Evaluation of Five Commercial Real-Time PCR Assays for Detection of *Mycoplasma pneumoniae* in Respiratory Tract Specimens[∇]

A. Touati,[1] A. Benard,[2] A. Ben Hassen,[1] C. M. Bébéar,[3]* and S. Pereyre[3]

Service des Laboratoires, Centre National de Greffe de Moelle Osseuse, Tunis, Tunisia[1]; Unité de Soutien Méthodologique à la Recherche Clinique et Epidémiologique, INSERM U897, CIC-EC 7, Université Victor Segalen Bordeaux 2 and CHU de Bordeaux, Bordeaux, France[2]; and Laboratoire de Bactériologie EA 3671, Université Victor Segalen Bordeaux 2 and CHU de Bordeaux, Bordeaux, France[3]

The performances of five commercial TaqMan real-time PCR assays for the detection of *Mycoplasma pneumoniae* in respiratory tract specimens were evaluated in comparison with an in-house real-time PCR. All kits allowed prompt and specific results, validated by the use of an internal control. The Nanogen kit showed the best clinical sensitivity.

Mycoplasma pneumoniae is a frequent human respiratory tract pathogen that causes 15 to 20% of community-acquired pneumonia cases in children and adults (2, 16). Since the symptoms are nonspecific, rapid and reliable laboratory diagnosis is necessary. The frequently used serological methods often allow a retrospective diagnosis only (4). Moreover, it was recently shown that PCR is superior to serology for diagnosis of *M. pneumoniae* during the early phase of infection (11). In addition, due to the fastidious nature of the organism, direct detection of *M. pneumoniae* by culture is difficult and time-consuming, and the method lacks sufficient sensitivity (13). Consequently, PCR has been increasingly used for *M. pneumoniae* detection. Several genes have been targeted for conventional amplification, including the 16S rRNA, ATPase, and P1 cytadhesin genes and repetitive elements located within the last group (5, 8, 9, 15). Moreover, several reports described real-time protocols using TaqMan probes (3, 7, 12–14, 18). In-house methods usually show excellent performance (6) but can be difficult to transfer successfully to other laboratories due to variability in suppliers, thermocyclers, nucleic acid extraction methods, or technician skills (10). Commercial kits represent an alternative that can guarantee reproducibility of results. Moreover, they are a way to provide reliable diagnostic tools to laboratories that cannot develop in-house PCR. Recently, several commercially available real-time PCR kits for the detection of *M. pneumoniae* have been developed, but only two have been investigated using a limited number of clinical specimens (6).

The aim of this study was to evaluate the performances of five commercialized TaqMan real-time PCR assays for the detection of *M. pneumoniae* in respiratory samples in comparison with our in-house real-time PCR.

Seventy nonredundant clinical specimens, collected between January 2007 and March 2008, were retrospectively selected from the Department of Bacteriology, University Hospital of Bordeaux (France), including 18 throat swab specimens, 8 nasopharyngeal aspirate specimens, 11 sputum specimens, 6 tracheal aspirate specimens, 4 bronchial aspirate specimens, 22 bronchoalveolar lavage specimens, and 1 pleural fluid specimen. Among them, specimens from 50 patients with a diagnosis of *M. pneumoniae* infection were systematically collected during this period, including 44 specimens *M. pneumoniae* positive by a former in-house PCR and/or by culture (17). The six other specimens were negative by PCR and culture but were obtained from patients with a high anti-*M. pneumoniae* immunoglobulin M titer in a serum sample collected at the same time. The last 20 specimens, negative for *M. pneumoniae* by culture and PCR but positive for other pathogens involved in respiratory tract infections (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia marcescens*), were obtained from patients without *M. pneumoniae*-specific antibodies. The Qiagen QIAamp DNA minikit (Qiagen SA, Courtaboeuf, France) was used for nucleic acid extraction from 200 µl of respiratory specimen according to the body fluid extraction protocol in the manufacturer's instructions. The elution volume was reduced to 100 µl, and 7-µl aliquots were stored at −80°C. To determine the detection limit of each real-time PCR assay, DNA was extracted from a pure culture of the reference strain *M. pneumoniae* M129 (ATCC 29342), using the Qiagen QIAamp DNA minikit, and its concentration was measured photometrically. For each kit, a 10-fold serial dilution was prepared from one DNA aliquot and amplifications were performed in duplicate with each dilution. Since a limited number of commercial tests were available for this study due to cost considerations, the analytical specificity was assessed on the 20 specimens that were *M. pneumoniae* negative but positive for other respiratory tract pathogens (see above).

Each nucleic acid extract from the 70 respiratory specimens was tested with our in-house real-time PCR assay. This method, targeting a 125-bp fragment of the *M. pneumoniae* P1 cytadhesin gene, was performed using the primers P1-204 (5′-GTGAACGTATCGTAACACGAGCTTT-3′), P1-328 (5′-TGGTTTGTTGACTGCCACTGC-3′), the TaqMan probe P1-284R (5′-6-carboxyfluorescein-TTGTCGCGCACTAAGGCCCACG-BHQ1-3′), and

* Corresponding author. Mailing address: Laboratoire de Bactériologie EA 3671, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux cedex, France. Phone: (33) 5 57 57 16 25. Fax: (33) 5 56 93 29 40. E-mail: cecile.bebear@u-bordeaux2.fr.

TABLE 1. Clinical sensitivities and mean C_T values of five evaluated commercial kits in comparison to results of in-house real-time PCR for detection of *M. pneumoniae* in 42 confirmed-positive respiratory tract specimens

Real-time PCR assay	No. of positive specimens[a]	Sensitivity (%)	95% CI[b]	P value[c]	Mean $C_T \pm$ SD	95% CI	P value[d]
In-house	42/42	100.0	[91.6–100.0]		31.8 ± 5.7	[30.0–33.5]	
Nanogen kit	41/42	97.6	[87.4–99.9]	0.32	31.6 ± 6.3	[29.7–33.6]	0.61
Simplexa kit	37/42	88.1	[74.4–96.0]	0.025	34.2 ± 6.3	[32.2–36.1]	<0.001
Diagenode kit	36/42	85.7	[71.5–94.6]	0.014	35.7 ± 6	[33.9–37.6]	<0.001
Cepheid kit	35/42	83.3	[68.6–93.0]	0.008	34.9 ± 7.7	[32.5–37.3]	<0.001
Venor kit	26/42	61.9	[45.6–76.4]	<0.001	36.0 ± 8.7	[33.3–38.7]	<0.001

[a] No. positive/no. tested.
[b] CI, confidence interval.
[c] Determined by McNemar's test.
[d] Determined by paired t test.

a commercial internal control (IC), the Universal Inhibition Control real-time PCR (Diagenode, Liège, Belgium). The PCR mixture consisted of 12.5 μl of 2× LightCycler 480 probe master mix (Roche Diagnostics, Meylan, France), 0.3 μM of each primer, 0.2 μM of probe, 1.25 μl of IC DNA, 2.5 μl of IC primers/probe, and 5 μl of template DNA. Amplifications were performed using a LightCycler 480 thermocycler under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Five commercially available PCR kits, based on the TaqMan PCR technology, all targeting the P1 cytadhesin gene and including an internal control, were evaluated: the Nanogen Mycoplasma pn Q-PCR Alert kit (Nanogen Advanced Diagnostics; purchased from Eurobio, Les Ulis, France); the Simplexa *Mycoplasma pneumoniae* kit (Focus Diagnostics, California, purchased from Eurobio, Les Ulis, France); the Diagenode detection kit for *Mycoplasma pneumoniae/Chlamydophila pneumoniae* (Diagenode SA, Liège, Belgium); the Cepheid *Mycoplasma pneumoniae* ASR kit (Cepheid; purchased from Instrumentation Laboratory, Paris, France), and the Venor Mp-Qp PCR detection kit (Minerva Biolabs GmbH; purchased from Biovalley, Marne La Vallée, France) used with the hot-start MB *Taq* DNA polymerase (Minerva Biolabs GmbH), not included in the kit.

Amplifications were performed with a LightCycler 480 instrument except for the Cepheid kit, which was evaluated using a SmartCycler II thermocycler (Cepheid) because of a fluorophore incompatibility. PCR mixtures of 20 μl were prepared according to each manufacturer's instructions, and 5-μl sample DNAs were added. Data were analyzed with the Roche LightCycler 480 software, version 1.2.9.11, using the absolute quantification-fit point method of analysis or with the SmartCycler system software. A respiratory tract sample was definitely considered positive for *M. pneumoniae* if at least two out of the five commercial or in-house assays produced a positive result. Unexpected negative results were confirmed by reamplification of the specimen DNA extract with the concerned assay. Cycle threshold (C_T) values of specimens were also collected for each assay. Mean and standard deviation were calculated by attributing an arbitrary C_T value of 45 if a specimen was found negative with a kit. The value of 45 was chosen since it corresponds to the maximum number of amplification cycles used by all assays. Comparisons were made using McNemar's test and a paired *t* test for qualitative and quantitative variables, respectively. Statistical analysis used the SAS software program, version 9.1 (SAS Institute Inc., Cary, NC). A *P* value of <0.05 was considered statistically significant.

The in-house assay and Nanogen and Cepheid kits showed the highest analytical sensitivity on *M. pneumoniae* DNA extracts, with a detection limit of 4.3×10^{-7} ng DNA/μl, whereas this limit was 2.4×10^{-6} ng DNA/μl for the Venor assay and 4.3×10^{-6} ng DNA/μl for both the Diagenode and Simplexa kits. These results were consistent with the sensitivity previously reported for the Venor kit (6), with a detection limit below 5.3×10^{-6} ng DNA/μl. The analytical specificity was 100% for all the assays since no amplification was obtained from negative specimens.

Of the 70 evaluated specimens, 42 specimens were included in the analysis as definitely positive ones. No specimen was found positive by a single assay only, and no specimen showed amplification inhibition. The best clinical sensitivities with patient specimens were found with the in-house and Nanogen assays, with 42 (100%) and 41 (98%) specimens identified as positive, respectively (Table 1). The Simplexa kit identified 88% of the confirmed-positive specimens, whereas 86%, 83%, and 62% were identified by the Diagenode, Cepheid, and Venor kits, respectively. There was no statistically significant difference between the sensitivity of the in-house method and that of the Nanogen kit ($P = 0.32$), but the remaining four kits detected statistically significantly fewer positive specimens ($P < 0.05$) than the in-house assay.

When C_T values were considered, the Nanogen kit exhibited the lowest C_T mean (Table 1), without a significant difference from the C_T mean of the in-house assay ($P = 0.61$). In contrast, the remaining four kits had statistically significantly higher C_T values ($P < 0.001$) than the in-house assay, with a difference in the C_T mean ranging from 2.4 cycles for the Simplexa kit to 4.2 cycles for the Venor kit. Interestingly, as previously described for another atypical bacterium (1), the analytical sensitivity of a kit did not predict its ability to detect the bacterial target in clinical specimens. Indeed, the lower analytical sensitivities of both the Diagenode and Simplexa assays were compensated by their overall efficiency in *M. pneumoniae* detection with clinical specimens. They had better sensitivity with clinical specimens (86% and 88%, respectively) than the Cepheid kit (83%), which had a 10-fold-better analytical sensitivity.

Although assay sensitivity and specificity are important considerations when evaluating the reliability of a kit, additional criteria, such as applicability, ease of use, handling time, and cost, should not be disregarded. Results with the LightCycler

TABLE 2. Costs and handling and testing times of evaluated real-time PCR commercial kits and in-house assay

Real-time PCR assay	No. of reagents per reaction mixture[a]	No. of tests per series	Thermal cycling time	Range of cost per sample[b] excl. tax. (euros)
In-house	7	96	1 h, 49 min	2–4
Nanogen kit	4	96	1 h, 40 min (+ color compensation file)[c]	12–14 (+6 euros/series for PC)
Simplexa kit	4	96	1 h, 14 min	10–12
Diagenode kit	5	96	1 h, 32 min	10–12
Cepheid kit	3	16 (independent)	50 min	22–24
Venor kit	6	96	1 h, 3 min	8–10

[a] The sample adding step was not counted.
[b] The range of cost includes reagent price required to conduct the amplification with the internal control. Consumables and DNA extraction reagents were not counted. excl. tax., excluding taxes; PC, positive control.
[c] A color compensation file, generated in an additional run, is required every 6 months.

480 instrument were available within a mean of 1 h, 28 min, ranging from 1 h, 3 min for the Venor kit to 1 h, 49 min for the in-house assay (Table 2). For the Cepheid kit, data were obtained in only 50 min. In addition, this kit showed the simplest and shortest workflow sequence, since two lyophilized beads, stored at 4°C, had only to be dissolved in water to prepare the mix. In contrast, the remaining tests needed four to seven handling steps. With the 16 independently programmable units of the Smart Cycler apparatus, the Cepheid kit allowed a very short time to result and proved to be more suitable for a single emergency than for routine series detection. Finally, the cost of each real-time assay was evaluated based on European available price lists of reagents. The Cepheid kit emerged as two-fold more expensive than the other kits. The in-house method was the cheapest one, with an estimated cost close to 2 euros per reaction. Concerning the Diagenode kit, it should be noted that the 10- to 12-euro price also included detection of *C. pneumoniae*, which was not evaluated in this study.

In conclusion, all kits allowed prompt and specific results, validated by the use of an internal amplification control. In comparison with the in-house assay, the Nanogen kit was shown to be the best commercially available kit evaluated in this study in terms of analytical sensitivity and performance with clinical specimens.

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