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Simultaneous and Sensitive Detection of Three Foodborne Pathogens by Multiplex PCR, Capillary Gel Electrophoresis, and Laser-Induced Fluorescence

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The simultaneous detection of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* spp. has been approached by a new multiplex PCR-based procedure followed by capillary gel electrophoresis with laser-induced fluorescence detection (multiplex-PCR-CGE-LIF). As compared to slab gel electrophoresis, the use of CGE-LIF improved from 10- to 1000-fold the sensitivity of the multiplex PCR analysis, allowing the detection of 2.6×10^3 cfu mL^{-1} of *S. aureus*, 570 cfu mL^{-1} of *L. monocytogenes*, and 790 cfu mL^{-1} of *Salmonella* in artificially inoculated food, without enrichment. Following 6 h of enrichment, as low as 260, 79, and 57 cfu mL^{-1} of *S. aureus*, *L. monocytogenes*, and *Salmonella*, respectively, were detected. The CGE-LIF method is shown to be reproducible, providing relative standard deviation (RSD) values lower than 0.8% for analysis time and lower than 5.8% for peak areas. The multiplex-PCR-CGE-LIF proved a powerful analytical tool to detect various food pathogens simultaneously in a fast, reproducible, and sensitive way.

KEYWORDS: Multiplex PCR; CGE; LIF; food analysis; foodborne pathogens; DNA analysis; *Salmonella* spp.; *Staphylococcus aureus*; *Listeria monocytogenes*

1. INTRODUCTION

Salmonella spp., *Staphylococcus aureus*, and *Listeria monocytogenes* are among the foodborne bacteria currently searched in a wide range of foods. These bacteria are human pathogens that have been reported as the most frequent agents in food poisoning. *Salmonella* may cause infections localized at the intestinal epithelium known as "nontyphoid salmonellosis" or gastroenteritis, and also a systemic infection "typhoid salmonellosis" or "enteric fever" with severe consequences (*1*). Staphylococcal food poisoning is due to the ingestion of foods or beverages containing one or more preformed enterotoxins (SE) produced by *S. aureus*. The disease is characterized by symptoms including nausea, vomiting, abdominal cramps, and diarrhea lasting from 24 to 48 h, and complete recovery usually occurs within 1−3 days (*2*). *L. monocytogenes* is associated with meningoencephalitis, septicaemia, and abortion in humans, specially in individuals at risk including pregnant women, newborn babies, the elderly, and the immunocompromised, but when ingested in high numbers, it may also cause noninvasive febrile gastroenteritis in otherwise healthy people (*3*). The three pathogens are considered of economic importance, even in the cases of mild, self-limited illnesses, due to the high sanitary costs as well as their negative repercussion in the food processing industry. Because for the three pathogens there is a potential contamination risk of fresh and ready-to-eat (RTE) foods, simultaneous detection of *L. monocytogenes*, *S. aureus*, and *Salmonella* spp. organisms can facilitate routine testing of food samples by reducing time and cost of labor and media. Bacterial pathogens may coexist, at different concentrations, in the same food sample, but they usually occur at low levels. Their detection is usually preceded by an enrichment step to increase cell numbers to the detection level. For simultaneous detection of more than one pathogen, differences in growth requirements and growth rates should be considered. Bailey and Cox described a method for the simultaneous detection of *Salmonella* and *Listeria* following a 24 h enrichment in universal pre-enrichment broth which allowed sublethally injured bacteria to resuscitate and multiply to sufficiently high numbers to be detected by secondary selective media for each specific bacteria (*4*). However, this type of procedure still relies on the use of selective media, biochemical reactions, and other parameters for bacterial identification and requires several days to obtain results, being therefore very labor- and time-consuming. As an alternative, PCR procedures have been revealed as rapid and highly specific methods by which detection and identification

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can be completed in less than 24 h without the need for isolating pure cultures (*5*). Multiplex PCR detection can also be considered, because the cost of reagents and the preparation time are less in multiplex PCR than in systems where several tubes of uniplex PCR are used. In fact, multiplex PCR procedures have already been described for the simultaneous detection of two pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7 (*6*), *Salmonella* spp. and *Shigella* spp. (*7*), *Salmonella* spp. and shiga-like toxin-producing *E. coli* (*8*), *S. aureus* and *Streptococcus* spp. (*9*), but also three (*S. typhimurium*, *E. coli*, and *Vibrio cholerae* (*10*)), four (*Campylobacter jejuni*, *Salmonella* spp., and *E. coli* O157:H7 (*11*)), five (*E. coli*, *S. typhimurium*, *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* (*12*)), and even six waterborne bacterial pathogens (*Aeromonas hydrophila*, *Shigella flexneri*, *Yersinia enterocolitica*, *S. typhimurium*, *V. cholerae*, *V. parahaemolyticus* (*13*)). Although different detection procedures were used in all of these applications for the amplified DNA (mostly slab gel electrophoresis), none of them reported the subsequent use of capillary electrophoresis. Other approximations for simultaneous detection of *L. monocytogenes* and *Salmonella* spp. combine the immunomagnetic separation and multiplex PCR reaction (IMS-PCR) (*14*) or even IMS-PCR and slot blot detection (*15*). Automation of simultaneous detection of these pathogens has also been approached by Peng and Shelef (*16*) using two BioSys instruments in parallel for specific detection of *L. monocytogenes* and *Salmonella* spp, after a common pre-enrichment step, and a PCR confirmation (30 h detection). With the same aim, Bhagwat adapted the BAX system to a real-time PCR protocol for simultaneous detection of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* that includes the use of a reaction tube for each pathogen and the melting-curve analysis of PCR products as a confirmatory test (*17*). Jothikumar et al. (*18*) developed a real-time multiplex SYBR green-based PCR assay for the simultaneous detection of *Salmonella* serovars and *L. monocytogenes* using a single tube and identification of PCR products by melting curve analysis.

To our knowledge, the simultaneous detection of *Salmonella* spp., *L. monocytogenes*, and *S. aureus* has not been approached yet. Moreover, the combined use of multiplex-PCR and capillary gel electrophoresis (CGE) has not been reported to detect pathogens either. Application of capillary electrophoresis to detect multiple DNA products should bring about analysis with higher sensitivity, separation efficiency (typically, several million theoretical plates/meter for DNA fragments of 80−1000 bp), and resolution. Besides, multiplex-PCR and CGE provide much faster separations than the traditional agarose gel procedure (*19*, *20*). In addition, PCR-based techniques combined with CGE and laser-induced fluorescence detection have demonstrated they are a powerful alternative for the rising need of species detection tools when fraudulent substitution, addition, or contamination are suspected in a foodstuff (*20*−*22*).

In this study, we have developed a multiplex PCR procedure combined with capillary gel electrophoresis and laser-induced fluorescence detection that allows rapid, sensitive, and simultaneous analysis of *Salmonella* spp., *S. aureus*, and *L. monocytogenes*. It includes a protocol for sample collection suitable for different food matrixes and a PCR procedure designed specifically for the assay. With this procedure, smaller amounts of reagents and shorter analysis times than in uniplex PCR are needed, making it more applicable to routine food microbial analysis and more cost-effective. It has been tested in artificially inoculated food.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Growth Conditions, and DNA Extraction. Twelve reference strains were used in this study that include four *Salmonella* strains, *S. typhi* CECT 409, *S. typhimurium* CECT 443, *S. paratyphi* CECT 554, and *S. typhi* CECT 725; three *L. monocytogenes* strains, CECT 4031^T (type strain, serovar 1a), CECT 4032 (serovar 4d), and CECT 940 (serovar 4d); three *S. aureus* strains, CECT 86 ^{T}, CECT 976, and CECT 4013; and two *Escherichia coli* strains, CECT 515 and CECT 4456. *Listeria* strains were grown on Brain Heart Infusion (BHI) or Agar (BHIA) (Merck, Darmstadt, Germany); *Salmonella*, *Staphylococcus*, and *E. coli* strains were grown on Tripticase Soja Agar (TSA) (Merck, Darmstadt, Germany) at 37 °C, 24 h. Strains *S. typhimurium* CECT 554 (hereafter *Salmonella*), *S. aureus* CECT 435, and *L. monocytogenes* CECT 4031^T were used as inocula for sensitivity assays in food. For this, one colony was grown in 5 mL of Buffered Peptone Water (BPW) (Merck, Darmstadt, Germany) at 37 °C, 6 h by agitation at 200 rev min^{-1}. Next, 5 μL of each culture was transferred to 5 mL of fresh BPW tube and incubated at 37 °C overnight. DNA was extracted and purified using the method of Pitcher et al. (*23*).

2.2. Sample Preparation. First, 40 g of meat cut in pieces of 2 g approximately was added to 360 mL of BPW, in a sterile plastic bag with lateral filter (BagPage S 400, BagSystem, Interscience, St-Nom-la-Breteche, France), and homogenized in a stomacher (Stomacher Lab-Blender 400, Seward Laboratories, London, UK) for 1 min. The resulting mixture was taken from the filter side, distributed in aliquots of 40 mL, and inoculated with 400 μL of 10-fold serial dilutions of each strain (previously enumerated by plate counting on TSA or BHIA) including a negative control without inoculation. They were incubated at 37 °C. Aliquots of 10 mL were used for DNA extraction after 0 and 6 h of incubation, and 1 mL aliquots were used after 24 h. Following centrifugation at 4000 rpm for 10 min, pellets were washed with 0.5 mL of TE buffer (10 mmol L^{-1} Tris-ClH; 1 mmol L^{-1} EDTA, pH 8), transferred to a microcentrifuge tube, and centrifuged at 10 000 rpm for 5 min in a microcentrifuge. Pellets were resuspended in 180 μL of lysis buffer (90 μL of 50 mg mL^{-1} lysozyme in TE buffer plus 90 μL of 50 μL mL^{-1} lysostaphin in phosphate buffer at pH 7), incubated for 30 min at 37 °C, and then DNA was purified by DNeasy Tissue Kit (Qiagen GmhH, Hilden, Germany) according to the manufacturer's manual. Purified DNA was recovered in 100 μL of elution buffer.

2.3. Multiplex PCR Reaction. The oligonucleotides used in this work were previously described in the literature: P1-P2 (*24*) for the amplification of a 163 bp fragment corresponding to the *oriC* of *Salmonella*, Pri1-Pri2 (*25*) for the amplification of a 270 bp fragment of *nuc* gene of *S. aureus*, and LMA-LMB (*26*) for the amplification of a fragment of 234 bp corresponding to the *HlyA* gene of *L. monocytogenes*. They were synthesized by Genset Oligos (Innogenetics Diagnóstica y Terapéutica, S.A., Barcelona, Spain).

The reaction mixture contained 200 μmol L^{-1} each dNTP, 1 μmol L^{-1} of each primer, 1 U of thermostable DNA polymerase (DyNAzyme II DNA polymerase, Finnzymes Oy, Finland), and 5 μL of DNA, in a PCR buffer (10 mmol L^{-1} Tris-HCl, pH 8.8; 1.5 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} KCl, 0.1% Triton X-100) in a total volume of 50 μL. Negative control of amplification was performed with 5 μL of water instead of DNA template.

A thermal program of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C, and a final extension of 5 min at 72 °C, in a GeneAmp PCR System 9700 (PE Applied Biosystems, Norwalk, CT) thermal cycler was used.

Specificity of the multiplex PCR reaction was assessed by using purified DNA of strains mentioned above and the selected primers under the multiplex PCR conditions established in this work. In addition, the multiplex PCR reaction was tested in the presence of a DNA mixture of the three species and *E. coli*.

2.4. Agarose Gel Electrophoresis. Fifteen microliters of PCR products was analized by agarose gel electrophoresis through 2% agarose (Pronadisa, Madrid, Spain) gel in TAE buffer (40 mmol L^{-1} Tris-acetate, pH 7.6 and 1 mmol L^{-1} Na_2EDTA). Amplified DNA was observed by UV transillumination after ethidium bromide staining.

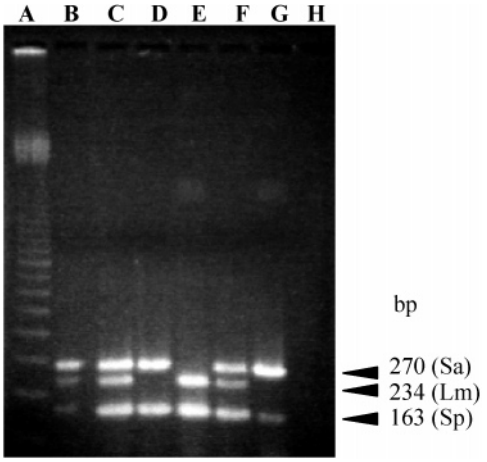

Figure 1. Multiplex PCR amplification of purified DNA with specific primers Pri1/Pri2 (*S. aureus*, 270 bp), LMA/LMB (*L. monocytogenes*, 234 bp), and P1/P2 (*Salmonella* spp., 163 bp). Lane A, 100 bp ladder molecular weight standard (Pharmacia); lanes B–F, mixed DNA from *E. coli* CECT 515 (1), *Salmonella typhimurium* CECT 443 (2), *S. aureus* CECT 86^T (3), *S. aureus* CECT 976 (4), and *L. monocytogenes* CECT 940 (5) as follows: lane B, 2, 4, and 5; lane C, 2, 3, and 5; lane D, 1, 2, and 3; lane E, 1, 2, and 5; lane F, 1, 2, 3, and 5; lane G, 2 and 3; lane H, no template negative control. (Sp) 163 bp *Salmonella* DNA, (Lm) 234 bp *L. monocytogenes* DNA, and (Sa) 270 bp *S. aureus* DNA fragments.

2.5. Capillary Gel Electrophoresis (CGE). Analyses of PCR samples were carried out in a PACE-MDQ (Beckman Instruments, Fullerton, CA) equipped with an Ar+ laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength). Bare fused-silica capillaries with 75 μm i.d. were purchased from Composite Metal Services (Worcester, England). Injections were made at the cathodic end using N_2 pressure of 1 psi for 12 s (1 psi = 6894.76 Pa). Data acquisition and integration were performed with 32 Karat Software (Beckman Instruments, Fullerton, CA).

Before first use, any uncoated capillary was preconditioned by rinsing with 0.1 M HCl for 30 min. Between injections, capillaries were physically coated using 0.1 M HCl for 4 min, 1% PVA (Mw 50 000; Aldrich, Spain) for 2 min, and separation buffer for 4 min. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside. The following conditions were used for PCR product separation: separation buffer (20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1 (Molecular Probes, Breda, The Netherlands) and 4.5% HEC (Mw 90 000; Aldrich, Spain) at pH 7.3); temperature of separation, 45 °C; running electric field, −217 V/cm. 100 bp ladder molecular marker was from Biotools (Madrid, Spain).

3. RESULTS

The multiplex PCR reaction was optimized by assaying different parameters. The primers under study were tested, individually each pair, for specificity and amplification efficiency at different annealing temperatures (50, 55, 56, 57, and 58 °C) using purified DNA of 12 reference strains. The best results were obtained at 56 °C, that allowed the amplification of the three specific bands with a reduction of unspecific amplification products. Further optimization assays were carried out by multiplex PCR reaction at 56 °C annealing temperature. Regarding the concentration of thermostable DNA polymerase, its increase from half to one unit resulted in a higher intensity of amplification bands. Similarly, the intensity of the amplification bands was higher by using 200 μmol L^{-1} of dNTP instead of 100 μmol L^{-1}. No differences were observed when the annealing time was increased to 50 s, nor by the addition of 2 and 3 times the primer concentration, nor by the addition of 10% glycerol to the reaction mixture. The increase in magnesium chloride concentration from 1.5 to 2.5 and 3 mmol L^{-1} resulted in a slight increase of amplification band intensity.

Figure 1 shows co-amplification of selected specific primers P1-P2, Pri1-Pri2, and LMA-LMB using the multiplex PCR conditions established in this work, using as target DNA either purified DNA or whole cells from the three target bacteria as well as *E. coli*.

Results of the multiplex PCR sensitivity tested in artificially inoculated raw beef at incubation times of 0, 6, and 24 h are displayed in **Figure 2**. Analysis of multiplex PCR reactions using agarose gel electrophoresis allowed simultaneous detection of the three pathogens, without enrichment, and detection levels were 5.7×10^5 cfu mL^{-1} for *L. monocytogenes*, 7.9×10^5 cfu mL^{-1} for *Salmonella*, and 2.6×10^6 cfu mL^{-1} for *S. aureus*. Following an enrichment step in BPW at 37 °C, *Salmonella*, *L. monocytogenes*, and *S. aureus* could be detected at 5.7×10^1, 7.9×10^1, and 2.6×10^2 cfu mL^{-1}, respectively, after 6 h of incubation, and at 6, 8, and 26 cfu mL^{-1}, respectively, after 24 h of incubation. These values correspond to the lowest cell concentrations tested in the assay.

A CGE separation method using dynamically coated fused silica capillaries together with a replaceable buffer with a

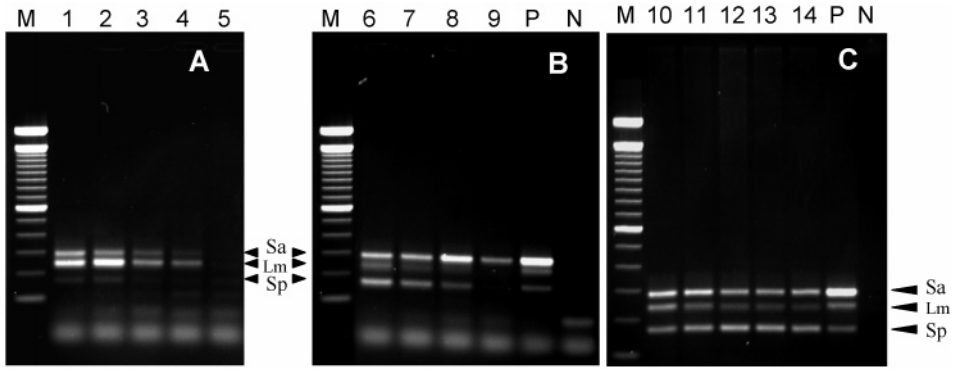

Figure 2. Sensitivity of the multiplex PCR reaction tested in artificially inoculated raw beef, (**A**) without enrichment; (**B**) 6 h of incubation time; (**C**) 24 h of incubation time. Lane M, molecular size marker (100 base-pair ladder, Pharmacia); N, noninoculated sample; P, positive control, 250 ng purified DNA of each strain; 1–14 samples inoculated with serial 10-fold dilutions of *L. monocytogenes* CECT 4031^T, *S. paratyphi* CECT 554, and *S. aureus* CECT 435, in cfu mL^{-1}, as follows: 5.7×10^6, 7.9×10^6, 2.6×10^7 (lane 1A); 5.7×10^5, 7.9×10^5, 2.6×10^6 (lane 2A); 5.7×10^4, 7.9×10^4, 2.6×10^5 (lanes 3A and 10C); 5.7×10^3, 7.9×10^3, 2.6×10^4 (lanes 4A, 6B, and 11C); 5.7×10^2, 7.9×10^2, 2.6×10^3 (lanes 5A, 7B, and 12C); 5.7×10^1, 7.9×10^1, 2.6×10^2 (lanes 8B and 13C); 5.7, 7.9, 2.6×10^1 (lanes 9B and 14C). (Sp) 163 bp *S. paratyphi* DNA, (Lm) 234 bp *L. monocytogenes* DNA, and (Sa) 270 bp *S. aureus* DNA fragments.

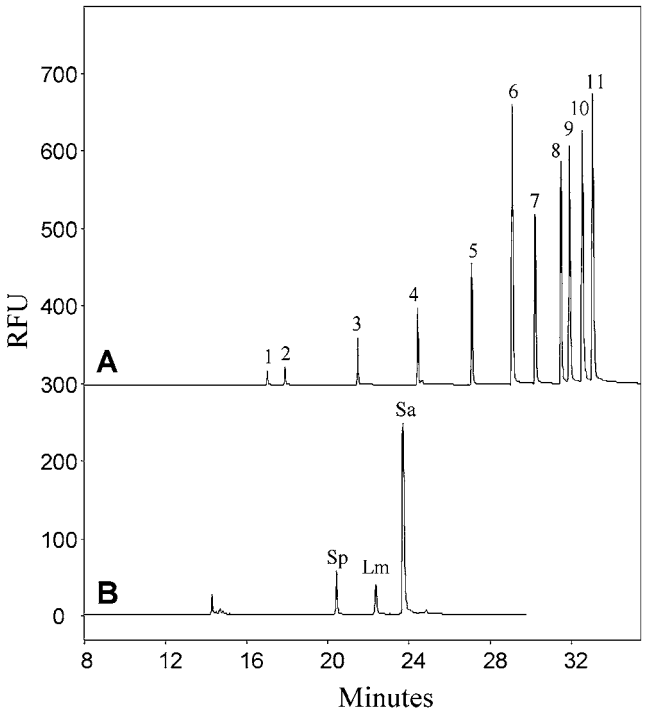

Figure 3. Separation of (**A**) a 100 bp DNA ladder (Biotools, Spain) and (**B**) multiplex PCR of 250 ng purified DNA of each strain. Separation conditions: uncoated fused silica capillary with 60 cm of total length, 50 cm of effective length, and 75 μm i.d. Separation voltage: –13 kV, 45 °C running temperature. Running buffer: 20 mM Tris, 10 mM ortophosphoric acid, 2 mM EDTA, 1.5 M Urea, 500 nM YOPRO-1, 4.5% HEC at pH 7.3. Injection for 12 s using N_2 pressure (1 psi). (1) 80 bp, (2) 100 bp, (3) 200 bp, (4) 300 bp, (5) 400 bp, (6) 500 bp, (7) 600 bp, (8) 700 bp, (9) 800 bp, (10) 900 bp, (11) 1000 bp DNA fragments, (Sp) 163 bp *S. paratyphi* DNA, (Lm) 234 bp *L. monocytogenes* DNA, and (Sa) 270 bp *S. aureus* DNA fragments.

fluorescent intercalating dye and laser-induced fluorescence (LIF) detection was used to analyze DNA samples. This CGE-LIF method, developed at our laboratory (*24*), allowed the detection of the three amplicons in 25 min (**Figure 3B**). Lengths of DNA fragments were verified by comparing theoretical and calculated sizes. Migration times (t_m) of the peaks were used to calculate the length of the PCR amplicons. To do this, an equation was obtained plotting the logarithm of length of DNA fragments (bp) ranging from 80 to 500 bp versus the logarithm of inverse t_m data of corresponding DNA fragments obtained from the separation of a 100 bp DNA ladder sample ($\log(bp) = 3.87 - 33.89/t_m$ ($R^2 = 0.999$, $n = 6$), see **Figure 3A**). By interpolation of migration times from the peaks displayed in electropherograms of PCR products separations, the calculated size values obtained were 161, 232, and 270 bp. The signal/noise ratios were calculated to determine positive detection. An arbitrary criterion was established considering as positive assay those samples that provide a signal/noise ratio higher than 3.

The described CGE-LIF separation method was used to analyze PCR amplifications of artificially inoculated raw beef and was compared to the more standard slab gel electrophoresis. Thus, **Table 2** summarizes the signal/noise ratios obtained for the three pathogens after 0 and 6 h of incubation using both analytical procedures. The negative control of amplification and negative control without inoculation were confirmed by both agarose slab gel and capillary gel electrophoresis analysis. As can be deduced from the results of **Table 2**, detection of the three pathogens in the dilution assayed from artificially inoculated raw beef without enrichment (i.e., 0 h of incubation) was only possible by using CGE-LIF. After 6 h of incubation, both procedures provide positive results; however, signal/noise ratios using CGE-LIF are up to 40 times higher than those from slab gel electrophoresis for the same sample. **Figure 4** shows the results of the analysis of these PCR amplifications of the same dilution (i.e., 5.7×10^2 cfu mL^{-1} *L. monocytogenes*, 7.9×10^2 cfu mL^{-1} *Salmonella*, and 2.6×10^3 cfu mL^{-1} *S. aureus*) of the artificially inoculated meat at different incubation times (0 and 6 h). The CGE-LIF electrophoregram of **Figure 4A** displays peaks of DNA amplified fragments from the pathogens tested, while no band could be visualized on the ethidium bromide-stained gel (see **Figure 4**). **Table 2** shows the signal/noise ratios calculated for 161, 232, and 270 bp peaks, at 0 and 6 h of incubation. The values corresponding to the CGE-LIF electropherogram of this sample at 0 h were 220, 29, and 37, respectively (**Figure 4A**). After 6 h of incubation, the signal/noise ratios calculated by CGE-LIF were 2868, 377, and 2533, respectively (see **Figure 4B**). Moreover, it could be observed that using very diluted concentrations of the three pathogens in artificially inoculated raw beef (8 cfu mL^{-1} of *Salmonella*, 6 cfu mL^{-1} of *L. monocytogenes*, and 26 cfu mL^{-1} of *S. aureus*), after only 6 h of incubation they could be detected by CGE-LIF (with signal/noise ratios of 198, 45, and 1637, respectively), obtaining negative results for *Salmonella* and *L. monocytogenes* when the same sample was analyzed by slab gel electrophoresis.

A reproducibility study of the separation CGE-LIF method was performed. The reproducibility of migration times and corrected peak areas was tested by injecting the same PCR reaction products from a given multiplex PCR reaction. **Table 1** shows the results of this reproducibility study using the GCE-LIF method.

Table 1. Reproducibility of Migration Times (t_m), Corrected Areas for the Same Day and for Three Different Days[a]

	same day ($n = 5$)	three days ($n = 15$)
t_m(min)[b]	20.4	20.5
% RSD[c]	0.4	0.8
corrected peak area[b]	26 281	25 212
% RSD	3.3	5.8

[a] All conditions are the same as those in **Figure 3**. [b] For 163 pb DNA fragment. [c] RSD, relative standard deviation calculated as the standard deviation $\sigma n - 1$ divided by the mean value and multiplied by 100.

Table 2. Signal/Noise Ratios Determined by Multiplex PCR Combined with Slab Gel Electrophoresis (SGE) or CGE-LIF for a Given cfu mL^{-1} Value of *Salmonella*, *L. monocytogenes*, and *S. aureus* after 0 and 6 h of Incubation

	CGE-LIF			SGE		
	Salmonella	*Listeria*	*Staphylococcus*	*Salmonella*	*Listeria*	*Staphylococcus*
starting cfu mL^{-1}	790	570	2600	790	570	2600
signal/noise ratios after 0 h of incubation	220[a]	29	37	ND[b]	ND	ND
signal/noise ratios after 6 h of incubation	2868	377	2533	62	20	104

[a] S/N ratio values calculated from CGE-LIF electropherograms or densitometric analysis of agarose gel. [b] Not detected.

4. DISCUSSION

The simultaneous detection of the three major foodborne pathogens considered as the most frequent causes for food

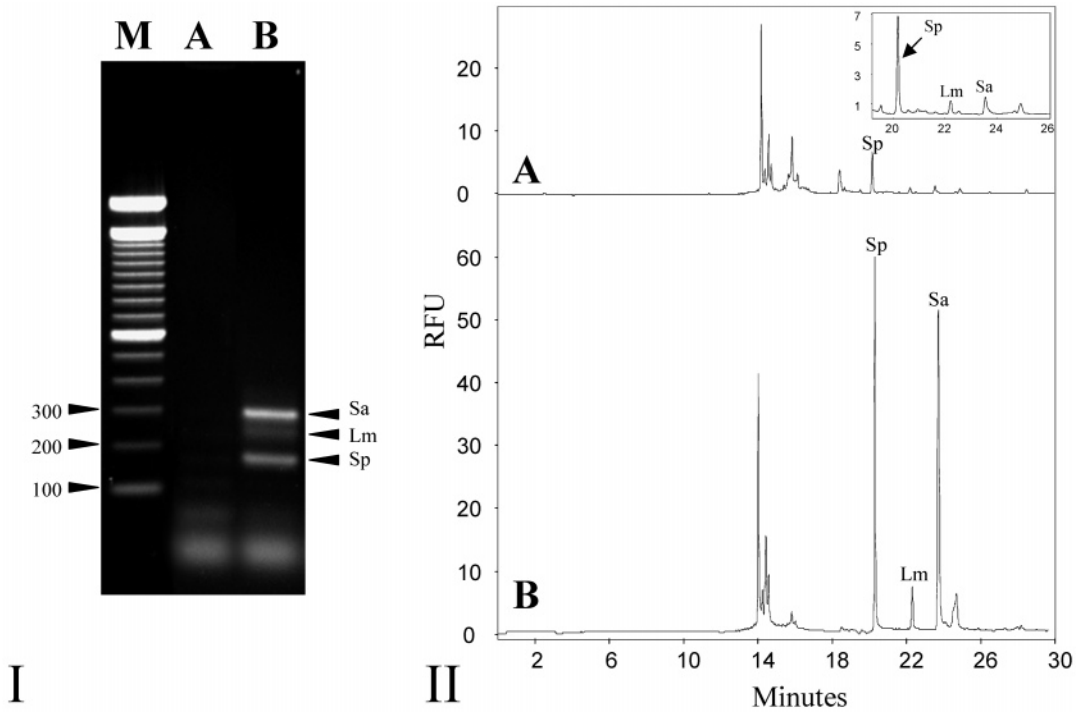

Figure 4. Electrophoretic analysis of multiplex PCR reactions of inoculated raw beef samples with 5.7×10^2, 7.9×10^2, 2.6×10^3 cfu mL^{-1} of *L. monocytogenes* CECT 4031^T, *S. paratyphi* CECT 554, and *S. aureus* CECT 435, respectively, incubated at different times. The analysis was performed by (I) slab agarose gel and (II) CGE-LIF. Samples: (M) 100 bp ladder molecular weight standard (Pharmacia); (A) sample at 0 h incubation time; (B) sample at 6 h incubation time. (Sp) 163 bp *S. paratyphi* DNA, (Lm) 234 bp *L. monocytogenes* DNA, and (Sa) 270 bp *S. aureus* DNA fragments. All of the separation conditions are the same as those in **Figure 3**.

poisoning (i.e., *Salmonella* spp., *S. aureus*, and *L. monocytogenes*) has been approached in the present work by a single sample processing procedure and a multiplex PCR reaction. Specific primers were selected, among those previously published, taking into account that they should have similar melting temperatures (T_m) and similar lengths of target DNA to prevent differential yields in band amplification products (*28*). The concentrations of the PCR reaction components were adjusted according to Henegariu et al. (*29*) to improve co-amplification and were standardized as one unit of thermostable DNA polymerase, 200 μmol L^{-1} dNTPs, and 1 mmol L^{-1} of each primer. The sample processing protocol was also adapted for co-detection of the three species by including both lysozyme and lysostaphin in a single incubation step at 37 °C to lyse cells. Under these conditions, the six specific primers have been adapted to work simultaneously in one PCR reaction tube, rendering specific amplification products for each pathogen.

Among the different factors that control the detectable level of PCR products (e.g., DNA quality and extraction procedure, adequate primers selection, PCR conditions optimization), the technique selected to analyze PCR products plays a crucial role. Thus, the most frequent procedure to analyze PCR products uses agarose gel electrophoresis, followed by ethidium bromide staining and recording the image with a digital imaging device. However, this procedure requires large volumes of sample, is time-consuming, and is essentially semiquantitative.

The procedure established in this study, that is, multiplex PCR-CGE-LIF, can be used for simultaneous detection of the three species. Besides, it is demonstrated that CGE-LIF provides accurate determination of the sizes of amplified fragments, as can be deduced from the good agreement achieved between the sizes of the DNA fragments experimentally obtained (161, 232, and 270 bp) and the theoretical sizes of these amplicons (163, 234, and 270 bp, respectively).

In this work, the use of CGE with LIF detection significantly improves the levels of detection of the three pathogens tested, allowing their simultaneous detection at concentrations of 570 cfu mL^{-1} for *L. monocytogenes*, 790 cfu mL^{-1} for *Salmonella*, and 2.6×10^3 cfu mL^{-1} for *S. aureus* in raw beef inoculated samples, without enrichment. That is, the use of CGE-LIF improves the sensitivity by at least 10-fold as compared to traditional slab gel electrophoresis. These detection levels are in accordance with those reported in the literature for uniplex PCR or multiplex PCR with a previous enrichment step in *S. aureus* (*25*, *30*), *Salmonella* spp. (*31*, *32*), and *L. monocytogenes* (*33*, *34*).

To improve the sensitivity of the multiplex PCR detection, the inclusion of an enrichment step was tested on three artificially inoculated raw beef samples. Enrichment was carried out in BPW because it is a generally recommended, nonselective media, in which the three bacteria are able to grow. Agarose gel electrophoresis showed PCR specific products of all pathogens tested in amplification reactions of samples incubated during 24 h, but positive simultaneous amplification could be only detectable above 57, 79, and 260 cfu mL^{-1} of *Salmonella*, *L. monocytogenes*, and *S. aureus*, respectively. CGE-LIF electropherograms of PCR amplification of enriched samples revealed the higher sensitivity of the CGE-LIF method showing levels of detection as low as 6, 8, and 26 cfu mL^{-1} of *Salmonella*, *L. monocytogenes*, and *S. aureus*, respectively, after only 6 h of incubation time. Moreover, for this dilution (the lowest assayed), the signal/noise ratios calculated from the CGE-LIF electrophoregram for the DNA fragments of 163, 234, and 270 bp were 198, 45, and 1637, respectively, indicating that even lower concentrations of these pathogens could theoretically be detected by this procedure. Interestingly, under identical conditions in agarose gel electrophoresis, bands of 163 and 234 bp, corresponding to *Salmonella* and *L. monocytogenes*, respec-

tively, could not be detected, which is an additional indication of the better sensitivity provided by CGE-LIF.

In addition, the different growth rates of the three pathogens investigated lead to a lower increase of *L. monocytogenes* with respect to *S. aureus* and *Salmonella*, after 6 h of enrichment, as it is shown in the electrophoregram of **Figure 4B**. After 24 h of enrichment, *Salmonella* showed the higher increase (**Figure 2C**). Thus, depending on the initial cell numbers and relative quantities of the three pathogens, the enrichment step could result in a false negative detection of *L. monocytogenes*. Yet in the case of *Salmonella*, the 24 h of enrichment would ensure the absence of *Salmonella* spp.

The reproducibility of the CGE-LIF procedure was good, as can be seen in **Table 1**, where the % RSD for migration times were 0.4% and 0.8% for the same day ($n = 5$) and three different days ($n = 15$), respectively. Moreover, % RSD values for corrected peak areas range from 3.3% to 5.8% for the same day ($n = 5$) and three different days ($n = 15$), respectively. These values confirm the good possibilities of the CGE-LIF procedure for quantitative purposes. In addition, the efficiency achieved was up to 1.1×10^6 plates/m calculated for the 163 bp peak in **Figure 3**. These values make evident that the proposed method is reproducible and efficient, and therefore can be used with confidence for analyzing multiplex PCR products to detect *Salmonella* spp., *L. monocytogenes*, and *S. aureus* simultaneously.

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