Detection of Legionellae in Hospital Water Samples by Quantitative Real-Time LightCycler PCR

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Contamination of hospital water systems with legionellae is a well-known cause of nosocomial legionellosis. We describe a new real-time LightCycler PCR assay for quantitative determination of legionellae in potable water samples. Primers that amplify both a 386-bp fragment of the 16S rRNA gene from Legionella spp. and a specifically cloned fragment of the phage lambda, added to each sample as an internal inhibitor control, were used. The amplified products were detected by use of a dual-color hybridization probe assay design and quantified with external standards composed of Legionella pneumophila genomic DNA. The PCR assay had a sensitivity of 1 fg of Legionella DNA (i.e., less than one Legionella organism) per assay and detected 44 Legionella species and serogroups. Seventy-seven water samples from three hospitals were investigated by PCR and culture. The rates of detection of legionellae were 98.7% (76 of 77) by the PCR assay and 70.1% (54 of 77) by culture; PCR inhibitors were detected in one sample. The amounts of legionellae calculated from the PCR results were associated with the CFU detected by culture (r = 0.57; P < 0.001), but PCR results were mostly higher than the culture results. Since L. pneumophila is the main cause of legionellosis, we further developed a quantitative L. pneumophila-specific PCR assay targeting the macrophage infectivity potentiator (mip) gene, which codes for an immunophilin of the FK506 binding protein family. All but one of the 16S rRNA gene PCR-positive water samples were also positive in the mip gene PCR, and the results of the two PCR assays were correlated. In conclusion, the newly developed Legionella genus-specific and L. pneumophila species-specific PCR assays proved to be valuable tools for investigation of Legionella contamination in potable water systems.

Legionnaires’ disease is normally acquired by inhalation or aspiration of legionellae from a contaminated environmental source. Water systems of large buildings, such as hospitals, are often contaminated with legionellae and therefore represent a potential danger to patients. Several reports have shown a clear association between the presence of legionellae in hot water systems and the occurrence of legionellosis (9, 11, 13, 16, 25). The degree of Legionella contamination in hospital water supplies has been shown to correlate with the incidence of nosocomial Legionnaires’ disease (6, 13).

For risk evaluation of nosocomial Legionella infection, surveillance of hospital water systems is needed. Isolation of legionellae from water samples by culture techniques is the method usually preferred, but it has limitations. Problems with culture detection include the fastidious growth requirements of the organisms, long incubation periods, overgrowth by other bacteria, and the presence of viable but nonculturable legionellae (4, 23) in some environmental samples. Recently, new methods for detection of legionellae in water samples by applying PCR techniques have been developed to overcome the limitations of culture. Several primer and probe systems targeting the 16S rRNA gene (19) and the 5S rRNA gene (12, 17, 24) of Legionella spp. and the mip gene of Legionella pneumophila (2, 12, 24), which codes for a protein of the FK506 binding protein class and is important for the intracellular survival of legionellae, have been applied. PCR techniques have the advantages of fast acquisition of results, detection of nonculturable legionellae, and easier handling of large sample amounts. However, quantitative determination of Legionella in water samples by PCR has not been available to date, and data about sensitivity and specificity of PCR compared to culture techniques are limited (17, 19, 24).

The introduction of rapid thermal cyclers combined with microvolume fluorimeters, e.g., the LightCycler instrument (Roche Diagnostics, Mannheim, Germany), now enables complete PCR in less than 45 min combined with real-time PCR product detection by sequence-specific hybridization probes. We utilized the LightCycler hybridization probe technology for the detection of legionellae in potable water samples and describe a new, highly sensitive, quantitative PCR assay targeting the 16S rRNA gene of Legionella spp. and an L. pneumophila-specific PCR assay targeting the mip gene. For detection of possible PCR inhibitors in the water samples, an internal inhibitor control is added to each sample and detected by use of a dual-color hybridization probe assay design. LightCycler PCR results are compared to Legionella counts obtained by culture.

MATERIALS AND METHODS

Bacteria, culture, and control DNA preparation. The Legionella strains used in this study and their sources are described in Table 1. Legionellae were grown at 37°C on buffered charcoal-yeast extract (BCYE) agar for 48 to 72 h before DNA extraction with a commercially available kit (QiAamp DNA Mini Kit; Qiagen, Hilden, Germany). DNA of L. pneumophila serogroup 1 (ATCC 33152) was used in all PCR runs as a positive control. For specificity control of the 16S rRNA gene PCR, the following bacteria were used (10 pg of bacterial DNA per PCR assay each): Acinetobacter junii (ATCC 17908), Acinetobacter baumannii (ATCC 19606), Acinetobacter ica (ATCC 15309), Citrobacter freundii (ATCC 8000), Citrobacter koseri (ATCC 27028), Enterobacter aerogenes (ATCC 13048), Klebsiella oxytoca (ATCC 43865), Proteus mirabilis (ATCC 29906), Proteus vulgaris (ATCC 29905), Serratia marcescens (ATCC 13880), Stenotrophomonas maltophilia (ATCC 13637), Alcaligenes faecalis, Brevundimonas vesicularis, Chryseemo-
A 100-ml portion of the water sample was centrifuged at 2,700 × g for 30 min. The sediment was transferred to a sterile 1.5-ml tube and centrifuged at 20,000 × g for 10 min, and the resulting sediment was resuspended in 100 µl of the supernatant. After another centrifugation step at 20,000 × g for 10 min, the resulting pellet was used for DNA extraction with a commercially available kit (QIAamp DNA Mini Kit; Qiagen). The method also extracts DNA from legionellae that reside in amoebae. Isolated DNA was eluted in 50 µl of elution buffer AE (supplied in the kit) and stored at 4°C until analysis by PCR. A negative control (i.e., sterile water without bacteria) was included in each batch of samples for DNA preparation and measured by PCR to exclude contamination of the buffer solutions.

Cloning of the internal inhibitor control. For detection of possible PCR inhibitors in the water samples, a 410-bp internal inhibitor control was cloned. It consists of lambda DNA with sequences at the ends that are complementary to the 16S rRNA gene primers used. Molecular cloning, restriction enzyme diges-
tions, DNA ligation, and transformation of E. coli were done by standard pro-
tocols. Briefly, for generating the internal control, lambda DNA (MBI Fermen-
tas, St. Leon-Rot, Germany) was amplified with the universal primer pair E. coli (5'-CTC GGA TCC AAC AGC TAG TTG ACA TCG AGC AGC GCA CTG AG) and Bam R (5'-CTC GGA TCC AAC AGC TAG TTG ACA TCG AGC AGC GCA CTG AG) (TIB MOLBIOL, Berlin, Germany) containing restriction sites for EcoRI and BamHI. Underlined bases correspond to our Legionella-specific primers (see below). The resulting fragment was cloned into the plasmid pUC18 and transformed into E. coli DH5α by heat shock treatment. The correct clone was named pNWLeg2. Plasmid DNA was isolated with a commercially available kit (QIAfilter Plasmid Midi Kit; Qiagen) and stored in small aliquots at −20°C until analysis by PCR.

Primers, probes, and PCR assay. The LightCycler PCR and detection system (Roche Diagnostics) was used for amplification and real-time quantification. For the quantitative, genus-specific PCR assay, oligonucleotide primers that amplify a 386-bp portion of the 16S rRNA gene from base 451 to base 837 of L. pneumophila ATCC 33152 were used. Forward primer JFP (5'-AGG GTT GAT AGG TTA AGA AGC GAG GCC ATC CGT C) and reverse primer JRP (5'-CCA ACA GCT AGT TGA AGG GTT GAT AGG TTA AGA AGC GCA CTG AG) (TIB MOLBIOL) were previously described by Jonas et al. (10). For amplicon detection, the LightCycler DNA Master Hybridization Probes Kit (Roche) was used as described in the manufacturer’s manual. Briefly, two different oligonucleotide probes hybridize to an internal genus-specific sequence of the amplified 16S rRNA gene of legionellae. Probe Leg LC has been labeled at the 5’ end with the LightCycler Red 640 fluorophore (5’-LC Red640-TAC TGA CAC TGA GGC AAA GCG GT) and probe Leg FL has been labeled at the 3’ end with fluorescein (5’-AGT GGC GAA GGC GGC TAC CT) (TIB MOLBIOL). During amplification, fluorescein is excited by the light source of the LightCycler instrument. The excitation energy is transferred to the acceptor fluorophore, LightCycler Red 640, and the emitted fluorescence after annealing is measured by the photobridist of the hybrid.

The internal inhibitor control was added to each sample in order to control for PCR inhibitors. It can be amplified with the primers JFP and JRP and can be detected by lambda-specific hybridization probes LAM FL (5'-AGT GGC GAA GGC GGC TAC CT) and LAM LC (5'-AGT GGC GAA GGC GGC TAC CT) (TIB MOLBIOL). The excitation energy is transferred to the acceptor fluorophore, LightCycler Red 640, and the emitted fluorescence after annealing is measured by the photobridist of the hybrid.

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For the *L. pneumophila*-specific PCR, forward primer Lp-mip-PT09 (5'-GCA TTG GTG CCG ATT TGG) and reverse primer Lp-mip-PT70 (5'-GCTTTTT TGG CCC AAT GTT TCT GAA) were used (sequences are from the formerly commercially available EnviroAmp Legionella Amplification Kit by Perkin-Elmer Cetus, Rodgau-Jüdesheim, Germany). They amplify a 168-bp fragment of the *L. pneumophila* *mip* gene. The amplified fragment was detected with mip-specific hybridization probes LPneu FL (5'-CCCC CTC ATA GCG TCT TGC ATG CCT TTA) and LPneu LC (5'-GCG TGT CACT GAT TAA CAT CTA TGC C) (TIB MOLBiol). The PCR mixture for the *mip* gene PCR contained 5 µl of sample DNA, 1 µl (10 pmol) of primers Lp-mip-PT09 and Lp-mip-PT70, 2 µl (0.2 pmol) of probes LPneu FL and LPneu LC, 2.4 µl of MgCl2 (final concentration, 4 mM), 2 µl of Master Hybridization Probes reaction mix (Roche), and PCR-grade sterile water to a final volume of 20 µl. Cycling conditions were the same as for the 16S rRNA gene PCR.

**Validation of the external standard curve for quantification.** For external standards, genomic DNA was extracted from *L. pneumophila* serogroup 1 (ATCC 33152) and stored in AE buffer. After photometric determination of the DNA concentration, MS2 RNA (Roche; final concentration, 10 ng/µl) was added (ATCC 33152) and stored in AE buffer. After photometric determination of the DNA concentration, MS2 RNA (Roche; final concentration, 10 ng/µl) was added.

**Statistical analysis.** LightCycler data were analyzed by the LightCycler software version 3.4 (Roche Diagnostics). Statistical correlations were calculated with the Spearman rank correlation test.

**RESULTS**

**Specificity of the 16S rRNA gene PCR assay.** The 16S rRNA gene primers used have been reported earlier to detect all medically important legionellae (8). We have further tested the primers and the newly developed hybridization probes on a panel of human-pathogenic and environmental legionellae and have obtained positive PCR results with all strains (described in Table 1), as well as with *Legionella*-like amoebal pathogen (LLAP) 10. Additionally, all known serogroups of *L. pneumophila* could be detected. The specificity of the primers for the genus *Legionella* has been described previously (8). We have investigated an expanded control panel of bacteria (see Materials and Methods) and have not found any cross-reactivity. Discrimination was achieved mainly by the forward primer, which had at least seven mismatches with the control bacteria, and even a strain with a target sequence of the reverse primer identical to that of *L. pneumophila* could be discriminated (Table 2). A faint signal at 40 PCR cycles was seen with 10 pg of DNA from a *Methylobacterium* sp., isolated from one of the water samples, but since a 1-pg DNA dilution remained repeatedly negative, this cross-reactivity at high concentrations can be disregarded. The target sequences of the 16S rRNA gene primers for *Methylobacterium* *extorquens* are depicted in Table 2 for illustration.

**Determination of the external standard curve.** In order to establish a quantitative PCR, we first developed a standard curve of serial 10-fold dilutions of *L. pneumophila* serogroup 1 genomic DNA from 1 ng to 1 fg per PCR (Fig. 1A). Statistical analysis revealed high reproducibility of the standards in the range of 1 ng to 10 fg per PCR, since the coefficients of variation were between 0.047 and 0.035 (interassay variance; n = 10) and between 0.034 and 0.006 (intra-assay variance; n = 10) and thus within known limits of the LightCycler (7). The standard curve was created by the second-derivative maximum method of the LightCycler software. This is a software algorithm which identifies the first turning point of the fluorescence curve (i.e., the first maximum of the second-derivative curve in terms of curve discussion), which serves as the crossing point in the calculation of the standard curve. The standard curve is the linear regression line through the data points on a plot of the crossing point versus the logarithm of the standard sample concentration (Fig. 1B). Since the crossing points are determined by a software algorithm, this method has the advantage that interpretation of the data values at different time points and by different investigators will give identical results. With the aid of the standard curve, the concentrations of unknown samples have been calculated. In 6 of 10 experiments a lower limit of detection of 1 fg of DNA/PCR (which corresponds to less than one *Legionella* organism) was achieved, but due to the
FIG. 1. Generation of the standard curve for the 16S rRNA gene PCR. (A) Serial 10-fold dilutions of \textit{L. pneumophila} serogroup 1 DNA were amplified with the primers JFP and JRP and detected by the hybridization probes Leg FL and Leg LC (results from 1 representative experiment out of 10 are shown). (B) The standard curve was generated by linear regression of the threshold cycle where the fluorescence signal first exceeds the level of background noise versus the logarithm of the sample concentration, using the second-derivative maximum method of the LightCycler software (see Results). Fluorescence intensity is in units obtained by the LightCycler.
low reproducibility this concentration was not considered in the calculation of the standard curve.

In order to determine the sensitivity of our PCR assay with respect to the copy number of our amplicon, we also measured a dilution series of purified 16S rRNA gene PCR products and repeatedly found a positive signal at 0.5 ag (i.e., \(0.5 \times 10^{-18}\) g), which corresponds to one copy of our target gene (data not shown). To confirm the accuracy of our photometric DNA concentration determination, a limiting-dilution assay was done with 20 repeats of a calculated one-copy standard, and 7 of these samples were detected as positive in the PCR. Although the sample size is very small for statistical calculation, sufficient accuracy of the photometric DNA concentration measurement can be assumed [calculated copy number by limiting-dilution assay = \(-\ln(\text{negative samples/total amount of samples tested}) = -\ln(7/20) = 1.05\)]

Quantitative determination of legionellae in water samples by 16S RNA gene PCR. For quantitative determination of legionellae, 77 water samples were investigated by culture and LightCycler PCR. Legionellae were detected by culture in 54 of 77 water samples (70.1%), with a mean CFU \(\pm\) standard deviation of 19 \(\pm\) 29.3 (median, 10.0; range, 1 to 150) per 100 ml. With the LightCycler PCR assay, DNA of Legionella spp. was detected in 76 of 77 water samples (98.7%). The one sample which remained negative was shown to contain PCR inhibitors. Those were detected by an absence of amplification of the internal inhibitor control in our dual-color multiplex PCR assay. Legionella DNA in the water samples was quantified with an external standard curve, generated as described above. A representative example of the PCR for four unknown water samples with external standard curve and internal inhibitor controls is shown in Fig. 2.

As the genome of one Legionella organism consists of approximately 4.3 fg of DNA (5), we could calculate the quantity of legionellae in the water sample. Under our assay conditions, 10 fg of Legionella DNA per PCR corresponds to approximately 23 legionellae per 100 ml of potable water. Thus, 23 legionellae/100 ml was our lower limit for quantification (Fig. 3). Compared to the results obtained by culture, no false-negative PCR results were obtained in the water samples without PCR inhibitors. However, all culture-negative samples (23 of 77) revealed Legionella-specific signals in the PCR (Fig. 3).

The specific amplification of legionellae has been assumed, since the PCR products had comparable melting temperatures as determined in the LightCycler melting-curve analysis (data not shown). Median PCR results for the culture-negative samples, however, were lower than those for the culture-positive samples (58 fg [mean, 174.6 fg; range, 5 to 1,194 fg] versus 157 fg [mean, 230.7 fg; range, 3 to 1,092 fg]). Overall, there was an association between the amounts detected by culture and those determined by PCR (Fig. 3), but the statistical correlation was weak \((r = 0.57; P < 0.001)\). Most Legionella counts calculated from the PCR standard curve were higher than those detected by culture by a median factor of 25 (mean, 68). Lower Legionella counts in the PCR than in culture have not been detected (Fig. 3).

Specific detection of L. pneumophila in water samples by mip gene PCR. Since L. pneumophila is the most pathogenic Legionella species and a frequent contaminant in environmental water sources, we further established an L. pneumophila-specific PCR hybridization probe assay based on amplification of the mip gene. The mip gene PCR was specific for L. pneumophila and detected all serogroups listed in Table 1, but it detected none of the non-L. pneumophila legionellae. As in the 16S rRNA gene PCR, the mip gene was detected quantitatively by means of external standards of L. pneumophila serogroup 1 DNA. The mip gene PCR had a sensitivity equal to that of the 16S rRNA gene PCR, and therefore, a similar range of the standard curve was used. All but one of the 16S rRNA gene PCR-positive water samples showed positive signals in the mip PCR (Fig. 4), thus suggesting the presence of L. pneumophila.

Attempts to sequence the 16S rRNA gene PCR product of the one culture- and mip PCR-negative sample were unsuccessful, since this sample contained minimal amounts (2 CFU/100 ml) of the above-mentioned Methylobacterium sp. The Legionella sp. and the Methylobacterium sp. could, however, be distinguished by melting-curve analysis of the PCR product of another LightCycler PCR assay (unpublished data). In all culture-positive samples (53 of a total of 76), the mip PCR results could be confirmed by L. pneumophila-specific immunofluorescence testing of the cultured legionellae. The sequence of the 16S rRNA gene PCR product of two mip PCR-positive but culture-negative samples (from 23 such samples of a total of 76) revealed identity with the published L. pneumophila sequence. In comparison to the results obtained by 16S rRNA gene PCR, mip PCR results had a tendency to show smaller DNA amounts and thus lower calculated numbers of CFU per 100 ml but showed a correlation with the 16S rRNA gene PCR results (Fig. 4) \((r = 0.66; P < 0.001)\).

DISCUSSION

Contamination of hospital water with legionellae is a well-known cause of nosocomial legionellosis. Rapid identification of the source of infection is essential to prevent further cases. While L. pneumophila is the main causative agent of legionellosis, other species, such as L. micdadei, L. dumoffii, and L. bozemaniae, are also known to cause disease in humans (13, 14, 20, 22).

In order to overcome the difficulties and limitations of Legionella detection by culture, we developed a new real-time LightCycler PCR assay for quantitative determination of legionellae in potable water samples that can be run in less than 45 min. Taking into account the time taken for sample preparation and DNA isolation, the total assay time is less than 5 h. Our genus-specific 16S rRNA gene PCR assay was specific for Legionella spp. and detected all 31 Legionella species tested, including all human-pathogenic legionellae. The 16S rRNA gene is exceptionally suited as a target gene since it exists in multiple copies per genome and thus allows a high sensitivity of the PCR (27). The sensitivity of our assay was 1 fg of Legionella DNA per PCR. Quantification was done with external standards consisting of 10-fold dilutions of L. pneumophila serogroup 1 genomic DNA. By means of this method, statistically validated quantification was possible at concentrations of as low as 10 fg per PCR. Another possibility for generation of an external standard is the use of purified PCR products of the target gene. We also tested this method and found a very high sensitivity of one copy of our target gene. However, since the exact copy number of the 16S rRNA gene in Legionella is not
known and may vary from species to species, this method may not be suitable to determine the exact concentration of legionellae. Third, a standard curve generated by dilution of viable legionellae with subsequent DNA isolation closely reflects the amount of viable legionellae in the samples. When using this method, we found a correlation between CFU and DNA quantities but insufficient reproducibility, and therefore we used 10-fold DNA dilutions to construct the standard curve. Quantification with external standards requires that the amplification efficiencies of the standards and the sample be identical. Since we used homologous standards (*L. pneumophila* DNA) and since fluorescence signals of the samples showed parallel increments compared to the standards (Fig. 2A), identical efficiencies can be assumed in our experiments.

As the presence of PCR inhibitors in environmental water samples is well known and remains a limiting factor for PCR detection techniques (12, 17, 24, 27), they have to be ruled out in each PCR assay. Therefore, we developed an internal inhibitor control that is measured simultaneously with our target gene in a dual-color multiplex LightCycler PCR assay. This method has the advantage of rapid detection of both the target gene and possible PCR inhibitors, while sensitivity of the PCR assay was not affected to a relevant extent. With this PCR assay, legionellae were detected in all but one of the water samples. In the one PCR-negative sample, PCR inhibition was identified.

Compared to culture results, the median numbers of CFU per 100 ml calculated from the PCR results were 25-fold.

FIG. 3. Comparison of culture results with the 16S rRNA gene PCR results. Water samples (*n* = 76 [one sample contained PCR inhibitors and was disregarded]) were investigated by culture and 16S rRNA gene PCR as described in Materials and Methods. The detection limit for culture was 1 CFU/100 ml, and the lowest limit for quantification in the PCR was 10 fg of *Legionella* DNA/PCR, i.e., 23 CFU/100 ml (detection limit of 1 fg of DNA/PCR, i.e., 2.3 CFU/100 ml). The ratios between the CFU counts detected by culture and those calculated by 16S rRNA gene PCR are indicated by the dotted lines. Symbols on the line indicating the lowest limit for quantification (i.e., 23 CFU/100 ml) represent samples in which the number was less than or equal to this limit but different from zero.

FIG. 2. Dual-color multiplex PCR of the *Legionella* 16S rRNA gene with external standards and internal inhibitor control. Standards (*L. pneumophila* serogroup 1 DNA; amounts per PCR assay are shown) and water samples were amplified according to the protocol described in Materials and Methods. The internal inhibitor control (1 fg of DNA/PCR assay) was added to each reaction mixture. Amplifications of the *Legionella* DNA and the inhibitor control were detected in fluorescence channels F2 (A) and F3 (B), respectively. A positive signal from the control signifies the absence of PCR inhibitors in the sample. The lack of signals from standards 1, 2, and 3 is caused by inhibition of the control DNA by the large amounts of standard DNA. Results from one representative experiment with 4 water samples out of a total of 77 samples are shown; fluorescence intensity is in units obtained by the LightCycler.
higher. This finding is not surprising, since rates of recovery in culture are usually noticeably less than 100% (24) due to fastidious growth requirements, overgrowth by other bacteria, and damage to the legionellae by the concentration steps. In contrast, PCR detection methods also include nonculturable legionellae and have been shown earlier to exceed sensitivity of culture (3, 12, 21, 27). In our study, overgrowth by contaminating bacteria has been overcome by the use of the selective GVPC medium, which was superior to other Legionella-selective media in previous experiments (data not shown). This medium also permits the best recovery of legionellae from water samples compared to other selective and nonselective media (data not shown), but the exact recovery rate is not estimable. Damage to the bacteria during the concentration steps most probably plays an important role, since our own experiments revealed a recovery rate of only 10 to 20% after centrifugation and concentration of spiked water samples (unpublished data). Concentration of water samples by filtration methods may be less hazardous to the bacteria, but growth on the filter may be more demanding for the bacteria, since we found comparable recovery rates for centrifugation and filtration methods (unpublished data). CFU may also underestimate the true number of bacteria, since legionellae can occur in clumps or may have been concentrated in amoebae. Nevertheless, the large amounts of legionellae detected by PCR may also represent nonviable cells or only Legionella DNA which is not infectious to humans. Even amplification and detection of nonlegionella DNA cannot be completely excluded, since alignment of the target sequence of the primers can never include all environmentally occurring, known and unknown, bacteria. Therefore, the high PCR signals should be critically interpreted and do not necessarily represent a health risk for exposed persons.

Although an association between the degree of Legionella contamination and the occurrence of legionellosis has been described (6, 13), an association with the exact concentration of legionellae remains unclear (6, 13). Fluctuations in the amount of legionellae as well as nonreliable culture detection

FIG. 4. Comparison of 16S rRNA gene PCR results with mip gene PCR results. Water samples (n = 75 [one sample contained PCR inhibitors and was disregarded, and one sample was mip PCR negative]) were investigated by 16S rRNA gene PCR and mip gene PCR as described in Materials and Methods. CFU counts per 100 ml were calculated from the PCR results. The dotted line represents a 1:1 ratio between the CFU counts per 100 ml detected by the 16S rRNA gene PCR and the mip gene PCR. Symbols on the lines indicating the lowest limit for quantification (i.e., 2³ CFU/100 ml) represent samples in which the number was less than or equal to these limits but different from zero.
methods may be possible reasons. In our experiments, exact amounts of legionellae per water sample have been calculated from the PCR results. However, due to the low variability of the standard curve and inevitable minimal inaccuracies during DNA isolation and dilution, it may be more appropriate to show the results in categories rather than as exact values. The invention and implementation of new PCR techniques, such as the method described in this paper, may provide a tool for the assessment of an association between Legionella concentration and risk of disease.

Since L. pneumophila is the main cause of legionellosis and can provoke the often life-threatening Legionnaires’ disease, we also developed a quantitative L. pneumophila-specific PCR assay, based on the mip gene of L. pneumophila. This PCR detects all 14 serogroups of L. pneumophila but none of the non-L. pneumophila legionellae and had sensitivity equal to that of the 16S rRNA gene PCR. All but one of the 16S rRNA gene PCR-positive water samples were also positive in the mip gene PCR, thus suggesting the presence of L. pneumophila. This could be confirmed by culture, sequencing of the PCR product, and detection of exclusively L. pneumophila during the previous years. Unfortunately, the mip-negative sample could not be further identified by sequencing. The presence of L. pneumophila alone might be characteristic of the three water systems investigated. However, the design of the study would not allow detection of a mixed culture of L. pneumophila and non-L. pneumophila strains or LLAP. According to surveys of the German National Reference Laboratory for Legionella during the past three decades, L. pneumophila is by far the most abundant Legionella species in potable and environmental water samples in Germany (15, 16; P. C. Luck and J. H. Helbig, Abstract. 5th Int. Conf. Legionella, abstr. 76, 2000), but other species, such as L. bozemanii and L. dumoffii, also occur (14, 22).

In conclusion, the newly developed Legionella genus-specific and L. pneumophila species-specific quantitative LightCycler PCRs with an internal PCR inhibitor control proved to be sensitive and specific for quantitative determination of legionellae in potable water samples. It may therefore be particularly useful both for routine testing for Legionella contamination and for screening of large sample amounts during outbreak investigations (18) in as short a time as possible. A possible benefit for investigation of clinical samples may be proven in further studies.

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