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Note

Multi-center evaluation of one commercial and 12 in-house real-time PCR assays for detection of *Mycoplasma pneumoniae*

Roger Dumke [a,*], Alvaro J. Benitez [b], Victoria Chalker [c], Karolina Gullsby [d], Birgit Henrich [e], Carlos Hidalgo-Grass [f], Theo Hoogenboezem [g], Darja Kese [h], Katherine Loens [i], Jolanda Maaskant [j], Ayelet Michael-Gayego [k], Allon E. Moses [k], Ran Nir-Paz [k], Suzan D. Pas [j], Sabine Pereyre [l], Randi F. Petersen [m], Meike Rosenblatt [e], Annemarie M.C. van Rossum [n], Søren A. Uldum [m], Wendy W.J. Unger [g], Dominique Ursi [i], Jonas M. Winchell [b], Cecile Bebear [l]on behalf of ESCMID Study Group for Mycoplasma Infections

[a] *TU Dresden, Dresden, Germany*
[b] *Centers for Disease Control and Prevention, Atlanta, GA, USA*
[c] *Public Health England, London, United Kingdom*
[d] *Centre for Research and Development, Uppsala University/Region Gävleborg, Sweden*
[e] *Universität Düsseldorf, Düsseldorf, Germany*
[f] *HGC Biomedical Research Diagnostics S.L., Huelva, Spain*
[g] *Laboratory of Pediatrics, Department of Pediatrics, Erasmus MC University Medical Center-Sophia Children's Hospital, Rotterdam, The Netherlands*
[h] *University of Ljubljana, Ljubljana, Slovenia*
[i] *Universitair Ziekenhuis Antwerpen, Edegem, Belgium*
[j] *Viroscience, Erasmus MC University Medical Center, Rotterdam, The Netherlands*
[k] *Hadassah-Hebrew University Medical Center, Jerusalem, Israel*
[l] *University of Bordeaux, Bordeaux, France*
[m] *Statens Serum Institut, Copenhagen, Denmark*
[n] *Division of Infectious Diseases and Immunology, Department of Pediatrics, Erasmus MC University Medical Center–Sophia Children's Hospital, Rotterdam, The Netherlands*

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ABSTRACT

Detection of *Mycoplasma pneumoniae* by real-time PCR is not yet standardized across laboratories. We have implemented a standardization protocol to compare the performance of thirteen commercial and in-house approaches. Despite differences on threshold values of samples, all assays were able to detect at least 20 *M. pneumoniae* genomes per reaction.

Mycoplasma pneumoniae is a leading agent of human respiratory infections. Symptoms range from mild and often undetected forms of tracheobronchitis to interstitial pneumonia (Atkinson et al., 2008). *M. pneumoniae* is responsible for 2–10% of all cases of community-acquired pneumonia. In epidemics which occur every 3 to 7 years, the rate of pneumonia due to *M. pneumoniae* increased to more than 25% (Dumke et al., 2015; Jain et al., 2015; Klapdor et al., 2012; Torres et al., 2014). Additionally, extra-pulmonary manifestations of infections are frequently described (Narita, 2016). Severe cases of pneumonia requiring intensive treatment of patients (Khoury et al., 2016) and the intrinsic resistance of the cell wall-less mycoplasmas to beta-lactam antibiotics require an early, specific and sensitive detection of *M. pneumoniae* infections for targeted treatment. Unfortunately, cultivation of the micro-organisms is time consuming. Detection of specific antibodies is hampered by problems of test specificity, variability of immunoglobulin responses in different age groups and after re-infections, the need of paired sera for the reliable interpretation of a seroconversion, and is often not suitable to confirm an infection at the onset of symptoms (Loens et al., 2010a). Nucleic acid amplification techniques are currently considered the most reliable alternative for the appropriate diagnosis of *M. pneumoniae* infections and were characterized as superior to serological methods (Chang et al., 2014), allowing a better interpretation of results of serodiagnosis (Loens and Ieven, 2016). Despite the development of further test approaches such as the LAMP assays (Ratliff et al., 2014), real-time PCR applications are the most

* Corresponding author. Tel.: +49-351-4586577; fax: +49-351-4586310.
E-mail address: roger.dumke@tu-dresden.de (R. Dumke).

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commonly used approach for detection of *M. pneumoniae* clinically. However, the variety of PCR instruments available, the use of varying reaction components and conditions, as well of the numerous targets described for amplification make the quantitative comparison of results between laboratories difficult. Previous studies confirmed differences between real-time PCR approaches regarding clinical sensitivity, limit of detection and crossing threshold (C_T)-values (Chou and Zheng, 2016; Dumke and Jacobs, 2009; Dumke and Jacobs, 2014; Schmitt et al., 2013; Touati et al., 2009; Winchell et al., 2008; Zhao et al., 2012). In the 2 multi-centre studies for PCR detection of *M. pneumoniae* published up to now, most participants reported qualitative data (Loens et al., 2010b) or the number of participating laboratories was limited (Loens et al., 2012). In the present study, the quantitative performance of current real-time PCR approaches for detection of *M. pneumoniae* in thirteen laboratories (Europe, Israel and USA) was investigated by using comparable samples and a standardized protocol.

The freeze-dried stocks of DNA standards of *M. pneumoniae* strain FH^T (ATCC 15531), each containing 1.0×10^8 genomes, were purchased from Minerva Biolabs (Berlin, Germany). Rehydration, combining of all stocks, aliquotation and pre-testing of sample were done in laboratory no. 3. During November 2015, frozen aliquots with 1.0×10^8 genomes in 100 μL DNA-free water (DNA concentration: 114.4 μg/mL) were distributed by courier to the laboratories taking part. Only samples that arrived to the participating laboratories in frozen condition were further processed. In parallel, an instruction manual was sent to all participants of the study. For determination of the standard curve, the sample was diluted tenfold with DNA-free water (1:10 to 1:10,000; avoidance of C_T-values >35), divided to enable 7 separate runs and stored at −20 °C. Moreover, 2 additional 1:10,000 dilutions were prepared. For determination of sensitivity, these 1:10,000 dilutions were diluted with DNA-free water to compass a reduced positivity of test results (at least: 1:10; 1:50; 1:100; 1:500; further dilution steps depend on the PCR approach used), aliquoted for 7 runs and stored at −20 °C (between 2 and 8 weeks). Tubes with low DNA binding (Eppendorf, Hamburg, Germany) were used and repeated freezing/thawing of samples was avoided. Thawed samples were kept on ice and processed as soon as possible. Testing was done within 2 month after receipt of samples. In all laboratories, real-time PCR runs for determination of standard curves or of test sensitivity were carried out at 5 different days with 3 replicates of each dilution. For all runs, separate sample sets were thawed. Together with a filled questionnaire (summarizing the characteristics of PCR method used), results were reported to laboratory no. 3. Mean C_T-values and standard deviations for each dilution were calculated and the number of genomes investigated was related to the sample volume used per reaction. Standard curve of each real-time PCR approach based on 5 tenfold dilutions of the DNA standard was determined and PCR efficiency was calculated ($10^{1/slope}$). Assay sensitivity was defined as range of number of genome copies of the highest dilution showing 100% positivity of the 15 replicates and of the next higher dilution.

The characteristics of the real-time PCR methods included in the study are summarized in Table 1. Despite the availability of different commercial assays (Loens and Ieven, 2016), most of the laboratories (12 out of 13) used in-house assays for the detection of *M. pneumoniae* (for assay, target and corresponding reference, see Table 1). Cost requirements in combination with the confirmed sensitivity and specificity are reasons for using in-house approaches. Furthermore, in 11 out of 13, the target for amplification was the well-characterized P1 adhesin although the suitability of many other sequences for specific amplification targets have been described (Loens et al., 2010a; Waites et al., 2012). It should be noted, that *M. pneumoniae* have been found as genetically stable micro-organism (Lluch-Senar et al., 2015; Xiao et al., 2015) and the approaches tested in the study amplify conserved regions in the genome. Different reports recommended the use of duplex or multiplex assays allowing the detection of several agents of respiratory tract infections (Diaz and Winchell, 2016; Loens and Ieven, 2016). In the present study, 4 laboratories (no. 1, 5, 10 and 11, respectively) applied such an approach. In laboratories no. 6 and 7 as well as in no. 12 and 13, the

Table 1
Characteristics of real-time PCR assays investigated.

Lab no.	Assay (manufacturer or reference)	Target	Thermocycler	Internal control	Sample volume/reaction	No. of cycles	Reagent mix
1	In-house (Thurman et al., 2011)	CARDS toxin[a]	ABI7500	Yes	5 μL	45	PerfeCTa multiplex qPCR supermix (Quanta Biosciences)
2	In-house (Touati et al., 2009)	P1 adhesin[b]	LightCycler 480	Yes	5 μL	50	LightCycler 480 probes master kit (Roche)
3	In-house (Dumke et al., 2007)	RepMP1[c]	LightCycler 1.2	No	5 μL	45	LC FastStart DNA master HybProbe (Roche)
4	In-house (Hardegger et al., 2000)	P1 adhesin	iCycler (CFX96)	Yes	5 μL	45	qPCR mastermix-no ROX (Eurogentec)
5	In-house (Gullsby et al., 2008)	P1 adhesin	Rotor-Gene 3000	No	5 μL	50	Express qPCR supermix universal (Invitrogen)
6	In-house (Averbuch et al., 2011)	P1 adhesin	ABI7500	No	6 μL	42	SYBR FAST qPCR master mix (Kapa Biosystems)
7	In-house (Averbuch et al., 2011)	P1 adhesin	Rotor-Gene 6000	No	2 μL	42	SYBR FAST qPCR master mix
8	In-house (n.a.[d])	P1 adhesin	ABI7500	Yes	5 μL	50	Platinum hot start PCR master mix (Invitrogen)
9	In-house (Ursi et al., 2003)	P1 adhesin	LightCycler 480	Yes	4 μL	45	LightCycler 480 probes master kit
10	Commercial (Argene)[e]	P1 adhesin	LightCycler 2.0	Yes	10 μL	45	r-gene amplification premix (Argene)
11	In-house (Ling and McHugh, 2013)	P1 adhesin	Rotor-Gene 6000	Yes	5 μL	45	Rotor-Gene multiplex PCR kit (Qiagen)
12	In-house (Spuesens et al., 2010)	P1 adhesin	ABI7900HT	No	5 μL	40	Taqman mastermix (Applied Biosciences)
13	In-house (Spuesens et al., 2010)	P1 adhesin	LightCycler 480	Yes	20 μL	45	Universal mastermix (Lifetech)

[a] Gene: *mpn372* (community-acquired respiratory distress syndrome toxin).
[b] Gene: *mpn141*.
[c] Copies of repetitive element RepMP1.
[d] No reference available.
[e] Chla/Myco pneumo r-gene.

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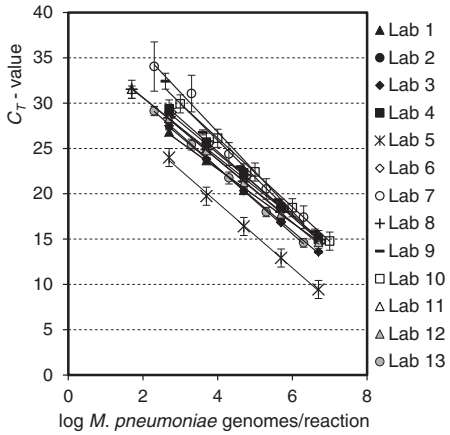

Lab no.	Standard curve		Assay sensitivity (genomes per reaction)
	r^2	PCR efficiency	
1	0.9955	2.171	1 - 5
2	0.9999	1.955	0.5 - 1
3	0.9995	1.936	1 - 5
4	0.9996	1.891	1 - 5
5	0.9980	1.898	1 - 5
6	0.9996	1.891	1 - 5
7	0.9822	1.692	0.4 - 2
8	0.9997	1.964	1 - 5
9	0.9896	1.760	1 - 4
10	0.9999	1.834	2 - 10
11	1	2.019	1 - 5
12	0.9995	1.955	1 - 5
13	0.9998	1.878	4 - 20

Fig. 1. Illustration of standard curves of real-time PCR approaches obtained after investigation of 5 tenfold dilutions of *M. pneumoniae* FH DNA standard (means and standard deviation of C_T-values), kinetics of standard curves and sensitivity of assays (given range represents lowest number of genomes resulted in 100% positive replicates and the number of genomes in the next lower dilution investigated).

same primers were used with different reaction conditions and cycler types. Eight laboratories included an internal control in their assays and the sample volume per reaction ranged from 2 to 20 μL. As expected, a broad spectrum of PCR instruments was used for amplification. However, standard curves covering 5 log units (corresponding to numbers of genomes between 50 and 10 000,000 per reaction) resulted in excellent correlation coefficients between 0.9822 and 1 (Fig. 1). In contrast, greater differences between the calculated PCR efficiencies were determined varying between 1.692 (laboratory 7) and 2.171 (laboratory 1), respectively. Furthermore, substantial differences of mean C_T-values of comparable numbers of genomes were measured. Interestingly, these differences had a limited influence on sensitivities since the method in laboratory no. 5 (lowest mean C_T-values; Fig. 1) demonstrated a sensitivity of 1 to 5 genomes per reaction which was comparable to the other approaches included in the study. Overall, the estimated sensitivities ranged between 0.4 and 20 genomes per reaction and are largely in agreement with the sensitivities reported previously for the assays (Averbuch et al., 2011; Dumke and Jacobs, 2014; Gullsby et al., 2008; Thurman et al., 2011; Touati et al., 2009).

Despite a relatively limited number of amplification targets, the approaches included here represents the broad spectrum of assays in use worldwide. Many of laboratories in this study are well-experienced in the detection of *M. pneumoniae* and some of which serve as reference centers. Further studies are necessary to determine the quantitative performance of methods used outside of these specialized laboratories. Several reports (Dumke and Jacobs, 2009; Dumke and Jacobs, 2014; Touati et al., 2009; Winchell et al., 2008; Zhao et al., 2012) described significant differences of the measured C_T-values if different target sequences were amplified under relatively comparable conditions (PCR instrument, master mix), complicating the comparison of C_T-values between laboratories. Bacterial load may be important to correspond to the severity of the *M. pneumoniae* infections clinical course (Jiang et al., 2014; Nilsson et al., 2010). Additionally, quantitative data may be used to distinguish between asymptomatic carriers and clinically relevant infections (Spuesens et al., 2013), thus our results are practically important if C_T-values of different real-time PCR assays will be compared. Interestingly, the amplification of a multi-copy target (repetitive element RepMP1; laboratory no. 3) was not found to be more sensitive than many approaches using single-copy sequences and confirmed the importance of further variables for test performance.

The results of the multicenter study for detection of *M. pneumoniae* by real-time PCR revealed differences of C_T-values obtained from samples with same number of genomes, of PCR efficiency and of assay sensitivity. Nevertheless, we found that all test systems are able to detect at least 20 genomes per reaction which can be considered as acceptable for the routine diagnostics. The results of the present report could serve as bench mark for epidemiological and clinical studies. Data are helpful to evaluate quantitatively the sensitivity and the extent of variation of C_T-values which should be expected when using different real-time PCR approaches to detect *M. pneumoniae* in human respiratory samples.

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References

Atkinson TP, Balish MF, Waites KB. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of *Mycoplasma pneumoniae* infections. FEMS Microbiol Rev 2008;32:956–73.

Averbuch D, Hidalgo-Grass C, Moses AE, Engelhard D, Nir-Paz R. Macrolide resistance in *Mycoplasma pneumoniae*, Israel, 2010. Emerg Infect Dis 2011;17:1079–82.

Chang HY, Chang LY, Shao PL, Lee PI, Chen JM, Lee CY, et al. Comparison of real-time polymerase chain reaction and serological tests for the confirmation of *Mycoplasma pneumoniae* infection in children with clinical diagnosis of atypical pneumonia. J Microbiol Immunol Infect 2014;47:137–44.

Chou RC, Zheng X. A comparison of molecular assays for *Mycoplasma pneumoniae* in pediatric patients. Diagn Microbiol Infect Dis 2016;85:6–8.

Diaz MH, Winchell JM. The evolution of advanced molecular diagnostics for the detection and characterization of *Mycoplasma pneumoniae*. Front Microbiol 2016;7:232.

Dumke R, Jacobs E. Comparison of commercial and in-house real-time PCR assays used for detection of *Mycoplasma pneumoniae*. J Clin Microbiol 2009;47:441–4.

Dumke R, Jacobs E. Evaluation of five real-time PCR assays for detection of *Mycoplasma pneumoniae*. J Clin Microbiol 2014;52:4078–81.

Dumke R, Schnee C, Pletz MW, Rupp J, Jacobs E, Sachse K, et al. *Mycoplasma pneumoniae* and *Chlamydia* spp. infection in community-acquired pneumonia, Germany, 2011-2012. Emerg Infect Dis 2015;21:426–34.

Dumke R, Schurwanz N, Lenz M, Schuppler M, Lück C, Jacobs E. Sensitive detection of *Mycoplasma pneumoniae* in human respiratory tract samples by optimized real-time PCR approach. J Clin Microbiol 2007;45:2726–30.

Gullsby K, Storm M, Bondeson K. Simultaneous detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* by use of molecular beacons in a duplex real-time PCR. J Clin Microbiol 2008;46:727–31.

Hardegger D, Nadal D, Bossart W, Altwegg M, Dutly F. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. J Microbiol Methods 2000;41:45–51.

Jain S, Williams DJ, Arnold SR, Ampofo K, Bramley AM, Reed C, et al. Community-acquired pneumonia requiring hospitalization among U.S. children. N Engl J Med 2015;372:835–45.

Jiang W, Yan Y, Ji W, Wang Y, Chen Z. Clinical significance of different bacterial load of *Mycoplasma pneumoniae* in patients with *Mycoplasma pneumoniae* pneumonia. Braz J Infect Dis 2014;18:124–8.

Khoury T, Sviri S, Abu Rmeileh A, Nubani A, Abutbul A, Hoss S, et al. Increased rates of intensive care unit admission in patients with *Mycoplasma pneumoniae*: a retrospective study. Clin Microbiol Infect 2016;22:711–4.

Klapdor B, Ewig S, Pletz MW, Rohde G, Schütte H, Schaberg T, et al. Community-acquired pneumonia in younger patients is an entity on its own. Eur Respir J 2012;39:1156–61.

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Ling CL, McHugh TD. Rapid detection of atypical respiratory bacterial pathogens by real-time PCR. Methods Mol Biol 2013;943:125–33.
Lluch-Senar M, Cozzuto L, Cano J, Delgado J, Llórens-Rico V, Pereyre S, et al. Comparative "-omics" in *Mycoplasma pneumoniae* clinical isolates reveals key virulence factors. PLoS One 2015;10:e0137354.
Loens K, Goossens H, Ieven M. Acute respiratory infection due to *Mycoplasma pneumoniae*: current status of diagnostic methods. Eur J Clin Microbiol Infect Dis 2010a;29:1055–69.
Loens K, Ieven M. *Mycoplasma pneumoniae*: current knowledge on nucleic acid amplification techniques and serological diagnostics. Front Microbiol 2016;7:448.
Loens K, Mackay WG, Scott C, Goossens H, Wallace P, Ieven M. A multicenter pilot external quality assessment programme to assess the quality of molecular detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. J Microbiol Methods 2010b;82:131–5.
Loens K, van Loon AM, Coenjaerts F, van Aarle Y, Goossens H, Wallace P, et al. Performance of different mono- and multiplex nucleic acid amplification tests on a multipathogen external quality assessment panel. J Clin Microbiol 2012;50:977–87.
Narita M. Classification of extrapulmonary manifestations due to *Mycoplasma pneumoniae* infection on the basis of possible pathogenesis. Front Microbiol 2016;7:23.
Nilsson AC, Björkman P, Welinder-Olsson C, Widell A, Persson K. Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. BMC Infect Dis 2010;10:39.
Ratliff AE, Duffy LB, Waites KB. Comparison of the illumigene mycoplasma DNA amplification assay and culture for detection of *Mycoplasma pneumoniae*. J Clin Microbiol 2014;52:1060–3.
Schmitt BH, Sloan LM, Patel R. Real-time PCR detection of *Mycoplasma pneumoniae* in respiratory specimens. Diagn Microbiol Infect Dis 2013;77:202–5.
Spuesens EBM, Fraaij PLA, Visser EG, Hoogenboezem T, Hop WCJ, van Adrichem LNA, et al. Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. PLoS Med 2013;10:e1001444.
Spuesens EB, Hoogenboezem T, Sluijter M, Hartwig NG, van Rossum AM, Vink C. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* by pyrosequencing. J Microbiol Methods 2010;82:214–22.
Thurman KA, Warner AK, Cowart KC, Benitez AJ, Winchell JM. Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. in clinical specimens using a single-tube multiplex real-time PCR assay. Diagn Microbiol Infect Dis 2011;70:1–9.
Torres A, Blasi F, Peetermans WE, Viegi G, Welte T. The aetiology and antibiotic management of community-acquired pneumonia in adults in Europe: a literature review. Eur J Clin Microbiol Infect Dis 2014;33:1065–79.
Touati A, Benard A, Ben Hassen A, Bebear CM, Pereyre S. Evaluation of five commercial real-time PCR assays for detection of *Mycoplasma pneumoniae* in respiratory tract specimens. J Clin Microbiol 2009;47:2269–71.
Ursi D, Dirven K, Loens K, Ieven M, Goossens H. Detection of *Mycoplasma pneumoniae* in respiratory samples by real-time PCR using an inhibition control. J Microbiol Methods 2003;55:149–53.
Waites KB, Xiao L, Paralanov V, Viscardi RM, Glass JI. Molecular methods for the detection of *Mycoplasma* and *Ureaplasma* infections in humans: a paper from the 2011 William Beaumont Hospital symposium on molecular pathology. J Mol Diagn 2012;14:437–50.
Winchell JM, Thurman KA, Mitchell SL, Thacker WL, Fields BS. Evaluation of three real-time PCR assays for detection of *Mycoplasma pneumoniae* in an outbreak investigation. J Clin Microbiol 2008;46:3116–8.
Xiao L, Ptacek T, Osborne JD, Crabb DM, Simmons WL, Lefkowitz EJ, et al. Comparative genome analysis of *Mycoplasma pneumoniae*. BMC Genomics 2015;16:610.
Zhao F, Cao B, He L, Yin Y, Tao X, Song S, et al. Evaluation of a new real-time PCR assay for detection of *Mycoplasma pneumoniae* in clinical specimens. Biomed Environ Sci 2012;25:77–81.

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