Evaluation of five real-time PCR assays for detection of *Mycoplasma pneumoniae*

Running title: Real-time PCR for detection of *M. pneumoniae*

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Abstract

Four commercial real-time PCR assays to detect *M. pneumoniae* were tested and the results compared with an in-house approach. Despite differences of crossing threshold values up to 4, assays were able to detect at least 20 cfu/5 μl (52 fg DNA/5 μl) of sample with best clinical sensitivity of Diagenode kit.
Mycoplasma pneumoniae is a common agent of infections of the human respiratory tract ranging from mild cases of tracheobronchitis to severe pneumonia requiring hospitalization of the patient. Furthermore, extra-pulmonary manifestations of M. pneumoniae infections are described (1, 2, 3). Especially in epidemic periods which are reported every 3 to 7 years, up to 40% of community-acquired pneumonia (CAP) cases are caused by M. pneumoniae (1, 2) affecting mainly older children but also adults (4, 5). Early and rapid diagnosis of interstitial pneumonia due to M. pneumoniae is of importance since beta-lactam antibiotics are recommended as first-line therapy in CAP which is ineffective against the cell wall-less mycoplasmas. Unfortunately, culture of these bacteria from clinical specimens is time-consuming and of low sensitivity. Serological tests are not helpful in early stages of infection, requiring in many cases paired sera for the definite interpretation of the results and showing problems with regard to specificity as well as to sensitivity (6, 7). Real-time PCR assays have been developed as sensitive and specific option for detection of microorganisms in respiratory samples. In the last years, a broad spectrum of targets for amplification was described (8, 9, 10). Among these, use of copies of repetitive elements repMP1 was demonstrated in different studies as a very sensitive approach (9, 10, 11). Since a number of variables can influence the results of amplification, there exists the need for inter- and intra-laboratory validation of different assays under defined conditions (6). Furthermore, the permanent dynamic on the market of laboratory diagnostics requires a testing of real-time PCR kits which are commercially available at given time. The aim of the present study was to compare the performance of selected commercial real-time PCR assays with a characterized in-house approach regarding the results from a set of standardized samples of different origin.
M. pneumoniae strain M129 (ATCC 29342) was grown in cell culture flasks with PPLO medium (Becton Dickinson, Sparks, MD, USA) at 37°C. After the color of the medium changed, attached cells were washed twice with PBS and harvested using a cell scraper.

Four commercially available PCR kits were included in the study and the results were compared to the in-house approach amplifying the repMP1 copies as multi-copy target (11). Of the fourteen repMP1 elements scattered all over the genome of M. pneumoniae (12), amplification of at least 10 copies can be expected. To limit the influence of different platforms on the results, we tested assays which are recommended for LightCycler 1.5 and 2.0 (Roche, Rotkreuz, Switzerland) since these instruments are widely used. The followed real-time PCR assays were selected: Diagenode Mycoplasma pneumoniae/Chlamydia pneumoniae real-time PCR (Europe Diagenode sa, Liege, Belgium), GeneProof Mycoplasma pneumoniae (GeneProof a.s., Brno, Czech Republic), BactoReal Mycoplasma pneumoniae (Ingenetix GmbH, Vienna, Austria) and LightMix kit Mycoplasma pneumoniae (TIB MOLBIOL GmbH, Berlin, Germany), respectively. Characteristics of the in-house and commercial kits are summarized in Table 1.

Respiratory samples (34 pharyngeal swabs, 2 sputa, one broncho-alveolar lavage) were taken between 2011 and 2013 from adult and pediatric patients with symptoms of CAP. Primary testing of samples was done with the repMP1-based real-time PCR. Positive samples (n=37) were aliquoted and frozen at -20°C until re-testing with all real-time PCR assays investigated. Amplification of M. pneumoniae-specific targets was done in duplicate with each assay and Ct-values were averaged.

For determination of kinetics and sensitivity of the real-time PCR assays evaluated, freshly prepared M. pneumoniae suspension was homogenized with a 27G syringe to reduce cell aggregations. An aliquot of the suspension was used to determine the cfu on PPLO agar with 10-fold dilutions of the bacteria in PPLO bouillon. In parallel, DNA in a further 200 µl aliquot was
prepared with QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer (blood and body fluids protocol) and collected in 100 µl elution buffer. DNA concentration was measured in a spectral photometer by standard procedure. After ten days of incubation, colonies on PPLO plates were counted. Before using as sample in real-time PCR, DNA was ten-fold diluted with HPLC water, aliquoted and stored at -20°C. PCR efficiency was determined by investigation of a standard curve of the quantified DNA sample diluted over six logs and PCR efficiency of the real-time PCR approaches was calculated (10^-slope). Sensitivities of the assays were estimated by determining the positivity rate after investigation of three DNA dilutions representing 20, 2 and 0.2 cfu/reaction, respectively.

To minimize degradation of DNA, none of the samples aliquoted was freeze/thawed more than once before use in real-time PCR. Real-time PCR assays were carried out according the recommendations of the different manufacturers or as published before (11) and for all runs the same LightCycler 1.5 or 2.0 instrument was used.

Comparison of results of testing real-time PCR approaches to detect *M. pneumoniae* is complicated. Differences in the target amplified, the special test conditions and the real-time PCR instrument used can influence the Ct-values measured (6). In addition, difficulties with the precise definition of cfu or colour-changing-units (ccu) in dilutions of the fastidious growing mycoplasmas is a further problem. As a consequence, a limited number of studies are available testing not only a single *M. pneumoniae*-specific target for quantitative amplification (9-11, 13-15). To our knowledge, beside the in-house repMP1-based approach, only the Diagenode kit was tested previously under comparable conditions with other commercial real-time PCR systems (14).

The standard curves obtained from all five real-time methods tested show excellent statistical characteristics (Table 2). Best PCR efficiency (2.008) was demonstrated with the repMP1-based
in-house approach. Lowest efficiency was measured with the Diagenode kit (1.914). Using the in-house assay, a broad range of Ct-values from 23.29 to 35.75 was found in respiratory samples of pneumonia patients (Table 3). Since Ct-values in respiratory tract samples of at least 20 up to 39 were detected in other reports (10, 11, 13, 15), this range can be considered as typical spectrum of M. pneumoniae-positive specimens in microbiological laboratories. Negative results in the GeneProof, Ingenetix and LightMix kits correspond to the samples (all were pharyngeal swabs) with the highest Ct-values (35.25, 35.27 and 35.75, respectively) detected in the in-house PCR assay. The Diagenode test with these three specimens was positive in one of the two parallel runs indicating that the detection limit of the procedure was achieved. However, the analytical sensitivity of at least 92% confirmed a good performance of all approaches tested. This is in contrast to a study by Touati *et al.* (14) reporting sensitivities of < 90% (88 to 62%) of four of five commercial real-time PCR assays in comparison with an in-house test demonstrating a similar mean Ct-value as the repMP1-based assay in the present study. Here, best results with a commercially available kit were obtained with the Diagenode assay showing a positive test result in all human respiratory samples investigated. Discrepancies of results in comparison to other studies (14) could be explained by differences in the instrument used, concentration of *M. pneumoniae*-DNA in samples tested and sample volume included. No amplification was observed with DNA from phylogenetically related or clinically relevant bacterial species *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma hominis* (ATCC 23114), *Mycoplasma orale* (ATCC 23714), *Mycoplasma salivarium* (ATCC 23064), *Ureaplasma urealyticum* (ATCC 27618), *Chlamydia pneumoniae* (strain TW-183), *Legionella pneumophila* (ATCC 33152), *Haemophilus influenzae* (ATCC 49247), *Streptococcus pneumoniae* (ATCC 6305), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas*
aeruginosa (ATCC 27853), and human DNA confirming the specificity of the real-time approaches included.

It should be noted that pre-testing of clinical samples with the real-time PCR targeting the repMP1 copies can lead to a selection of positive specimens and is a limitation of the present study. However, results of the repMP1-based system as sensitive approach to detect M. *pneumoniae* in culture and clinical samples confirmed findings of previous studies (9, 10, 13).

Regarding Ct-values, in comparison with the repMP1-based approach significantly higher mean values were measured with the Ingenetix and the LightMix kits. In contrast, the difference to the results of the Diagenode and the GeneProof test is statistically not significant confirming that under optimized test conditions, mono-copy targets can be amplified with comparable efficiency to repetitive elements (9). Interestingly, the good clinical sensitivity of the GeneProof kit corresponds not in all cases to the analytical sensitivity. This discrepancy was also reported in other studies (14). Providing that the efficiency of the used DNA preparation corresponds to nearly 100%, all five test kits were able to detect 20 cfu/5 µl of sample in the eight parallel runs tested (Table 2). Whereas the in-house, Diagenode and LightMix approach demonstrated positive signals in eight experiments after testing of specimens with 2 cfu/5 µl, amplification products were found in 7 out of 8 (Ingenetix) and 3 out of 8 parallels (GeneProof) with the two further kits. Only the repMP1-based approach was able to detect 0.2 cfu/5 µl in all parallels.

Results of studies indicated that the bacterial load might influence the severity of diseases after infection with *M. pneumoniae* (16, 17). The data of the present and other comparison reports confirmed that the PCR approach used will influence the positivity rate of tested samples as well as the measured load of *M. pneumoniae* in the specimens. Not only the mean results from a single sample can differ by Ct-values of more than 3 between the real-time PCR approaches used (represent a difference of genome copies of around one log-unit) also the mean Ct-values of a
complete panel of tested samples can show this range of difference and was also reported in other studies (10, 11, 13-15). Furthermore, cultural and molecular detection of *M. pneumoniae* in healthy individuals as well long-term carriage of bacteria after the period of typical respiratory symptoms and adequate antibiotic therapy is a known fact (18, 19). Future studies have to prove if a range of bacterial load is associated with this phenomenon and might be used to differentiate patients with acute illness due to *M. pneumoniae* from symptom-less carriers or *M. pneumoniae*-positive patients with symptoms of CAP caused by other micro-organisms (20). In this context, knowledge of sensitivity of the real-time PCR approach used is a pre-condition for the evaluation of the significance of the concentration of genome copies measured.

In conclusion, with the real-time PCR approaches tested a rapid (<1.5 h) detection of *M. pneumoniae*-specific targets was demonstrated. Despite differences among the methods regarding PCR-efficiency, Ct-values, analytical and clinical sensitivity, all systems detect at least 20 cfu/5 µl of sample. With regard to the quantitative results of other studies based on real-time PCR (16, 17, 20, 21), this can be considered as an acceptable level of test sensitivity for most clinical questions.

**Acknowledgements**

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**References**


Table 1 Characteristics of the in-house and commercial real-time PCR assays investigated.

<table>
<thead>
<tr>
<th>Assay</th>
<th>LightCycler</th>
<th>Target</th>
<th>Internal control</th>
<th>Sample volume/test</th>
<th>Reagent mix</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house</td>
<td>1.5</td>
<td>repMP1-copies</td>
<td>No</td>
<td>5 µl</td>
<td>LC FastStart DNA Master HybProbe (Roche, Mannheim, Germany)</td>
<td>0.2 cfu a</td>
</tr>
<tr>
<td>Diagenode</td>
<td>2.0</td>
<td>P1 adhesin</td>
<td>Yes</td>
<td>10 µl</td>
<td>LC TaqMan Master (Roche)</td>
<td>50 ccu b</td>
</tr>
<tr>
<td>GeneProof</td>
<td>2.0</td>
<td>CARDS toxin</td>
<td>Yes</td>
<td>10 µl</td>
<td>included</td>
<td>no information</td>
</tr>
<tr>
<td>Ingenetix</td>
<td>1.5</td>
<td>16S rRNA</td>
<td>Yes</td>
<td>5 µl</td>
<td>LC FastStart DNA Master HybProbe</td>
<td>0.9 cfu b</td>
</tr>
<tr>
<td>LightMix</td>
<td>1.5</td>
<td>P1 adhesin</td>
<td>Yes</td>
<td>5 µl</td>
<td>LC FastStart DNA Master HybProbe</td>
<td>10 copies b</td>
</tr>
</tbody>
</table>

a - according to Dumke et al. (11).

b - as specified by the manufacturer.
Table 2

Kinetics and sensitivity of the evaluated in-house and commercial real-time PCR assays using DNA extract of bouillon cultures of *M. pneumoniae* M129.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Standard curve $^a$</th>
<th>% positive samples (mean Ct of positive samples±SD) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>PCR 52 fg DNA/5 µl 5.2 fg DNA/5 µl 0.5 fg DNA/5 µl</td>
</tr>
<tr>
<td></td>
<td>efficiency</td>
<td>(20 cfu/5 µl) (2 cfu/5 µl) (0.2 cfu/5 µl)</td>
</tr>
<tr>
<td>In-house</td>
<td>0.9994</td>
<td>2.008 100 (30.55±0.06) 100 (33.30±0.18) 100 (34.71±0.67)</td>
</tr>
<tr>
<td>Diagenode</td>
<td>0.9995</td>
<td>1.914 100 (31.76±0.16) 100 (35.02±0.49) 75 (38.02±1.19)</td>
</tr>
<tr>
<td>GeneProof</td>
<td>0.9988</td>
<td>1.994 100 (31.07±0.37) 37.5 (32.96±0.52) 0</td>
</tr>
<tr>
<td>Ingenetix</td>
<td>0.9999</td>
<td>1.938 100 (34.60±0.27) 87.5 (38.35±1.15) 0</td>
</tr>
<tr>
<td>LightMix</td>
<td>0.9995</td>
<td>1.975 100 (32.20±0.19) 100 (36.00±0.93) 50.0 (37.65±1.17)</td>
</tr>
</tbody>
</table>

$^a$ - crossing thresholds determined after investigation of six dilutions of a *M. pneumoniae* M129 stock (DNA concentration: 169.8 to 0.002 ng/5 µl, n=4 each).

$^b$ - investigation of eight parallel runs of DNA of each dilution.
Table 3

Results of four commercial real-time PCR assays used for investigation of 37 *M. pneumoniae*-positive respiratory tract samples in comparison to the in-house real-time PCR.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Number of positive samples/number of samples tested (%)</th>
<th>Mean crossing thresholds of positive samples ± SD</th>
<th>Range of mean crossing thresholds</th>
<th>95% CI a</th>
<th>P value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house</td>
<td>37/37 (100)</td>
<td>30.80±2.72</td>
<td>23.29–35.75</td>
<td>29.92–31.68</td>
<td>-</td>
</tr>
<tr>
<td>Diagenode</td>
<td>37/37 (100)</td>
<td>31.50±3.28</td>
<td>23.29–39.31</td>
<td>30.44–32.56</td>
<td>n.s.</td>
</tr>
<tr>
<td>GeneProof</td>
<td>34/37 (91.9)</td>
<td>30.80±1.98</td>
<td>24.44–33.72</td>
<td>30.13–31.47</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ingenetix</td>
<td>35/37 (94.6)</td>
<td>34.13±2.94</td>
<td>26.41–40.85</td>
<td>33.16–35.10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LightMix</td>
<td>35/37 (94.6)</td>
<td>32.29±3.05</td>
<td>24.32–37.86</td>
<td>31.28–33.30</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

a - confidence interval.
b - calculated by paired student’s t-test (only results of samples positive in both in-house and commercial assay were compared). n.s. - not significant.