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Detection of *Escherichia coli* O157 by Peptide Nucleic Acid Fluorescence *In Situ* Hybridization (PNA-FISH) and Comparison to a Standard Culture Method

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Despite the emergence of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) infections, *E. coli* serotype O157 is still the most commonly identified STEC in the world. It causes high morbidity and mortality and has been responsible for a number of outbreaks in many parts of the world. Various methods have been developed to detect this particular serotype, but standard bacteriological methods remain the gold standard. Here, we propose a new peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) method for the rapid detection of *E. coli* O157. Testing on 54 representative strains showed that the PNA probe is highly sensitive and specific to *E. coli* O157. The method then was optimized for detection in food samples. Ground beef and unpasteurized milk samples were artificially contaminated with *E. coli* O157 concentrations ranging from 1×10^{-2} to 1×10^{2} CFU per 25 g or ml of food. Samples were then preenriched and analyzed by both the traditional bacteriological method (ISO 16654:2001) and PNA-FISH. The PNA-FISH method performed well in both types of food matrices with a detection limit of 1 CFU/25 g or ml of food samples. Tests on 60 food samples have shown a specificity value of 100% (95% confidence interval [CI], 82.83 to 100), a sensitivity of 97.22% (95% CI, 83.79 to 99.85%), and an accuracy of 98.33% (CI 95%, 83.41 to 99.91%). Results indicate that PNA-FISH performed as well as the traditional culture methods and can reduce the diagnosis time to 1 day.

Escherichia coli strains include a genetically heterogeneous group of bacteria which are typically nonpathogenic (1, 2). However, a considerable number of strains are recognized as important pathogens. There are 6 classes of pathogenic *E. coli*: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC) (1). Among pathogenic *E. coli* strains, EHEC strains are perhaps the most important because of their virulence and association with life-threatening complications (1, 2). Within the EHEC group, *E. coli* serotype O157:H7 (the serotype is based on the O [Ohne] antigen, determined by cell wall lipopolysaccharide, and the H [Haunch] antigen due to the flagellum protein) is the most commonly isolated (2, 3).

The infectious dose of *E. coli* O157:H7 is reported to be as few as 10 cells, lower than that of most enteric pathogens (4, 5). Three major virulence factors have been identified in this bacterium, including the production of Shiga toxins, a pathogenicity island called the locus of enterocyte effacement, and an F-like plasmid, pO157 (4, 6). Among these virulence factors, the role of pO157 is the least understood (7). The most critical is the production of one or two phage-encoded Shiga toxins, called Stx1 and Stx2. These Shiga toxins are among the most potent cytotoxins currently known to affect eukaryotic cells (4, 6).

The effects of an EHEC infection range from asymptomatic to lethal. In severe cases the patients can develop serious diseases, such as hemolytic-uremic syndrome (HUS) and thrombocytopenic thrombotic purpura (TTP), or even die (4). For *E. coli* O157:H7 outbreaks reported in the United States, 25% of affected persons were hospitalized, 5 to 10% developed HUS or TTP, and 1% died (3).

Regarding the environmental reservoirs, cattle are considered the primary and natural reservoir, but other animals, such as goats, sheep, and pigs, may be carriers as well (8). Results from a study of 90 outbreaks occurring between 1982 and 2006 showed that in 42% of cases the source of transmission to humans was associated with food (such as ground beef, ready-to-eat products, and vegetables), 12.2% with dairy products (such as cheese and milk), 7.8% with animal contact, 6 to 7% with water, and 2.2% with the environment. The transmission source was unknown in 28.9% of the outbreaks (3).

Because of the importance of quality control in the food industry, the methods used to detect bacterial contaminants must be rapid, sensitive, and reliable, as well as versatile, in order to accommodate the dynamic needs of the food processing plant. Initially testing for sorbitol fermentation has been suggested as a simple means to screen for *E. coli* O157:H7, because it lacks the β-glucuronidase enzyme (9–11). Most of the existing culture methods were developed based on the above-mentioned feature, as well as the strain's inability to ferment rhamnose and its tolerance to tellurite (9, 10, 12). These plating techniques remain an integral aspect of quality control during food processing, because they are

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cost-effective and technically simple, with a high level of accuracy and sensitivity. However, they are very time-consuming and laborious, and they fail to detect *E. coli* O157:H7 bacteria that ferment sorbitol or are susceptible to tellurite. They also fail in the detection of samples with low numbers of pathogens (<200 CFU/g sample) (11, 13–15). Moreover, culture-based methods, such as ISO 16654:2001 (horizontal method for the detection of *Escherichia coli* O157), usually include an agglutination assay (detecting the O157 or H7 antigen) that is not specific, since the O157 and H7 antigens are present in other *E. coli* species. These antibodies can also cross-react with other *E. coli* serotypes, *Escherichia* species, and other members of the *Enterobacteriaceae* family (11, 16).

Fluorescence *in situ* hybridization (FISH) is a molecular assay that is widely applied for bacterial identification and localization within samples. This method is based on the specific binding of small oligonucleotides (probes) to particular rRNA regions due to its high cellular abundance, universal distribution, and use as a phylogenetic marker. More recently, peptide nucleic acid probes (PNA) have been developed for microbial detection (17, 18). These molecules mimic DNA and establish a stronger bond, since they have a neutrally charged repeated N-(2-aminoethil) glycine unit instead of the negatively charged sugar-phosphate backbone. The adequate use of this molecule in FISH technology has made the procedure more robust, quicker, and more efficient and allowed the development of several PNA-FISH methods for the detection of important pathogenic organisms (reviewed in reference 18).

Here, we have developed a new PNA-FISH-based method for the specific detection of *E. coli* O157 in food samples, and we have compared its performance to that of the traditional bacteriological method. To the best of our knowledge, this is the first PNA-FISH method developed to detect a specific serotype of *E. coli*.

MATERIALS AND METHODS

Bacterial growth and culture media. The bacterial strains and species used in this study are listed in Table 1. All bacterial species were maintained on tryptic soy agar (TSA) (VWR, Portugal) at 37°C and streaked onto fresh plates every 48 h.

PNA probe design. To identify potentially useful oligonucleotides to use as probes, 16S and 23S rRNA gene sequences available at the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) were chosen. This selection contained 6 *Escherichia coli* O157:H7 strains, 6 *Escherichia coli* non-O157:H7 strains, and 4 other strains from related species belonging to the *Enterobacteriaceae* family (see Fig. S1 in the supplemental material). The possible regions of interest were selected by sequence alignment using the ClustalW program, available from the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/). A conserved region in the 23S rRNA of all *E. coli* O157:H7 isolates was identified. The criteria for the selection of the final probe sequence included Gibbs free energy, percentage of GC, lack of self-complementary structures, and melting temperature higher than 50°C. The selected sequence was synthesized (Panagene, Daejeon, South Korea), and the oligonucleotide N terminus was attached to Alexa Fluor 594 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker.

Theoretical evaluation of the PNA probe performance. After the design of the probe, its performance was evaluated to determine the theoretical values for sensitivity and specificity. These parameters were evaluated with ProbeCheck software, available in the ARB Silva database (http://www.arb-silva.de/). For this theoretical estimation, only the good-quality sequences with at least 1,900 bp were considered along with *E. coli* strain sequences with the designated serotype. The probe was tested against the large-subunit ([LSU]; 23/28S) database and the small-subunit ([SSU]; 16/18S) database. Theoretical values were determined as previously reported by Almeida et al. (19). Briefly, specificity was calculated as (nECs/TnECs) × 100, where nECs is the number of non-*Escherichia coli* O157:H7 strains that did not react with the probe and TnECs is the total number of non-*Escherichia coli* O157:H7 strains examined. Sensitivity was calculated as (ECs/TECs) × 100, where ECs is the number of *E. coli* O157:H7 strains detected by the probe and TECs is the total number of *E. coli* O157:H7 strains present in the database. Accuracy was determined as the number of correct results divided by the number of all returned results (20).

Hybridization protocol optimization. The PNA-FISH protocol was performed on glass slides as previously described (19), with some modifications. In order to understand the behavior of the probe and infer the best hybridization conditions, the hybridization temperature ranged between 53 and 61°C; the fixation step using ethanol was tested between 50 and 80%, and different hybridization times (30, 45, 60, and 90 min) were assessed.

After the optimization of all parameters described above, the procedure that was found to result in the strongest fluorescent signal was as follows. 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) Ficoll (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 EcoPNA1169 probe. Samples were covered with coverslips, placed in moist chambers, and incubated for 45 min at 59°C. Subsequently, the coverslips were removed and the slides were submerged in a prewarmed (59°C) washing solution containing 15 mM NaCl (Sigma), 1% (vol/vol) Triton X-100 (Sigma), and 5 mM Tris base (pH 10; Sigma). Washing was performed at 59°C for 30 min, and the slides were allowed to air dry. The slides were stored in the dark for a maximum of 24 h before microscopy. The experimental specificity and sensitivity of the probe were evaluated with the equations used for the theoretical calculation of specificity and sensitivity described above.

Characterization of the *E. coli* isolates. The *E. coli* isolates were evaluated for the presence of the O157 and H7 antigens and also for the presence of verotoxin genes stx_1 and stx_2. For the serological evaluation, the latex agglutination test Wellcolex *E. coli* O157:H7 (Oxoid) was used according to the manufacturer's instructions. Briefly, the bacteria were grown in TSB until the exponential phase. Forty-μl drops then were placed in two circles on the reaction cards for each sample. For each test sample, one drop of the O157 or H7 test latex was added to one circle and one drop of the O157 or H7 test latex was added to the other circle. The contents of the samples were mixed and spread over the entire circle area. Samples were inspected for the presence of agglutination after 1 min.

The presence of the verotoxin genes was evaluated as previously described (21, 22). Briefly, 1-ml samples of TSB cultures, grown overnight, were pelleted, and genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. DNA was screened for the presence of stx_1 and/or stx_2 genes by PCR (Peltier thermal cycler [PTC-200]) using the primers and conditions previously reported (21, 22). PCR products were separated by electrophoresis on a 1.5% (wt/vol) agarose gel and visualized under UV light (GelDoc 2000 system; Bio-Rad Laboratories, Hercules, CA) by ethidium bromide staining (10 mg/ml).

Cell inactivation treatment. *E. coli* O157:H7 (CECT 4267) overnight culture aliquots (adjusted to an optical density at 600 nm [OD_{600}] of ~0.1) were used in the experiments of cell inactivation. The aliquots (1 ml) were heat treated in a thermoblock for 20 min at 72°C or autoclaved (20 min at 121°C) (23). Cells were immediately processed. For PNA-FISH analysis, 20 μl of both suspensions was fixed and hybridized according to the procedure described above. Loss of cell viability was evaluated by the

TABLE 1 Results of the EcoPNA1169 probe specificity and sensitivity test[c]

Strain[a]	Serotype	Isolation origin	Verotoxin production	PNA FISH outcome
E. coli CECT 4267	O157:H7	Human stool from outbreak of hemorrhagic colitis	Stx1, Stx2	+
E. coli CECT 4782	O157:H7	Human stool from outbreak of hemorrhagic colitis	Stx1, Stx2	+
E. coli CECT 4783	O157:H7	Raw hamburger meat implicated in hemorrhagic colitis outbreak	Stx1, Stx2	+
E. coli CECT 5947	O157:H7		stx_2 gene has been replaced	+
E. coli NCTC 12900	O157:H7		NT	+
E. coli CCC-1-12	O157:H7	Fecal swab	Stx2	+
E. coli CCC-5-12	O157:H7	Fecal swab	Stx2	+
E. coli CCC-7-12	O157:H7	Fecal swab	Stx2	+
E. coli CCC-10-12	O157:H7	Fecal swab	Stx2	+
E. coli CCC-11-12	O157:H7	Milk filter	Stx2	+
E. coli CCC-12-12	O157:H7	Milk filter	Stx2	+
E. coli CCC-13-12	O157:H7	Milk filter	Stx2	+
E. coli CCC-14-12	O157:H7	Bovine milk filter	Stx2	+
E. coli CCC-15-12	O157:H7	Bovine milk filter	Stx2	+
E. coli CCC-16-12	O157:H7	Caprine milk filter	Stx2	+
E. coli CCC-18-12	O157[b]	Bovine milk filter	Stx2	+
E. coli CCC-23-12	O157:H7	Milk filter	NT	+
E. coli CCC-24-12	O157:H7	Milk filter	NT	+
E. coli CCC-25-12	O157:H7	Milk filter	NT	+
E. coli CCC-26-12	O157[b]	Milk filter	NT	+
E. coli CECT 352	O127a:K63(B8):H-		EPEC	−
E. coli CECT 504	O141:K85(B):H4	Swine edema	ND	−
E. coli CECT 515T	O1:K1(L1):H7	Human urine–cystitis	ND	−
E. coli CECT 533	O103:K-:H-		ND	−
E. coli CECT 727	O111:K58(B4):H-	Infantile gastroenteritis	EPEC	−
E. coli CECT 730	O55:K59(B5):H-		ND	−
E. coli CECT 736	O28a,28c:K73(B18):H-	Feces	ND	−
E. coli CECT 740	O125a,125b:K70(B15):H19	Gastroenteritis	ND	−
E. coli CECT 744	O158:K-:h23	Feces of infant with diarrhea	ND	−
E. coli CECT 832	O111:K58(B4):H-	Infantile gastroenteritis	ND	−
E. coli CECT 4537	O10:K5(L5):H4	Human peritonitis	ND	−
E. coli CECT 4555	O97:K-:H-		ND	−
E. coli CCC-2-12	O103	Fecal swab	NT	−
E. coli CCC-3-12	O26	Fecal swab	Stx1, Stx2	−
E. coli CCC-4-12	O26	Fecal swab	NT	−
E. coli CCC-8-12	O26	Fecal swab	NT	−
E. coli CCC-9-12	O26	Fecal swab	NT	−
E. coli CCC-19-12	O26	Caprine milk filter	Stx1	−
E. coli CCC-20-12	O26	Caprine milk filter	Stx1	−
E. coli CCC-21-12	O26	Bovine milk filter	Stx1	−
E. coli CCC-22-12	O26	Bovine milk filter	Stx1	−
E. coli CECT 434	O6	Clinical isolate	ND	−
E. coli N9	ND	Porcine feces	ND	−
E. coli N5	ND	Bovine feces	ND	−
E. coli ATCC 29425 (K12)	OR:H48:K-		ND	−
Escherichia hermannii ATCC 33650	NR	Human isolate		−
Escherichia vulneris ATCC 29943		Human wound		−
Shigella boydii ATCC 9207			ND	−
Salmonella enterica serovar Typhimurium NCTC 12416				−
Salmonella enterica serovar Typhi SGSC 3036				−
Salmonella enteritidis SGSC 2476				−
Cronobacter sakazakii CECT 858		Child's throat		−
Cronobacter sakazakii		Milk		−
Klebsiella pneumoniae ATCC 11296				−

[a] SGSC, *Salmonella* Genetic Stock Centre; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CECT, Spanish Type Culture Collection.
[b] *E. coli* O157 strains that tested negative for the presence of H7 antigen.
[c] NT, nontoxigenic *E. coli*; EPEC, enteropathogenic *E. coli* (epidemiologically implicated as a pathogen, but the virulence mechanism is not related to the excretion of enterotoxins); ND, not determined; NR, nonrelevant information for the present study.

inoculation of 100 μl of the treated cell suspensions into 10 ml of TSB (incubated at 37°C for 24 h, 120 rpm) and by agar plating of 100 μl of the same suspensions (TSA plates incubated at 37°C for 24 h).

Food sample inoculation and preenrichment. For artificial food contamination, a loopful of *E. coli* O157:H7 CECT 4267 was transferred to 20 ml of tryptic soy broth (TSB) and incubated overnight (~18 h) at 37°C and 120 rpm in an orbital incubator. Cells were then suspended in a phosphate-buffered saline (PBS) solution and adjusted to a cell density corresponding to approximately 1×10^8 cells/ml. Cells were further diluted in PBS to obtain the desired cell concentration for inoculation into food samples. Cell concentrations were confirmed by plating on TSA. For the testing on food samples, two matrices obtained from a local retailer (Pingo Doce, Braga, Portugal) were selected, ground beef and unpasteurized milk. Twenty-five g or ml from each type of food was mixed with 225 ml of prewarmed buffered peptone water (BPW; Liofilchem) or mTSB+N (modified tryptic soy broth supplemented with novobiocin; Oxoid) in sealed stomacher bags (with filters). The samples were then artificially contaminated with *Escherichia coli* O157:H7 at concentrations ranging from 0.01 to 100 CFU/25 g or ml of food. Samples were then homogenized with a stomacher (Seward 3500) for 1 min and transferred to 500-ml flasks. A noninoculated food sample was included for each experiment to check for any possible natural contamination with *E. coli* O157. This experiment was repeated with a different strain, the isolate *E. coli* O157:H7 CCC-05-12. Three independent assays were performed for each experiment and each strain.

Detection in food samples using conventional bacteriological methods. The detection of *E. coli* O157:H7 by culture-based methods was performed according to ISO 16654:2001. Briefly, the artificially contaminated samples, prepared in mTSB+N as described above, were incubated overnight at 37 or 41.5°C with agitation at 120 rpm. After preenrichment, 1-ml samples were taken for the immunomagnetic separation step using a Dynabeads MAX *E. coli* O157 kit (Invitrogen). The samples were mixed with a 20-μl suspension of microspheres coated with anti-*E. coli* O157 antibodies, followed by a 3-min separation phase. After the separation phase, the enrichment medium is withdrawn and the coated microspheres washed and then resuspended in 100 μl of buffer (provided with the kit). This suspension was then inoculated in selective media, CT-SMAC (cefixime-tellurite sorbitol MacConkey agar; Oxoid) and CHROmagar O157, for 24 h at 37°C. Suspect colonies were inoculated in TSA and then tested by the Kovac reagent (Remel) and, in the case of a positive outcome, tested for immunoagglutination in latex (Oxoid).

Detection in food samples using PNA-FISH. After an overnight preenrichment (18 to 24 h) at 37 or 41°C in BPW or mTSB+N, 20-μl samples were taken and placed directly in the microscope slide. Alternatively, 15-μl samples were mixed with 15 μl of a Triton X-100 solution (1%) directly on the microscope slides. A quick centrifugation step (10,000 × *g* for 5 min) of a 1-ml sample was also tested to try to remove some autofluorescence particles. Twenty μl then was also placed on the slide. All samples were dried (approximately 5 min at 59°C), and then hybridization was performed as described above.

Microscopy visualization. The smears were mounted with one drop of nonfluorescent immersion oil (Merck) and analyzed 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 EcoPNA1169 probe (excitation, 530 to 550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). Other filters present in the microscope that were not capable of detecting the EcoPNA1169 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 steps described above were carried out, but no probe was added during the hybridization procedure. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of ×1,000.

RESULTS AND DISCUSSION

Probe design and testing. Based on the selection criteria described in Materials and Methods, the following probe sequence was selected: 5′-CAA CAC ACA GTG TC-3′. This sequence hybridizes between positions 1169 and 1183 of *E. coli* O157:H7 strain TW14359 (accession number CP_001368); hence, it was named EcoPNA1169.

The theoretical parameters of the probe were evaluated *in silico* using the probeCheck program coupled with the ARB SILVA rRNA database. The probe was aligned with a total of 180,344 sequences present in the large-subunit ([LSU]; 23/28S) database. EcoPNA1169 matched 80 *E. coli* O157:H7 sequences (all O157 sequences present in the database) and 11 nontarget sequences, with a total of 91 matches (last accession date, August 2012). The 11 non-*E. coli* O157 strains matched by EcoPNA1169 included 2 *E. coli* O55:H7 strains, 1 *Escherichia hermannii* strain, 7 *Salmonella* sp. strains, and 1 *Cronobacter sakazakii* strain. Despite the alignment of 7 *Salmonella* strains, this number represents less than 2% of the *Salmonella* sequences present in the database. Moreover, none of the *Salmonella* strains tested in this work have shown cross-hybridization with EcoPNA1169 (Table 1). The match of two *E. coli* O55:H7 isolates may represent a drawback, since this serotype, despite also being diarrheagenic, is an EPEC strain. This cross-hybridization with O55 strains has been reported for several other PCR and enzyme-linked immunosorbent assay (ELISA) methods developed to detect O157 (24, 25) and may happen because O55:H7 is the serotype most closely related to O157:H7 (14, 26). Both pathogens express the locus of enterocyte effacement (LEE) island that induces diarrhea via an attachment-effacement mechanism (27). *E. coli* (STEC) O157:H7 is believed to descend from *E. coli* O55:H7 that, during evolution, acquired bacteriophage encoding Stx2 and/or Stx1 toxins. Despite the importance of the O55:H7 serotype, EPEC strains are no longer an important cause of diarrhea in developed countries as they were a few decades ago (28).

Concerning the remaining nontarget sequences, despite the alignment with 1 *E. hermannii* and 1 *Cronobacter* sp. strain, no cross-hybridization was observed for any *Escherichia* or *Cronobacter* species included in the probe experimental test (Table 1).

Based on this *in silico* evaluation, the estimated theoretical specificity and sensitivity for the EcoPNA1169 probe were 99.95 and 100%, respectively.

To test the probe experimental specificity and sensitivity, the PNA-FISH procedure was applied to a total of 54 strains (Table 1). Twenty *E. coli* O157 and 25 *E. coli* non-O157 strains were included. Additionally, 9 strains belonging to the same genus and family (*Escherichia*, *Salmonella*, *Enterobacter*, *Shigella*, and *Klebsiella*) were also included. Results show that the hybridization only occurs with *E. coli* O157 (Table 1); therefore, specificity and sensitivity values for both were 100% (95% CI for specificity, 87.02 to 100%; 95% CI for sensitivity, 79.95 to 100%). As such, EcoPNA1169 has proven to be highly specific and sensitive for the detection of *E. coli* O157. On the other hand, 5 nontoxigenic O157 strains and two O157 non-H7 strains (both tested negative for H7 antigen) were detected. This indicates that the procedure is specific for O157 despite the H7 and toxin presence. Actually, this might be an important advantage for the identification of other *E. coli* (EHEC) O157 non-H7 strains. For instance, O157:H- strains, which are EH sorbitol-fermenting strains frequently isolated in

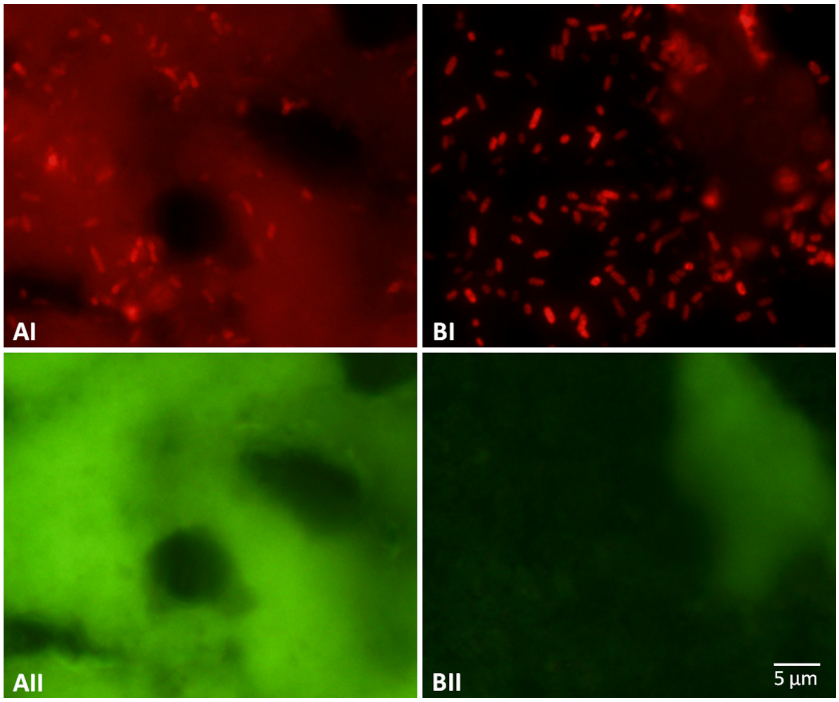

FIG 1 PNA-FISH outcome for ground beef samples artificially inoculated with 10 CFU/25 g of *E. coli* O157:H7 CCC-05-12. Results were obtained using a direct hybridization protocol without any additional sample pretreatment (A) or using a pretreatment with 1% Triton X-100 (B). It is possible to observe a decrease in the autofluorescence intensity for panel B in both red (I) and green (II) channels.

Europe, are commonly associated with large outbreaks of HUS in Germany (15, 29, 30). Although they have not been included in this study, the detection of O157:H- strains is likely to occur.

Another issue in the development of molecular detection methods is the ability to distinguish dead from viable cells (31). This is not a crucial evaluation for this method, since a preenrichment step will be used to amplify the *E. coli* O157 population. Nonetheless, the heat-inactivated bacteria showed a weak fluorescence signal (see Fig. S2 in the supplemental material), indicating that misdetection is unlikely to occur.

Reduction of autofluorescence signal. An important feature to bear in mind when optimizing FISH protocols is that some food components may present a strong autofluorescence signal, which can interfere with bacterial detection (32). To eliminate/reduce this phenomenon, an additional step can be added before the hybridization procedure. We have tested two different approaches: a centrifugation step (to remove some autofluorescent food particles) and the use of a detergent (1% [vol/vol] Triton X-100) to emulsify the fatty compounds. Both steps decreased the autofluorescence signal, but the detergent presented a stronger reduction and also seems to improve the fluorescence signal (Fig. 1). This may happen because detergents can also assist in cell permeabilization (33). Subsequent analyses included this additional step.

Preenrichment optimization. The preenrichment step is also recognized as a limiting step in several microbiological detection methods, mainly due to low numbers of the target bacteria, high levels of competing microflora, and technique detection limit (34, 35). A careful optimization of this step is of great important to achieve high values for sensitivity.

The food samples containing *E. coli* O157:H7 usually present low contamination levels. As the described PNA-FISH detection limit is approximately 10^5 cells per ml (27), an enrichment step is recommended. This enrichment step can be performed using several types of culture media, from complex rich media (such as TSB or BPW) to selective media, such as Gram-negative (GN) broth, R&F enrichment broth (R&F-EB), or *E. coli* (EC) broth (34).

TSB is reported as the most frequently used enrichment broth. Additionally, antibiotics, such as novobiocin (the most commonly used), cefixime, cefsulodin, and vancomycin, as well as other selective compounds (e.g., bile salts to inhibit the non-*Enterobacteriaceae* strains), are often added to enrichment broths. These media then are incubated for a period that usually ranges between 16 and 24 h (overnight growth) at 35 to 42°C. However, results relating to the enrichment protocol efficacy are rare and differ from one study to another.

Regarding the enrichment temperature, it appears that the incubation temperature is not related to the type of serogroup searched (34). Nevertheless, some authors have shown that O157:H7 strains usually present an optimal average temperature around 40°C, which means that temperature can be used to limit the background microflora and favor *E. coli* O157 growth. Actually, the ISO recommended for O157 detection in food samples (ISO 16654:2001) includes a preenrichment step in mTSB+N at 41.5°C.

In order to evaluate the influence of the enrichment medium and incubation temperature on the detection level of the PNA-FISH method, two different media (the selective mTSB+N and the complex BPW), at 37 and 41.5°C, were used. Additionally, the PNA-FISH method probe described in this work was tested in two different types of food samples: ground beef and unpasteurized milk (two matrices commonly associated with O157:H7 infec-

TABLE 2 PNA-FISH results obtained for the detection of *E. coli* O157:H7 on different food matrices inoculated with concentrations between 0.01 and 100 CFU per 25 g or ml[c]

Concn (CFU/25 g or ml)	PNA-FISH result (PP/TP[a]) for:					
	Ground beef					
	37°C		41.5°C		Unpasteurized milk	
	mTSB+N[b]	BPW	mTBS+N	BPW	37°CmTBS+N[b]	41.5°CmTBS+N
100	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)	+ (6/6)
10	+ (6/6)	+ (4/6)	+ (6/6)	+ (2/6)	+(6/6)	+ (6/6)
1	+ (5/6)	− (0/6)	+ (5/6)	− (0/6)	+ (6/6)	+ (6/6)
0.1	− (0/0)	− (0/0)	− (0/0)	− (0/0)	− (0/0)	− (0/0)
0.01	− (0/0)	− (0/0)	− (0/0)	− (0/0)	− (0/0)	− (0/0)

[a] PP/TP, number of samples that tested positive by PNA-FISH/total number of positive samples as determined by the culture method.
[b] Results were considered for the determination of the method sensitivity, specificity, and accuracy.
[c] Results for the three independent assays, performed for two *E. coli* O157:H7 strains, are provided.

tions) artificially contaminated with low *E. coli* O157:H7 concentrations (Table 2).

As observed in Table 2, mTSB allowed the best detection limit, and the use of higher temperatures (41.5°C) did not seem to improve the detection rate of *E. coli* O157. The preenrichment in mTSB allowed a positive detection of 5 out of 6 samples for the lower concentration, while the BPW was not able to provide a concentration above the detection limit. The better performance of mTSB may be related to the selective nature of this medium, which includes novobiocin and bile salts that partially inhibit the growth of the competing microflora. However, after growth rate determination for both *E. coli* strains in BPW and mTSB, it was observed that the medium composition (not the selective factors) was the determinant factor. Growth rates between 1 and 0.8 h^{-1} were observed for the strains grown on mTSB, while for those grown on BPW the values ranged between 0.2 and 0.4 h^{-1} for both temperatures (data not shown).

Although the best performance was observed for mTSB+N, when a multiplex approach is desired, it should be possible to standardize the enrichment step to allow the simultaneous detection of different food-borne pathogens.

Performance in food samples. After the selection of the best enrichment conditions, the PNA-FISH method performance was evaluated in two different matrices. For this, results obtained for 60 food samples evaluated by both techniques (see Table S1 in the supplemental material, samples enriched in mTSB+N at 37°C) were used to determine the sensitivity, specificity, and accuracy values for the PNA-FISH method. ISO 16654:2001 was considered the gold standard. Results were consistent with the inoculation levels for both techniques, with only one discrepant result observed (Table 2; see also Table S1 in the supplemental material). Based on these results, PNA-FISH specificity and sensitivity were 100% (95 CI%, 82.83 to 100%) and 97.22% (95 CI%, 83.79 to 99.85%), respectively. Based on these two values, an accuracy of 98.33% (95 CI%, 83.41 to 99.91%) was observed.

Regarding other molecular methods developed to detect *E. coli* O157, PCR protocols are probably the most widely used. It has recognized advantages over culture and other standard methods for the detection of microbial pathogens, such as specificity, rapidity, accuracy, and capacity to detect small amounts of target nucleic acid in a sample (36). However, a major drawback of PCR detection methods is the occurrence of false-negative results. Works comparing the performance of different PCR-based protocols for O157 detection have shown that some protocols failed to detect a number of samples that were positive by standard culture methods (24, 37, 38). This might be related to inefficient cell lysis (necessary for nucleic acid extraction), nucleic acid degradation, and, more commonly, PCR-inhibitory substances that might be present in food samples (24, 36, 37, 39). Usually PCR inhibition can be solved by diluting the sample 1:10. However, the majority of the published PCR-based approaches for detection of O157 lack an internal amplification control (IAC), which is required in order to monitor the presence of PCR inhibitors. Additionally, they usually are only relatively specific to O157, giving some cross-reactions with O26, O125, O126, O145, and especially with O55 (24, 37).

PNA-FISH has emerged more recently, but it is already established as a robust microbial identification/detection technique (17, 18). As it is not based on an amplification step, this technique is not susceptible to inhibitors. However, similar to what happens with PCR-based protocols, it can present some cross-hybridization with the O55 serotype. An important feature of the PCR protocol is its ability to evaluate the presence of Shiga toxin genes and to specifically identify EHEC. As PNA-FISH targets rRNA sequences, which are universal phylogenetic marks, EcoPNA1169 will have to be combined with a probe targeting a specific sequence of the EHEC group. Regarding the assay time and detection limit, the reported PCR detection limit varies between 10^4 CFU/ml and 1 CFU/35 g of food sample, but to achieve the desired detection limit of 1 CFU an enrichment step is also required (usually of 16 to 24 h) (24, 34, 40, 41). Sensitivity values are not present in some publications, but they seem to be diverse depending on the length of the enrichment step and PCR protocol. Arthur and coworkers have compared 3 PCR protocols and found sensitivity values between 53 and 98% (24). PCR procedures were not subjected to evaluation in this work; however, based on the reported results, it seems that the PNA-FISH method developed here performs at least as well as the existing PCR protocols.

Concluding remarks. EcoPNA1169 is highly specific and sensitive to *E. coli* O157 strains; however, some cross-hybridization may occur with the closely related O55:H7 serotype. The use of selective compounds and higher temperature provided only a limited improvement on the method detection limit. Even so, the use of mTSB+N allowed the PNA-FISH method to reach the desired detection limit of 1 CFU/25 g or ml of food samples.

PNA-FISH performed well in the different matrices tested, and

the inclusion of an additional step with Triton X-100 can significantly reduce the interference of autofluorescent food particles. The implementation of the PNA-FISH method can save at least 2 days in the detection of *E. coli* serotype O157 compared to the traditional bacteriological method; however, the effectiveness of the method detecting the O157:H7 serotype has not been proven. Finally, comparison of results to those of the standard culture method have shown high specificity and sensitivity, with an estimated accuracy of 98.33% (95 CI%, 83.41 to 99.91%).

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C.A., J.M.S., R.R., N.F.A., and M.J.V. have submitted a patent request for the PNA probes used in this study (INPI patent request no. 20131000032694, June 2013). L.C. and R.R. were employees of Biomode S.A. at the time of the study.

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