

Targeting Species-Specific Low-Affinity 16S rRNA Binding Sites by Using Peptide Nucleic Acids for Detection of Legionellae in Biofilms

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Using fluorescence in situ hybridization to detect bacterial groups has several inherent limitations. DNA probes are generally used, targeting sites on the 16S rRNA. However, much of the 16S rRNA is highly conserved, with variable regions often located in inaccessible areas where secondary structures can restrict probe access. Here, we describe the use of peptide nucleic acid (PNA) probes as a superior alternative to DNA probes, especially when used for environmental samples. A complex bacterial genus (*Legionella*) was studied, and two probes were designed, one to detect all species and one targeted to *Legionella pneumophila*. These probes were developed from existing sequences and are targeted to low-binding-affinity sites on the 16S rRNA. In total, 47 strains of *Legionella* were tested. In all cases, the *Legionella* spp. PNA probe labeled cells strongly but did not bind to any non-*Legionella* species. Likewise, the specific *L. pneumophila* PNA probe labeled only strains of *L. pneumophila*. By contrast, the equivalent DNA probes performed poorly. To assess the applicability of this method for use on environmental samples, drinking-water biofilms were spiked with a known concentration of *L. pneumophila* bacteria. Quantifications of the *L. pneumophila* bacteria were compared using PNA hybridization and standard culture methods. The culture method quantified only 10% of the number of *L. pneumophila* bacteria found by PNA hybridization. This illustrates the value of this method for use on complex environmental samples, especially where cells may be in a viable but noncultivable state.

Fluorescence in situ hybridization (FISH) is a valuable technique and has been widely used (1, 3, 10, 21). It does, however, have a number of limitations which have restricted its applicability in certain areas. These are mainly related to obtaining sufficient signal strength while maintaining target specificity (21, 37). Sequences on the 16S rRNA are often chosen as target sites for probe development due to their high copy numbers in all cells (4, 6, 13, 21, 37). The 16S rRNA molecule has been shown to have a high degree of evolutionary stability. There are areas of high variability providing species specificity, but these are often located in highly inaccessible regions of the 16S rRNA structure. This is clearly illustrated in the *Escherichia coli* 16S rRNA structure model given by Fuchs et al. (13). From this model, it becomes clear that many of the specific sequences which are useful for probe design (i.e., showing the required variability between separate species) are located in these inaccessible areas of the 16S rRNA molecule (7, 8, 13).

The use of peptide nucleic acid (PNA) probes may provide a suitable alternative to traditional DNA probes. PNAs are DNA analogues with a 2-aminoethyl-glycine linkage replacing the phosphodiester backbone found in DNA (11, 22). The synthetic PNA molecule remains able to bind in a sequence-specific manner to both DNA and RNA, obeying Watson-Crick hydrogen bonding rules (11, 12). Their novel chemistry provides resistance to enzymatic cleavage, and once bound, they demonstrate high thermal stability and are nonionic (25, 26). In practical terms, these characteristics allow them to be

used in hybridization studies with less-stringent hybridization conditions, including those for melting temperature, formamide, and salt concentrations (5, 26, 31, 36, 37).

In the current study, members of the genus *Legionella* were used in a FISH assay. This is a complex genus, with several species being serious human pathogens (32, 34). *Legionella* spp. are widespread in the environment and are often found in association with other microorganisms, for example, coexisting inside amoebae and/or forming part of the biofilm community (15). There is a requirement to improve the available detection methods to enable a more accurate understanding of environmental populations and their potential to cause outbreaks. A number of studies have developed DNA probes for use in FISH assays (14, 20), but these have not been extensively used for detection. Manz et al. (20) designed a DNA probe, LEG226, to detect all *Legionella* spp. LEGPNE1 was developed to specifically target *Legionella pneumophila* (14). Using these published probes as a template, PNA probes were designed and developed. The aim of the current study was to use PNA probes based on these existing DNA probe sequences to assess binding to low-affinity (but highly variable) sites on the 16S rRNA and subsequently improve and develop a standardized FISH protocol. To ensure that this method could be successfully applied to environmental samples, spiked potable water biofilms were also tested, comparing the PNA hybridization method to standard culture detection methods. This builds on previous work by Lehtola et al. (17, 18), where probes were developed to detect *Campylobacter* and *Mycobacterium* species.

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MATERIALS AND METHODS

Bacterial strains and cultivation. Bacterial strains used in this study and their sources are indicated in Table 1. A total of 47 strains of *Legionella* were tested. These were supplied by Microgen Bioproducts (United Kingdom) and were stored at -80°C on microbeads (Protect system; Fisher Scientific, United King-

TABLE 1. Characteristics of *Legionella* strains and probes tested in this study^a

Strain no.	DNA probes		PNA probes	
	LEG 226	LEGPNEI	Class ^b	<i>L. pneumophila</i> spp.
<i>Legionella</i> sp.				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 1				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 1				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 1				
<i>L. pneumophila</i> sgp 1				
<i>L. pneumophila</i> sgp 1				
<i>L. pneumophila</i> sgp 1				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 2				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 3				
<i>L. pneumophila</i> subsp. <i>fraseri</i> sgp 4				
<i>L. pneumophila</i> subsp. <i>fraseri</i> sgp 5				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 6				
<i>L. pneumophila</i> sgp 7				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 8				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 9				
<i>L. pneumophila</i> sgp 10				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 11				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 12				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 13				
<i>L. pneumophila</i> subsp. <i>pneumophila</i> sgp 14				
<i>L. pneumophila</i> subsp. <i>fraseri</i> sgp 15				
<i>L. anisa</i> sgp 1				
<i>L. micdadei</i> sgp 1				
<i>L. dumoffi</i> sgp 1				
<i>L. gormanii</i> sgp 1				
<i>L. bozemanae</i> sgp 1				
<i>L. bozemanae</i> sgp 2				
<i>L. feeleii</i> sgp 1				
<i>L. rubrilucens</i> sgp 1				
<i>L. sainthelensii</i> sgp 1				
<i>L. erythra</i>				
<i>L. jamestowniensis</i> Sgp 1				
<i>L. hackeliae</i> sgp 1				
<i>L. hackeliae</i> sgp 2				
<i>L. macleodensis</i>				
<i>L. parisiensis</i> sgp 1				
<i>L. jordanis</i> sgp 1				
<i>L. spiritensis</i> sgp 1				
<i>L. longbeachae</i> sgp 1				
<i>L. longbeachae</i> sgp 2				
<i>L. wadsworthii</i> sgp 1				
<i>L. oakridgensis</i> sgp 1				
<i>L. cherrii</i>				
<i>L. quinlivanii</i>				
<i>L. israelensis</i>				
<i>L. steigerwalii</i>				
<i>L. birninghamensis</i>				
<i>L. cincinnatiensis</i>				

^a For strains listed in the ribosomal databases, the target sites of the DNA and PNA probes are given along with the classification of relative fluorescence based on the *E. coli* 16S rRNA map by Fuchs et al. (8), sgp group.

^b Six classes of relative fluorescence: class I (100 to 81% relative fluorescence compared to Eco1482), class II (80 to 61%), class III (60 to 41%), class IV (40 to 21%), class V (20 to 6%), and class VI (5 to 0%).

dom). All *Legionella* strains were grown on buffered charcoal-yeast extract (α BCYE) agar (Oxoid, United Kingdom) at 37°C for 2 to 4 days. Non-*Legionella* strains were grown under the following conditions: *E. coli* K12 and *E. coli* O157 (NCTC 12900) were grown on nutrient agar at 37°C for 16 to 24 h; *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* were grown for 7 days on mycobacteria 7H10 agar at 37°C; *Helicobacter pylori* was grown on Columbia blood agar plates in a microaerophilic atmosphere at 37°C for 2 to 4 days. These are all pathogenic species which have been found in or are known to present a risk to potable water supplies (32).

Probe design and synthesis. DNA probes specific to all *Legionella* spp. (LEG226) (20) and only to strains of *L. pneumophila* (LEGPNE1) (14) were synthesized (Eurogentec, Belgium). LEG226 has the sequence 5'-TCG GAC GCA GGC TAA TCT-3' and was synthesized with the fluorophore 6-carboxyfluorescein (excitation, 494 nm; emission, 525 nm) linked at the 5' end. The specific *L. pneumophila* probe, LEGPNE1, has the sequence 5'-ATC TGA CCG TCC CAG GT T-3' and was synthesized with the fluorophore 6-carboxytetramethylrhodamine (excitation, 565 nm; emission, 580 nm) linked at the 5' end. The specificities of these probes were checked using the Ribosomal Database Project II (<http://rdp.cme.msu.edu/>) (9) and National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/blast/>) ribosomal databases. The target positions of each probe on all strains listed in these ribosomal databases are given in Table 1.

PNA probes were designed based on the sequences of these preexisting DNA probes. The optimum probe length of PNA is 15-mer, and there should be no self-complementary structures (31). For the detection of all *Legionella* spp., the sequence 5'-GAC GCA GGC TAA TCT-3' (i.e., omitting the first three 5' nucleotides of the DNA probe) was used for synthesis with the fluorophore 6-carboxyfluorescein, attached at the 5' end (Eurogentec, Belgium) (PLEG200). Ribosomal databases indicated that this sequence would target sites on all the *Legionella* spp. currently listed with at least a 1-mer difference from any other bacterial group. For the specific detection of *L. pneumophila*, the sequence 5'-CTG ACC GTC CCA GGT-3' (i.e., omitting the first two 5' nucleotides and the last nucleotide of the DNA probe) was used (PLPNE620). This was synthesized with the fluorophore 6-carboxytetramethylrhodamine, attached at the 5' end (Eurogentec, Belgium). Database analysis indicated that this probe would target sites on all the *L. pneumophila* strains listed with at least a 1-mer mismatch with non-*pneumophila* strains. The target sites of these two PNA probes for all strains listed in the ribosomal databases are listed in Table 1.

Cell fixation. A loopful (10- μ l inoculation loop) of test bacteria was removed from an agar plate and resuspended in 350 μ l sterile phosphate buffered saline (PBS) and vortex mixed. A 25- μ l aliquot of this suspension was placed on a clean glass microscope slide (polytetrafluoroethylene [Teflon]-coated multispot slides) (C. A. Hendley Ltd., United Kingdom). This resulted in approximately 10³ cells per slide. The samples were allowed to air dry. Two fixation methods were tested, one used in the DNA probe protocols (14, 20) and the other used for PNA hybridization (6, 17, 18). Following the method given for the DNA probes (14, 20), the bacteria were fixed by covering of the slides with 4% (wt/vol) paraformaldehyde-PBS solution and leaving them at room temperature for 1 h. Slides were then washed once with PBS and dehydrated in an aqueous ethanol series (50, 80, and 96%, 10 min in each). The alternative fixation method involved gently flaming the slides with the dried bacterial suspension, covering them with 90% ethanol, and leaving them for 10 min before air drying.

Hybridization methods. Three separate hybridization methods were tested. Two followed the DNA hybridization protocols given for the preexisting DNA probes (14, 20), and one was modified from protocols specified for PNA probes (6, 17, 18, 30).

DNA hybridization for LEG226. Following fixation, 25- μ l aliquots of hybridization buffer (20% [vol/vol] formamide, 0.9 M NaCl, 0.01% [vol/vol] sodium dodecyl sulfate (SDS), 20 mM Tris-HCl, pH 7.6) with or without 200 nM probe were added to the slide preparations (20). Slides were placed in a hybridization chamber and incubated at 46°C for 90 min. Following incubation, slides were covered with prewarmed washing buffer (180 mM NaCl, 0.01% [vol/vol] SDS, 20 mM Tris-HCl, pH 7.2) and incubated at 46°C for a further 15 min. Slides were rinsed with filter-sterilized distilled water and left to dry in the dark.

DNA hybridization for LEGPNE1. After fixation, 25 μ l of hybridization buffer (25% [vol/vol] formamide, 0.9 M NaCl, 0.01% [vol/vol] SDS, 20 mM Tris-HCl, pH 7.6) with or without 200 nM probe was added to the samples. Slides were incubated in a hybridization chamber at 43°C for 2 h, after which they were covered with prewarmed washing buffer (5 mM EDTA-160 mM NaCl, 0.01% [vol/vol] SDS, 20 mM Tris-HCl, pH 7.6) and left at 43°C for a further 20 min (14). Slides were then rinsed with filter-sterilized distilled water and left to dry in the dark.

PNA hybridization. A 25- μ l aliquot of hybridization buffer (30% [vol/vol] formamide, 0.10 M NaCl, 10% [wt/vol] dextran sulfate, 0.1% [wt/vol] sodium pyrophosphate, 0.2% [wt/vol] polyvinylpyrrolidone, 0.2% [wt/vol] Ficoll, 5 mM

disodium EDTA, 0.1% [vol/vol] Triton X-100) with or without 200 nM probe was added to each slide. Slides were placed in a hybridization chamber for 90 min and incubated at selected temperatures between 50 and 65°C. Following hybridization, slides were covered with prewarmed washing buffer (15 mM NaCl, 0.1% [vol/vol] Triton X-100, 5 mM Tris-HCl, pH 10) and incubated at the same temperature for 30 min. Slides were then rinsed with filter-sterilized distilled water and left to dry in the dark.

Microscopic examination. Once the slides were dry, a drop of nonfluorescent immersion oil (Fluka, United Kingdom) or ProLong antifade reagent (Molecular Probes) was put onto slide preparations and a coverslip placed on top. A further drop of nonfluorescent immersion oil was placed on top of the coverslips. All slides were examined using a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope, under oil, using 40 \times Plan APO, 60 \times Plan APO, and 100 \times Plan APO objectives (Best Scientific, United Kingdom) (16).

Biofilm experiments. In order to validate the PNA hybridization method for use with environmental samples, tests were conducted on laboratory-generated potable water biofilms spiked with *L. pneumophila*. A Propella reactor (Xenard, Mechanique de Precision, France) (24, 33) was run with potable water at 20°C for 10 days at a flow rate of 183 ml h⁻¹. Polyvinylchloride (PVC) coupons were removed from the reactor and a known concentration of *L. pneumophila* serogroup 1 (Philadelphia) bacteria added. *L. pneumophila* bacteria were grown on α BCYE agar plates at 30°C. The spiked coupons were then allowed to air dry at room temperature. Coupons were then prepared for PNA hybridization or for culture analysis. PNA hybridization was carried out using the method previously described for slide hybridizations, with some modifications. Following air drying, the coupons were flamed gently and covered with 90% ethanol (10 min, room temperature). After this fixation step, the surfaces of the coupons were covered with the probe/hybridization solution and incubated at 57°C for 90 min. Coupons were then immersed in PNA washing buffer for a further 30 min (56°C) before being rinsed with filter-sterilized water and kept in the dark prior to analysis. The coupons were examined using the EDIC/EF microscope system. Coupons to be used for culture analysis were placed in 50-ml centrifuge tubes containing 5 ml filter-sterilized tap water. These were then vortex mixed for 2 min to remove the biofilm and cells from the surfaces. This method follows the instructions of international standard ISO 11731 (14a) on the preparation of environmental samples for quantification of legionellae. Serial dilutions of this mixed bacterial suspension were made using filter-sterilized tap water and aliquots plated onto α BCYE and glycine-vancomycin-polymyxin B-cycloheximide (GVPC) agar plates (Oxoid, United Kingdom). These were incubated for up to 14 days at 30°C (28). For each analysis method, at least six coupons were used.

RESULTS

A comprehensive set of experiments was completed to test whether PNA probes can improve labeling when targeted to low-binding-affinity sites on the 16S rRNA. In all cases, reference slides were examined as controls; these were exposed to identical treatments but did not have the appropriate probe added to the hybridization buffer. For all probes tested, no labeling of non-*Legionella* species was observed, indicating the specificity of the probes.

Initially, two different cell fixation and dehydration methods were assessed. In all cases, it was observed that diffuse background fluorescence in all fluorescence channels on the microscope system was greatly increased when paraformaldehyde was used. In contrast, the simple flaming and single dehydration step did not increase this fluorescence or disrupt the cells in any noticeable way. This is illustrated in Fig. 1, where *L. pneumophila* serogroup 8 cells were fixed using the two methods. No probes were added to these preparations. The general fluorescence of the cells was clearly increased following fixation with paraformaldehyde (Fig. 1a), compared to that of cells fixed by flaming (Fig. 1b). In addition, there was increased clumping of cells, resulting in areas of diffuse fluorescence. For all subsequent experiments, the PNA fixation method was employed.

The results for each probe and hybridization method will be

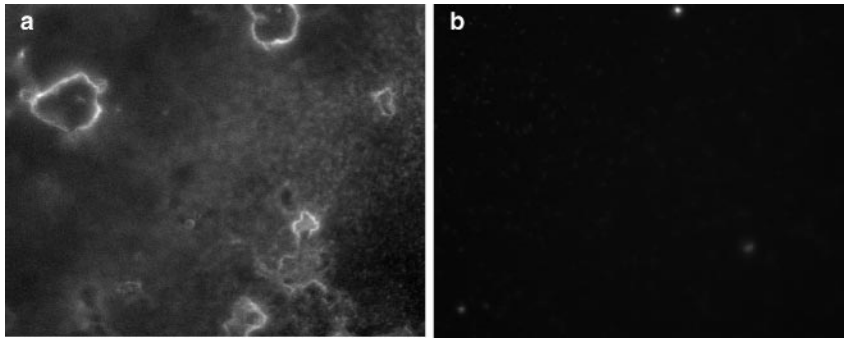


FIG. 1. Comparison of fixation methods using unlabeled *L. pneumophila* serogroup 8 bacteria. (a) Fluorescence visible after fixation with 4% paraformaldehyde-PBS and ethanol dehydration series. (b) Fixation by flaming and dehydration in 90% ethanol, with no fluorescence visible. Immersion oil was used as described in the text. Magnification, $\times 600$.

described separately. A summary of the results for each hybridization method (following fixation using the PNA method) is given in Table 2.

LEG226 DNA probe. A total of 20 non-*pneumophila* *Legionella* species were tested with the DNA probe LEG226 in addition to 11 strains of *L. pneumophila* (Table 2). Using this probe with the method described by Manz et al. (20), results indicating differing levels of binding efficiency were obtained (Table 2). If binding correctly, this probe should label all species tested (total of 31) (including all *L. pneumophila* strains); however, clear, positive results were observed for only 21 strains (an example is shown in Fig. 3e, where there was strong labeling of *L. pneumophila* serogroup 1 [Philadelphia]). Within this group, reference slides (where no probe had been added) of three strains (*Legionella spiritensis*, *Legionella wadsworthii*, and *Legionella maceachernii*) also gave strong positive signals, indicating a degree of autofluorescence either from the cells or caused by the treatment process. For the remaining 18 positive strains, eight of the reference slides also gave a faint positive signal. This is illustrated in Fig. 2a, where *Legionella jordanis* was tested. The bacteria shown in Fig. 2b were hybridized with LEG226, and although the cells fluoresced, there was no clear noticeable difference with the reference slide (where no probe was added [Fig. 2a]). The 10 strains which did not give a clear positive signal upon addition of the probe gave two distinct responses; 4 showed strong labeling of a proportion of the cells while others remained unlabeled, and 6 strains exhibited only very faint labeling. A general observation in all samples was that there was clumping of bacteria, making it difficult to see individual cells (Fig. 2a and b and 3e).

LEGPNE1 DNA probe. The DNA probe LEGPNE1 was tested here on a total of 31 strains of *Legionella*, including 11 strains of *L. pneumophila* (Table 2). The binding efficiencies of LEGPNE1 varied between strains, and if working correctly, positive signals should have been observed for only the 11 *L. pneumophila* strains and not for the remaining 20 non-*pneumophila* species. However, only six of the *L. pneumophila* strains gave clear positive signals, and one of these (*L. pneumophila* serogroup 1 [Philadelphia]) gave a similar positive reaction for the reference sample (where no probe had been added) (Fig. 3a and b). Of the remaining *L. pneumophila* strains, two (*L. pneumophila* serogroup 1 [Bellingham] and *L. pneumophila* serogroup 11) gave only faint signals, and three

(*L. pneumophila* serogroup 9, *L. pneumophila* serogroup 10, and *L. pneumophila* serogroup 13) showed positive labeling of some cells but no visible labeling of others.

In addition, for the 20 non-*pneumophila* species, strong positive labeling was observed for 8 (e.g., *L. jordanis*, as shown in Fig. 2e) and faint labeling for 3 species. Of the eight showing strong labeling, three showed similar levels of fluorescence in the reference samples (where no probe was added), indicating a degree of autofluorescence from the bacteria or an artifact of the method. In general, all preparations exhibited clumping of the cells (Fig. 2e and 3a and b) and diffuse fluorescence around the bacteria (clearly seen in Fig. 3a and b).

PNA hybridizations. The PNA probes, PLEG200 and PLPNE620, were tested over a range of hybridization temperatures (from 50 to 65°C), and at temperatures higher than 55°C, no differences could be seen between samples; all hybridizations showed equally strong signals. At hybridization temperatures less than 55°C, only a proportion of the bacterial population showed labeling with the probes.

The results for all strains are summarized in Table 2, which shows the reactions with each probe and describes any observed autofluorescence. A total of 20 strains of *L. pneumophila* were tested with both PLEG200 and PLPNE620. Positive reactions were observed for all these strains with both probes (e.g., Fig. 3d and f, whereas the reference slide in Fig. 3c shows no labeling in comparison to a strong signal when the probes have been added). Of the 20 strains of *L. pneumophila* tested, 2 (*L. pneumophila* serogroup 1 [Allentown] and *L. pneumophila* serogroup 10) showed noticeable degrees of autofluorescence in all fluorescence channels. The other *Legionella* spp. tested all showed positive reactions with PLEG200 (e.g., Fig. 2c and d, where reference and treatment slides with a suspension of *L. jordanis* are shown), and in most cases, negative results were observed with PLPNE620 (e.g., Fig. 2f, showing no labeling of *L. jordanis* with the PLPNE620 probe). Some cells of *Legionella anisa* were labeled by PLPNE620, though not the entire population, suggesting that this could be due to an inefficient hybridization. Positive labeling was also observed for *Legionella longbeachae* serogroup 1 and *Legionella quinlivanii*. A high degree of autofluorescence was seen for *L. quinlivanii* in the red tetramethyl rhodamine isocyanate (TRITC) channel, and this has therefore masked the results. In contrast, *L. longbeachae* serogroup 1 did not exhibit any clear autofluores-

TABLE 2. Observations following hybridization with both DNA and PNA probes^a

<i>Legionella</i> sp. and sgp	Autofluorescence	Result for DNA probe		Result for PNA probe		
		LEG 226	LEGPNE1	Autofluorescence	PLEG200	PLPNE620
<i>L. pneumophila</i> sgp 1 (Olda)				–	+	+
<i>L. pneumophila</i> sgp 1 (Bellingham)	–	Faint +	Faint +	Faint	+	+
<i>L. pneumophila</i> sgp 1 (Philadelphia)	– Faint (TRITC)	+	+	–	+	+
<i>L. pneumophila</i> sgp 1 (Knoxville)	Faint (FITC/TRITC)	+	+	–	+	+
<i>L. pneumophila</i> sgp 1 (Benidorm)				–	+	+
<i>L. pneumophila</i> sgp 1 (Allentown)	+ (FITC/TRITC)	Faint +	+	Strong	+	+
<i>L. pneumophila</i> sgp 2	Faint (FITC/TRITC)	+	+	Faint	+	+
<i>L. pneumophila</i> sgp 3	Faint (FITC)	Faint +	+	–	+	+
<i>L. pneumophila</i> sgp 4				–	+	+
<i>L. pneumophila</i> sgp 5				–	+	+
<i>L. pneumophila</i> sgp 6				–	+	+
<i>L. pneumophila</i> sgp 7				–	+	+
<i>L. pneumophila</i> sgp 8	–	Faint +, but not all	+	–	+	+
<i>L. pneumophila</i> sgp 9	–	+	+, but not all	Faint	+	+
<i>L. pneumophila</i> sgp 10	–	+	+, but not all	Strong	+	+
<i>L. pneumophila</i> sgp 11	Faint (FITC) –	+	Faint +	–	+	+
<i>L. pneumophila</i> sgp 12				–	+	+
<i>L. pneumophila</i> sgp 13	–	Faint +	+, but not all	–	+	+
<i>L. pneumophila</i> sgp 14				–	+	+
<i>L. pneumophila</i> sgp 15				–	+	+
<i>L. anisa</i> sgp 1	–	+	–	–	+	+/-, inconclusive
<i>L. micdadei</i> sgp 1	Faint (FITC), strong (TR)	+	+	–	+	–
<i>L. dumoffi</i> sgp 1	Faint (FITC)	+	–	–	+	–
<i>L. gormanii</i> sgp 1	–	+	+	–	+	–
<i>L. bozemanæ</i> sgp 1	Faint (FITC) –	+	–	–	+	–
<i>L. bozemanæ</i> sgp 2	–	+	–	–	+	–
<i>L. feeleii</i> sgp 1	– Faint (TRITC)	+	Faint +	–	+	–
<i>L. rubrilucens</i> sgp 1				–	+	–
<i>L. sainthelensi</i> sgp 1	Faint (FITC), some (TR)	+	+, but not all	–	+	–
<i>L. erythra</i>				–	+	–
<i>L. jamestowniensis</i> sgp 1	–	Faint +, but not all	+, faint	–	+	–
<i>L. hackeliae</i> sgp 1	Faint (FITC) –	+, but not all	–	–	+	–
<i>L. hackeliae</i> sgp 2	–	+, but not all	–	–	+	–
<i>L. maceachernii</i>	+ (FITC/TRITC)	+	+, but not all	–	+	–
<i>L. parisiensis</i> sgp 1	Faint (FITC) –	Faint +	–	–	+	–
<i>L. jordanis</i> sgp 1	Faint (FITC) –	+	+	–	+	–
<i>L. spiritensis</i> sgp 1	+ (FITC), faint (TRITC)	+	–	Strong in FITC	+	–
<i>L. longbeachae</i> sgp 1				–	+	+
<i>L. longbeachae</i> sgp 2				–	+	–
<i>L. wadsworthii</i> sgp 1	+ Bright	+	+	–	+	–
<i>L. oakridgensis</i> sgp 1				Strong in FITC	+	–
<i>L. cherrii</i>				–	+	–
<i>L. quinlivanii</i>	Faint (FITC), strong (TR)	+	+	Strong in TRITC	+	+
<i>L. israelensis</i>	– Some (TRITC)	+	Faint +	–	+	–
<i>L. steigerwaltii</i>	–	+, but not all	Faint +	–	+	–
<i>L. birminghamensis</i>	–	+	+, diffuse	–	+	–
<i>L. cincinnatiensis</i>				–	+	–

^a The autofluorescence values from samples treated only with buffer (no probe added) were noted. FITC refers to fluorescence in the green FITC channel; TRITC refers to fluorescence in the red TRITC channel. +, positive signal; –, negative signal; sgp, serogroup.

cence, but BLAST searches showed that it was the next-closest relative to the *L. pneumophila* strains listed. *L. longbeachae* is also a well-documented human pathogen (32).

Autofluorescence was assessed by examination of the bacteria which had been exposed to the hybridization buffer only. A number of *Legionella* spp. did show some degree of autofluorescence, in either one or all three of the selected fluorescence

channels (4',6'-diamidino-2-phenylindole [DAPI], fluorescein isothiocyanate [FITC], and TRITC) of the microscope. However, in comparison to experiments using DNA probes and hybridization methods, it was found that differences between autofluorescence and probe labeling could be easily distinguished; probe labeling was much stronger, and any autofluorescence faded rapidly.

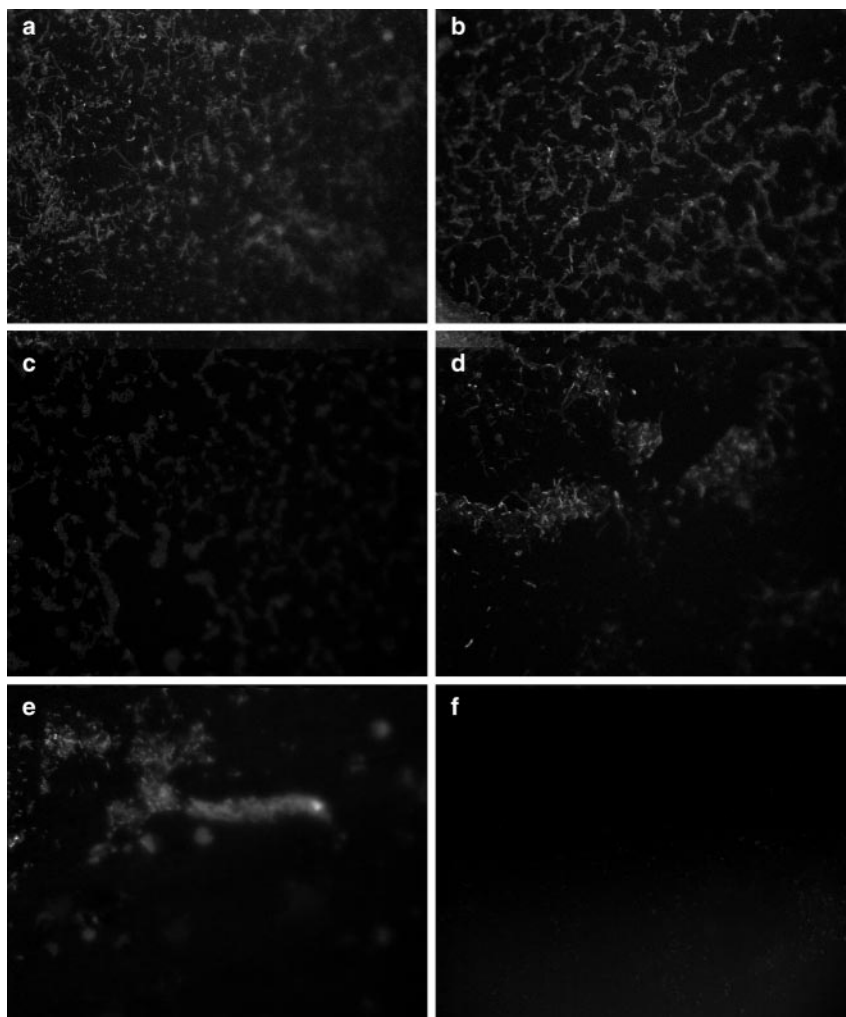


FIG. 2. *L. jordanis* after various hybridization treatments. (a) Following hybridization with buffers only (no probe added), using protocol designed for the DNA probe LEG226 (20). (b) Hybridization with LEG226 DNA probe. (c) Hybridization with PNA protocol buffers only (23, 24). (d) Hybridization with PLEG200. (e) Hybridization with DNA probe LEGPNE1, using the protocol designed for this probe (21). (f) Hybridization with PNA probe PLPNE620. Immersion oil was used as described in the text. Magnification, $\times 600$.

Biofilm studies. Potable water biofilms spiked with *L. pneumophila* were used to directly compare the PNA hybridization procedure with standard culture methods for the detection of legionellae in environmental samples. Cultures were grown at 30°C (28); this lower temperature was used to ensure recovery as the biofilm coupons were being removed from a reactor run at 20°C. In addition, the *L. pneumophila* inoculum was also grown at 30°C. Prior to the spiking, the coupons were examined under the EDIC/EF microscope to confirm the presence of biofilm (in the EDIC channel) (Fig. 4a) and assess any autofluorescence of the biofilm or coupon (in the EF channels). No visible autofluorescence could be detected (data not shown). The PNA hybridization procedure was successfully used on the PVC coupons. Under the TRITC channel of the EDIC/EF microscope, it was possible to see labeled *L. pneumophila* bacteria clearly (Fig. 4b). As a control for nonspecific binding, biofilms not spiked with *L. pneumophila* showed no probe binding (data not shown). The absence of any labeling of the general biofilm indicates no cross-reactivity of the probes with the natural water biofilm species from this reactor.

The number of labeled legionellae could be easily quantified using this method (Table 3). The culture used had been diluted 1:10⁴, with approximately 10⁶ cells being added to each coupon. The results given show the mean values per cm² recovered from the coupon. These data were compared to numbers obtained from standard culture analysis. Using the culture method, fewer *L. pneumophila* bacteria could be detected (Table 3), with approximately 10% of the bacteria detected by use of the PNA hybridization method. The detection limit of the direct PNA-FISH method is difficult to determine because it is dependent upon the number of fields of view examined. In theory, it would be possible to detect a single labeled cell on a coupon.

DISCUSSION

The use of PNA probes in FISH assays to detect bacterial species has been reported previously (6, 17, 18, 23, 25, 27, 30, 31, 36), but the use of DNA probes remains the preferred

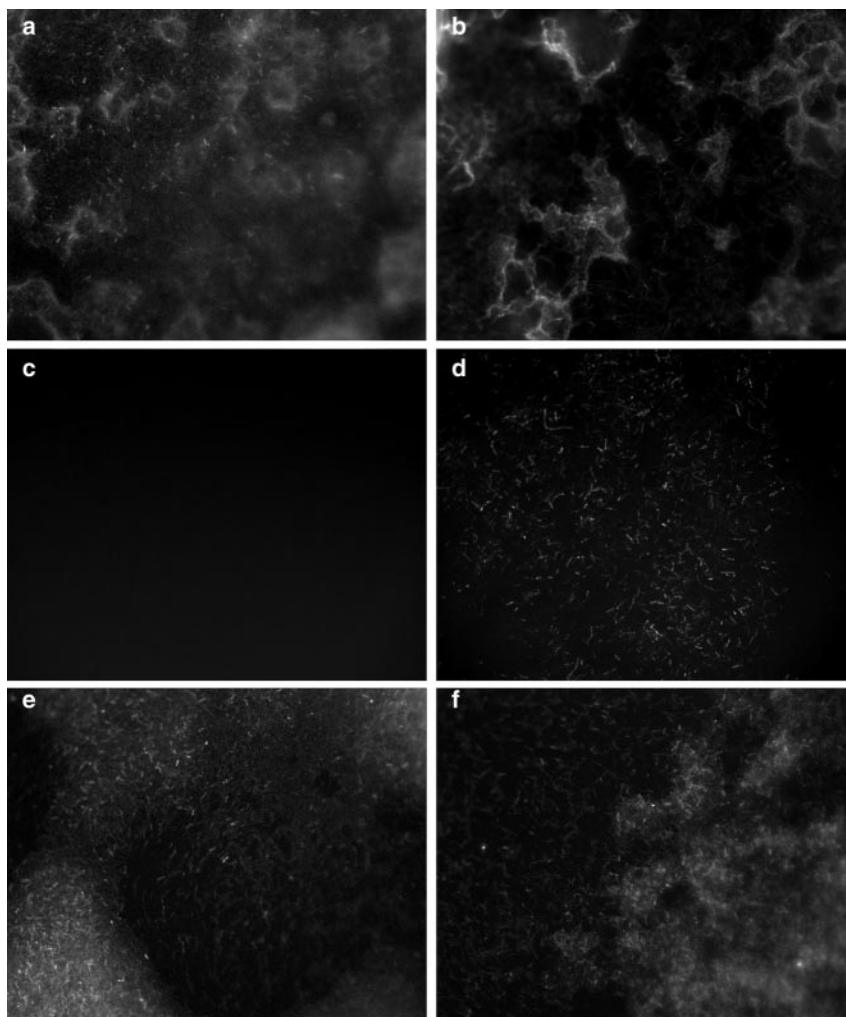


FIG. 3. *L. pneumophila* serogroup 1 (Philadelphia) after various hybridization treatments. (a) Following hybridization with buffers only (no probe added), using protocol designed for the DNA probe LEGPNE1 (21). (b) Hybridization with LEGPNE1 DNA probe. (c) Hybridization with PNA protocol buffers only (23, 24). (d) Hybridization with PLPNE620. (e) Hybridization with DNA probe LEG226, using the protocol designed for this probe (20). (f) Hybridization with PNA probe PLEG200. Immersion oil was used as described in the text. Magnification, $\times 600$.

approach. DNA probes do have limitations which have been discussed previously (37). The use of PNAs to overcome these problems has been mentioned, but this is the first study to conduct an extensive comparison of the efficiency of published

DNA probes with PNA probes developed from the same target sequences. The focus is on low-binding-affinity target sites, which develops further the work by Lehtola et al. (17, 18). The complex genus *Legionella* was used as an experimental model.

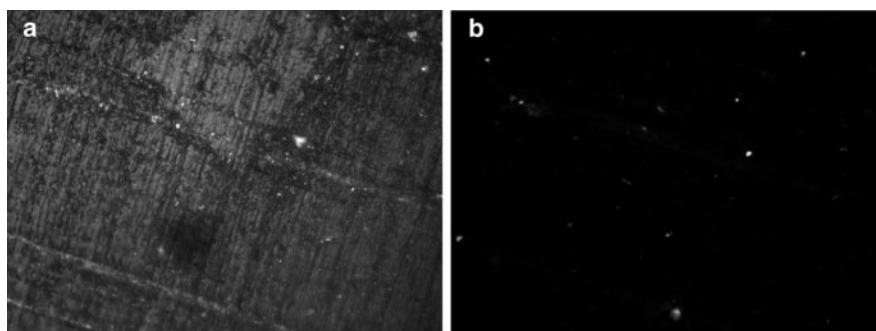


FIG. 4. EDIC photomicrograph of potable water biofilm on PVC coupon from the Propella reactor (a) and equivalent EF photomicrograph of PNA labeling of *L. pneumophila* spiked onto the biofilm (b). Magnification, $\times 1,000$.

TABLE 3. Numbers of *L. pneumophila* bacteria recovered from spiked biofilms on PVC coupons^a

Method of analysis	Mean no. of <i>L. pneumophila</i> organisms (cm ⁻²)	SE
PNA-FISH analysis	8.57×10^5	4.45×10^4
Culture method	8.38×10^4	5.08×10^3

^a The PNA-FISH method is compared with standard culture methods (ISO 11731), and mean data and standard errors are given. For each analysis, six separate reactor coupons were used. For PNA-FISH analysis, a minimum of 25 fields of view from across each coupon were quantified. For culture analysis, a dilution series was used, with three plates assessed from each dilution.

There is a requirement to improve detection methods for this pathogen.

Ribosomal database analysis indicates that the DNA probe LEG226 should bind only to members of the family *Legionellaceae* with at least one mismatch with any bacterial group (20). A single mismatch has been shown to be sufficient to prevent labeling of a nontarget organism (2, 19, 29), and this is understood to be stronger if a PNA probe is used (12). However, in the study by Manz et al. (20), it was found that LEG226 (used in a FISH assay) did not label all species of *Legionella* tested and that fluorescence intensity was poor in some cases. This could be due to a number of reasons related to the hybridization conditions. However, it may be caused by the location of the target sites on the 16S rRNA molecule. In the study by Manz et al. (20), it is stated that LEG226 targets sites between 226 and 243 on the 16S rRNA; according to the classification developed by Fuchs et al. (13), these sites are all class IV (hence, an expected fluorescence intensity of 21 to 40%). In the current study, we found LEG226 to target positions between 134 and 230 on the 16S rRNA of the different species (based on information in the ribosomal sequence databases), which corresponds to classes II to VI (an expected fluorescence intensity of 0 to 80%). The majority of target sites in this analysis are class IV, giving a binding affinity of only 21 to 40% for this probe. The PNA probe that was designed and developed (PLEG200) was based on the sequence of LEG226 but shortened in length by three nucleotides, as the optimum length for a PNA probe is 15-mer (31). PNA has a very strong binding affinity and so can be synthesized from shorter sequences, whereas the binding affinities of DNA probes are reduced at these shorter lengths. The ribosomal sequence databases indicated that specificity to only *Legionella* spp. was maintained with this shortened sequence. The results presented here show a clear improvement in specific labeling with PLEG200, compared to results obtained with LEG226. The PNA probe gave no false negative or false positive results, labeling all *Legionella* spp. tested but none of the non-*Legionella* species. Labeling was bright and consistent in contrast to results obtained with hybridizations using LEG226. The PNA probe could effectively and confidently be used in whole-cell FISH assays, indicating a distinct advantage over the DNA probe LEG226. LEG226 is known to give a weak hybridization signal, but here we have demonstrated that a PNA probe, based upon the same sequence and targeting the same sites, can provide a substantial improvement and permit accurate labeling of legionellae without generating potentially false negative results.

Similar observations were made when the DNA and PNA probes specific to *L. pneumophila* were compared (LEGPNE1 and PLPNE620, respectively). The original *L. pneumophila* DNA probe, LEGPNE1, was tested on six strains of *L. pneumophila* and five non-*pneumophila* species (14). In the original study (14), it is stated that LEGPNE1 is complementary to a highly variable region of the 16S rRNA. These target sites are located close to helix 23, which is known to be a less evolutionarily conserved region (13). LEGPNE1 labeled bacteria well, but as discussed by Grimm et al. (14), this was probably due to the high copy number of 16S rRNA within these actively growing cells (the probe was specifically used to detect cells surviving with amoeba). The PNA probe (PLPNE620) was designed from LEGPNE1 and targets sites on the same regions of the 16S rRNA. In the current study, both LEGPNE1 and PLPNE620 were tested on a large number of *Legionella* species, including several strains of *L. pneumophila*. This direct comparison clearly shows the improved performance of PLPNE620. Hybridization with PLPNE620 labeled all strains of *L. pneumophila* strongly but did not label the majority of non-*pneumophila* species. *L. quinlivanii* gave a positive signal, but this was found to be caused by strong autofluorescence from the bacteria themselves. A positive signal was also obtained for *L. longbeachae* serogroup 1; this was not due to autofluorescence, but this species is closely related to *L. pneumophila* (according to ribosomal database analysis), and it is also known to be a human pathogen (32). Autofluorescence is a potential limitation of the method, but this fades rapidly and can easily be distinguished from probe labeling. Additionally, the careful choice of fluorophore can prevent any interference from autofluorescence.

There are other characteristics of PNAs which are advantageous when they are used in FISH assays. Fixation has traditionally been done by the application of paraformaldehyde followed by dehydration (14, 20), and in this study, we have shown how this procedure can cause clumping of cells and an increase in diffuse background fluorescence. The fixation step is necessary to preserve cellular structure and also helps to permeabilize the cells. However, due to the shorter length of PNA probes, movement across the cell membranes is easier, and hence, less permeabilization is required (31). The simple fixation technique of flaming the samples followed by a single dehydration step can therefore be used (6, 17, 18).

The PNA probes did not label any of the non-*Legionella* species tested. The species chosen have all been detected in or are known to present a risk to potable water supplies (32). Further work is required to confirm that there is no cross-reactivity with related species, such as those of *Pseudomonas* and *Bacillus*.

The PNA-FISH method was then validated for use on environmental samples. Potable water biofilms were generated on PVC coupons in a Propella reactor (24, 33). These were spiked with *L. pneumophila*. Two quantification methods, direct analysis of the coupons using PNA-FISH and the removal of cells followed by culture analysis, were compared. Use of an EDIC/EF microscope permits the direct examination of biofilms on the coupon surface (16). Using the PNA-FISH protocol, it was possible to quantify the number of *L. pneumophila* bacteria on the coupons. Comparing the data with culture analysis revealed a 10-fold increase with the use of the PNA-

FISH method. The PNA-FISH method is a direct quantification method, whereas the culture analysis requires the physical removal of cells. This could lead to differences; however, control experiments with known numbers of legionellae have indicated that this method is efficient at cell removal (unpublished data). In addition, there was no nonspecific labeling of the biofilm, indicating no cross-reactivity with neighboring potable water biofilm species. The PNA-FISH method is particularly advantageous due to the limitations of the existing culture method. The culture method relies on the removal of cells from the biofilm; it also may not support the growth of potentially viable but noncultivable bacteria, and it takes up to 14 days for colonies to appear. In contrast, the PNA-FISH method is highly specific and rapid (data can be obtained within 4 h) and, combined with the use of the EDIC/EF microscope, provides information on the location of legionellae within the biofilm. This will aid our understanding of the spatial distribution of populations and their environmental growth requirements. These experiments were conducted on PVC coupons, but work is ongoing to test this combined PNA-FISH method on other substrates, such as cast iron (a common material used for main drinking-water supply pipes). In addition, preliminary work has indicated that this method can be successfully used to locate and quantify legionellae surviving in biofilms rather than spiked samples.

In summary, in all experiments, the PNA probes outperformed the DNA probes in terms of both target specificity and signal intensity. In the current study, an extensive list of strains was tested and clearly indicated the limitations of the original DNA probes. The use of PNA probes can permit the targeting of individual species/strains by allowing access to the highly variable but often inaccessible regions of the 16S rRNA molecule. The technique can also be used successfully on complex environmental samples, which have mixed bacterial populations. Such an approach will improve our understanding of the prevalence of these bacteria and help in the development of adequate control measures.

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