Evaluation of Three Real-Time PCR Assays for Detection of *Mycoplasma pneumoniae* in an Outbreak Investigation

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We compared the performances of three recently optimized real-time PCR assays derived from distinct genomic regions of *Mycoplasma pneumoniae* during an outbreak. Comprehensive evaluation established that a newly described toxin gene represents a superior target for detecting *M. pneumoniae* DNA in clinical specimens, although use of multiple targets may increase testing confidence.

*Mycoplasma pneumoniae* accounts for approximately 15% to 20% of all community-acquired pneumonia cases and is a common cause of outbreaks (10, 16, 18). Outbreaks have been reported to occur in 3- to 7-year intervals, and although all age groups are susceptible, incidence rates vary with age and may occur more frequently in certain settings (10, 16, 17). *M. pneumoniae* infection spreads efficiently within households and close living quarters, with incubation periods as long as 3 weeks (17). The insidious nature of this infection and its protracted disease course make this agent a predominant cause of “walking pneumonia” that can persist within the population and cause community or institutional outbreaks. Upper- and lower-respiratory-tract symptoms are often mild, resulting in tracheobronchitis, headache, and cough. Occasionally, severe cases with extrapulmonary involvement can result in hospitalization and death due to neurological disease, such as encephalitis (1, 2, 4, 15, 17). The high rate of morbidity and the occasional mortality reinforces the need for timely diagnosis for administering proper antibiotic treatment (7, 9).

Conventional tests for detecting *M. pneumoniae* are fraught with limitations (3). *M. pneumoniae* culture can often take several weeks, requires special media and expertise, and is insensitive and prone to contaminants and inhibitors. Serological assays such as complement fixation and commercially available immunoglobulin detection kits are by nature retrospective, requiring paired serum samples from both acute and convalescent phases, and provide questionable specificity and sensitivity results. In sum, these approaches are impractical for a rapid diagnosis. A variety of nucleic acid-based tests based upon PCR have been developed for the rapid and sensitive detection of *M. pneumoniae* (5, 11, 13, 14, 16). The range of variables within each PCR study (specimen type, nucleic acid extraction and amplification procedures, target selection, definitions used in calculating data, etc.) makes it difficult to compare results and draw a single, comprehensive approach for reliable detection.

Recent community outbreaks of *M. pneumoniae* infection underscore a need among public health departments and local hospitals for a rapid and reliable diagnostic assay (1, 10, 12, 17, 18). Moreover, this test should be highly specific and sensitive and should be evaluated in an outbreak setting. The aim of the current study was to evaluate the use of three recently optimized real-time PCR assays for the detection of *M. pneumoniae* in respiratory samples from a recent outbreak. To our knowledge, this is the first prospective and comparative study of real-time PCR targets used to identify cases during an outbreak investigation of *M. pneumoniae* and the first report of a study targeting the recently identified ADP-riboylsoting toxin gene encoding the CARDS (community-acquired respiratory distress syndrome) toxin for real-time PCR detection.

Multiple TaqMan primer-probe sets targeting the ATPase gene (GenBank accession no. U43738) and the CARDS toxin gene (GenBank accession no. DQ447750) of *M. pneumoniae* were designed using Primer Express version 3.0 (Applied Biosystems, Foster City, CA) (8). The real-time PCR mixture was prepared in a total volume of 25 μl. Each PCR mixture contained the following per reaction: 12.5 μl of Platinum quantitative PCR SuperMix-UDG (catalog no. 11730-025; Invitrogen), 1.5 μl of 50 mM MgCl₂, 0.5 μM final concentrations of each primer, a 0.1 μM final concentration of the probe, 1.25 U Taq DNA polymerase (catalog no. 10966-034; Invitrogen) (5 U/μl), 1 μl of 10 mM PCR nucleotide mix (catalog no. C1141; Promega), 5 μl of extracted nucleic acid from each specimen, and nuclease-free water (catalog no. P1193; Promega) to achieve a 25-μl final volume. Real-time PCR for each target was performed using an ABI 7500 system (Applied Biosystems) under the following conditions: initial activation of 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. After thorough analysis and evaluation of the primer-probe sets, which involved BLAST searches, one toxin (Mp181) and two ATPase (Mp3 and Mp7) gene targets were selected based upon specificity and sensitivity performance and optimized to achieve maximum efficiency (Table 1).

Each assay demonstrated >99% efficiency, as calculated using a standardized dilution series of quantitated DNA samples of *M. pneumoniae* tested using six replicates over six logs (100 pg to 1 fg). The average from these data is reported as the square of the coefficient of regression values (efficiency) in Table 1. The sensitivity of each assay was determined by extracting a...
trans, fermentans
Mycoplasma buccale
Mycoplasma pirum
rium
Mycoplasma lipophilum
coplasma faecium
and viral pathogen panel consisting of the following targets:
assay was also tested for specificity with at least 15 ng of nucleic
detected between 1 and 5 CFU, while assays using both Mp3 and
detected within each real-time PCR. Use of Mp181 consistently
in six replicates; values are reported in Table 1 as total CFU
mined using a hemolytic plaque formation procedure as previ-
CS11301; Invitrogen) following the manufacturer’s instruc-
salivarius
strains of
M. pneumoniae
series of dilutions from quantitated stocks of both prototypical
the “(T).”
ticipants (mean, 21.7) were tested in triplicate with each
age (mean, 21.7) were tested in triplicate with each
RNase P internal control to ensure proper nucleic acid extrac-
diagnosed by chest X-ray or clinical examination during a 12-
side outbreak within a college setting. A total of 54 respiratory
parainfluenza virus 2, human parainfluenza virus 3, human ade-
Moraxella catarrhalis,
mophilus influenzae
Escherichia coli
cans
Chlamydia trachomatis,
Staphylococcus epidermidis,
Streptococcus pyogenes
Pseudomonas aeruginosa,
Ureaplasma parvum
Mycoplasma salivarium
Mycoplasma pneumoniae
Legionella
Hae-
series of dilutions from quantitated stocks of both prototypical
strains of
M. pneumoniae
(M129 [type I] and FH [type 2]) by
use of a ChargeSwitch gDNA Mini Bacteria kit (catalog no.
CS11301; Invitrogen) following the manufacturer’s instruc-
tions. The CFU value per milliliter of each dilution was deter-
moved using a hemolytic plaque formation procedure as previously
described (6). Each dilution was then tested with each assay
in six replicates; values are reported in Table 1 as total CFU
detected within each real-time PCR. Use of Mp181 consistently
detected between 1 and 5 CFU, while assays using both Mp3 and
Mp7 were slightly less sensitive, detecting 5 to 50 CFU. Each
assay was also tested for specificity with at least 15 ng of nucleic
acid and showed no cross-reactivity with an extensive bacterial
and viral pathogen panel consisting of the following targets:
Mycoplasma faecium, Mycoplasma lipophilum, Mycoplasma saliva-
rium, Mycoplasma pirum, Mycoplasma orale, Mycoplasma pene-
trans, Mycoplasma genitalium, Mycoplasma hominis, Mycoplasma
fermentans, Mycoplasma buccale, Mycoplasma arginini, Myco-
plasma hyorhinis, Mycoplasma amphiorme, Lactobacillus plani-
tartum, Staphylococcus epidermidis, Coxiella burnetii, Streptococcus
salivarius, Bordetella pertussis, Legionella pneumophila, Legionella
longbeachae, Streptococcus pneumoniae, Ureaplasma urealyticum,
Neisseria meningitidis, Chlamydia trachomatis, Chlamylophila
psitacci, Chlamydophila pneumoniae, Streptococcus pyogenes, Hae-
mophilus influenzae, Neisseria elongata, Pseudomonas aeruginosa,
Moraxella catarrhalis, Mycobacterium tuberculosis, Candida albici-
cans, Escherichia coli, Staphylococcus aureus, Ureaplasma parvum,
human DNA, human coronavirus, human rhinovirus, human para-
influenza virus 2, human parainfluenza virus 3, human ade-
novirus, influenza virus A, influenza virus B, respiratory syncytiat
virus A, and respiratory syncytial virus B.

These assays were used to identify a recent
M. pneumoniae
outbreak within a college setting. A total of 54 respiratory
samples (oropharyngeal and nasopharyngeal swabs) from pa-
tients (n = 35) and negative controls (n = 19) 18 to 35 years of
age (mean, 21.7) were tested in triplicate with each
M. pneumoniae-specific assay (Mp3, Mp7, and Mp181) along with an
RNase P internal control to ensure proper nucleic acid extrac-
tion and integrity. The data in Table 2 demonstrate that 18 of
35 pneumonia cases (~51%), where a pneumonia case is de-
defined as exhibiting fever (≥100.4°F) and cough or pneumonia
diagnosed by chest X-ray or clinical examination during a 12-
week outbreak period, were positive with all three signature
sequences. Of note, the Mp181 assay routinely exhibited cross-

ing-threshold values earlier than both the Mp7 and the Mp3
assays (P < 0.0001 and P = 0.06, respectively, following Stu-
dent’s t test), which is concordant with the sensitivity data of
Table 1. The crossing-threshold values ranged from ~26 to
~39, depending on the target, and displayed a typical sigmoi-
dal curve similar to that seen with the positive controls, as did
all RNase P assays (data not shown). All negative controls
(defined as age-matched asymptomatic subjects within the
same population) and samples from 16 patients demonstrated
no reactivity with any of the
M. pneumoniae-specific markers
but gave positive RNase P signals (data not shown). The lack
of PCR reactivity in the 16 cases may reflect the presence of an

<table>
<thead>
<tr>
<th>Primer or probea</th>
<th>Sequence (5’→3’)</th>
<th>Gene target</th>
<th>Assay product (bp)</th>
<th>Assay efficiency</th>
<th>Assay sensitivity (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mp181-F</td>
<td>TTTGTTAGCTGGTACGGGAAAT</td>
<td>CARDs toxin</td>
<td>73</td>
<td>0.9954</td>
<td>~1–5</td>
</tr>
<tr>
<td>Mp181-R</td>
<td>GGTCCGACAGAATTTCCATATAAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp181-P</td>
<td>TGTCACAGAAGACCCAGAACGGCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp3-F</td>
<td>CGATCTATGCGCCAGTGATGA</td>
<td>ATPase</td>
<td>68</td>
<td>0.9931</td>
<td>~5–50</td>
</tr>
<tr>
<td>Mp3-R</td>
<td>AGCATCCAGGTTGGTTAAAGGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp3-P</td>
<td>TTGACTGACCCCCGTCCGGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp7-F</td>
<td>ACTAACAATTCGATCGTTACAATGAA</td>
<td>ATPase</td>
<td>106</td>
<td>0.9932</td>
<td>~5–50</td>
</tr>
<tr>
<td>Mp7-R</td>
<td>CCACCTTGTTGCTTGATCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp7-P</td>
<td>ACTCTT(T)GCCAACCAACAAAAACGAGTCTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Probes were labeled with 5′ 6-carboxyfluorescein and 3′ Black Hole Quencher-1 except for Mp7-P, which was internally quenched with Black Hole Quencher-1 on the “(T).”

Table 1. Primers and probes for real-time amplification of M. pneumoniae targets

<table>
<thead>
<tr>
<th>Case</th>
<th>Crossing threshold ± SD for indicated gene target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.86 ± 0.84  99.11 ± 0.42  31.96 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>30.79 ± 0.86  36.21 ± 2.35  34.53 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>27.66 ± 0.71  21.19 ± 0.11  31.06 ± 0.07</td>
</tr>
<tr>
<td>4</td>
<td>26.12 ± 0.83  27.40 ± 0.02  29.52 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>35.70 ± 0.81  35.76 ± 0.54  38.58 ± 0.55</td>
</tr>
<tr>
<td>6</td>
<td>30.78 ± 0.76  34.7 ± 0.23   34.26 ± 0.67</td>
</tr>
<tr>
<td>7</td>
<td>28.66 ± 1.08  30.03 ± 0.12  32.10 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>30.91 ± 1.31  33.11 ± 0.1    34.79 ± 0.23</td>
</tr>
<tr>
<td>9</td>
<td>28.59 ± 0.21  28.53 ± 0.01  30.18 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>35.03 ± 0.61  34.42 ± 0.04  36.49 ± 0.36</td>
</tr>
<tr>
<td>11</td>
<td>35.64 ± 0.45  34.04 ± 0.11  38.90 ± 0.69</td>
</tr>
<tr>
<td>12</td>
<td>28.16 ± 0.10  28.24 ± 0.03  29.81 ± 0.27</td>
</tr>
<tr>
<td>13</td>
<td>29.07 ± 0.44  28.34 ± 0.36  30.20 ± 0.14</td>
</tr>
<tr>
<td>14</td>
<td>32.43 ± 1.21  32.14 ± 0.15  34.21 ± 0.44</td>
</tr>
<tr>
<td>15</td>
<td>33.68 ± 1.36  32.31 ± 1.43  34.7 ± 0.30</td>
</tr>
<tr>
<td>16</td>
<td>32.68 ± 1.08  31.87 ± 0.16  33.65 ± 0.35</td>
</tr>
<tr>
<td>17</td>
<td>30.91 ± 0.57  33.29 ± 0.18  31.91 ± 0.07</td>
</tr>
<tr>
<td>18</td>
<td>29.99 ± 0.74  30.04 ± 0.08  29.16 ± 0.10</td>
</tr>
<tr>
<td>19</td>
<td>36.04 ± 0.26  ND*          ND*</td>
</tr>
</tbody>
</table>

* Total number of samples tested, 54; total number of pneumonia cases, 35; total number of negative controls, 19; total number of PCR-negative cases, 16. All samples were tested in triplicate for each marker except the case 19 sample, which contained limited sample volume.

* ND, not done.

Table 2. Average crossing-threshold values for Mp181, Mp3, and Mp7 gene targets from clinically defined cases of pneumonia with positive real-time PCR data.
infection with a different pathogen or poor sample quality. Serum samples were collected from a limited number of subjects and proved to be of little value for diagnosis. Serological assays are often unreliable due to specificity and sensitivity limitations and the documented persistence of antibodies in patients (3).

This study evaluated three real-time PCR assays targeting the ATPase and newly described CARDS toxin genes during a recent outbreak for the purpose of assessing its utility in such instances. Although other real-time PCR assays for the detection of *M. pneumoniae* have been reported, none have been applied to an investigation involving an outbreak. Interestingly, the assay targeting the CARDS toxin gene (Mp181) proved to be the most sensitive in identifying positive specimens during this outbreak and has been subsequently used to positively identify *M. pneumoniae* DNA in other specimens (respiratory and cerebrospinal fluid) in sporadic cases. These data support the use of the Mp181 assay as an initial screening marker for detecting the presence of *M. pneumoniae* DNA in respiratory clinical specimens, although the inclusion of Mp3 and Mp7 may provide an increased level of confidence for the reporting of results. The use of these assays may allow the rapid identification of an *M. pneumoniae* outbreak at the local and state levels when testing is implemented in a timely manner. Further investigation of each assay may be warranted for possible use in clinical practice.

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**REFERENCES**