Development of Conventional and Real-Time PCR Assays for Detection of *Legionella* DNA in Respiratory Specimens

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The development and validation of a PCR assay based on the use of new 16S ribosomal DNA (rDNA)-targeted primers to detect *Legionella* DNA in respiratory specimens are described. The assay was originally developed as conventional PCR followed by electrophoretic detection and was then adapted to Lightcycler format with SYBR Green I detection and melting curve analysis. The 73 *Legionella pneumophila* strains tested were amplified with both applications. In addition, 21 and 23 out of 27 other *Legionella* strains were found positive by conventional and real-time PCR assays, respectively, including the clinically important species *L. micdadei*, *L. bozemaniae*, and *L. dumoffii*. Two DNA purification methods were compared using artificially seeded clinical specimens: a standard organic extraction method and a commercial kit based on adsorption of DNA to silica particles. The detection limit of the assay varied from 2 CFU to >200,000 CFU per ml of clinical specimen, depending on the background sample (i.e., pooled sputa or BAL fluids) and the DNA purification method, the silica method achieving lower detection limits. Analysis of 77 clinical samples (66 bronchoalveolar lavage fluid and 11 sputum samples) by conventional PCR yielded results that were consistent with *Legionella* culture results. The melting curve analysis in the Lightcycler system readily detected the specific amplification products. However, run-to-run variations in the measured melting temperatures required normalization against the standard sample in each run. The results obtained with the clinical specimens were similar to those obtained with conventional PCR, but more samples are required to determine whether the system can be applied to routine screening of samples for the presence of *Legionella* DNA.

Bacteria of the genus *Legionella* cause community-, travel-, and hospital-acquired pneumonia in humans, usually via inhalation of aerosols formed from man-made water systems, in which the bacteria have been enriched. *Legionella pneumophila* serogroup 1 is the most important causative agent, especially in community outbreaks and travel-acquired infections. Among the 1,442 cases of Legionnaires' disease reported from 28 European countries in 1998, *L. pneumophila* serogroup 1 accounted for 60%, other or undetermined serogroups of *L. pneumophila* accounted for 34.4%, and other species (*L. micdadei* and *L. bozemaniae*) accounted for the remaining 5.6% of cases (24).

In Finland, with a population of 5 million, about 10 cases of *Legionella* pneumonia are reported annually (http://www.ktl.fi/ttr/tt9599_33_63.pdf). Apart from a hospital outbreak involving four patients in 1995, the cases have been sporadic. According to a study of 52 Finnish legionellosis cases from 1982 to 1992 (21), 44% of patients were immunosuppressed due to ongoing or recent therapy with immunosuppressive agents, 23% had other underlying diseases, and 33% had no predisposing conditions. Seventy percent of the infections in previously healthy persons were associated with travel, and all were caused by *L. pneumophila* serogroup 1. Legionellosis was nosocomial in 73% of the immunosuppressed patients. Among those patients, *L. pneumophila* serogroup 1 caused only 26% of the cases; *L. pneumophila* serogroup 6 was recognized as the

causative agent in 35%, and non-*L. pneumophila* *Legionella* species were so recognized in 9%.

The diagnosis of legionellosis is optimally confirmed by isolation of *Legionella* spp. from lower-respiratory-tract specimens. Although absolutely specific, this approach is rather slow and insensitive, as in only 21.6% of the reported European cases in 1998 was the diagnosis based on successful culturing of *Legionella* organisms (24). Serologic analysis can be used only retrospectively and may be of limited value in immunocompromised patients. During recent years, screening kits for *Legionella* antigens in urine have become available (5). Alternatively, many groups have developed and evaluated assays to detect *Legionella* DNA in respiratory tract specimens and sometimes also in serum or urine (1, 6–8, 9–12, 14–16, 23). PCR is more time-consuming and complex to perform than antigen detection tests but, especially if culturing fails, offers the possibility to use the same sample for identification and/or typing of the agent by molecular methods.

Here we describe the development and validation of a PCR assay for the detection of *L. pneumophila* DNA in respiratory specimens. In addition to validating this assay, we adapted the procedure to a new real-time capillary PCR format (Lightcycler; Roche Molecular Diagnostics, Mannheim, Germany), which allows the amplification and detection to take place in less than 1 h and therefore significantly accelerates the screening of clinical specimens.

MATERIALS AND METHODS

Bacteria. Unless stated otherwise, the experiments were done using the *L. pneumophila* type strain (ATCC 33152), which was used for quality control throughout. A total of 47 other *L. pneumophila* serogroup 1 strains were used in this study, including 40 strains of the phase II panel of the European Working

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TABLE 2. Primers and probe used in this study^a

Name	Use	Target bases	Sequence (5' to 3')
Lpneu2	PCR (forward)	618–639	GCT TAA CCT GGG ACG GTC AGA T
Lpneu3	PCR (reverse)	862–839	GCG CCA CTA ATT ATT TTC ATA TAA
Lpneuprb	Hybridization	801–820	GGT AGT CCA CGC TGT AAA CG

^a The target gene encodes *L. pneumophila* 16S rRNA.

Group on Legionella Infections (EWGLI) (4), 10 of which were studied as duplicates. The strain collection also included 25 *L. pneumophila* strains representing serogroups 2 to 12, as well as 27 non-*L. pneumophila* strains representing 20 *Legionella* species. In addition, 21 bacterial strains either phylogenetically related to the genus *Legionella* or possibly present in sputa or bronchoalveolar lavage fluids were used in specificity tests. The *Legionella* strains were a kind gift from Hannele Jousimies-Somer, National Public Health Institute, Helsinki, Finland. The sources of the other strains are listed in Table 1. *Legionella* strains were grown on buffered charcoal-yeast extract (BCYE; Oxoid Ltd., Basingstoke, United Kingdom) agar plates at 37°C for 48 to 72 h. Other bacteria were cultivated on standard media supporting optimal growth. The clinical strains were identified by standard methods.

DNA purification from bacteria. Cultured bacterial cells were suspended in 200 µl of phosphate-buffered saline and digested with proteinase K (0.1 mg/ml, 56°C, 2 to 17 h). DNA was extracted with two phenol-chloroform-isoamyl alcohol extractions, washed once with ether, and precipitated with sodium acetate-ethanol. Purified DNA was dissolved in 100 µl of sterile water. The DNA concentration was determined by measuring the absorption at 260 nm and adjusted to 100 µg/ml.

Primer design and PCR optimization. To find oligonucleotide sequences specific for *L. pneumophila*, a multiple alignment was done from the complete 16S rRNA and rDNA sequences at EMBL database release 52.0 (22) for six *L. pneumophila* strains (accession numbers M59157, M36023, M36024, X36025, M36026, and X73402) and the type strains of *L. longbeachae*, *L. bozemaniae*, and *L. micdadei* (accession numbers M36029, M36031, and M36032, respectively). The specificity of the candidate primers for all bacterial sequences in the database was verified by FastA analysis (18). Primers were purchased from Medprobe AS, Oslo, Norway.

The PCR conditions used with the selected primers (Table 2) were optimized by titration of the annealing temperature (range, 50 to 65°C) and MgCl₂ concentration (range, 1 to 5 mM). Conventional PCR was performed with 50-µl reaction mixtures containing each primer at 0.2 µM, each deoxynucleoside triphosphate (dNTP; Promega, Madison, Wis.) at 0.2 mM, MgCl₂ at 4 mM, 1 U of DNA polymerase (Dynazyme II F-501L [Finzymes, Espoo, Finland] or AmpliTaq Gold [Applied Biosystems, Foster City, Calif.]) with appropriate buffer, and 5 µl of the template DNA. In preliminary experiments, 500 ng of DNA isolated from *L. pneumophila* was used as the template, and final optimization was done with serial dilutions of *L. pneumophila* cells used to spike pooled sputum samples that had originally remained negative for *Legionella* bacteria in cultures. In addition to the routine negative sample and reagent controls (19), DNA preparations from *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were used as controls to confirm the specificity of amplification under the tested conditions. The final amplification program included activation of the enzyme at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. Ten microliters of the PCR product was run on 4% agarose gels and stained with ethidium bromide. The presence of a visible 245-bp band was interpreted as a positive result. The specificity of the amplicon was confirmed by Southern hybridization on nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) with γ-³²P-labeled probe Lpneuprb as described earlier (17).

The reagents and capillaries for Lightcycler PCR were purchased from Roche Molecular Diagnostics. The 20-µl reaction mixture contained 2 µl of Lightcycler FastStart Reaction mix (including FastStart Taq DNA polymerase with buffer, dNTP mix, SYBR Green I dye, and 10 mM MgCl₂), additional MgCl₂ to achieve the optimal final concentration of 4 mM, each primer at 0.5 µM, 1 U of uracil-DNA-glycosylase (UNG), and 2 µl of template DNA. Before amplification, the capillaries were kept at room temperature for 10 min to allow UNG to break down the possible contaminating amplicons and then were heated to 95°C and held at that temperature for 10 min to deactivate UNG and activate the polymerase enzyme prior to the start of cycling. Amplification steps (15 s at 95°C, 5 s at 53°C, 9 s at 72°C, and 1 s at 82°C) were repeated 45 times. Fluorescence was measured at channel F1 (gain set at 5) during the last step of each cycle at 82°C.

Melting curves were constructed by lowering the temperature to 63°C, raising the temperature by 0.1°C/s to 95°C, and measuring the fluorescence continuously. Reactions with purified *L. pneumophila* DNA at three concentrations (100, 20, and 4 ng per capillary) were included in each run to construct the standard curve. Quantification was performed according to the instrument manual by setting the noise band over the background fluorescence and determining the crossing points arithmetically with the use of two fit points. Melting points were calculated by the instrument. In order to normalize the run-to-run variations in the measured melting points, a melting temperature (*T_m*) ratio was calculated by dividing the melting point of each sample by the melting point of the 20-ng standard sample. After each run, the presence of 245-bp amplification products was also verified on 4% agarose gels.

The MgCl₂ concentration was optimized in the same way as that described above for conventional PCR. During the optimization, the cycle during which the fluorescence signal from a given specimen exceeded the background signal (crossing point) was used as an indicator of the efficiency of the amplification at different MgCl₂ concentrations.

Strict precautions were taken to avoid contamination as described previously (17). In the conventional PCR system, pre-PCR and post-PCR steps were carried out by different persons in dedicated rooms. As one person performed the whole Lightcycler PCR procedure and occasional breakage of capillaries caused the risk of amplicon contamination, the UNG decontamination system was incorporated into the protocol.

Sample preparation. About 1 ml of sputum samples was treated with proteinase K, and 200 µl was used for DNA extraction. BAL fluids were concentrated (8,000 × g, 5 min), and DNA was extracted from 200 µl of the concentrate. Two DNA extraction systems were applied: the standard phenol-ether purification described above (without the precipitation step) and a commercial kit based on the adsorption of DNA to silica particles (High Pure PCR template preparation kit; Roche Molecular Diagnostics).

Determination of the analytical sensitivity of the assay. To determine the lowest number of *L. pneumophila* cells detectable by the assay, serial dilutions of *L. pneumophila* cells were used to spike sputum samples or BAL fluids. The artificially seeded sputum samples were prepared by pooling sputum samples from four or five different patients, adding an equal volume of 0.1% dithiothreitol (Sigma), vortexing for 5 min, and incubating for 15 min at room temperature. The homogenate was divided into 1-ml aliquots, which were spiked with 2 to 200,000 *Legionella* cells. One-milliliter aliquots of pooled and homogenized sputum or of pooled BAL fluid were concentrated as described above, and DNA was extracted with either the phenol-ether procedure or the High Pure PCR template preparation kit. To directly compare the ability of the two systems to release and purify template DNA for amplification, each spiked sample was divided in two, an equal volume was treated with each DNA preparation procedure, and 5 µl was used as a template in the PCR (or 2 µl for the Lightcycler PCR). Statistical analysis of the detection limits for the two DNA extraction methods was performed with the Kruskal-Wallis test.

Reproducibility of Lightcycler PCR. The within-run and between-run variations in the Lightcycler PCR system were estimated by running samples of one dilution series in two different sputum backgrounds twice, two replicates in each run, with purified *L. pneumophila* DNA as the standard. The run-to-run variations in the Lightcycler system were also assessed by calculating the average crossing points and *T_m*s from the three standard samples in 20 consecutive runs.

Clinical specimens. Seventy-seven respiratory specimens (11 sputum samples and 66 BAL fluid samples) from 71 hospitalized patients with symptoms of acute pneumonia were analyzed by *L. pneumophila* PCR. DNA was isolated by the phenol-ether method described above. Eighteen specimens were divided on arrival in the laboratory, and about 1 ml was processed with the High Pure PCR template preparation kit. All specimens were tested for PCR inhibition by amplification of the human growth hormone gene as described previously (19). All specimens were cultivated on BCYE agar plates with MWY and BMA-α supplements (Oxoid) at 37°C for 14 days to screen for the presence of *Legionella*.

TABLE 1. Bacterial strains tested by the described PCR methods

Bacterial species	Sero-group	No. of strains	Strain identification ^a	Results of ^b :		
				Conventional PCR	Hybridization	Real-time PCR
<i>Legionella pneumophila</i>						
Type strain	1	1	ATCC 33152	+	+	+
EWGLI phase II strains	1	40	EUL 1, 2, 19, 31, 36, 40, 47, 48, 51, 56, 71, 73, 76 to 79, 86, 96, 101, 121, 124 to 143	+	+	+
Other	1	7	IHL 103211, IHL 103226, IHL 10322, IHL 103229, IHL 103244, IHL 33501, IHL 33553	+	+	+
	2	1	ATCC 33154	+	+	+
	3	2	ATCC 33155, IHL 33913	+	+	+
	4	2	ATCC 33156, IHL 33879	+	+	+
	5	9	ATCC 33216, IHL 103154, IHL 103213, IHL 103227, IHL 103253, IHL 33625, IHL 33675, IHL 33679, IHL 33896	+	+	+
	6	4	ATCC 33215, IHL 103234, IHL 33512, IHL 33531	+	+	+
	7	1	ATCC 33823	+	+	+
	8	1	ATCC 35096	+	+	+
	9	1	ATCC 35289	+	+	+
	10	2	ATCC 43283, IHL 33592	+	+	+
	11	1	ATCC 43130	+	+	+
	12	1	ATCC 43290	+	+	+
Other <i>Legionella</i> species						
<i>L. anisa</i>		1	ATCC 35292	+	± ^c	+
<i>L. birminghamensis</i>		1	IHL 33850	± ^c	—	—
<i>L. bozemaniae</i>	1	2	ATCC 33217, IHL 33817	+	+	+
<i>L. cherrii</i>		1	ATCC 35252	+	± ^c	+
<i>L. dumoffii</i>		2	ATCC 33279, IHL 103232	+	—	+
<i>L. erythra</i>		1	ATCC 35303	—	—	—
<i>L. feeleii</i>		2	ATCC 35072, ATCC 35849	+	—	—
<i>L. gormanii</i>		1	IHL 103212	+	+	+
<i>L. hackeliae</i>		1	ATCC 33250	—	—	± ^d
<i>L. jamestowniensis</i>		1	ATCC 35298	+	—	± ^d
<i>L. jordanis</i>		1	ATCC 33623	+	—	± ^d
<i>L. longbeachae</i>	1	1	ATCC 33462	—	—	+
	1	2	IHL 33697, IHL 33865	+	+	+
	2	1	ATCC 33484	—	—	± ^d
<i>L. maceachernii</i>		1	ATCC 35300	+	—	± ^d
<i>L. micdadei</i>		2	ATCC 33218, IHL 33628	+	+	+
<i>L. oakridgensis</i>		1	ATCC 33781	—	—	± ^d
<i>L. rubrilucens</i>		1	ATCC 35304	± ^c	—	± ^d
<i>L. sainthelensi</i>		1	ATCC 35248	+	—	—
<i>L. santicrucis</i>		1	ATCC 35301	—	—	+
<i>L. spiritensis</i>		1	ATCC 33249	+	± ^c	± ^d
<i>L. steigerwaltii</i>		1	ATCC 35302	+	—	+
Other bacteria						
<i>Acinetobacter calcoaceticus</i>		1	ATCC 23055	—	Not done	—
<i>Aeromonas hydrophila</i>		1	Local isolate	—	Not done	—
<i>Bordetella bronchiseptica</i>		1	Local isolate	—	Not done	—
<i>Eikenella corrodens</i>		1	Local isolate	—	Not done	—
<i>Escherichia coli</i>		1	ATCC 25922	—	Not done	—
<i>Haemophilus influenzae</i>		1	ATCC 49766	—	Not done	— ^e
<i>Haemophilus parainfluenzae</i>		1	Local isolate	—	Not done	— ^e
<i>Klebsiella pneumoniae</i>		1	ATCC 27786	—	Not done	—
<i>Moraxella catarrhalis</i>		1	ATCC 25238	—	Not done	— ^e
<i>Neisseria meningitidis</i>		1	NCTC 10025	—	Not done	— ^f
<i>Proteus vulgaris</i>		1	Local isolate	—	Not done	—
<i>Pseudomonas aeruginosa</i>		1	ATCC 27853	—	Not done	—
<i>Pseudomonas putida</i>		1	CCUG 264	—	Not done	— ^f
<i>Pseudomonas stutzeri</i>		1	CCUG 16590	—	Not done	— ^f
<i>Salmonella enteritidis</i>		1	Local isolate	—	Not done	— ^f
<i>Staphylococcus aureus</i>		1	ATCC 25923	—	Not done	—
<i>Stenotrophomonas maltophilia</i>		1	Local isolate	—	Not done	—
<i>Streptococcus milleri</i>		1	Local isolate	—	Not done	—
<i>Streptococcus mutans</i>		1	ATCC 25175	—	Not done	— ^e
<i>Streptococcus oralis</i>		1	ATCC 35037	—	Not done	—
<i>Streptococcus pneumoniae</i>		1	ATCC 49619	—	Not done	—

^a ATCC, American Type Culture Collection, Manassas, Va.; EUL, European Union Legionella Culture Collection, PHLS Central Public Health Laboratory, London, United Kingdom; IHL, Isolate Helsinki Legionella, Collection of National Public Health Institute, Helsinki, Finland; NCTC, National Collection of Type Cultures, London, United Kingdom; CCUG, Culture Collection of University of Gothenburg, Gothenburg, Sweden.

^b For conventional PCR, 500 ng of purified DNA was used as a target, and *Legionella* strains also were tested with 50 ng of DNA as a target. Conventional PCR and hybridization were considered positive when a 245-bp band was seen. For real-time PCR, all strains were screened with 20 ng of DNA as a target, and bacteria of *Legionella* species other than *L. pneumophila* and other bacteria also were screened with 200 ng of DNA as a target. Real-time PCR was considered positive when the T_m ratio was between 0.993 and 1.005 (average and 3 SDs for *L. pneumophila* strains). +, positive result; —, negative result.

^c Weak band.

^d Positive with 200 ng only.

^e The 200-ng template yielded PCR products with a T_m of $\geq 84^\circ\text{C}$ (T_m ratio, ≤ 0.973).

^f The 200-ng template yielded PCR products with a T_m of $\geq 88^\circ\text{C}$ (T_m ratio, ≤ 1.040).

DNA. The identification of *Legionella* species was based on typical cellular fatty acid profiles in gas-liquid chromatography (3).

RESULTS

Primers and optimization of PCRs. Primers Lpneu2 and Lpneu3 (Table 2) produced the expected 245-bp amplicon from *L. pneumophila* DNA, while the control reactions with DNA from *P. aeruginosa* and *S. maltophilia* remained negative. Originally, a polymerase isolated and purified from an *Escherichia coli* strain carrying a plasmid with the cloned DNA polymerase gene from *Thermus brockianus* was used (DyNAzyme II F-501L DNA polymerase). Optimized PCR with DNA from bacteria produced either a single 245-bp band or no band at all. However, when DNA from simulated sputum samples was used as a template, bands of about 500 bp were detected, especially when no or few *L. pneumophila* cells were used for spiking. No cross-amplification of DNA from normal flora was detected when AmpliTaq Gold enzyme was used.

Amplification of DNA from bacterial strains. (i) Conventional PCR. DNA from all 73 *L. pneumophila* strains was amplified in the optimized PCR, and all PCR products also hybridized with the probe Lpneuprb (Table 1). In addition, DNA from 21 out of 27 other *Legionella* strains yielded the expected 245-bp band on an agarose gel. The bands were often weaker with non-*L. pneumophila* *Legionella* strains, and some remained negative for hybridization with the probe. DNA from other bacteria was not amplified.

(ii) Real-time PCR. All *L. pneumophila* strains were amplified by the Lightcycler system. With 20 ng of purified DNA as a template, serogroup 1 strains had an average T_m of 85.58°C (standard deviation [SD], 0.18), with a mean T_m ratio (T_m of sample divided by T_m of the 20-ng standard) of 0.999 (SD, 0.002). The respective values for the other *L. pneumophila* serogroups were 85.37°C (SD, 0.21) and 1.000°C (SD, 0.002). The amplification of non-*L. pneumophila* *Legionella* strains was variable. They were considered positive when the T_m ratio was between 0.993 and 1.005 (average and 3 SDs for *L. pneumophila* serogroup 1 strains). The results were in accord with the presence or absence of the 245-bp band on the gel. The average T_m for positive non-*L. pneumophila* *Legionella* strains was 85.31°C (SD, 0.30). The results obtained by Lightcycler PCR were not always consistent with those obtained by conventional PCR (Table 1). With a high template DNA concentration (200 ng), some bacteria other than *Legionella* spp. were amplified, but the products were readily distinguished from *Legionella*-specific products by different melting points (Table 1).

Detection limits of the assays. Table 3 shows the detection limits of the assays when serial dilutions of *L. pneumophila* were used to spike simulated clinical specimens and then were purified by different DNA extraction methods. For sputa, the background sample had a profound effect on the detection limits, as the lowest and the highest limits were obtained with the same dilution series and with the same samples by both DNA isolation methods. When aliquots of each simulated sample were processed by the two DNA isolation methods, the detection limits achieved with the silica method were statistically significantly lower than those obtained by the phenol-ether method ($P = 0.0218$; Kruskal-Wallis test). In general, the sensitivity of the Lightcycler system in detecting small amounts

TABLE 3. Detection limits of the conventional and real-time PCR assays with dilution series of *L. pneumophila* serogroup 1 (ATCC 33152) cells used to spike simulated clinical specimens^a

Background	DNA isolation method	Detection limit (CFU/ml) of PCR	
		Conventional	Real-time
BAL fluid	Phenol-ether	20,000	20–2,000
	High Pure kit	2	200
Sputum	Phenol-ether	2,000–>200,000	2,000–>200,000
	High Pure kit	20–200,000	200–2,000

^a The bacteria were used to spike 1 ml of simulated clinical sample, which was concentrated to 200 μ l and used for DNA isolation. Two independent dilution series were used, and each was used to spike two sputum pools and two BAL fluid pools. Samples of 5 and 2 μ l of the 200- μ l DNA preparation were used for conventional and Lightcycler PCRs, respectively. At least two independent amplifications were done with each DNA preparation by both assays, and the most dilute sample positive in both runs was regarded as the detection limit. High Pure, High Pure PCR template preparation kit.

of target DNA was comparable to that of conventional PCR. As in conventional PCR, the variations observed in the detection limits with the Lightcycler system were mainly due to the background sample, but there were also run-to-run variations in the most dilute sample detected as positive.

Reproducibility of Lightcycler PCR. Table 4 shows the results obtained from an experiment evaluating the within-run and between-run variations of the Lightcycler PCR. As determined from values obtained in 20 consecutive runs, the coefficient of variation (CV) for the crossing point was about 16% with all three standard samples. The average T_m for the standards was 85.71 (SD, 0.54; CV, 0.62), and the calculated T_m of the 20-ng standard varied from 84.97°C to 87.02°C.

Detection of *Legionella* DNA in clinical specimens. (i) Conventional PCR. None of the samples showed total PCR inhibition, as judged by amplification of the human growth hormone gene in a separate reaction. None of the 11 sputum samples was PCR positive for *Legionella* DNA. Two (3%) out of the 66 BAL fluid samples were positive for *Legionella* DNA in the PCR. Both bands also hybridized with the probe Lpneuprb. The PCR results were consistent with those of culturing, as *L. pneumophila* was isolated from both PCR-positive BAL fluid samples. One of the positive samples was processed by

TABLE 4. Reproducibility of Lightcycler PCR

No. of target cells per sample	CV (%)					
	Intra-assay ^a			Interassay ^b		
	Concn	Crossing point	T_m	Concn	Crossing point	T_m
200,000	23.0	1.56	0.01	11.0	6.08	0.03
20,000	49.5	1.50	0.03	78.0	4.81	0.23
2,000	52.0	7.88	0.03	123	8.98	0.18

^a Dilution series of *L. pneumophila* cells were used to spike aliquots of two different sputum pools. DNA was isolated using the High Pure PCR template preparation kit. Two replicates of each sample were analyzed in each of two runs. The CVs represent the mean CVs for the results obtained from the four pairs of replicates with the same number of target cells analyzed in the same run.

^b The CVs represent the mean CVs for the results obtained in two separate runs from two pairs of samples with the same number of target cells but different sample backgrounds. The mean of the two replicates for each sample was used as the value for the concentration, crossing point, and T_m obtained in each run.

both tested DNA purification methods; one was a scant sample and was purified only by the routine phenol-ether method.

(ii) Real-time PCR. Clinical samples were considered positive when the T_m ratio was between 0.993 and 1.005 (average and 3 SDs for *L. pneumophila* serogroup 1 strains). Sixty-five BAL fluid samples and 11 sputum samples treated with phenol-ether were screened. The two BAL fluid samples growing *L. pneumophila* were positive, with T_m s of 86.97 and 86.45°C (T_m ratios of 0.999 and 0.994, respectively). The crossing points were 17.99 and 28.49, corresponding to about 1 ng and 2 pg of DNA in the 2- μ l preparation, respectively. In terms of CFU, the concentrations would have been more than 2×10^6 CFU/ml and about 20,000 CFU/ml of BAL fluid, respectively (Fig. 1). Silica-purified DNA from 18 BAL fluid samples yielded consistent results.

DISCUSSION

An optimal PCR assay for the diagnosis of legionellosis should detect *L. pneumophila* as well as about 20 other *Legionella* species that have been associated with infections in humans. The importance of *Legionella* spp. other than *L. pneumophila* is emphasized when a major portion of referred samples originate from hospitalized patients with underlying diseases. On the other hand, these patients are often colonized by other gram-negative rods (20), which must not give false-positive signals in the assay.

The first PCR systems for the detection of the genus *Legionella* were based on primers targeted at 5S rDNA (13), which amplified a wide range of *Legionella* spp. but also some *Pseudomonas* species. Specificity problems also were reported in attempts to detect *Legionella* DNA in urine with the use of modified 5S rRNA primers (14).

16S rDNA is more variable than 5S rRNA, and large databases with thousands of bacterial sequences offer a firm background for the rational design of primers for genus- or species-specific PCRs. However, we were unable to design generic *Legionella* sp. primers based on 16S rRNA sequences, and previously published primers have covered only part of the genus. Lisby and Dessau (12) described a PCR assay with primers partly overlapping ours and a membrane hybridization confirmation system. *L. pneumophila* and some other *Legionella* spp. were detected, but the clinically important *L. micdadei* and *L. bozemanii* were not. Only two out of seven PCR-positive results could be confirmed by culturing or serologic analysis. Jonas et al. (8) developed a PCR-enzyme-linked immunosorbent assay with 16S rRNA primers and probe and reported a slightly different range of detected *Legionella* species. Eight of the 14 PCR-positive results were confirmed by culturing, and 5 out of 6 culture-negative, PCR-positive samples came from patients on high-dose erythromycin therapy which, according to our experience, explains such results (19). Cloud et al. (1) used the same primers in less stringent conditions to allow the amplification of, e.g., *L. micdadei* and obtained specificities of 93% with PCR alone and 98% after confirmation by sequencing.

An alternative approach for a clinical PCR, the use of the macrophage infectivity potentiator (*mip*) virulence gene as a PCR target, has also been applied to detecting *L. pneumophila* in respiratory samples (7, 10) and serum (11). Jaulhac et al. (7)

FIG. 1. Amplification profiles (A), melting curves (B), and agarose gel electrophoresis patterns (C) of Lightcycler PCR with SYBR Green I. Samples: 1, water (reagent control); 2 to 4, *L. pneumophila* DNA standards at 100 ng (2), 20 ng (3), and 4 ng (4); 5 to 9, aliquots of BAL fluid pools spiked with 200,000 (5), 20,000 (6), 2,000 (7), or 200 (8) *L. pneumophila* cells before DNA extraction and the same BAL fluid pool without added bacteria (9); 10 to 13, patient samples (BAL fluids). (A) Fluorescent emissions were measured as the last step of each thermal cycle at 82°C and plotted against the cycle number. The number associated with each curve is the sample number. For quantification, the noise band was set above the background fluorescence at about 1, and the cycle at which the curve of each sample crosses the noise band is proportional to the amount of *L. pneumophila* DNA in the sample. (B) After amplification, fluorescence was measured while the temperature was increased at 0.1°C/s from 63°C to 95°C. The change in fluorescence with the change in temperature [$-d(F1)/dT$] was plotted as a function of temperature. The T_m of the *L. pneumophila* PCR product is about 86.75°C, and nonspecific products melt at between 74 and 83°C. (C) PCR products were run on a 4% agarose gel, stained with ethidium bromide, and visualized under UV light. Lane L, 1-kb DNA ladder (Gibco); lanes 1 to 13, samples (see panel A). In addition to the specific 245-bp bands, some faint bands of other lengths are seen in BAL fluid samples with little or no *L. pneumophila* DNA; these correspond to the small melting curve peaks in panel B. F1, fluorescence at wavelength of the instruments channel 1.

used primers targeted to the conserved area of the *mip* gene and successfully amplified not only *L. pneumophila* but also *L. bozemanii* and *L. micdadei*. *L. dumoffii* is the clinically most important species that remains unamplified in this assay, but it can be detected in our system. The reported detection limit in BAL fluid (25 CFU per ml after Southern blot hybridization) is comparable to ours. A commercial kit using a combination of 5S rRNA and *mip* primers (EnviroAmp; Applied Biosystems) and designed for the detection of *Legionella* DNA in water samples has been successfully applied to respiratory specimens (9, 15, 23) and to urine specimens with somewhat reduced sensitivity (6). The kit is, however, currently not commercially available.

Our PCR design was successful in the sense that the clinically most relevant species, *L. pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. dumoffii*, were amplified. Furthermore, a positive PCR result could be interpreted rather reliably as indicative of the presence of *Legionella* DNA in the sample and thus reported to the clinician in a timely manner. However, the development of a rapid confirmatory assay seems necessary. Confirmation could be based on capillary sequencing or on a hybridization probe assay by use of Lightcycler with a small panel of probes for different species.

Optimal sample processing should concentrate the possible target organisms in the specimen, release their DNA, and wash away the inhibitory compounds. Of the two DNA purification systems tested, the High Pure PCR template preparation kit achieved higher analytical sensitivity, although it remains unclear whether this result also affects clinical sensitivity. With both DNA purification methods, the detection limits for PCR remained relatively high in comparison to those reported for culturing (1 to 60 cells per ml in the artificially seeded sputum samples) (2). In clinical samples, the lower analytical sensitivity of PCR is partly compensated for by the ability of PCR to detect DNA from nonreplicating bacterial cells as well.

In the Lightcycler system, the accumulation of amplicons in the reaction capillaries can be monitored cycle by cycle using either SYBR Green I dye, which binds any double-stranded DNA, or sequence-specific detection with two fluorogenic hybridization probes. Since *Legionella* organisms are not considered to be part of the normal human flora, we were interested in detecting the presence or absence of *Legionella* DNA rather than its quantification and chose to start with the simpler and less expensive SYBR Green I format with T_m analysis of the PCR product. Adaptation of this particular PCR to Lightcycler was easy, and optimization was even easier than with conventional PCR, as the whole reaction rather than the end product could be monitored. Analysis of T_m s readily distinguished the specific and nonspecific PCR products, even if they were visualized as bands of almost equal sizes in electrophoresis. Determination of melting points was very stable in a given run and in consecutive runs, as observed by analysis of strain collections, but the long-term variation makes it difficult to set fixed limits for accepted melting points without normalization to a standard sample. According to our experiments, reliable quantification of *Legionella* DNA in clinical samples with this assay would be difficult due to variable sample background and a tendency to form primer-dimers and nonspecific amplification products when the concentration of the specific target is decreasing, i.e., variable amplification efficiency in different sam-

ples. However, the assay shows promise for simple screening of samples for the presence of *Legionella* organisms provided that the concentrations of *Legionella* DNA in samples from infected patients are at the level of the two positive samples in our collection and that the variation in the normalized T_m of the PCR product is as low as in this study.

The real-time assay can be completed 2 to 3 h after the sample has arrived in the laboratory, whereas the conventional assay takes at least 6 h (without hybridization). Even with a confirmatory test, the results of the real-time PCR assay could be reported during the same or the next day, depending on the transportation of the samples and the routine work flow of the laboratory. The cost of reagents, plasticware, and capillaries for the Lightcycler PCR is about \$5 per reaction, as opposed to about \$2 for the conventional PCR and electrophoresis detection. Labor savings via elimination of the need for electrophoresis compensate for the higher reagent cost of the real-time system. However, a larger number of clinical samples should be analyzed to ensure that annealing and amplification in the Lightcycler assay are stringent enough to be applied to the bacterially complex environment of human respiratory specimens and that the melting curve analysis reliably separates the possible by-products of the amplification reaction.

In conclusion, we describe a PCR assay which detects the presence of the clinically most important *Legionella* species in respiratory samples. We have included the conventional assay in the repertoire of our routine diagnostic PCR laboratory. Although the amplification seemed specific, the development of a rapid confirmatory system will improve the clinical utility of the assay. The primers are also applicable to capillary real-time PCR with SYBR Green I detection and melting point analysis of the PCR product, but further experience is needed to assess the reliability of that kind of assay as a screening tool for the presence of *Legionella* DNA in clinical specimens.

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