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Legionella Confirmation Using Real-Time PCR and SYTO9 Is an Alternative to Current Methodology

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The currently accepted culture techniques for the detection of *Legionella* spp. in water samples (AS/NZS 3896:1998 and ISO 11731 standard methods) are slow and laborious, requiring from 7 to 14 days for a result. We describe a fully validated rapid confirmation technique that uses real-time PCR incorporating the intercalating dye SYTO9 for the direct identification of primary cultures, significantly decreasing turnaround time and allowing faster remedial action to be taken by the industry.

Legionellae are ubiquitous organisms and are commonly found in lakes and rivers, usually at low levels (17). These organisms infiltrate water distribution systems and multiply in a variety of man-made constructions, including cooling towers, spa pools, misters, fountains, showers, and ice machines (4). The conditions in these environments make human infection possible via inhalation (or microaspiration in the case of ice machines [9]) of contaminated aerosols. The majority of human infections are caused by *Legionella pneumophila* serogroup 1, although in Australia and New Zealand, numerous cases have been attributed to *Legionella longbeachae* found in potting mixes, supposedly due to the use of pine and eucalypt products (11, 16).

There is still no consensus regarding the infectious dose and environmental levels of *Legionella* that are necessary for the spread of disease. Data of *Legionella* counts from cooling towers implicated in outbreaks are not readily available, but counts between 1,000 CFU/ml (3) and 100,000 CFU/ml (6) have been found in suspected sources, whereas counts found in potable water supplies in nosocomial settings have been very low (18).

Regardless of outbreak source or infective dose, there is a need for a faster culture and confirmation technique for *Legionella*. *Legionella* detection methods adopted in most testing laboratories are based upon the ISO 11731 (2) or the AS/NZS 3896:1998 (3) method in Australia and New Zealand, which are considered the "gold standards." These culture methods are similar and require traditional confirmation of *Legionella*-like isolates by subculture on media that primarily challenge the cysteine requirement of the microorganism. This approach in itself presents interpretational challenges, particularly for slow-growing and unusual species of bacteria. For example, the ISO 11731 method requires subculture onto buffered charcoal-yeast extract (BCYE) agar minus cysteine, but *Legionella oakridgensis* will grow on this medium (13). Several other methodologies exist, each with advantages and disadvantages. The direct fluorescent-antibody (DFA) assay is tedious, lacks sensitivity, can be cross-reactive with non-*Legionella* isolates, and cannot discriminate between culturable and nonculturable cells (5). Numerous direct PCR methodologies have been reported, and despite obvious speed advantages, they cannot discriminate between culturable and nonculturable cells. Previously, a commercially available PCR kit, the EnviroAmp *Legionella* kit (Perkin Elmer), was used for *Legionella* detection in water samples and was adapted for direct colony confirmation using PCR (14) but was subsequently withdrawn from the market due to specificity concerns. Another limitation of alternative methodologies is that the interpretation of the significance of the results is difficult because action levels for the detection of *Legionella* are based on counts derived using the standard culture techniques. This is particularly the case for individuals who now employ the AS/NZS 3666.3 part 3 method (1) as part of performance-based maintenance procedures which dictate remedial actions based on reported colony counts.

We describe here a colony-based confirmatory assay for the rapid identification of *Legionella pneumophila* and *Legionella* spp. using real-time PCR and a double-stranded-DNA-intercalating dye, SYTO9, recently described by Monis et al. (12). In total, 148 isolates from 144 samples (potable waters, evaporative tower water, and cooling tower water) were included in this evaluation. This assay delivered cost and time savings and also allowed the culture, confirmation, and serogrouping of *L. pneumophila* in as few as 3 days.

Isolation of *Legionella* from water samples and latex agglutination assays. Water samples (500 ml) were examined without preconcentration in accordance with the AS/NZS 3896:1998 method (3). In brief, 0.1 ml was inoculated onto buffered charcoal-yeast extract agar base (code CM0655; Oxoid, Basingstoke, Hampshire, United Kingdom) with MWY selective supplement (code SR0118; Oxoid), and 0.01 ml was inoculated onto BCYE agar with BMPA selective supplement (code SR0111; Oxoid). An aliquot was heat treated at 50°C for 30 min, and 0.1 and 0.01 ml were inoculated onto MWY agar. Additionally, 1 ml of sample was acid treated in 9 ml of HCl-KCl acid buffer (pH 2.2) for 5 min, and 0.1 ml was inoculated onto BMPA agar. All plates were incubated at 35°C. Our laboratory protocol was to examine plates for *Legionella*-like organisms on days 3, 5, and 7, and the suspect isolates were subcultured onto BCYE and horse blood agar (Medvet,

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TABLE 1. ATCC strains used for testing the specificities of the *mip* and 16S rRNA gene assays

Organism	ATCC accession no.	16S rRNA PCR result	*mip* PCR	
			Take off[a]	Result
Legionella pneumophila serogroup 1	ATCC 43111	+	<25	+
Legionella pneumophila serogroup 13	ATCC 43736	+	<25	+
Legionella pneumophila serogroup 9	ATCC 35298	+	<25	+
Legionella pneumophila serogroup 1	ATCC 33152	+	<25	+
Legionella pneumophila serogroup 6	ATCC 33215	+	<25	+
Legionella pneumophila serogroup 1	ATCC 33153	+	<25	+
Legionella anisa	ATCC 35292	+	>25	−
Legionella micdadei	ATCC 33218	+	>25	−
Legionella longbeachae serogroup 1	ATCC 33462	+	>25	−
Legionella longbeachae serogroup 2	ATCC 33484	+	>25	−
Legionella cincinnatiensis	ATCC 43753	+	>25	−
Legionella sainthelensi	ATCC 35248	+	>25	−
Legionella santicrucis	ATCC 43119	+	>25	−
Legionella oakridgensis	ATCC 33761	+	>25	−
Legionella bozemanii	ATCC 33217	+	>25	−
Legionella birminghamensis	ATCC 43702	+	>25	−
Legionella bozemanii serogroup 1	ATCC 33217	+	>25	−
Legionella bozemanii serogroup 2	ATCC 35545	+	>25	−
Legionella brunensis	ATCC 43878	+	>25	−
Legionella cherrii	ATCC 35252	+	>25	−
Legionella dumoffii	ATCC 33279	+	>25	−
Legionella erythra	ATCC 35303	+	>25	−
Legionella fairfieldensis	ATCC 49588	+	>25	−
Legionella feeleii serogroup 1	ATCC 35489	+	>25	−
Legionella feeleii serogroup 2	ATCC 358449	+	>25	−
Legionella gormanii	ATCC 33297	+	>25	−
Legionella gratiana	ATCC 49413	+	>25	−
Legionella hackeliae	ATCC 35999	+	>25	−
Legionella israelensis	ATCC 43119	+	>25	−
Legionella jamestownensis	ATCC 35298	+	>25	−
Legionella jordanis	ATCC 33623	+	>25	−
Legionella lansingensis	ATCC 49751	+	>25	−
Legionella maceachernii	ATCC 35300	+	>25	−
Legionella moravica	ATCC 43877	+	>25	−
Legionella parisiensis	ATCC 35299	+	>25	−
Legionella quinlivanii	ATCC 43830	+	>25	−
Legionella rubrilucens	ATCC 35304	+	>25	−
Legionella spiritensis	ATCC 35249	+	>25	−
Legionella steigerwaltii	ATCC 35302	+	>25	−
Legionella tucsonensis	ATCC 49180	+	>25	−
Legionella wadsworthii	ATCC 33877	+	>25	−
Legionella worsliensis	ATCC 49508	+	>25	−
Aeromonas hydrophila	ATCC 7966	−	>25	−
Enterobacter aerogenes	ATCC 13048	−	>25	−
Escherichia coli	ATCC 1175	−	>25	−
Citrobacter freundii	ATCC 8090	−	>25	−
Bacillus subtilis	ATCC 6333	−	>25	−
Klebsiella pneumoniae	ATCC 13883	−	>25	−
Pseudomonas aeruginosa	ATCC 10145	−	>25	−
Pseudomonas fluorescens	ATCC 13523	−	>25	−

[a] "Take off" is defined as the cycle at which exponential amplification starts.

Adelaide, South Australia) and incubated for a further 3 days. For this study, isolates were also subcultured on either MWY or BMPA, depending on their origins during primary isolation. Gram-negative organisms that grew on BCYE (and on MWY/BMPA) and not on horse blood agar were reported to be *Legionella*. Serogrouping with the *Legionella* latex kit (code DR0800; Oxoid) was performed according to the manufacturer's instructions with isolates subcultured onto BCYE and onto MWY or BMPA. An alternate isolate confirmation method (real-time PCR) was run concurrently with selective media using the methods described below.

Control organisms and DNA template preparation. Experiments to optimize and validate PCR were performed using the bacterial strains listed in Table 1. Single colonies of control strains or environmental isolates were used to prepare colony suspensions with a McFarland standard of 0.5 (in phosphate-buffered saline), and 5-μl volumes of the suspensions were added directly to each PCR.

Confirmation of isolates using real-time PCR and melting curve analysis. *Legionella* 16S rRNA gene PCR was performed as described previously (7, 10, 19) except that Ampli*Taq* Gold was used as the DNA polymerase, the reaction volume was

25 μl, and the cycling conditions were changed to an initial hold at 95°C for 10 min, followed by 40 cycles consisting of 94°C for 20 s, 60°C for 20 s, and 72°C for 25 s. *L. pneumophila*-specific PCR was performed using primers *mip* 99F (5′ TGT CTTATAGCATTGGTGCC 3′) and *mip* 213R (5′ CAATTG AGCGCCACTCATAG 3′) (8) under the same cycling conditions. For both *mip* and 16S rRNA assays, 5 μl of template DNA was used in a 25-μl reaction mixture that included 1× PCR buffer II (Applied Biosystems, New Jersey), 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate mix (Promega Corporation, Madison, WI), 0.5 μM each of the forward and reverse primers, 3.34 μM SYTO9 (Molecular Probes, OR), and 1 U AmpliTaq Gold (Applied Biosystems, New Jersey). All reactions were carried out in a RotorGene 3000 (Corbett Research, Sydney, Australia) with data acquisition at 72°C on the 6-carboxyfluorescein channel (excitation at 470 nm, detection at 510 nm) at a gain of 5. Amplification takeoff (defined as the cycle at which exponential amplification starts) was determined using the comparative quantitation feature of the RotorGene software for the amplification data acquired at a gain of 5. Following amplification, melting curve data were acquired on the 6-carboxyfluorescein channel (at gains of 2 and 5) using a ramping rate of 1°C/60 s from 75°C to 95°C. The differentiated data were analyzed using RotorGene software with the digital filter set as "none." When required, samples were analyzed by 1% agarose gel electrophoresis with the addition of Gelstar nucleic acid stain (Cambrex Bio Science, Rockland, Inc.) using standard methods (15).

Results and discussion. The specificity of the real-time PCR was determined by challenging the assays using the organisms listed in Table 1. The specificity of the 16S rRNA gene assay has been described previously (7, 10, 19) and was confirmed by melting curve analysis, producing a characteristic melting temperature (T_m value) of 88 ± 1°C that corresponded to the detection of a 386-bp fragment by gel electrophoresis (data not shown). PCR using the *mip* primers amplified a 114-bp product with a T_m value of 82.5 ± 1°C from *L. pneumophila* and also from some non-pneumophila *Legionella* species such as *L. longbeachae* serogroups 1 and 2, *L. anisa*, *L. micdadei*, *L. cincinnatiensis*, *L. sainthelensi*, and *L. santicrucis*, but there was a notable difference in the cycles at which amplification started for pneumophila and non-pneumophila species (based on cycle takeoff value). As shown in Fig. 1A, amplification of DNA from *L. pneumophila* was detected within 12 to 21 cycles for the isolates tested. In comparison, amplification of DNA from other *Legionella* species occurred after 25 cycles. This difference was supported by melting curve analysis, which found that *L. pneumophila* samples had relative peak heights threefold greater than those of non-pneumophila *Legionella* species (Fig. 1B). These observations for the *mip* reactions can be attributed to differences in primer binding efficiency. The primers are exact matches for *L. pneumophila*, whereas there are between 3 and 7 base mismatches between the *mip* 99F or *mip* 213R primer and the corresponding regions of non-pneumophila *Legionella* species, including mismatches at the 3′ end of each primer. These mismatches cause poor priming from the genomic DNA of non-pneumophila *Legionella* species, resulting in an increase in the cycle number at which amplification is detected. Therefore, based on the differential takeoff values, any amplification of *mip* that is detected after cycle 25 is classed as negative for *L. pneumophila*.

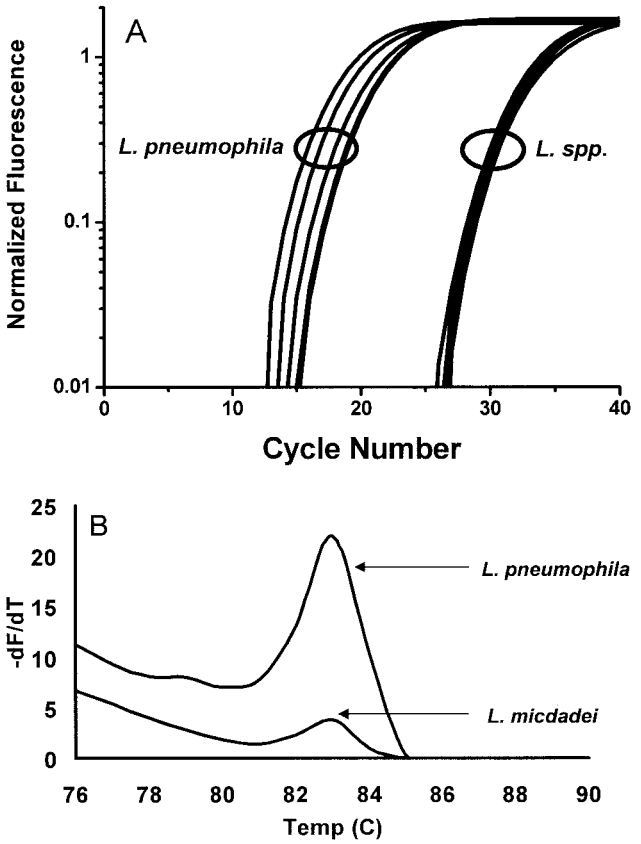

FIG. 1. A. Raw cycling data for *mip* PCR using SYTO9. B. Typical *mip* melting curve analysis of *L. pneumophila* and some *Legionella* spp. (such as *L. micdadei*) that may produce T_m values similar to those produced by *L. pneumophila* after 40 cycles. The relative peak height difference is used for differentiation in conjunction with the take-off value.

Direct addition of isolate suspensions into the PCR was not significantly different to extraction by boiling at 100°C for 10 min, suggesting that the initial denaturation step of 95°C for 10 min used in the PCR is sufficient to effectively lyse the cells and release template DNA (Fig. 2). Additionally, storage of boiled isolate suspensions at 4°C and −10°C for 1 week did not affect the performance of the assay. The inherent variations seen with the preparation of a suspension with a McFarland standard of 0.5 did not seem to affect the performance of this assay, eliminating the need to quantify DNA inoculums by other methods. It is important, however, to visually compare the isolate suspension to those in 0.5 McFarland standard comparator tubes that are commercially available in order to ensure standardization.

A total of 144 environmental samples yielding 148 isolates were analyzed by the rapid real-time PCR confirmation method and the traditional confirmation method. Of the 148 isolates tested, the standard method classed 57 as *Legionella* sp., 36 as *L. pneumophila*, and 55 as non-*Legionella* organisms. The rapid assay described here showed complete correlation with the standard method, with no disparities observed (Table 2). All PCR-negative

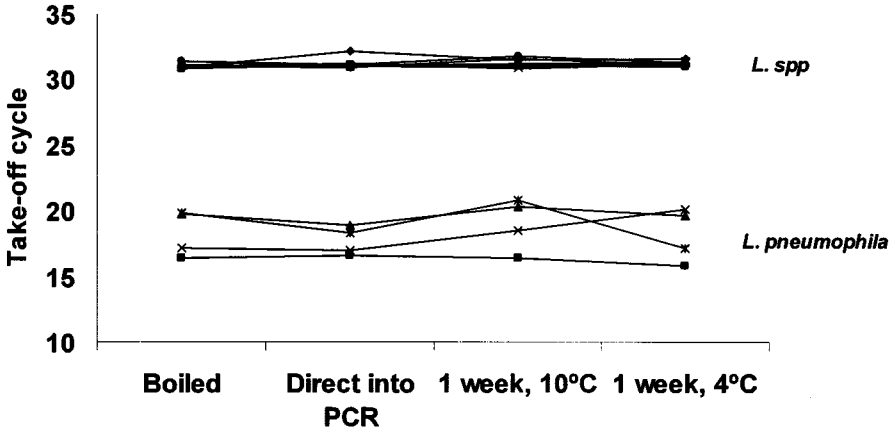

FIG. 2. Comparison of *mip* take-off cycles for template DNA preparation and storage.

samples were subjected to a repeat PCR (under similar conditions, except approximately 200 copies of *L. pneumophila* serogroup 1 DNA were incorporated into the PCR master mix) to monitor for PCR inhibition. In all cases the spiked DNA master mix plus the previously negative sample returned a positive result, demonstrating the absence of PCR inhibitors in the original isolate suspensions (data not shown).

Fifty *Legionella* isolates were included in a comparative study of latex agglutination assays from selective (MWY and BMPA) and nonselective (BCYE) media. Twenty-seven *L. pneumophila* serogroup 1, 9 *L. pneumophila* serogroup 2-14, and 14 latex agglutination-positive *Legionella* species were included in this comparison. There was no difference in performance between the latex agglutination assays when isolates were picked from selective and nonselective media. These results therefore indicate that *L. pneumophila* strains can be serogrouped directly from the primary isolation medium once confirmed by positive reactions for 16S rRNA and *mip* by real-time PCR.

The combined method of standard culture and real-time PCR confirmation is therefore capable of significantly decreasing turnaround times for *Legionella* identification and quantitation. For example, the current standard method may yield visible *L. pneumophila* colonies after 3 to 4 days and will require an additional 2 to 4 days to confirm by standard methods. A confirmed result in this case is available in 5 to 8 days. With the method we describe, the same colony can be used for real-time PCR confirmation and subsequent serogrouping, with a confirmed result available in 3 to 4 days; i.e., isolates can be identified as *Legionella* or *L. pneumophila* on the same day that they are visible. Similarly, slower-growing species such as *L. anisa* or *L. micdadei*, which typically appear after 4 to 6 days of incubation, can be confirmed and quantitated on the day that colonies are visible, compared to a further 2 to 4 days for traditional confirmation.

TABLE 2. Comparison of *Legionella* identifications of 16S rRNA and *mip* PCR with those of the AS/NZS 3896:1998 method

Species detected	No. of isolates identified (n = 148)		
	AS/NZS 3896:1998 method	Real-time PCR	
		16S rRNA gene	*mip*
Legionella species	57	57	0
L. pneumophila	36	36	36
Non-*Legionella* spp.	55	0	0

The incorporation of SYTO9 instead of the conventionally used dye SYBR green I required minimal optimization and did not lead to any interpretative difficulties. SYTO9 is an exciting alternative to SYBR green I in the diagnostic setting since the assays employing this dye seem more robust and insensitive to changes in DNA concentration, which is in direct contrast to SYBR green I for selected amplicons as described by Monis et al. (12). Monis et al. compared the performance of SYTO9 to that of SYBR green I in a number of PCR targets in both prokaryotic and eukaryotic systems, including the 16S rRNA gene and *mip* gene described in this assay, and concluded that the use of SYTO9 in real-time PCR melting curve analysis is superior to the use of SYBR green I. The assay described here follows from the work of Monis et al. and is the first reported use of SYTO9 in a diagnostic setting that has been extensively validated with field samples and isolates, and it seems that the use of SYTO9 may lend itself to real-time PCR users wishing to fast track optimization and implementation of real-time PCR assays.

In conclusion, we have described a rapid assay for *Legionella* that complements the current culture-based standard methods. This rapid method is easy to employ and could be implemented by most water testing laboratories. The results from this assay can be used in the same context as data generated using the current methods, therefore allowing rapid response to a confirmed quantitative count. The need to respond to counts is highlighted by prescriptive standards (e.g., AS/NZS 3666.3) where remedial action is based upon a confirmed colony count. Direct PCR from samples, therefore, continues to be of little use, as little information aside from the presence or absence (of viable, nonviable, or mixed cells) is obtained. As real-time PCR technology becomes more accessible and adopted in routine testing laboratories, rapid-PCR methods such as that described here will eventually supersede traditional methods for confirmation of bacterial identification. In a time when swift responses are not only required but demanded, the uptake of

such a method will facilitate the administration of remedial action in a much more timely fashion.

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