were determined with reference to iMD assay. MP prevalence was also determined by age and gender across each of the three sites.
Analytical statistics: Two by two data tables were utilized to determine the positive and negative percent agreement between iM and iMD assays. The performance characteristic (positive and negative percent agreement) analysis along with the 95% confidence intervals (CI) was performed using the Vassarstats website: http://vassarstats.net/clin1.html.

Results

A total of 456 subjects with symptoms of upper respiratory tract illness from three different sites were enrolled in the study. The highest prevalence was observed at the Missouri site (10.2%), followed by Texas (8.1%) (Table 1). There was approximately equal distribution of male and female subjects enrolled in this study (Females: n=230, 50.4%; Males: n=226, 49.6%) (Table 2). The median age of the patients included in the study was 5 (0.06-97) years. Highest MP prevalence was observed in the age group 3-12 years (22/147; 15%) compared to the rest of the study population (P value<0.001). Association of age with MP prevalence has been documented in previous studies (9-11). MP prevalence among females and males (7.0% vs. 8.0%) was found to be comparable (P value= 0.7). Detailed age and gender specific prevalence across all sites is listed in Table 2.

Among the 456 specimens tested, the iMD and iM assays detected MP in 34 (7.5%) and 25 (5.5%) specimens, respectively. There were 10 false positive specimens and one false negative specimen detected by the iMD assay. The positive and negative percent agreements between the iMD and iM assay were 96.0% and 97.7%, respectively. Discrepant analysis was performed by repeat testing of a second aliquot of the discrepant sample with the iM assay. Four out of 10 false positive specimens were identified positive by the iM assay, after retesting with an additional
frozen sample. Repeat testing of one false negative specimen by the iM assay, with the original patient sample and an additional frozen sample both produced negative results.

Detailed performance parameters for all study subjects and specific parameters for the study population are presented in Table 3. There were no invalid runs observed during the study.

Standard of care tests for MP detection (EIA / PCR for the 1st site, iM assay for the second site and Filmarray Biofire RP for the 3rd site) was available for 70 out of the 456 subjects enrolled in the study resulting in 11 positive detections.

**Discussion**

The objective of this multisite clinical trial was to evaluate the performance characteristics of the iMD as compared to those of the iM assay. This is the first study evaluating the performance of the iMD assay.

A previous study (8) in the United States compared the iM assay, Prodesse ProPneumo-1 assay (Hologic Gen-Probe, CA, USA) and *Mycoplasma pneumoniae* P1 LightMix Kit (TIB Molbiol, NJ, USA) with FilmArray® Respiratory Panel (FilmArray RP; Biomerieux, France) for detection of MP from pediatric clinical nasopharyngeal swab specimens. All of these three commercially available NAAT assays had similar sensitivity, specificity (iM, Prodesse assay, and LightMix Kit was 100%, 100%, and 96%, respectively) and hands-on time. Another study compared the iM assay with the culture using frozen respiratory specimens from adults and children for which historic culture results were available (1). The sensitivity and specificity of the iM assay after discrepant analysis were observed to be 100% and 99%, respectively. In our study, the overall percent agreement between the iMD and iM assay was found to be 97.6%. Given the study
results, it is expected that the iMD assay will perform comparable to the existing diagnostic platforms.

Current FDA cleared sample-to-answer molecular methods for MP detection are nested multiplex PCR panel by Biofire Inc. and standalone LAMP assays (iM and iMD) by Meridian Biosciences, Inc. The iMD assay targets the intracellular protease-like protein gene by utilizing LAMP technology (no thermocycler needed) and provides results in less than an hour. By comparison, the FilmArray Respiratory Panel (RP) targets the tox gene for MP detection. Advantages of iMD over the FilmArray RP for MP detection are that both instrument and cost/test are lower for the iMD test and it has a higher throughput as it can process 10 specimens vs. single specimen at one time making it ideal for testing in outpatient setting. One study evaluated the performance of the FilmArray RP for detection of MP against the laboratory developed real time TaqMan PCR targeting the p1 gene (12) and determined the positive percent agreement to be 100% (70.1-100) which was comparable to 96.0% [95% CI: 80.5-99.3] for both iM and iMD assays obtained in our study. We evaluated 25 true MP positive isolates as compared with 9 positives utilized in FilmArray RP study; this resulted in a relatively more robust estimates as indicated by the 95% CI. Several studies conducted in Europe have compared various commercially available NAAT assays for MP detection (13-15). A summary of commercially available NAAT assays for detection of MP also is described in a recent mini-review article (16). These NAAT assays need specialized trained personnel, expensive setup with thermal cycling amplification platforms, and a molecular biology facility. By contrast, the LAMP technology utilized in the iMD assay allows specific and continuous DNA amplification under isothermal conditions. The iMD is a sensitive assay that does not require an additional DNA extraction step, making it faster and more
economical than other NAAT assays that require the DNA extraction step. Overall, iMD is a
simple, convenient, and rapid molecular assay for detection of MP from throat swab specimens.

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Table 1. Total subjects enrolled and MP prevalence across the three sites

| Location     | Total | Total positive | Prevalence (%) |
|--------------|-------|----------------|----------------|
| Site 1       | Florida | 152  | 4             | 2.6            |
| Site 2       | Texas  | 49   | 4             | 8.1            |
| Site 3       | Missouri | 255 | 26            | 10.2           |
| **Total**    |        | 456  | 34            | 7.5            |
Table 2. MP prevalence by age and gender across all three sites with the iMD assay

| Location       | Site 1 | Site 2 | Site 3 | Total | Total positive | Prevalence (%) |
|----------------|--------|--------|--------|-------|----------------|----------------|
| 0-1 month      | .      | .      | 7      | 7     | 0              | 0.0            |
| 2 months to 2 years | 9      | 12     | 136    | 157   | 5              | 3.2            |
| 3 – 12 years   | 20     | 28     | 99     | 147   | 22             | 15.0           |
| 13 – 21 years  | 16     | 9      | 13     | 38    | 5              | 13.2           |
| 22 – 65 years  | 86     | .      | .      | 86    | 2              | 2.3            |
| >65 years      | 21     | .      | .      | 21    | 0              | 0.0            |
| Gender         |        |        |        |       |                |                |
| Male           | 63     | 24     | 139    | 226   | 18             | 8.0            |
| Female         | 89     | 25     | 116    | 230   | 16             | 7.0            |
| Total          | 152    | 49     | 255    | 456   | 34             | 7.5            |
Table 3. Performance characteristics of illumigene Mycoplasma Direct assay as compared with illumigene Mycoplasma assay (Qiagen Extraction)

| illumigene Mycoplasma Direct (iMD) | illumigene Mycoplasma Predicate (iM) |
|------------------------------------|-------------------------------------|
| Positive                           | Positive   | 24 | 10* | 34  |
| Negative                           | Negative   | 1* | 421 | 422 |
| Total                              | Total      | 25 | 431 | 456 |

Performance parameters (95% CI)

|                               | Overall Percent Agreement | 97.6 (95.7 - 98.6) |
|                               | Positive Percent Agreement | 96.0 (80.5 - 99.3) |
|                               | Negative Percent Agreement | 97.7 (95.8 - 98.7) |

* 4/10 samples were identified positive by illumigene Mycoplasma, after testing with an additional frozen swab collected from patients for discrepant analysis.

* Repeat testing by illumigene Mycoplasma, with the original patient sample and an additional frozen sample, all produced negative results.