

Utility of Real-Time PCR for Diagnosis of Legionnaires' Disease in Routine Clinical Practice[†]

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The main aim of our study was to determine the added value of PCR for the diagnosis of Legionnaires' disease (LD) in routine clinical practice. The specimens were samples submitted for routine diagnosis of pneumonia from December 2002 to November 2005. Patients were evaluated if, in addition to PCR, the results of at least one of the following diagnostic tests were available: (i) culture for *Legionella* spp. on buffered charcoal yeast extract agar or (ii) detection of *Legionella pneumophila* antigen in urine specimens. Of the 151 evaluated patients, 37 (25%) fulfilled the European Working Group on Legionella Infections criteria for a confirmed case of LD (the "gold standard"). An estimated sensitivity, specificity, and overall percent agreement of 86% (32 of 37; 95% confidence interval [CI] = 72 to 95%), 95% (107 of 112; 95% CI = 90 to 98%), and 93% (139 of 149), respectively, were found for 16S rRNA-based PCR, and corresponding values of 92% (34 of 37; 95% CI = 78 to 98%), 98% (110 of 112; 95% CI = 93 to 100%), and 97% (144 of 149), respectively, were found for the *mip* gene-based PCR. A total of 35 patients were diagnosed by using the urinary antigen test, and 34 were diagnosed by the 16S rRNA-based PCR. With the *mip* gene PCR one more case of LD ($n = 36$; not significant) was detected. By combining urinary antigen test and the *mip* gene PCR, LD was diagnosed in an additional 4 (11%) patients versus the use of the urinary antigen test alone. The addition of a *L. pneumophila*-specific *mip* gene PCR to a urinary antigen test is useful in patients with suspected LD who produce sputum and might allow the early detection of a significant number of additional patients.

Since the initial description of Legionnaires' disease (LD) in 1976, *Legionella pneumophila* has been increasingly recognized as a pathogen causing both community-acquired and nosocomial pneumonia (9, 10, 31). *Legionella* spp. are responsible for 1 to 5% of cases of community-acquired pneumonia (CAP) (31, 35, 39). Infection of humans usually occurs via inhalation of aerosols from a variety of manufactured water systems. Although currently more than 50 species of *Legionella* (9, 10) are recognized, only 19 *Legionella* species have been documented as human pathogens on the basis of their isolation from clinical material. Although potentially all *Legionella* spp. may cause human disease, the majority (92%) of clinical cases are caused by *L. pneumophila* (39). Microbiological diagnosis is warranted, since LD is clinically not distinguishable from other pneumonias. Timely and appropriate treatment improves the prognosis and can be achieved by rapid diagnosis (15).

Legionellae are slow-growing fastidious bacteria, and successful culture requires selective media and prolonged incubation periods. Culture on buffered-charcoal yeast extract (BCYE) plates is the gold standard for the laboratory diagnosis of LD. Considerable interlaboratory variation has been documented for the ability to culture legionellae (4, 9). Serological diagnosis is also commonly used, and a sensitivity of 41 to 91% has been reported (4, 10). Unfortunately, a diagnosis

by a fourfold immunoglobulin G or immunoglobulin M titer increase can only be made retrospectively and rarely influences the initial treatment of the patient (2, 10, 13). Therefore, there is a need for additional tests to diagnose LD in the early stages of disease.

The urinary antigen test and nucleic acid amplification tests (NAAT), most often PCR, are useful for this purpose. Urinary antigen testing is now an established and valuable rapid tool for the diagnosis of LD, particularly in regions where *L. pneumophila* serogroup 1 is the most common cause of the disease. These tests provide results within 30 min and have sensitivities of between 60 and 100% for patients with LD due to *L. pneumophila* serogroup 1 (7, 8, 40). However, because these assays only detect a limited number of serogroups of *L. pneumophila*, some authors suggest that total dependence on this diagnostic assay may miss up to 40% of cases of LD (10).

NAATs are attractive tools for the detection of legionellae in clinical samples, since they detect all *Legionella* spp. and provide rapid results. Most diagnostic PCR assays have specific target regions within the 16S rRNA genes (1, 18, 28, 30, 32, 36, 37), the 23S-5S spacer region (16), 5S rRNA gene (14), or the macrophage inhibitor potentiator gene (*mip*) (1, 19, 20, 21, 26, 29, 30, 37, 38). Thus far, results obtained from in vitro evaluations and small patient series have been encouraging. When samples from the lower respiratory tract are tested, PCR has repeatedly been shown to have a sensitivity equal to or greater than culture (3, 30, 32, 33). However, false-positive results have been reported (3), and the potential of false-positive results may have hindered a more widespread application of PCR. A problem with the interpretation of these so-called false-posi-

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tive results is the question as to whether these are truly false positive or whether the reference method failed, e.g., because less-common legionellae are not as easily detected by conventional methods (25). It is difficult to solve this issue and, at present, there are no well-designed studies available that have determined the exact sensitivity and specificity of *Legionella* PCR in patients with pneumonia of unknown etiology.

At the Regional Public Health Laboratory, located at the St. Elisabeth Hospital, Tilburg, The Netherlands, we have used a repertoire of conventional diagnostic tests (serology, culture, and urine antigen testing), as well as *Legionella*-specific PCR (targeted at the 16S rRNA gene and the *mip* gene), for several years. The present study compares the results obtained with PCR to those obtained by conventional testing of samples from patients suspected of having LD. The two main objectives of our study were (i) to determine the performance of PCR compared to that of conventional diagnostic tests in terms of sensitivity and specificity and (ii) to evaluate the additional value of a *Legionella*-specific PCR for the diagnosis of LD compared to the urinary antigen test.

MATERIALS AND METHODS

Patients. All data were collected retrospectively from the laboratory information system of the Regional Public Health Laboratory, located at the St. Elisabeth Hospital, Tilburg, The Netherlands. The conventional diagnostic testing (urinary antigen test and culture), PCR (16S rRNA- and *mip* gene-based), and the DNA extraction procedures were performed with identical protocols on all patient samples during this time period. The following lower respiratory samples were included: sputa, endotracheal aspirates, lung biopsy samples, and bronchoscopic specimens. PCR results obtained from throat swabs, pleural fluid, or serum samples were not included for the present analysis. The patients were all hospitalized, had symptoms compatible with pneumonia, and showed radiological signs of infiltration. Specimens consisted of samples that were submitted for routine diagnosis of pneumonia from December 2002 to November 2005, which included PCR analysis of lower respiratory tract samples for the detection of *Legionella* spp. Samples were included from patients for whom, in addition to PCR, the results of at least one of the following diagnostic tests for *Legionella* infection were available: (i) culture for *Legionella* spp. on BCYE agar supplemented with α -ketoglutarate or (ii) detection of *L. pneumophila* antigen in urine specimens. Only the first available sample from a particular patient was included, and all other additional results (if available) were excluded from the analysis. All cases of suspected LD in the present study were sporadic and not part of an outbreak.

A case of confirmed *Legionella* pneumonia (the gold standard) was defined according to the European Working Group on Legionella Infections (EWGLI) criteria. An LD-positive patient was defined as a patient with laboratory evidence for LD and included at least one of the following criteria: (i) Isolation of *Legionella* spp. from a respiratory sample and/or (ii) the presence of *L. pneumophila* antigens in urine specimens. The LD-negative population was defined as including patients that had no laboratory evidence for LD in culture and/or urinary antigen testing.

Culture. Each specimen was divided into two aliquots; one was sent to the molecular biology laboratory for PCR, and the other was applied to BCYE agar and BCYE agar with polymyxin B, anisomycin, and vancomycin (Oxoid, Haarlem, The Netherlands). Lower-respiratory-tract specimens were plated undiluted. Specimens were processed upon receipt. The plates were incubated at 35°C with 70 to 80% relative humidity. All cultures were examined daily for 7 days before the final reporting of results. Visible colonies were subcultured onto a BCYE agar plate and a blood agar plate with 5% sheep blood (Oxoid, Haarlem, The Netherlands). Bacteria that grew on BCYE agar but not on a blood agar plate were considered possible *Legionella* spp. and were tested with a slide agglutination test (Dryspot *Legionella* Latex test; Oxoid, Haarlem, The Netherlands). The latex test allows separate identification of *L. pneumophila* serogroup 1 and serogroups 2 to 14 and detection of seven other non-*pneumophila* *Legionella* spp. (*L. longbeachae* 1 and 2, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanii*, *L. micdadei*, and *L. anisa*). If the agglutination with the isolate was negative, the organism was sent to the molecular laboratory for further identification.

Urinary antigen detection. The presence of *L. pneumophila* antigens in unconcentrated urine specimens was determined with a qualitative immunochromatographic assay (Binax NOW; Binax, USA).

Extraction of DNA from respiratory samples. (i) **Sample preparation.** Prior to DNA isolation, all respiratory samples were processed with MagNALyser (Roche Diagnostics) according to the manufacturer's instructions.

(ii) **DNA extraction.** After treatment with MagNALyser, 200 μ l of sample was processed in the MagNA Pure LC isolation robot by using a Total Nucleic Acid isolation kit (Roche Diagnostics) with an elution volume of 50 μ l. Then, 5 μ l of the eluate was used as a template in PCR. The remainder of the eluate was stored at -20°C; the remainder of the original sample was stored at -70°C.

(iii) **Control material.** As an internal control, Phocid herpesvirus was added to the samples to monitor processing, DNA extraction, and inhibition of PCR (27). Detection of Phocid herpesvirus was included in the *mip* gene PCR. In each run a no-template control (mix control) was included. Sensitivity controls in PCR consisted of 10-fold dilutions of *L. pneumophila* DNA ranging from 1,000 to 10 fg (approximately 230 to 2.3 genome equivalents). A total of 1,000 fg of *L. bozemanii* DNA served as a control in the 16S rRNA-based PCR for discrimination between *L. pneumophila* and non-*pneumophila* *Legionella* spp.

Real-time PCR. Between December 2002 and November 2005, two *Legionella*-specific PCR assays were used, targeted at specific regions within the 16S rRNA gene and the *mip* gene, as described previously (6). In the dual-color two-probe 16S rRNA-based PCR assay the primers *Leg1* (forward, 5'-TACCTACCTTG ACATACAGTG-3') and *Leg2* (reverse, 5'-CTTCTCCGGTTTGTCAC-3') were used to obtain a 200-bp amplicon. Real-time detection was done with a *Legionella*-specific fluorescent probe (LSPP, VIC-5'-GGTTGCGTCGTTACG-3') conjugated to a minor groove binder. An *L. pneumophila*-specific fluorescent minor groove binder probe (LPN, FAM-5'-GAGTCCCCACATCACATG-3') was used on the cDNA strand. In the *L. pneumophila*-specific PCR based on the sequences of the *mip* gene, the primers *Mip-F1* (forward, 5'-GCCAAGTGGT TTGCAATACAAA-3') and *Mip-R1* (reverse, 5'-CTCGACAGTGACTGTATC CGATTT-3') were used to obtain an 80-bp amplicon. Real-time detection was done with a TaqMan probe, *Lpn-mip* (FAM-5'-TAATCAATGCTGGAAATG GTGTTAAACCCG-3'-TAMRA). When inhibition of the PCR was observed, the DNA extraction and PCR were repeated. If inhibitors of the PCRs could not be removed, the result was reported as inhibited. Primers and probes were synthesized by Applied Biosystems (ABI, Nieuwerkerk a/d IJssel, The Netherlands). The TaqMan Universal Master Mix (ABI) was used in all PCR assays.

PCR conditions. Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). DNA was amplified according to the following parameters: after 2 min of incubation at 50°C and 10 min of denaturation at 95°C, amplification consisted of 50 cycles of 15 s of denaturation at 95°C, followed by 1 min of annealing and extension at 60°C.

Interpretation of PCR results. The results of the real-time PCR were expressed as threshold cycle (C_T) values corresponding to the cycle at which PCR enters the exponential phase. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative. The results obtained with conventional testing for *Legionella* spp. were compared to those found in real-time PCR. Two PCR results were available: (i) the result of the 16S rRNA-based PCR assay and (2) the result of the *mip*-based PCR assay. The dual-color two probe 16S rRNA-based PCR assay is positive for *L. pneumophila* if double amplification curves are obtained (both probes react). When non-*pneumophila* *Legionella* spp. are amplified, only one amplification curve is present since the *L. pneumophila*-specific probe cannot bind. A positive result in the *mip* gene PCR is considered positive for *L. pneumophila*. All positive results in *mip* and/or 16S rRNA PCR were confirmed in a second PCR run (both 16S rRNA- and *mip* gene-based PCRs were retested) after a second DNA isolation of the respiratory sample. If a positive PCR could not be confirmed, it was assumed to be negative for *Legionella* spp.

The PCR results were interpreted as follows: (i) "negative," both the 16S rRNA-based PCR assay and the *mip* gene-based assay tested negative; "positive for *L. pneumophila*," a positive result in the *mip* gene- and/or 16S rRNA-based PCRs (both probes react) after confirmation in a second PCR run after a second DNA isolation of the respiratory sample; and (iii) "positive for non-*pneumophila* *Legionella* spp.," a negative result in the *mip* gene-based PCR and a positive result in the 16S rRNA-based PCR (*Legionella* genus probe reacts) after confirmation in a second PCR run after a second DNA isolation of the respiratory sample.

Discrepant analysis. Discrepant resolution focuses on specimens for which the new test and the gold standard gave different results. Discrepant resolution in the present study is described as a two-stage testing process: stage 1 was to test all specimens using the new test and the gold standard and, for stage 2, in case of a

TABLE 1. Positive diagnostic test results for 37 confirmed cases of LD according to EWGLI criteria (the gold standard)

Test	No. of positive results/no. of cases ^a evaluated as determined by:		
	Culture	Urinary antigen test	PCR
Culture	21/35	19/31	19/30
Urinary antigen	19/21	35/37	32/34
PCR	17/19	32/35	34/37

^a The table is read in columns as follows: 21 patients had a positive culture out of a total of 35 confirmed LD patients tested; of these 21 culture-positive patients, 19 were positive by urinary antigen out of 21 tested, 17 were positive by PCR out of 19 tested.

PCR-positive but gold standard-negative sample, we used additional molecular tests to determine which one was "correct." A maximum of four additional tests, depending on the amount of sample available, were performed to determine whether the positive PCR test results could be confirmed. The following two approaches were taken.

(i) **Approach 1: use of a different PCR for confirmation.** For the confirmation of *Legionella* positivity in respiratory samples, various PCR assays were used, including a *Legionella* spp. assay targeted at specific regions within the 5S rRNA gene (6) and two different assays targeted at the *mip* and the 16S rRNA gene, performed at a different laboratory (33).

(ii) **Approach 2: confirmation of 16S rRNA PCR-positive discrepant results by sequencing.** Identification of discordant non-*pneumophila* *Legionella* spp. PCR-positive results was attempted by PCR reamplification and sequence analysis of the 200-bp 16S rRNA gene fragment. The primers used for sequencing analysis of DNA samples with discrepant results were the same as those outlined above: *Leg1* and *Leg2*. Sequence analysis of the 16S rRNA gene fragment was performed at Base Clear, Leiden, The Netherlands. The sequences obtained were compared to those submitted to the GenBank database by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

A result was considered confirmed (true positive) if it was reproducible in approach 1 or approach 2.

Statistical analysis. The clinical sensitivity and specificity of the assays were determined by using two-by-two contingency tables. Diagnostic sensitivity was defined as the fraction of the patients correctly identified by the PCR as having LD compared to determination by the gold standard. Diagnostic specificity was defined as the fraction of the patients correctly identified in PCR as not having LD compared to determination by the gold standard. The overall percent agreement represents the proportion of samples similarly classified by the gold standard and PCR. Samples that were inhibited in PCR were not included in the calculations. Confidence intervals (CIs) were determined by using the adjusted Wald method.

RESULTS

Patient characteristics. Samples were obtained between December 2002 and November 2005. A total of 151 patients fulfilled the criteria outlined in Materials and Methods were available for evaluation in the present study. Of the 151 included patients, 37 (25%) fulfilled the EWGLI criteria for a confirmed case of LD. Of the included patients, 92 (61%) were men, with a mean age of 57 years (range, 23 to 85 years) and 59 were women with a mean age of 60 years (range, 25 to 85 years). The median age of the 37 confirmed LD patients was 54 years (range, 23 to 74 years), and the male/female ratio was 2.1:1 (25 men, 12 women). The median age of non-LD patients was 59 years (range, 24 to 85 years), and the male/female ratio was 1.4:1 (67 men, 47 women).

Microbiological tests. Of the 151 included patients, 37 (25%) fulfilled the EWGLI criteria for a confirmed case of LD. The diagnosis of LD was confirmed by culture in 21 cases and urine antigen test in 35 cases (Table 1). In 19 cases both culture

TABLE 2. Results of samples tested by PCR for the detection of *Legionella*-specific DNA in patients with or without LD

Finding	No. of samples (<i>n</i> = 149) ^a			
	16S rRNA PCR		<i>mip</i> PCR	
	Positive	Negative	Positive	Negative
LD positive (<i>n</i> = 37)	32	5	34	3
LD negative (<i>n</i> = 112)	5	107	2	110

^a Samples were tested for *Legionella* sp. DNA in a 16S rRNA-based PCR and in an *mip* gene-based PCR. Estimated sensitivity, specificity, and overall percent agreement values of 86% (32 of 37; 95% CI = 72 to 95%), 95% (107 of 112; 95% CI = 90 to 98%), and 93% (139 of 149), respectively, were found for the 16S rRNA-based PCR and corresponding values of 92% (34 of 37; 95% CI = 78 to 98%), 98% (110 of 112; 95% CI = 93 to 100%), and 97% (144 of 149), respectively, were found for the *mip* gene-based PCR.

and the urinary antigen test were positive, in 12 cases only the urinary antigen test was positive, in 4 urinary antigen-positive cases a culture had not been performed, and in 2 cases the culture was positive but the urinary antigen test was negative. In 2 (1.3%) of the 151 samples tested with PCR, inhibitors of the PCR could not be removed, despite repeated DNA extraction. Thus, a total of 149 patients were included in the calculations. A total of 40 of 149 patients (27%) tested were positive in PCR: 36 *L. pneumophila* and 4 non-*pneumophila* *Legionella* spp. In patients positive for *L. pneumophila*, both the *mip*- and the 16S rRNA-based PCRs were positive in 33 samples and only *mip* gene PCR positive in 3 samples.

Estimated sensitivity, specificity, and overall percent agreement of real-time PCR compared to conventional test results. Results of samples tested by PCR were compared in patients with or without LD, according to EWGLI criteria (Table 2). Among all 149 patients included, 37 tested positive in the 16S rRNA gene based PCR, with a mean C_T value of 32 (range, 19 to 42), and 36 tested positive in the *mip* gene-based PCR, with a mean C_T value of 31 (range, 21 to 39). Three samples initially tested positive for non-*pneumophila* *Legionella* spp. in 16S rRNA PCR, but these results could not be confirmed in a second PCR run after a second DNA isolation of the respiratory sample and are therefore interpreted as negative in PCR.

An estimated sensitivity, specificity, and overall percentage agreement of 86% (32 of 37; 95% CI = 72 to 95%), 95% (107 of 112; 95% CI = 90 to 98%), and 93% (139 of 149), respectively, were found for 16S rRNA based PCR. An estimated sensitivity, specificity, and overall percentage agreement of 92% (34 of 37; 95% CI = 78 to 98%), 98% (110 of 112; 95% CI = 93 to 100%), and 97% (144 of 149), respectively, were found for the *mip* gene-based PCR.

Discrepant analysis. Discrepant analysis was performed for samples determined to be positive by PCR analyses but negative by conventional testing (Table 3). Using additional tests, two of five potential false positives in the 16S rRNA PCR are reclassified and would shift from cell B (false positive) to cell A (true positives) in Table 2, and two of two are reclassified as true positives in the *mip* gene PCR.

Results of PCR in comparison to the urinary antigen test. (i) 16S rRNA-based PCR. A total of 135 patients were tested by both the urinary antigen test and PCR. A total of 35 patients tested positive for *Legionella* urinary antigen; 30 of these patients were also positive in the 16S rRNA-based PCR. A total

TABLE 3. Data from samples with discrepant results (*Legionella* negative, PCR positive)^a

Sample	Gender, age (yr) ^b	16S rRNA (C _T)	<i>mip</i> (C _T)	Interpretation ^c	Other etiology	Test result ^d				Interpretation ^e
						5S rRNA PCR (C _T)	16S rRNA PCR (C _T)	<i>mip</i> PCR (C _T)	Sequencing (% match)	
1	M, 68	Pos (24.5)	Neg	LNP	No	Pos (24)	Pos (25)	Neg	<i>L. longbeachae</i> (100%)	TP
2	M, 68	Pos (33.1)	Pos (32.8)	LP	No	Pos (27)	ND	ND	ND	TP
3	F, 85	Pos (33.5)	Neg	LNP	No	Neg	Neg	Neg	<i>Firmicutes</i> bacterium clone m0027.115 (98%)	FP
4	M, 37	Pos (34.1)	Neg	LNP	No	Neg	Neg	Neg	ND	FP
5	M, 61	Pos (32.9)	Neg	LNP	CAP, <i>S. pneumoniae</i>	Neg	Neg	Neg	<i>Lactobacillus gasseri</i> (98%)	FP
6	F, 25	Neg	Pos (36.0)	LP	No	Pos (35)	Pos (33)	Pos (32)	ND	TP

^a Pos, positive; Neg, negative; ND, not determined.^b M, male; F, female.^c LP, *L. pneumophila*; LNP, *Legionella non-pneumophila* spp.^d The 16S rRNA and *mip* PCRs were performed according to the method of Templeton et al. (33).^e FP, false positive; TP, true positive.

of 100 patients tested negative for *Legionella* urinary antigen; 93 of these patients were also negative in the PCR tests. Of the seven urinary antigen-negative and PCR-positive patients, two fulfilled the criteria of a confirmed case of LD (culture positive). Five urinary antigen test-negative, PCR-positive patients were not confirmed by conventional diagnostics. After discrepant analysis, two patients were considered true positives and three samples were determined to be false positives (Table 3). Thus, 35 patients were diagnosed by using the urinary antigen test, and 34 patients were diagnosed by 16S rRNA PCR. Two of the reclassified discrepant specimens were associated with negative urine tests.

(ii) ***mip* gene-based PCR.** A total of 35 patients tested positive for *Legionella* urinary antigen; 32 of these patients were also positive in the *mip* gene-based PCR. A total of 100 patients tested negative for *Legionella* urinary antigen, 96 of these patients were also negative in PCR. Of the four urinary antigen-negative, PCR-positive patients, two fulfilled the criteria of a confirmed case of LD (culture positive). Two urinary antigen test-negative, PCR-positive patients were not confirmed by conventional diagnostics. After discrepant analysis, both patients were determined to be true positives. Thus, 35 patients were diagnosed by using the urinary antigen test, and 36 patients were diagnosed in the *mip* gene-based PCR. The combination of both tests (urinary antigen test and *mip* gene PCR) diagnosed a total of 39 patients with LD.

DISCUSSION

From the late 1970s until the late 1980s, options for testing for LD were limited to culture, serology, and urinary antigen testing. Since the 1990s, many NAATs have been described for the detection of *Legionella* spp. in respiratory specimens (3, 16, 17, 18, 19, 20, 21, 28, 30, 31, 32, 33, 36, 38). In addition to in-house PCR tests, commercial kits are becoming available as well (11). PCR offers several theoretical advantages, such as a high sensitivity, rapid availability of results, and the potential to detect infections caused by various serogroups of *L. pneumophila*, as well as non-*pneumophila* *Legionella* spp. However, few studies have addressed the practical value of PCR in routine

clinical microbiology laboratories. We performed a retrospective, laboratory-based study in which results obtained by PCR were compared to those of conventional testing for *Legionella* on samples sent to our laboratory from patients suspected of having LD.

Of the 151 patients tested, two samples (1.3%) were shown to inhibit the PCR. Of the 149 patients from whom noninhibiting samples were obtained, 37 (25%) fulfilled the EWGLI criteria for a confirmed case of LD. The estimated sensitivity and specificity of the 16S rRNA-based PCR were 86 and 95%, respectively. The sensitivity and specificity of the *mip*-gene based PCR assay were 92 and 98%, respectively. Five potential false-positives were detected in the 16S rRNA-based PCR, and two were detected in the *mip*-gene based PCR. The interpretation of the performance of PCR as a diagnostic test in LD is hindered by the lack of a perfect gold standard. Because the sensitivity of conventional testing is not 100%, we suspected that estimates of PCR specificity might be biased downward since a number of PCR-positive specimens from infected persons would be misclassified as uninfected if the conventional test failed to detect *Legionella*. Discrepant analysis involves the performance of one or more additional tests with these specimens, reclassification as infected those persons for whom the new-test-positive results are confirmed, and recalculation of the estimates of new-test sensitivity and specificity by using the revised classification. This approach has been criticized because of the bias introduced by the selective use of confirmation testing (22). We acknowledge that discrepant analysis is an inherently biased and unsatisfactory approach to statistical parameter estimation. However, it has been shown that for the detection of *Chlamydia trachomatis* the bias in estimates of NAAT specificity based on discrepant analysis is small: test specificities of ca. 96%, based on a culture reference standard, are probably underestimates due to false-negative cultures (12). In the present study, a proportion of PCR-positive results were confirmed, and these results might be considered true *Legionella* infections; two patients were positive for *L. pneumophila*, and one patient was positive for *L. longbeachae*. Although statistically incorrect, if we were to use the results of the discrepant analysis, and assuming a prevalence of 5% of

Legionella spp. in patients with pneumonia, recalculated sensitivity, specificity, and positive and negative predictive values would be 87% (34 of 39), 97% (107 of 110), and 60 and 99% for the 16S rRNA-based PCR assay and 92% (36 of 39), 100% (110 of 110), and 100 and 99.6% for the *mip* gene-based PCR assay, respectively.

Despite discrepant analysis, at least three false-positive results occurred in 16S rRNA PCR. Because LD is a relatively rare disease, 5% false-positive test results might be considered unacceptable for a diagnostic test, since this results in a positive predictive value of 60%. PCR quality assessment studies have also recorded false-positive results. The quality performance of 46 participating laboratories for the detection of *Legionella* spp. by two quality control exercises was investigated in 2004 and 2005 (24). In-house methods were used by 93% of participants. The rate of false positivity (panel members negative for *Legionella* spp.) ranged from 4.0% in 2004 to 8.2% in 2005. Laboratories should comply with stringent quality control requirements, and this quality control study underlines that NAATs have not yet been properly standardized in all laboratories. Laboratory workers and clinicians must be cautious when interpreting results obtained from these types of assays and should not hesitate to question results which are unexpected based on clinical presentation and local epidemiology. The occurrence of false-positive *Legionella* testing described here also demonstrates the value of routine confirmatory testing procedures, because such protocols can be beneficial in rapidly detecting problems with diagnostic assays.

Since routine diagnostics are more directed to the detection of *L. pneumophila*, the importance of non-*pneumophila* *Legionella* spp. may be underestimated. Based on serological studies, some authors suggest that sporadic non-*pneumophila* *Legionella* spp. may cause a considerable proportion of CAP (23). However, the results obtained in our study show that *L. pneumophila* is the predominant species and that the detection of non-*pneumophila* *Legionella* spp. is rare. One patient, admitted with a severe CAP with unknown etiology, tested positive for a non-*pneumophila* *Legionella* spp. in both 16S rRNA- and a 5S rRNA-based PCRs (5). Nucleotide sequence analysis of the 16S rRNA gene amplification product showed a 100% match with *L. longbeachae*. So, in a group 151 patients that was thoroughly investigated by conventional and various NAATs there was probably one case (0.7%) of infection with a non-*pneumophila* *Legionella* spp.

The usefulness of a diagnostic test is strongly influenced by local *Legionella* epidemiology. *L. pneumophila* serogroup 1 is the predominant cause of LD in The Netherlands, and infection with this organism is easier to diagnose than is infection with non-*pneumophila* *Legionella* spp. PCR and the urinary antigen test are able to provide a diagnosis in a time frame that is helpful in the acute stage of the disease. Because urinary antigen assays only detect a limited number of serogroups of *L. pneumophila*, some authors have suggested that total dependence on this diagnostic assay in LD may miss a significant proportion of cases (10). Although this may be true in distinct geographic regions where only a minority of infections are caused by *L. pneumophila* serogroup 1, urinary antigen testing is a fast, easy-to-use test with high sensitivity and optimal degree of specificity in regions where *L. pneumophila* serogroup 1 is the most common cause of the disease. With the

urinary antigen test, LD was diagnosed in 35 patients, and 34 patients of these were diagnosed by 16S rRNA-based PCR. A problem with 16S rRNA PCR, however, was a relative high proportion of false-positive results, making confirmation (using a different PCR target or sequence analysis) necessary. Although with the *mip* gene PCR we detected more cases of LD, this difference was not found to be statistically significant. However, with the combination of a urinary antigen test and the *mip* gene PCR LD was diagnosed in 39 patients, e.g., in 11% (4 of 35) more compared to the use of the urinary antigen test alone (i.e., 35 of 135 versus 39 of 135; $P = 0.7$).

The amount of laboratory and microbiological work-up needed should be determined by the severity of the pneumonia. Mild pneumonia does not usually require any further microbiological studies (34). For patients with mild LD, sensitivities for the urinary antigen test range from 40 to 53%, whereas for patients with severe LD, who need immediate special medical care, the sensitivities reach 88 to 100% (40). In regions such as, for instance, The Netherlands, where *L. pneumophila* serogroup 1 are the most frequent *Legionella* species causing disease, urinary antigen detection in urine is recommended for patients with severe CAP and in patients where this infection is clinically or epidemiologically suspected. In the case of a negative antigen test, *Legionella*-specific PCR should be considered in severe pneumonia of unknown etiology. In regions where legionellae other than *L. pneumophila* serogroup 1 are important pathogens, current urinary antigen tests are still useful but should not be used as the sole diagnostic tool.

The present study has some important limitations. We performed a retrospective, laboratory-based evaluation. The PCR assays have been evaluated in a selected patient population, and therefore the performances of the tests that were evaluated may have been confounded. Ideally, these tests should be studied prospectively and performed in all patients to make a more reliable estimate of the added value of each test. In addition, there is no clinical information available on the described patients, including a lack of information on treatment type and duration before testing. This information could be important for interpreting the laboratory results, especially that of culture, which can be adversely affected by prior antimicrobial therapy. In addition, we performed confirmatory NAAT and sequence analyses. The logic behind confirmatory testing is based upon two assumptions. The assumptions are that failure to confirm a positive result means the initial positive result was likely to be a false positive and that confirming the initial positive result increases the confidence that it was a correct result. A problem is that by applying discrepant analysis, the test under evaluation is used to define a true-positive result, and new tests under evaluation should ideally be compared to an independent gold standard. In addition, discrepant analysis involves post-hoc testing of specimens that were positive in the initial evaluation, and such selective testing of specimens is biased in favor of the new test. However, discrepant analysis allowed us to identify positive specimens as actually containing multiple properties (detection of multiple genes, sequence analysis) of the organism under question, which in our view meets a reasonable criterion for detecting the organism.

The use of diagnostic molecular techniques has been so widely publicized that increasing pressure has been placed on

clinical microbiology laboratories to apply these techniques, especially since kits are being made commercially available. The great enthusiasm aroused by nucleic acid detection methods in LD is tempered by the knowledge that the expectations concerning their superior sensitivity and specificity have not yet been fulfilled for all pathogens in all PCR assays. Although the 16S rRNA-based PCR performed well in an in vitro evaluation, the results from the present study suggest a clinical specificity lower than that reported previously (6). The *mip* gene-based PCR used in the present study is a reliable, sensitive, and highly specific technique, suitable for the detection of *L. pneumophila* in respiratory samples. The combination of a urinary antigen test and *mip* gene PCR diagnosed more patients with LD compared to the use of the urinary antigen test alone. Early recognition of patients with LD is essential; the recognition of a single case may be the sentinel event that leads to the recognition of other cases and the contaminated point source. We therefore conclude that the addition of an *L. pneumophila*-specific *mip* gene PCR is useful in patients with suspected LD who produce sputum and might allow the early detection of a significant number of extra patients.

Prospective studies are needed that directly compare multiple PCR assays available against reference tests in larger samples. The results of such comparisons would be more helpful to clinicians and microbiologists, who are faced with having to choose among many new tests, and might help to establish standard PCR methods that are robust enough to be used outside the setting of a research laboratory.

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