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NOTES

Comparison of Commercial and In-House Real-Time PCR Assays Used for Detection of *Mycoplasma pneumoniae*[▽]

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We tested two commercial and three in-house PCR assays under standardized conditions to detect *Mycoplasma pneumoniae*. All five procedures were able to demonstrate *M. pneumoniae* DNA in a concentration comparable to 1 CFU/μl, but the mean crossing points resulted in differences in the concentration of the genome copies of a factor of 20.

Recent studies have shown that the cell wall-less mollicute species *Mycoplasma pneumoniae* is the causative agent of about 5 to 20% of all cases of community-acquired pneumonia in humans (2). In closed populations like those in army bases or in periods showing epidemic peaks of *M. pneumoniae* infections (occurring every 3 to 7 years) (8, 14), the incidence of infections might increase up to 50% (2). The primarily respiratory tract infection can be followed by extrapulmonary complications (22). The specific antibiotic treatment of the disease, restricted to macrolides, tetracyclines, or fluoroquinolones, accounts for the search for sensitive, specific, and fast methods for the detection of *M. pneumoniae* infections in routine bacteriological practice. Cultivation is time consuming (up to 3 weeks), insensitive, and requires confirmation that the colonies grown are *M. pneumoniae* bacteria. Serological methods, such as the complement fixation test, enzyme-linked immunosorbent assay, and Western blot, are commonly used in the clinical laboratory but have practical limitations. The age- and time-dependent formation of specific immunoglobulin A and immunoglobulin M antibodies, the persistence of detectable antibody levels resulting from previous contacts with *M. pneumoniae*, and the variable specificity and sensitivity of the test kits used influence the significance of the results of serodiagnosis (2). Therefore, PCR approaches extended the spectrum of available routine methods. Recent results reported the superiority of PCR over serology for the confirmation of a *M. pneumoniae* infection in the clinically important first week after the onset of pneumonia symptoms (17).

In recent years, different PCR systems targeting a wide range of genes of *M. pneumoniae* (e.g., P1 adhesin, 16S rRNA, and ATPase operon gene) have been developed (16). Real-time PCR especially is characterized by rapidity, practicability, reduced risk of contamination, and high specificity and sensitivity of detection (3, 7, 9, 13, 15, 19, 23, 24, 25). However, the increasing number of real-time PCR approaches stresses the need for comparative validation of the performance of the different test procedures. Up to now, studies dealing with the evaluation of more than a single quantitative PCR system to detect *M. pneumoniae* have been very rare (7, 25) and have not included commercial test kits. To our knowledge, commercial quantitative PCR systems for the detection of *M. pneumoniae* are not available in the United States (2), whereas kits from different manufacturers are widely used in Europe. The aim of the present study was to investigate the sensitivities of different commercial and in-house real-time PCR approaches for the detection of *M. pneumoniae* under standardized conditions.

M. pneumoniae reference strains M129 (subtype 1; ATCC 29342) and FH (subtype 2; ATCC 15531) and patient isolates M3896 (subtype 3; kindly provided by S. Dégrange, Université Victor Segalen, Bordeaux, France), 4817 (variant 1), and ST (variant 2a) were propagated as described previously (5). For calculation of the standard curves, a freshly grown culture of *M. pneumoniae* M129 was harvested and sheared through a 27-gauge needle to reduce bacterial aggregates. Aliquots of the suspension were used to prepare the DNA and to calculate the CFU by spreading 10-fold phosphate-buffered saline dilutions in triplicate on PPLO agar (Becton Dickinson, Sparks, MD). *M. pneumoniae*-positive clinical samples were obtained from a collection of respiratory tract specimens (bronchoalveolar lavage fluids, nasopharyngeal or pharyngeal swabs, or sputa) of pneumonia patients in Germany and Switzerland. DNA from all *M. pneumoniae* culture and patient material samples (sample volume, 200 μl each) was extracted with a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer (protocol for blood and body fluids; elution volume, 150 to 200 μl). The DNA concentration was measured photometrically. DNA of patient samples was pretested by using the real-time PCR described recently in reference 7. *M. pneumoniae* bacteria in specimens confirmed as positive were subtyped by P1 sequencing according to the method of Dumke et al. (6). The DNAs of quantified dilutions of the *M. pneumoniae* M129 stock, of the different *M.*

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pneumoniae subtypes and variants, and of the *M. pneumoniae*-positive patient samples used for amplification with the different real-time PCR approaches were aliquoted and stored at −20°C until use. For each quantitative PCR test, freshly thawed DNA aliquots of the quantification standards, of the *M. pneumoniae* subtypes and variants, and of the patient materials were used in order to avoid an influence of DNA degradation on the crossing thresholds.

Five real-time PCR assays were selected for parallel testing of the *M. pneumoniae*-positive DNA samples. The three in-house real-time PCR systems were chosen according to the target used for amplification and the appropriate validation of the procedure in the literature. The RepMp1-based system (7) amplifies an 89-bp product within the 14 repetitive elements of RepMp1 scattered all over the genome of the sequenced *M. pneumoniae* strain M129 (20) and represents a multicopy target assay. The second approach targets a 76-bp part of the ATPase operon gene (MPN592) of *M. pneumoniae* (3), whereas the CARDS Tx assay (25) detected *M. pneumoniae* DNA by amplification of a 73-bp region located in the recently described community-acquired respiratory distress syndrome toxin gene (10). Primer and probes (Biomers, Ulm, Germany) were exactly as published. Probes were labeled with 6-carboxyfluorescein (5′) and 6-carboxytetramethylrhodamine (3′). The different real-time PCR assays were performed by using a LightCycler 1.5 instrument (Roche, Mannheim, Germany) with a final volume of 20.0 μl containing 4.6 μl water (PCR grade, Roche), 2.4 μl $MgCl_2$ (25 mM, Roche), 2.0 μl LightCycler FastStart DNA master HybProbe mix (Roche), 2.0 μl of each primer (5 pmol), 2.0 μl of probe (2 pmol), and 5.0 μl of DNA. The glass capillaries were incubated with the following cycling conditions: preincubation at 95°C (10 min), 45 cycles of denaturation at 95°C (10 s), hybridization at 53°C (RepMp1; 10 s) or 60°C (ATPase and CARDS Tx; 10 s), and elongation at 72°C (30 s). The reaction mixtures were cooled down to 40°C for 1 min. The commercial quantitative PCR assays selected for the study are the artus *M. pneumoniae* LC PCR kit (Qiagen), also targeting the different RepMp1 copies of *M. pneumoniae*, and the Venor Mp-QP *M. pneumoniae* diagnostic kit (Minerva Biolabs, Berlin, Germany), amplifying a part of the main P1 adhesin of *M. pneumoniae*. Both of the commercial quantitative PCR systems are expressly recommended for use with the LightCycler instrument. Performance of the commercial real-time PCR assays was carried out strictly according to the instructions of the manufacturer. This included the use of the recommended MB *Taq* DNA polymerase, which is not provided with the kit, in the Venor quantitative PCR. The data from all amplifications were analyzed with the LightCycler software, version 3.5 (Roche).

The in-house and commercial real-time PCR approaches were extensively tested with the DNA of related mycoplasma species and different agents commonly detected in patients with pneumonia and were found to be specific for the amplification of *M. pneumoniae*. According to the results in the published studies and the data in the handbooks of the manufacturers, the proven detection limits of the procedures investigated ranged from at least 0.2 copies or CFU per assay (RepMp1 and artus) to 1 to 5 CFU per assay (CARDS Tx) and 10 copies or CFU per assay (ATPase and Venor). The DNA samples (with one exception that was due to the limited volume of a single respiratory tract specimen) were tested in triplicate with each of the five real-time PCR approaches investigated. Parallel testing of all materials with the different procedures was carried out within three weeks to reduce the influence of long-term sample storage on the results.

Investigations of collections of patient isolates confirmed that *M. pneumoniae* is a very homogeneous species from a genetic point of view (5). However, since 1990 (21), two subtypes of *M. pneumoniae* showing sequence differences in the two copies of the repetitive elements RepMp4 and RepMp2/3 located in the main P1 adhesin have frequently been found in the human population. Furthermore, in recent years, three variants of *M. pneumoniae* showing sequence variations in one of these two repetitive elements were described (4, 6, 11). Variant 2a strains were reported with increasing numbers in Japan (12) and Europe (6), but only one isolate has been characterized as variant 1. Variant 2b isolates were detected in limited number with a molecular subtyping method in Europe only (6); therefore, DNA samples from cultures of this genotype could not be included in the present study. Recently, a new genetic composition of both repetitive elements in the P1 gene in a strain investigated in France led to the definition of a third subtype of *M. pneumoniae* (18).

Despite the distinct differences in the quantitative occurrence of the described genotypes, diagnostic real-time PCR procedures have to detect all known subtypes and variants of *M. pneumoniae*. Possible sequence variations in the target genes in the subtypes and variants are not reported but cannot be excluded. As indicated by the results summarized in Table 1, all real-time PCR approaches investigated demonstrated the occurrence of *M. pneumoniae*-specific DNA in bouillon cultures of the five available genotypes of *M. pneumoniae*. The standard curves (crossing thresholds versus log CFU) generated by six 10-fold dilutions of a quantified *M. pneumoniae* M129 stock (1 to 10^5 CFU/μl) yielded r^2 values of 0.999 (ATPase), 0.998 (RepMp1), 0.997 (CARDS Tx), 0.993 (Venor), and 0.977 (artus), respectively (data not shown). The PCR efficiencies ($10^{1/\text{slope}}$) varied between 1.9 (ATPase) and 2.3 (artus). All quantitative PCR procedures included in the study were able to detect at least one CFU per 1-μl sample. The results of testing the samples from the respiratory tract of patients with confirmed *M. pneumoniae* infection demonstrated that the five real-time PCR procedures investigated also recognized the occurrence of DNA of *M. pneumoniae* variant 2b strains, which are not available as culture material. The internal control amplification in both of the commercial assays confirmed that PCR inhibition in the tested samples can be excluded.

With regard to the crossing threshold values (arithmetic mean) of the 18 respiratory tract samples tested, the following order was calculated: 27.03 (RepMp1) > 27.30 (artus) > 28.92 (Venor) > 29.91 (ATPase) > 31.67 (CARD Tx). None of the real-time procedures amplifying a monocopy target (ATPase, CARD Tx, and Venor) yielded higher crossing points in any sample than the RepMp1-based systems. Statistically significant differences between the crossing threshold values of the RepMp1-based in-house assay and the RepMp1-based commercial assay (artus) could not be found, whereas the differences between the results of these multicopy targeting procedures and those of the three other assays are significant

TABLE 1. Results of testing different specimens with commercial and in-house real-time PCR assays for the detection of *M. pneumoniae*

Sample	Description	*M. pneumoniae* strain	Crossing threshold ± SD (n = 3) with indicated assay				
			Commercial		In-house		
			Venor	artus	RepMp1	CARDS Tx	ATPase
DNA extracts from bouillon cultures		M129 (subtype 1)	18.04 ± 0.12	13.73 ± 0.04	14.21 ± 0.47	18.83 ± 1.42	16.93 ± 0.03
		FH (subtype 2)	18.83 ± 0.22	16.71 ± 0.03	17.24 ± 0.54	21.44 ± 0.64	20.02 ± 0.09
		M3896 (subtype 3)	14.10 ± 0.36	10.70 ± 0.10	11.30 ± 0.17	15.35 ± 0.34	13.40 ± 0.09
		4817 (variant 1)	18.39 ± 0.38	15.32 ± 0.32	15.41 ± 0.17	20.51 ± 0.53	17.75 ± 0.05
		ST (variant 2a)	17.17 ± 0.18	14.53 ± 0.06	14.63 ± 0.12	19.97 ± 0.75	17.41 ± 0.11
	1 CFU/μl (5.3 fg DNA/μl)	M129 (subtype 1)	33.71 ± 0.17	31.59 ± 0.11	32.01 ± 0.11	36.85 ± 0.32	35.39 ± 0.10
	10 CFU/μl (52.6 fg DNA/μl)		31.48 ± 0.31	28.65 ± 0.06	29.07 ± 0.10	34.01 ± 0.08	31.99 ± 0.05
	100 CFU/μl (525.9 fg DNA/μl)		27.65 ± 1.10	25.41 ± 0.20	25.77 ± 0.01	30.88 ± 0.18	28.58 ± 0.06
	1,000 CFU/μl (5.3 pg DNA/μl)		25.24 ± 0.35	22.02 ± 0.01	22.20 ± 0.06	27.08 ± 0.02	25.21 ± 0.42
	10,000 CFU/μl (52.6 pg DNA/μl)		21.75 ± 0.09	18.32 ± 0.24	18.29 ± 0.01	23.21 ± 0.08	21.14 ± 0.10
	100,000 CFU/μl (525.9 pg DNA/μl)		17.74 ± 0.50	14.76 ± 0.16	15.01 ± 0.13	19.60 ± 0.04	20.97 ± 0.45
DNA extracts of respiratory tract samples of *M. pneumoniae*-positive pneumonia patients[a]	CAP 1	Subtype 1	20.41 ± 0.29	19.78 ± 0.38	19.30 ± 0.04	24.40 ± 0.09	22.24 ± 0.05
	CAP 2	Subtype 1	28.10 ± 0.04	27.42 ± 0.32	26.77 ± 0.03	31.80 ± 0.26	29.70 ± 0.06
	CAP 3	Subtype 1	25.44 ± 0.15	23.52 ± 0.41	22.82 ± 0.07	27.74 ± 0.09	25.77 ± 0.05
	CAP 4	Subtype 1	25.59 ± 0.10	24.23 ± 0.13	24.04 ± 0.18	28.62 ± 0.02	26.92 ± 0.11
	CAP 5	Subtype 1	31.48 ± 0.37[b]	27.14 ± 0.03	26.76 ± 0.04	31.59 ± 0.08	29.89 ± 0.01
	CAP 6	Subtype 1	27.25 ± 0.09	25.76 ± 0.24	25.62 ± 0.09	30.22 ± 0.07	28.54 ± 0.12
	CAP 7	Subtype 1	32.13 ± 0.14	31.43 ± 0.23	30.96 ± 0.17	35.94 ± 0.17	34.68 ± 0.57
	CAP 8	Subtype 2	24.87 ± 0.04	22.42 ± 0.40	22.42 ± 0.16	27.80 ± 0.17	25.60 ± 0.10
	CAP 9	Subtype 2	30.72 ± 0.41	26.51 ± 0.49	26.03 ± 0.24	30.90 ± 0.25	28.88 ± 0.27
	CAP 10	Subtype 2	32.64 ± 0.33	30.95 ± 0.16	30.63 ± 0.03	35.27 ± 0.09	33.90 ± 0.04
	CAP 11	Subtype 2	35.04 ± 0.11	33.37 ± 0.14	32.88 ± 0.06	36.95 ± 0.14	37.05 ± 0.84
	CAP 12	Subtype 2	36.27 ± 0.03	34.50 ± 0.23	33.48 ± 0.12	37.03 ± 0.13	37.27 ± 0.21
	CAP 13	Subtype 2	30.37 ± 0.25	28.97 ± 0.16	28.99 ± 0.09	33.18 ± 0.14	31.48 ± 0.04
	CAP 14	Variant 2a	31.83 ± 0.22	31.12 ± 0.12	31.51 ± 0.03	35.49 ± 0.30	33.60 ± 0.17
	CAP 15	Variant 2a	27.34 ± 0.17	25.25 ± 0.08	25.27 ± 0.12	30.09 ± 0.07	28.05 ± 0.05
	CAP 16	Variant 2a	22.89 ± 0.34	21.75 ± 0.18	21.20 ± 0.07	25.82 ± 0.19	24.08 ± 0.15
	CAP 17	Variant 2b	30.08 ± 0.10	29.19 ± 0.09	29.72 ± 0.03	34.39 ± 0.16	32.23 ± 0.12
	CAP 18	Variant 2b	29.04 ± 0.05	28.07 ± 0.33	28.21 ± 0.08	32.86 ± 0.25	30.96 ± 0.12

[a] *M. pneumoniae* DNA from samples was subtyped according to the method of Dumke et al. (6). CAP, community-acquired pneumonia.
[b] Mean of results for two replicate samples (sample volume was insufficient for three).

(Student's t test; $P < 0.05$). It should be noted that with the exception of the artus PCR system (10 μl), all procedures tested used a sample volume of 5 μl DNA. With regard to the fact that the decrease of the crossing thresholds by 3.3 resulted in an increase of the number of genome copies by a log unit, the reported differences in the mean crossing points of the tested procedures (up to 4.6) cause differences in the calculated mean concentrations of genome equivalents by a factor of up to 20. The results are in agreement with the data of a recent study comparing the RepMp1-based in-house system with a monocopy assay targeting a part of the P1 gene (7). In a previous study, the CARD Tx-based procedure resulted in significantly lower mean crossing points than two ATPase-based systems (not used in the present investigation) after analysis of *M. pneumoniae*-positive respiratory samples (25). In contrast, the CARD Tx approach showed the highest mean crossing thresholds of all assays.

This study minimized sources of variation evident in multicenter investigations in several ways. Unfavorable storage and transportation conditions of the samples, slight variations in the LightCycler instruments used, and different interpersonal sample handling can affect the comparability of the results of real-time procedures (1). These influences are reduced by the standardized performance of the assays tested in the present study. Furthermore, within the test systems investigated, the coefficients of variation of the mean crossing points of the three quantified dilutions of *M. pneumoniae* DNA used to calculate the concentration of the genome copies in the five independent runs ($n = 15$) ranged between 0.7 and 3.3% (data not shown), confirming a high level of intralaboratory reproducibility in all assays tested. These facts allow a direct comparison of the crossing thresholds. The results of real-time PCR procedures used to detect *M. pneumoniae* revealed significant differences in the sensitivities of the test systems. Nevertheless, all assays investigated were able to detect the known genotypes of *M. pneumoniae* bacteria with a sensitivity of at least one CFU per 1-μl sample and can be recommended for the diagnostic laboratory. Further studies are needed to prove if the higher sensitivity of the procedures amplifying a multicopy target (RepMp1; artus) is of any diagnostic relevance.

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