Applied and Environmental Microbiology, July 2010, p. 4476–4485 Vol. 76, No. 13
0099-2240/10/$12.00 doi:10.1128/AEM.01678-09
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Fluorescence *In Situ* Hybridization Method Using a Peptide Nucleic Acid Probe for Identification of *Salmonella* spp. in a Broad Spectrum of Samples[▽]

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Received 15 July 2009/Accepted 20 October 2009

A fluorescence *in situ* hybridization (FISH) method for the rapid detection of *Salmonella* spp. using a novel peptide nucleic acid (PNA) probe was developed. The probe theoretical specificity and sensitivity were both 100%. The PNA-FISH method was optimized, and laboratory testing on representative strains from the *Salmonella* genus subspecies and several related bacterial species confirmed the predicted theoretical values of specificity and sensitivity. The PNA-FISH method has been successfully adapted to detect cells in suspension and is hence able to be employed for the detection of this bacterium in blood, feces, water, and powdered infant formula (PIF). The blood and PIF samples were artificially contaminated with decreasing pathogen concentrations. After the use of an enrichment step, the PNA-FISH method was able to detect 1 CFU per 10 ml of blood ($5 \times 10^9 \pm 5 \times 10^8$ CFU/ml after an overnight enrichment step) and also 1 CFU per 10 g of PIF ($2 \times 10^7 \pm 5 \times 10^6$ CFU/ml after an 8-h enrichment step). The feces and water samples were also enriched according to the corresponding International Organization for Standardization methods, and results showed that the PNA-FISH method was able to detect *Salmonella* immediately after the first enrichment step was conducted. Moreover, the probe was able to discriminate the bacterium in a mixed microbial population in feces and water by counter-staining with 4′,6-diamidino-2-phenylindole (DAPI). This new method is applicable to a broad spectrum of samples and takes less than 20 h to obtain a diagnosis, except for PIF samples, where the analysis takes less than 12 h. This procedure may be used for food processing and municipal water control and also in clinical settings, representing an improved alternative to culture-based techniques and to the existing *Salmonella* PNA probe, Sal23S10, which presents a lower specificity.

Salmonella spp. are enteropathogenic bacteria that cause diseases that range from a mild gastroenteritis to systemic infections (5, 18) The disease severity is determined by the virulence characteristics of the *Salmonella* strain, host species, and host health condition. Phylogenetic analysis has demonstrated that the genus *Salmonella* includes two species: *Salmonella bongori* and *Salmonella enterica*. *Salmonella* strains are conventionally identified and classified according to the Kauffmann-White serotyping scheme, which is based on antigenic variation in the outer membrane (23). To date, more than 2,500 *Salmonella* serovars have been identified, and most of them are capable of infecting a wide variety of animal species and humans (33). *Salmonella* can be transmitted directly by person to person via the fecal-oral route or by contact with external reservoirs if fecal contamination of soil, water, and foods occurs. It is therefore necessary to develop robust detection methods for all of these sample types.

The diagnostic method currently used for *Salmonella* detection is bacterial culture (International Organization for Standardization [ISO] method 6579:2002), a time-consuming and laborious process (40). A rapid and reliable tool to assist disease control management should aim to reduce salmonellosis in both people and animals. For this purpose a number of assays, such as the enzyme-linked immunosorbent assay (ELISA), PCR, and fluorescence *in situ* hybridization (FISH), have been developed to decrease the time required to identify *Salmonella* in food, feces, water, and other clinical samples (8, 10, 14, 15, 25, 26, 31, 41).

Several authors have compared some of these approaches, especially culture-based, ELISA, and PCR methods, for *Salmonella* detection. Some authors found that PCR and ELISA-based methods failed to detect some samples that were positive by culture method (12, 13, 36, 39, 40). Even so, PCR-based methods have proved to be more accurate. Other work showed that when a selective enrichment step was performed before PCR, all *Salmonella* samples recovered by the culture method were detected. Moreover, the presence of *Salmonella* that was not recovered by the culture method could be detected by PCR (13, 35). These studies revealed that the enrichment step could increase the molecular assay sensitivity by eliminating problems such as the low numbers of bacteria and the presence of inhibitory substances in certain types of samples, such as food and fecal matter (11, 28, 36). However, PCR-based methods usually require a DNA extraction step, and none of the methods referred to above allows a direct, *in situ* visualization of the bacterium within the sample.

FISH is a molecular assay widely applied for bacterial iden-

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▽ Published ahead of print on 7 May 2010.

tification and localization within samples (2, 3). The method is usually based on the specific binding of nucleic acid probes to particular RNAs, due to their higher numbers of copies in the cells. There are already some studies reporting *Salmonella* detection by FISH using DNA probes (21, 29). A recently developed synthetic DNA analogue, named peptide nucleic acid (PNA), capable of hybridizing to complementary nucleic acid targets, has made FISH procedures easier and more efficient (38, 42). PNA-FISH methods have been successfully applied to the detection of several pathogenic microorganisms (6, 16, 17, 19, 22, 30, 34, 37, 42). For *Salmonella*, a PNA probe, designated Sal23S10, that targets the 23S rRNA of both *Salmonella* species has been already developed (31). However, the probe is also complementary to *Actinobacillus actinomycetemcomitans*, *Buchnera aphidicola*, and *Haemophilus influenzae* 23S rRNAs.

In this paper, we identify and describe the design of a new fluorescently labeled PNA probe for the specific identification of the *Salmonella* genus. A novel, rapid, and reliable PNA-FISH method that can be easily applied to a great variety of sample types, either clinical or environmental, has consequently been developed and optimized.

MATERIALS AND METHODS

Culture maintenance. The bacterial strains used in this study are listed in Table 1. All bacterial species, except for *H. pylori*, were maintained on tryptic soy agar (TSA) (VWR, Portugal) at 37°C and streaked onto fresh plates every 24 h. *H. pylori* strains were maintained on Columbia agar (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (Probiológica, Lisbon, Portugal). Plates were incubated at 37°C in a CO_2 incubator (HERAcell 150; Thermo Electron Corporation, Waltham, MA) set to 10% CO_2 and 5% O_2, and cells were streaked onto fresh plates every 2 or 3 days.

PNA probe design. To identify potentially useful oligonucleotides to use as probes, 17 23S rRNA gene sequences available at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/) were chosen. This selection contained 10 *Salmonella* sequences, including representative strains of each of the seven subspecies, and seven other strains from related species belonging to *Enterobacteriaceae* family (Fig. 1). The possible regions of interest were selected by sequence alignment using the ClustalW program available at the European Bioinformatics Institute (EBI) website (www.ebi.ac.uk/clustalw/).

The potentially useful oligonucleotide sequences were tested at the NCBI website to find the probe with the highest number of *Salmonella* sequences detected and the lowest number of non-*Salmonella* sequences detected. Criteria for the selection of the PNA probe also included the following: high GC percentage, no self-complementary structures, and melting temperature higher than 50°C.

Subsequently, the chosen sequence was synthesized (Panagene, Daejeon, South Korea), and the oligonucleotide N terminus was attached to Alexa Fluor 594 via a double AEEA linker.

Theoretical determination of sensitivity and specificity. The theoretical specificity and sensitivity of the probe were evaluated with the ProbeCheck program available on the Internet in Silva rRNA databases (24). It is important to note that for this theoretical estimation all 101 *Salmonella* strain sequences in the databases were considered, including only the good-quality sequences with >1,900 bp. The probe was aligned with a total of 11,124 sequences present in the large subunit ([LSU] 23S/28S) databases. It was also tested against the small subunit ([SSU] 16S/18S) databases to evaluate the existence of possible cross-hybridization with the 16S rRNA sequences. Specificity was calculated as nSs/(TnS) × 100, where nSs stands for the number of non-*Salmonella* strains that did not react with the probe and TnS is the total of non-*Salmonella* strains examined. Sensitivity was calculated as Ss/(TSs) × 100, where Ss stands for the number of *Salmonella* strains detected by the probe and TSs is the total number of *Salmonella* strains present in the databases.

Hybridization procedure on glass slides. Hybridization was performed as described in Guimarães et al. with some modifications (16). Smears of each strain were prepared by standard procedures and immersed in 4% (wt/vol) paraformaldehyde (Sigma) followed by 50% (vol/vol) ethanol for 10 min each and allowed to air dry. The smears were then covered with 20 μl of hybridization solution containing 10% (wt/vol) dextran sulfate (Sigma), 10 mM NaCl (Sigma), 30% (vol/vol) formamide (Sigma), 0.1% (wt/vol) sodium pyrophosphate (Sigma), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma), 0.2% (wt/vol) Ficol (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (vol/vol) Triton X-100 (Sigma), 50 mM Tris-HCl (pH 7.5; Sigma), and 200 nM PNA probe. Samples were covered with coverslips, placed in moist chambers, and incubated for 30 min at 57°C. Subsequently, the coverslips were removed, and the slides were submerged in a prewarmed (57°C) washing solution containing 5 mM Tris base (Sigma), 15 mM NaCl (Sigma), and 1% (vol/vol) Triton X (pH 10; Sigma). Washing was performed at 57°C for 30 min, and the slides were allowed to air dry. The smears were mounted with one drop of nonfluorescent immersion oil (Merck) and covered with coverslips. The slides were stored in the dark for a maximum of 24 h before microscopy.

Hybridization in suspension. The hybridization method was based on the procedure of Perry-O' Keefe et al. with slight modifications (31). For all strains, cells from 1-day-old cultures were harvested from TSA plates, suspended in sterile water, and homogenized by vortexing for 1 min. Subsequently, 1 ml of cell suspension was pelleted by centrifugation at 10,000 × *g* for 5 min, resuspended in 500 μl of 4% (wt/vol) paraformaldehyde (Sigma), and fixed for 1 h. The fixed cells were rinsed in autoclaved water, resuspended in 500 μl of 50% (vol/vol) ethanol, and incubated for 30 min at −20°C. Subsequently, 100 μl of the fixed-cell aliquot was pelleted by centrifugation and rinsed with sterile water, resuspended in 100 μl of hybridization solution with 200 nM PNA probe (as described above), and incubated at 57°C for 30 min. After hybridization, cells were centrifuged at 10,000 × *g* for 5 min, resuspended in 500 μl of wash solution (as described above), and incubated at 57°C for 30 min. Washed suspension was pelleted by centrifugation and resuspended in 500 μl of sterile water. Finally, 20 μl of the cell suspension was spread on a microscope slide, or 200 μl was filtered through a membrane (pore size, 0.2 μm; cellulose nitrate; Whatman). Samples were allowed to air dry; they were mounted with one drop of nonfluorescent immersion oil (Merck), and covered with coverslips. The slides were stored in the dark for a maximum of 24 h before microscopy.

***Salmonella* detection in artificially contaminated blood.** For the detection of *Salmonella* in artificially contaminated blood, 10 ml of horse blood (ProBiológica, Portugal) was mixed with 90 ml of tryptic soy broth (TSB) (VWR, Portugal) culture medium. TSB was inoculated at three different *Salmonella* contamination levels (1, 10, and 100 CFU/total volume) and incubated overnight at 37°C with agitation at 120 rpm. A noninoculated culture was prepared in parallel and exposed to the same conditions as a control. Samples of 1 ml were recovered from each culture to perform hybridization in suspension or on glass slides, as described above. The samples were diluted 1 to 10 before the hybridization procedure. *Salmonella* detection was also performed using selective and/or differential medium, such as MacConkey, xylose lysine desoxyscholate (XLD), or brilliant green phenol red agar (BGA), and also confirmative biochemical tests (triple sugar iron, urea agar, and Api 20E). This experiment was performed three times for two different strains, *S. enterica* serovar Enteritidis ATCC 13076 and *S. enterica* serovar Typhimurium LT2 (ATCC 43971).

The enriched culture concentration was determined by CFU count on TSA plates and by PNA-FISH. Quantification of the cell number by PNA-FISH was obtained by epifluorescence microscopy, as described bellow. A total of 15 fields with an area of 0.0158 mm^2 were counted using image analysis software, and the average was used to calculate total cells per ml of sample.

***Salmonella* detection in PIF.** The detection of *Salmonella* in powdered infant formula (PIF) was based on Almeida et al. (1). The infant formula (NAN 1 Premium; Nestle) was reconstituted by mixing 10 g in 80 ml of sterile distilled water. Serial 10-fold dilutions of a *S. enterica* ATCC 13076 culture were made in sterile distilled water, and 10-ml volumes were added to 90 ml of the reconstituted formula to obtain final concentrations of *Salmonella* ranging from 1×10^{-4} to 1×10^{7} CFU/ml (corresponding to 1×10^{-2} to 1×10^{9} CFU/10 g). After an 8-h enrichment step at 37°C, 1-ml samples were taken and diluted 1 to 10, and hybridization was performed in suspension or on glass slides, as earlier described. An uninoculated culture was prepared in parallel and exposed to the same conditions as the control. *Salmonella* detection was also performed using selective and/or differential medium and confirmative biochemical tests. This experiment was performed three times and repeated with the *S. enterica* LT2 strains. The enriched culture concentration was determined by counting the CFU on TSA plates and by PNA-FISH.

***Salmonella* detection in feces.** For the detection of *Salmonella* in feces, porcine feces (Ribeirense Lda., Braga, Portugal) were aseptically collected directly from the gut into a sterile flask, and 10 g was mixed with 100 ml of buffered peptone water (BPW) (VWR, Portugal) and incubated overnight at 37°C with agitation at 120 rpm. A 1-ml sample was collected to perform hybridization in suspension or

TABLE 1. Results of the *Salmonella* probe specificity test

Microorganism (no. of strains)	Origin (locality)	Source	PNA-FISH outcome
Salmonella enterica subsp. *enterica* (subsp. I) serovars			
Enteritidis (12) including ATCC 13076[b]	Human, food, poultry, and environment (Portugal)	J. Azeredo, University of Minho	+
	Unknown (Switzerland and Brazil)	*Salmonella* Genetic Stock Centre	
Heidelberg[a]	Poultry (Portugal)	J. Azeredo, University of Minho	+
Typhimurium (4) including LT2[b]	Parrot (California), human (England)	*Salmonella* Genetic Stock Centre	+
	Water (Basque country)	J. Garaizar, University of the Basque Country	
Kottbus[a]	Beach (Basque country)	J. Garaizar, University of the Basque Country	+
Glostrup[a]	Black pepper (Basque country)	J. Garaizar, University of the Basque Country	+
Virchow[a]	Water (Basque country)	J. Garaizar, University of the Basque Country	+
Litchfield[a]	Ereño river water (Basque country)	J. Garaizar, University of the Basque Country	+
Miami[a]	Water (Basque country)	J. Garaizar, University of the Basque Country	+
Cremieu[a]	Beach (Basque country)	J. Garaizar, University of the Basque Country	+
Anatum[a]	Beach (Basque country)	J. Garaizar, University of the Basque Country	+
Hadar[a]	Hamburger (Basque country)	J. Garaizar, University of the Basque Country	+
Goldcoast[a]	Beach (Basque country)	J. Garaizar, University of the Basque Country	+
Agona[a]	Unknown (Peru)	*Salmonella* Genetic Stock Centre	+
Saintpaul[a]	Human (Florida)	*Salmonella* Genetic Stock Centre	+
Brandenberg[a]	Unknown (Scotland)	*Salmonella* Genetic Stock Centre	+
Choleraesuis[a]	Swine (Minnesota)	*Salmonella* Genetic Stock Centre	+
Derby[a]	Swine (Minnesota)	*Salmonella* Genetic Stock Centre	+
Dublin[a]	Bovine (France)	*Salmonella* Genetic Stock Centre	+
Gallinarum[a]	Human (Connecticut)	*Salmonella* Genetic Stock Centre	+
Indiana[a]	Unknown (Scotland)	*Salmonella* Genetic Stock Centre	+
Infantis[a]	Human (North Carolina)	*Salmonella* Genetic Stock Centre	+
Montevideo[a]	Human (Florida)	*Salmonella* Genetic Stock Centre	+
Muenchen[a]	Human (France)	*Salmonella* Genetic Stock Centre	+
Newport[a]	Human (North Carolina)	*Salmonella* Genetic Stock Centre	+
Panama[a]	Human (North Carolina)	*Salmonella* Genetic Stock Centre	+
Paratyphi A ATCC 9150[b]		*Salmonella* Genetic Stock Centre	+
Paratyphi B[a]	Human (Africa)	*Salmonella* Genetic Stock Centre	+
Paratyphi C[a]	Human (France)	*Salmonella* Genetic Stock Centre	+
Stanley[a]	Unknown (Scotland)	*Salmonella* Genetic Stock Centre	+
Thompson[a]	Human (Florida)	*Salmonella* Genetic Stock Centre	+
Typhi (2), including IP E.88.374[b]	Unknown (Dakar)	*Salmonella* Genetic Stock Centre	+
Typhisuis[a]	Swine (California)	*Salmonella* Genetic Stock Centre	+
Salmonella enterica subsp. II		*Salmonella* Genetic Stock Centre	+
CDC 151-85[b]	Human (Massachusetts)		
CDC 3472-64[b]	Unknown		
Salmonella enterica subsp. *arizonae* (subsp. IIIa) (2)	Cotton seed (Basque country)	J. Garaizar, University of the Basque Country	+
CDC 409-85[b]	Human (California)	*Salmonella* Genetic Stock Centre	
Salmonella enterica subsp. IIIb		*Salmonella* Genetic Stock Centre	+
CDC 156-87[b]	Human (Oregon)		
CDC 678-94[b]	Human (California)		
Salmonella enterica subsp. IV		*Salmonella* Genetic Stock Centre	+
CDC 2584-68[b]	Animal (Canal Zone)		
CDC 287-86[b]	Human (Illinois)		
Salmonella enterica subsp. VI		*Salmonella* Genetic Stock Centre	−
CDC 1363-65[b]	Unknown (India)		
CDC 347-78[b]	Unknown		
Salmonella enterica subsp. VII		*Salmonella* Genetic Stock Centre	+
CDC 2439-64[b]	Unknown (Tonga-T1)		
CDC 5039-68[b]	Human (Florida)		
Salmonella bongori (subsp. V)		*Salmonella* Genetic Stock Centre	+
CDC 750-72[b]	Frog (unknown)		
CDC 2703-76[b]	Parakeet (United States)		
Shigella flexineri ATCC 12022[b]		*Salmonella* Genetic Stock Centre	−

Continued on following page

TABLE 1—*Continued*

Microorganism (no. of strains)	Origin (locality)	Source	PNA-FISH outcome
Shigella sonnei ATCC 25931[b]		*Salmonella* Genetic Stock Centre	Autofluorescence[c]
Shigella dysenteriae ATCC 11835[b]		*Salmonella* Genetic Stock Centre	Autofluorescence
Shigella boydii ATCC 9207[b]		*Salmonella* Genetic Stock Centre	Autofluorescence
Escherichia hermanii ATCC 33650[b]		*Salmonella* Genetic Stock Centre	–
Escherichia vulneris ATCC 29943[b]		*Salmonella* Genetic Stock Centre	–
Klebsiella pneumoniae subsp. *ozaenae* ATCC 11296[b]		*Salmonella* Genetic Stock Centre	–
Klebsiella oxytoca ATCC 13182[b]		*Salmonella* Genetic Stock Centre	–
Citrobacter freundii[a]		*Salmonella* Genetic Stock Centre	–
Citrobacter koseri[a]		*Salmonella* Genetic Stock Centre	–
Pantoea agglomerans[a]		*Salmonella* Genetic Stock Centre	–
Yersinia enterocolitica[a]		*Salmonella* Genetic Stock Centre	–
Yersinia kritensenii[a]		*Salmonella* Genetic Stock Centre	–
Enterobacter helveticus (2)[a]		S. Fanning, University College Dublin	–
Enterobacter turicensis (2)[a]		S. Fanning, University College Dublin	–
Enterobacter cloacae[a]		S. Fanning, University College Dublin	–
Enterobacter sakazakii (*Cronobacter sakazakii*)		S. Fanning, University College Dublin	–
ATCC 29544[b]			
ATCC 51321[b]			
Enterobacter aerogenes ATCC 12048[b]		S. Fanning, University College Dublin	–
Enterobacter amnigenus ATCC 33072[b]		S. Fanning, University College Dublin	–
Staphylococcus aureus		Spanish Type Culture Collection	–
ATCC 12600[b]			
ATCC 6538[b]			
ATCC 13565[b]			
Staphylococcus epidermidis		Spanish Type Culture Collection	–
ATCC 35983[b]			
ATCC 35984[b]			
ATCC 1798[b]			
Escherichia coli (4)		S. Fanning, University College Dublin, and J. Azeredo, University of Minho	–
ATCC 25922[b]			
K-12[b]			
Pseudomonas fluorescens (2), including ATCC 13525[b]		M. J. Vieira, University of Minho	–
Pseudomonas aeruginosa ATCC 10145[b]		M. J. Vieira, University of Minho	–
Serratia plymuthica[a]		M. J. Vieira, University of Minho	–
Listeria monocytogenes (5)[a]		J. Azeredo, University of Minho	–
Helicobacter pylori (5)		J. Solnick, University of California	–
ATCC 700824[b]			
ATCC 700392[b]			
NCTC 11367[b]			
Campylobacter coli[a]		J. Azeredo, University of Minho	–

[a] Isolate.
[b] Reference strain.
[c] Autofluorescence, strong autofluorescence signal.

on glass slides as described above. Other samples were also taken and mixed 1 to 100 with Rappaport Vassialidis soya (RVS) broth (Liofilchem, Italy) and 1 to 10 with Muller Kauffmann tetrathionate-novobiocin (MKTTn) broth (Liofilchem, Italy). Cultures were incubated overnight at 37°C for MKTTn broth and at 42°C for RSV broth. After the selective enrichment step, 1-ml samples were also collected to perform hybridization, and the protocol followed for *Salmonella* detection was according to ISO 6579:2002. For hybridization on slides, samples were diluted (1 to 10) before smear preparation. This experiment was also performed three times. Before samples were mounted with immersion oil, they were covered with 20 μl (10 μg/ml) of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and incubated for 10 min in the dark. Excess DAPI was gently removed, and the sample was allowed to air dry; it was then mounted with nonfluorescent immersion oil (Merck), and covered with coverslips. The percentage of *Salmonella* in the enriched cultures was determined by PNA-FISH counts as described above.

***Salmonella* detection in water.** For *Salmonella* detection in water, we aseptically collected natural water from a fountain in a highly eutrophic state, which makes it green, at Bom Jesus, Braga. Then, three different volumes were mixed with 50 ml of BPW as recommended by ISO 6340:1995. The water volumes used were 5, 25, and 100 ml. For the 5-ml sample, the 50 ml of BPW was prepared with

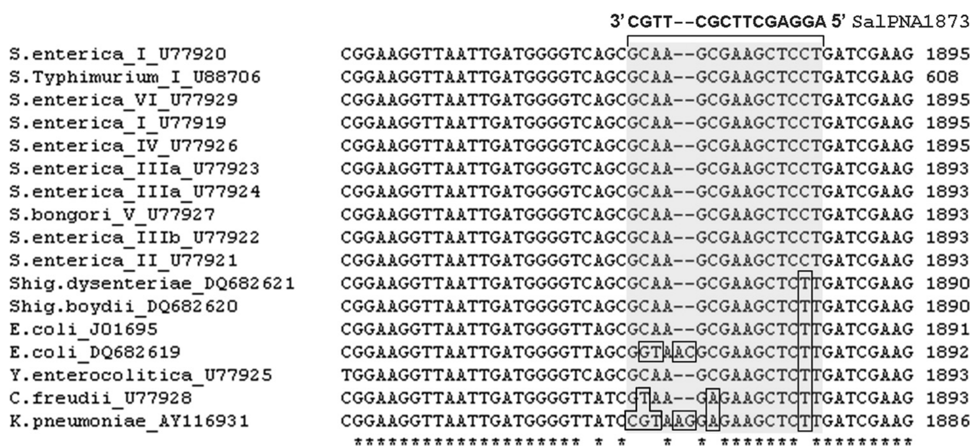

FIG. 1. Partial alignment of 23S rRNA gene sequences for probe selection. The antiparallel complementary sequence of the SalPNA1873 probe is shown above the alignment. Base differences between the *Salmonella* sequences and other species sequences are highlighted.

only 45 ml of distillated water. The 25-ml sample was mixed with 25 ml of BPW 2-fold concentrated. The 100-ml water sample was aseptically filtered, and then the filter was placed in 50 ml of BPW. The three water dilutions were incubated overnight at 37°C with agitation at 120 rpm. After this preenrichment step, 1-ml samples were taken to perform hybridization in suspension or on glass slides as described above. Subsequently, 1 ml of BPW cultures was mixed with 100 ml of RSV broth. Cultures were incubated overnight at 42°C with agitation at 120 rpm. After this selective enrichment step, 1-ml samples were also collected to perform hybridization. *Salmonella* detection continued with the confirmative tests, according to the ISO procedure. This experiment was also performed three times. Before samples were mounted with immersion oil, they were covered with 20 μl of DAPI and incubated for 10 min in the dark. The excess DAPI was removed, and the sample was allowed to air dry; it was then mounted with nonfluorescent immersion oil and covered with coverslips. The percentage of *Salmonella* bacteria in the enriched cultures was also determined by PNA-FISH counts.

Microscopy visualization. Microscopy visualization was performed using an Olympus BX51 (Olympus Portugal SA, Porto, Portugal) epifluorescence microscope equipped with one filter sensitive to the Alexa Fluor 594 molecule attached to the PNA probe (excitation, 530 to 550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). Other filters present in the microscope that are not capable of detecting the probe fluorescent signal were used in order to confirm that cells did not autofluoresce. For every experiment, a negative control was performed simultaneously for which all the steps described above were carried out, but no probe was added during the hybridization procedure. All the images were acquired using Olympus CellB software with a magnification of ×900.

RESULTS AND DISCUSSION

Probe design. The identification of useful oligonucleotides was performed by aligning 23S rRNA gene sequences of representative *Salmonella* sequences of each of seven subspecies and other sequences from species belonging to the *Enterobacteriaceae* family (Fig. 1). Six potential regions were selected, but only one region of 18 bp appeared capable of detecting all the *Salmonella* strains in the NCBI database. For this region, four possible probes were designed, and of these, one appeared to be better than the others as it did not hybridize with any non-*Salmonella* sequence and contained 60% of GC bases. According to these selection criteria, the following PNA oligomer sequence was obtained: 5′-AGGAGCTTCGCTTGC-3′. The sequence hybridizes between positions 1873 and 1887 of the *S. enterica* subsp. *enterica* serovar Typhimurium LT2 (ATCC 43971) 23S rRNA gene sequence (accession number U77920). The probe was designated SalPNA1873 based on the starting position of the target sequence in the LT2 strain.

Determination of theoretical specificity and sensitivity. The theoretical specificity and sensitivity of the probe were further evaluated with the LSU database using the ProbeCheck program. The search confirmed that SalPNA1873 detected only the 101 *Salmonella* spp. existing in the database. Therefore, a theoretical specificity and sensitivity of 100% were obtained (Table 2). In order to compare the probe developed in this study with probes developed earlier, the theoretical specificity and sensitivity of the probes Sal23S10 (31), Sal3 (29), and Salm63 (21) were also evaluated with the ProbeCheck program (Table 2). The search showed that all the probes presented acceptable levels of specificity and sensitivity, apart from the Sal23S10 that presents a lower specificity. This happens mainly because of the large number of *Yersinia* and *Haemophilus influenzae* sequences detected (119 and 13, respectively). Despite the 100% specificity, the Sal3 probe was not capable of detecting all the *Salmonella* sequences in the database (96 of 101 sequences), failing to detect mainly *S. bongori* and *S. enterica*

TABLE 2. Theoretical specificities and sensitivities of the existing probes for detection of *Salmonella* spp.

Probe	Type	Sequence (5′→3′)	No. of *Salmonella* strains detected[a]	No. of non-*Salmonella* strains detected[b]	Specificity (%)	Sensitivity (%)	Reference or source
Sal3	DNA	AATCACTTCACCTACGTG	96	0	100	95.05	29
Salm63	DNA	TCGACTGACTTCAGCTCC	73	3	99.97	72.23	21
Sal23S10	PNA	TAAGCCGGGATGGC	95	206	98.13	94.06	31
SalPNA1873	PNA	AGGAGCTTCGCTTGC	101	0	100	100	This work

[a] *Salmonella* strains detected in a total of 101 *Salmonella* sequences present in the database.
[b] Non-*Salmonella* strains detected in a total of 11,023 non-*Salmonella* sequences deposited in the database.

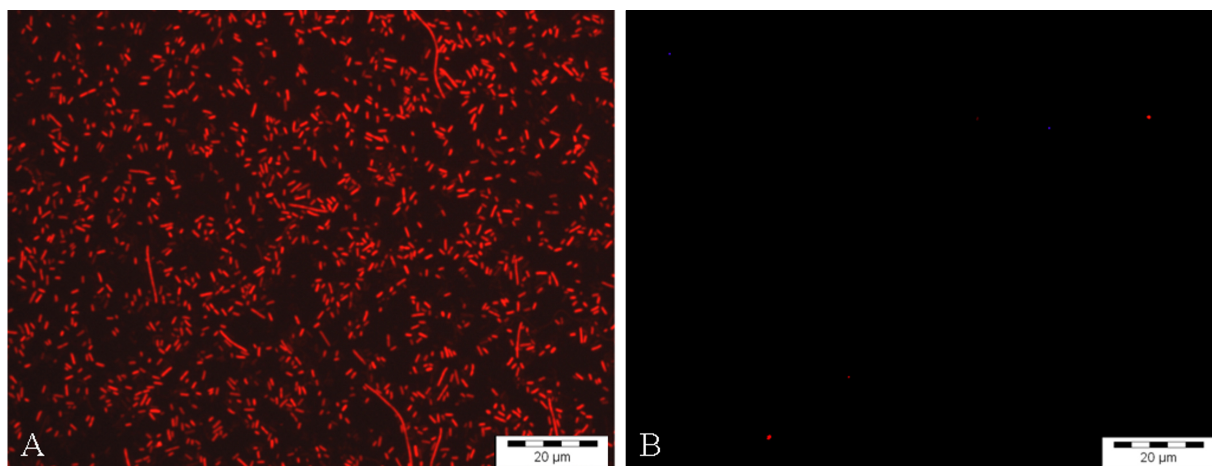

FIG. 2. Detection of *Salmonella* using the red fluorescent SalPNA1873 probe in a smear of *S. enterica* subsp. *enterica* serotype Enteritidis ATCC 13076 pure culture (A) and lack of signal in a smear of *E. coli* ATCC 25922 pure culture (B). The experiments were performed simultaneously, and images were obtained with equal exposure times.

subsp. *arizonae*, as previously reported. The Salm63 probe detected only 73 of the 101 *Salmonella* sequences in the database and also matched three strains belonging to different species (*Plesiomonas shigelloides*, *Yersinia enterocolitica* and *Enterobacter sakazakii*). None of the probes showed cross-hybridization with the 279,862 16S rRNA sequences presented in the SSU database. This theoretical evaluation shows that the SalPNA1873 probe improves *Salmonella* detection mainly because of two aspects: advantages of the PNA molecule and the specificity and sensitivity values. The PNA molecule makes the FISH procedure easier and faster than the Sal3 and Salm63 DNA FISH assays, while none of the existing probes can reach the theoretical values obtain for SalPNA1873.

Protocol optimization. Even though the hybridization protocol developed was largely based on that described by Guimarães et al. and Perry-O' Keefe et al. (16, 31), some aspects of the hybridization/fixation conditions had to be optimized. Different hybridization temperatures, between 53°C and 59°C, were tested. The strongest signal-to-noise ratio was obtained at 57°C, independent of whether the hybridization was performed on slides or in suspension (Fig. 2). Different ethanol concentrations (50% and 80%) in the fixation step were also tested, but no differences in signal intensity were found. A range of hybridization times (30, 45, 60, and 90 min) was tested, and the shorter times were found to be as efficient as the longer times. Hybridization in suspension was performed on cellulose nitrate membranes due to the lower autofluorescence signal for these membranes at this particular wavelength.

To make sure that the signal obtained was not related to autofluorescence, all samples were visualized with the other available filters, and no autofluorescence was observed. Samples were also counterstained with DAPI to confirm that SalPNA1873 was staining all cells present. In addition, for each experiment a negative control was performed simultaneously, following all the steps for standard hybridization but without the addition of the probe to the hybridization solution.

***Salmonella* probe specificity and sensitivity testing.** Once the hybridization method was fully optimized, the specificity and sensitivity of the PNA probe were tested. For this, the procedure was applied to 61 representative *Salmonella* strains from the two species and from the six *S. enterica* subspecies and to 46 other strains. The latter strains included 25 taxonomically related strains belonging to the same family (*Shigella*, *Klebsiella*, *Citrobacter*, *Pantoea*, *Yersinia*, *Enterobacter*, *Escherichia*, and *Serratia*) and 21 strains belonging to a different order (*Pseudomonas*), class (*Helicobacter* and *Campylobacter*), or even phylum (*Listeria* and *Staphylococcus*). As shown in Table 1, apart from the *S. enterica* subsp. VI that was not detected by the SalPNA1873, the remaining 59 *Salmonella* strains were detected, whereas no hybridization was observed for the other species used. It is also important that we could not assess the PNA-FISH outcome for three *Shigella* species. This happened because of the strong autofluorescence signal of these strains detected in both positive and negative (without probe) samples. In any case, this result is not related to the difference of only one nucleotide between the probe and the *Shigella* species because *Shigella flexneri*, which has a similar RNA sequence, did not hybridize with the probe. Moreover, other species such as *Y. enterocolitica* and *Escherichia coli* K-12 also have only one mismatch in exactly in the same position, and no cross-reaction was observed. This result supports the observation from other authors who have reported that with PNA probes it is simpler to distinguish sequences with only one mismatch (32). An experimental specificity of 100% (95% confidence interval [CI], 96.1 to 100%) and sensitivity of 96.7% (95% CI, 87.2 to 99.2%) were thus obtained.

***Salmonella* detection in artificially contaminated samples (blood and PIF).** After designing the probe and optimizing the FISH procedure both for glass slides and for cells in suspension, this method was adapted to the detection in artificially contaminated blood and feces.

For blood samples, hybridization was initially performed in a contaminated blood smear. This approach proved to be unsuccessful due to blood cell autofluorescence. On the other hand, blood pathogen load levels could be low (20, 27), which could make detection difficult in clinical samples. Because of this, a blood culture, a method currently used in clinical laboratories to enrich samples, was simulated by inoculating a rich

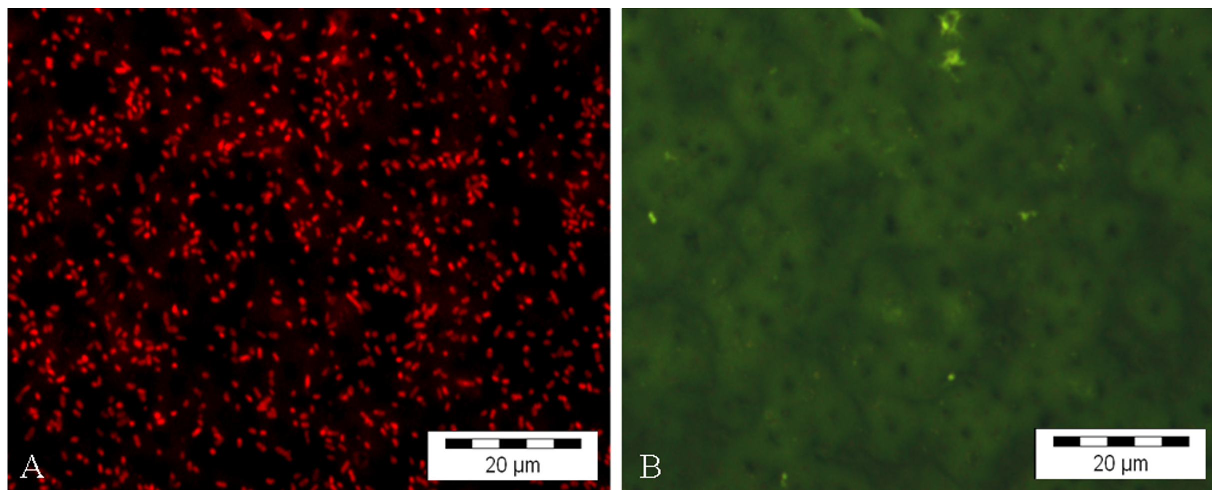

FIG. 3. (A) Detection of *S. enterica* subsp. *enterica* serovar Typhimurium LT2 in blood culture using the SalPNA1873 probe. (B) Visualization of the same microscopic field at the green channel, where it is possible to observe autofluorescence of the red blood cells and the absence of fluorescent cells. Images were obtained with equal exposure times.

medium with horse blood artificially contaminated with low levels of *Salmonella* bacteria. After culture enrichment overnight, pathogen detection was performed in suspension or in a slide test (Fig. 3). The blood autofluorescence remained detectable but did not interfere with bacterial detection. The procedure proved to be very sensitive, being able to detect the bacteria in samples with 1 CFU per 10 ml of blood in less than 20 h.

An expert meeting organized by the Food and Agriculture Organization of the United Nations and the World Health Organization concluded that *S. enterica* together with *E. sakazakii* are the microorganisms of greatest concern in PIF and that PIF contamination with *S. enterica* is an important cause of infection and illness in infants (7). It was therefore decided to apply the probe to this type of sample as previously performed for *E. sakazakii* (*Cronobacter* sp.) (1).

An experiment regarding *Salmonella* detection in PIF was planned based on an earlier work that also applied the PNA-FISH method to detect *Cronobacter* (1).

In this testing, an artificial contamination of 1×10^{-4} to 1×10^{7} CFU/ml of *Salmonella* was made, followed by an 8-h enrichment step. After culture enrichment, pathogen detection was performed in suspension to avoid autofluorescence of the infant formula proteins (Fig. 4). This procedure was able to detect PIF samples with 1 CFU per 10 g, a value similar to that found in the *Cronobacter* study mentioned above (1). *Salmonella* detection in PIF was performed in less than 12 h, which represents a time-saving of several days compared with the ISO 6579:2002 method, recommended for *Salmonella* detection in food and animal feeding stuffs. As the hybridization temperatures are similar for the *Cronobacter* probe, a multiplex assay can be easily developed.

***Salmonella* detection in natural samples (feces and water).** After the detection in monospecies/artificially contaminated samples, we attempted to apply SalPNA1973 to detect *Salmonella* in multispecies/natural samples, such as feces and water.

For detection of *Salmonella* in feces this experiment was based on the ISO 6579:2002 method, and colony isolation on XLD and BGA, together with the biochemical tests (triple sugar iron, urea agar, and Api 20E), confirmed the presence of

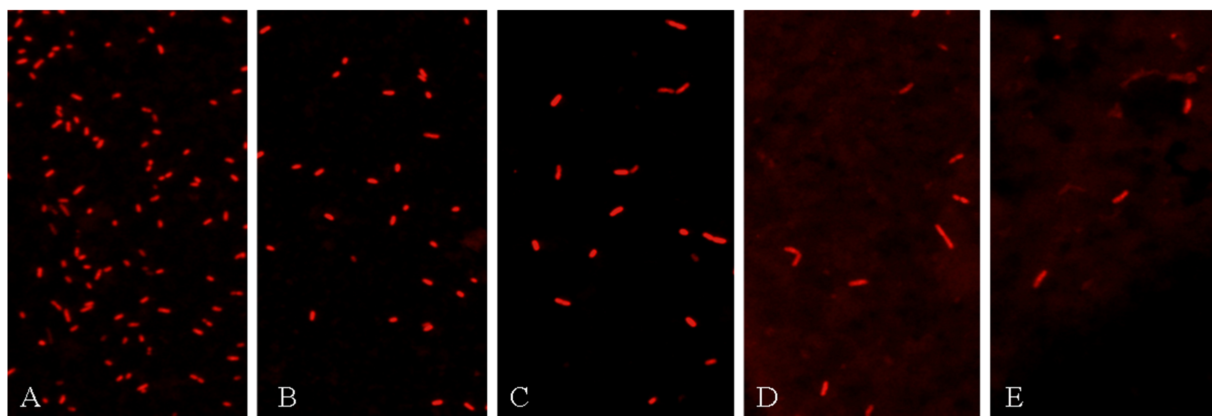

FIG. 4. *S.* Typhimurium (LT2) detection using the SalPNA1873 probe in an 8-h enriched culture (10% PIF), with 10,000 (A), 1,000 (B), 100 (C), 10 (D), and 1 (E) initial CFU per 10 g of PIF.

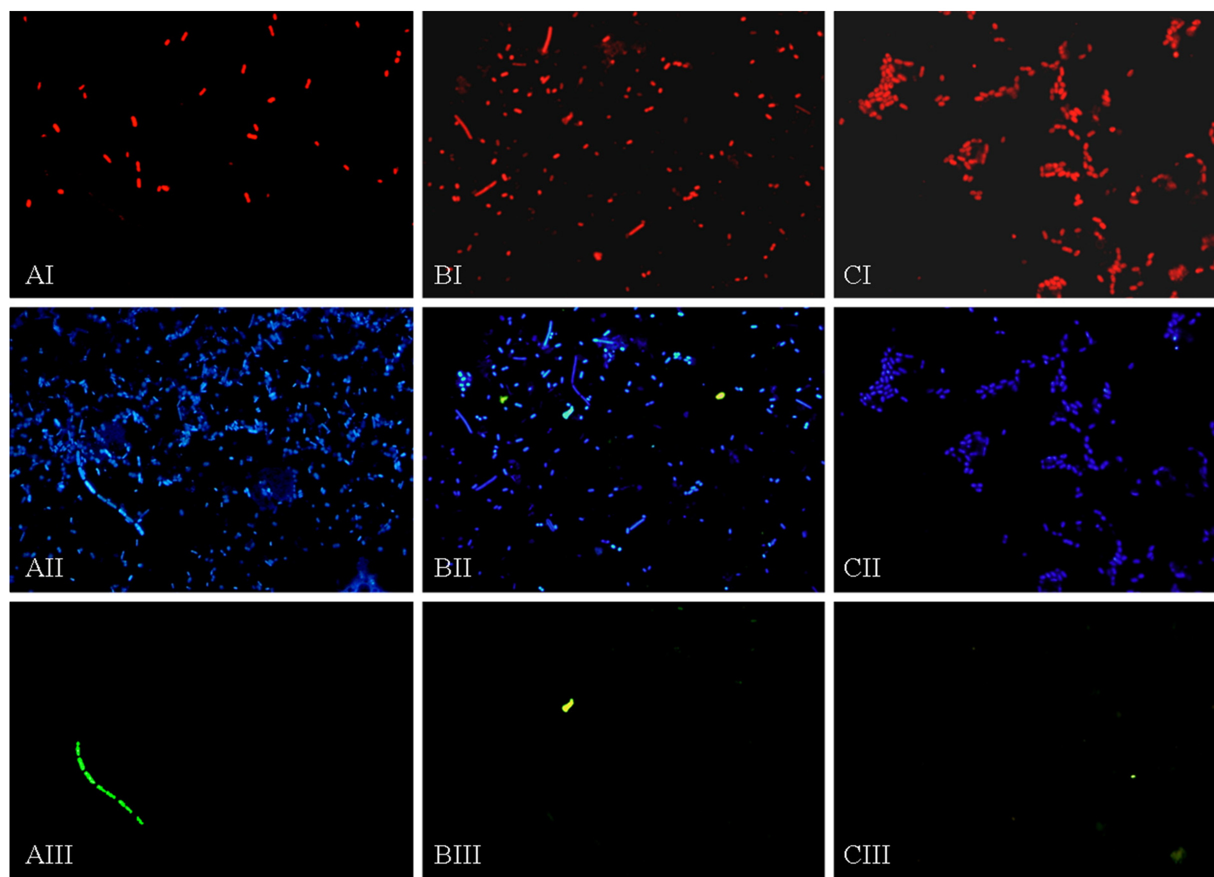

FIG. 5. *S.* Enteritidis (ATCC 13076) detection in feces, after an overnight preenrichment in BPW, using the SalPNA1873 probe. *Salmonella* was detected after preenrichment in BPW (A) selective enrichment in RVS broth (B), and selective enrichment in MKTTn broth (C). *Salmonella* was detected using the PNA probe (row I) and by counterstaining with DAPI (total population) (II). Row III shows the visualization of the same microscopic field at the green channel. Images were obtained with equal exposure times.

Salmonella. During the culture procedure, samples were taken at the end of each enrichment step and analyzed by PNA-FISH (Fig. 5). We verified that the PNA-FISH method was able to detect *Salmonella* at the end of the preenrichment step in BPW, avoiding the selective enrichment need. This means that this method is capable of detecting *Salmonella* in less than 20 h, resulting in the saving of at least 3 days compared to the ISO assay and matching the best times reported for PCR-based techniques (10, 13, 36, 40). Moreover, we did not have problems with inhibitory substances affecting the hybridization procedure, as reported in some studies using molecular approaches for *Salmonella* detection (28, 36). Direct detection (without an enrichment step) was not performed because of the low numbers of bacteria usually present in the samples (11, 28, 36). In this experiment, DAPI counterstaining allowed the determination of the percentage of *Salmonella* in the enriched sample in BPW, which was ~4.4% (± 0.8%) of the total population.

For detection in water, we aseptically collected natural water from a fountain, and then we performed *Salmonella* detection according to the ISO 6340:1995 method, which confirmed the pathogen's presence in the three water volumes used for all three experiments performed (all nine replicates were positive). During the ISO procedure, samples were taken after the preenrichment step in BPW and after the selective enrichment in RSV broth. As verified for detection in feces, the PNA-FISH method was able to detect *Salmonella* after the preenrichment step, with the *Salmonella* population representing approximately 11.2% (± 2.3%) of the enriched BPW total population (Fig. 6).

After these two experiments we can conclude that the PNA-FISH method proved to be capable of detecting *Salmonella* in natural samples in less than 20 h. In fact, even the samples using only 5 ml of water were positive after the preenrichment step in BPW. Moreover, we showed above for PIF samples that even 1 CFU can be detected using an enrichment step of only 8 h. It is also important to notice that in natural samples the effect of competing flora is much more important and may affect the *Salmonella* growth capacity. Nevertheless, other authors have shown that the recovery of *Salmonella* strains from a broad spectrum of samples using this universal enrichment broth, i.e., BPW, can be used as an effective enrichment medium for salmonella despite high levels of competing microorganisms (4, 9).

Concluding remarks. In conclusion, the PNA-FISH procedure using the SalPNA1873 probe has proved to be a very sensitive and specific method for *Salmonella* detection in different samples, such as blood, feces, water, and PIF. By per-

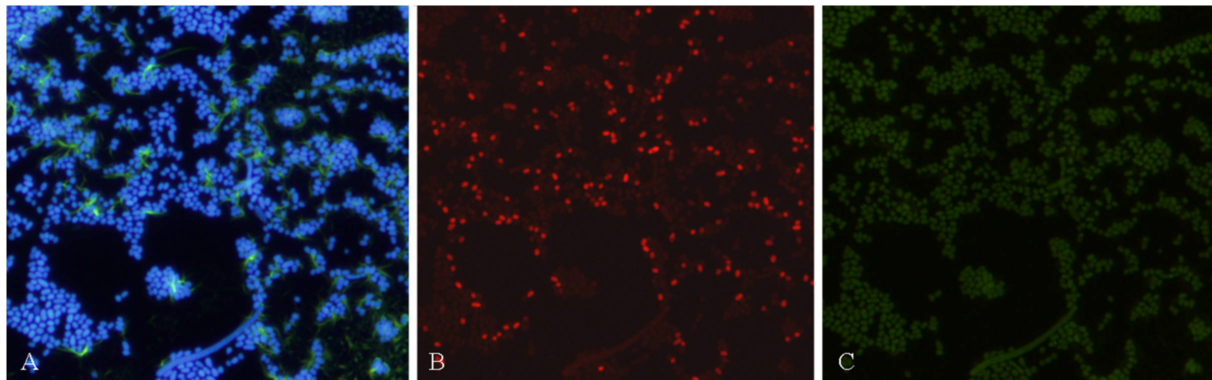

FIG. 6. *S.* Enteritidis (ATCC 13076) detection in water, after an overnight preenrichment in BPW, using the SalPNA1873 probe. (A) Counterstaining with DAPI (total population). (B) *Salmonella* detection using the red fluorescent SalPNA1873 probe. (C) Visualization of the same microscopic field at the green channel. Images were obtained with equal exposure times.

forming a preenrichment step in a rich medium, the PNA-FISH method using the SalPNA1873 probe is able to detect the pathogen even when it is outnumbered by other microorganisms.

Most studies reporting *Salmonella* detection are PCR-based or culture-based techniques. While the first type is more technically demanding and usually also involves a preenrichment step to improve the detection limit, the second type is time-consuming and could give inaccurate results (13, 35, 39). The PNA-FISH protocol presented in this work is technically less demanding. Although an enrichment step is needed, the total time required for the PNA-FISH assay (less than 20 h, except for PIF, which takes 12 h) is similar to or even better than the times reported for PCR-based methods (10, 13, 36, 40).

The PNA-FISH assay was demonstrated to be a reliable alternative to the currently used culture-based techniques, to the existing *Salmonella* probes, and even to PCR and ELISA protocols. It was shown that with the PNA-FISH method, detection takes at least 48 h less than with the culture technique; in addition, the probe developed presents a higher specificity than that reported by Perry-O'Keefe, and inhibitory substances do not interfere with PNA-FISH as happens for some molecular approaches.

Moreover, this work demonstrates that PNA-FISH could be easily adapted for the identification/quantification of *Salmonella* in several other clinical or environmental samples even if strong heterotrophic population profiles are present. Future work could involve testing a new universal approach reducing the enrichment step to 8 h as performed for PIF samples. Additionally, we can take advantage of the PNA suitability and the very narrow emission band of the fluorophore attached (e.g., Alexa Fluor family) to perform multiplex assays detecting numerous pathogens in a particular sample.

ACKNOWLEDGMENTS

We thank Joana Azeredo, Séamus Fanning, and Javier Garaizar for providing some of the strains used in this study.

This work was supported by the Portuguese Institute Fundação para a Ciência e Tecnologia (Postdoctoral Fellowship SFRH/BPD/20484/2004 and Ph.D. Fellowship SFRH/BD/29297/2006).

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