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Multiplex Real-time PCR for the Simultaneous Detection of *Salmonella* spp. and *Listeria monocytogenes* in Food Samples

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Abstract In this study, we have developed a rapid method for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in foods, combining culture enrichment and a multiplex real-time polymerase chain reaction (PCR). The assay used two pre-existing primer-probe sets, labelled with different reporter dyes to enable the direct distinction of the original contaminating agent. Amplification efficiency and inclusivity/exclusivity of the combined assay was successfully assessed. The overall process included the culture enrichment based on the ISO standard, consisting of 24 h incubation in appropriate media (Half Fraser Broth for *Listeria* and buffered peptone water (BPW) for *Salmonella*), followed by a single DNA extraction of mixed enrichment aliquots, and real-time PCR detection of the *hly* and *bipA* genes of *L. monocytogenes* and *Salmonella* spp., respectively. An internal amplification control, co-amplified during the PCR run, was included in the assay to verify the results. The tool was evaluated with a variety of artificially inoculated samples of fresh products and ready to eat and cooked dishes, allowing the identification of the target pathogens down to 5 CFU/25 g of food sample. Moreover, the analysis saved a considerable amount of time compared to the ISO standard, being performed in less than 2 working days. Specificity, sensitivity and accuracy were satisfactorily tested by comparison to the standard methods ISO 11290-2:1998 and ISO 6579:2002, suggesting that the tool has a great potential as a reliable alternative for food safety assurance providing rapid detection of both pathogens in food samples.

Keywords *Salmonella* · *Listeria monocytogenes* · Multiplex · Real-time PCR · Food quality control · Simultaneous detection

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Introduction

Salmonellosis and listeriosis are two of the most common foodborne diseases (Anonymous 2001; Mead et al. 1999), and its early and sensitive detection is a critical issue in public health policy (Rijpens and Herman 2002).

Ingestion of foods contaminated with *Listeria monocytogenes* can result in listeriosis, a severe infectious disease characterized by meningoencephalitis, abortion, septicemia and a high fatality rate (30%). Listeriosis predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients. Salmonellosis, on the other hand, is less severe, although it is also a major public health concern causing millions of human cases worldwide every year and resulting in thousands of deaths (Tirado and Schmidt 2001).

Food industry and public health services demand for faster methods than those currently in use, still based in laborious procedures involving the selective enrichment and isolation of pathogenic bacteria, which often require final biochemical or serological confirmation, taking several days until a result is confirmed.

Many PCR and real-time PCR assays targeting different genes have been already described, that combined with culture enrichment (either separate or simultaneous) have allowed a faster specific detection of *L. monocytogenes* or *Salmonella* spp. than that achieved with standard culture methods (Hoorfar 1999; Kawasaki et al. 2005; Malorny et al. 2003; O'Grady et al. 2008; Omiccioli et al. 2009; Rodriguez-Lazaro et al. 2004; Rudi et al. 2005; Somer and Kashi 2003). Multiplex PCR has also been successfully applied as an option to further reduce costs and turnaround time to results in food safety tests, enabling the simultaneous analysis of the presence of distinct foodborne pathogens in one single reaction (Elizaquível and Aznar 2008; Jothikumar et al. 2003; Nguyen et al. 2004; Wang et al. 2007). The simultaneous control of *Salmonella* spp. and *L. monocytogenes* has been accomplished either by conventional (Kawasaki et al. 2005, 2009; Germini et al. 2009; Jofré et al. 2005) or by real-time PCR technologies (Bhagwat 2003; Wang et al. 2004). In the latter case, the described systems rely on SYBRgreen as the reporter dye, requiring subsequent melt-curve analysis to distinguish each pathogen; although dual-labelled probes and high-resolution melting (HRM) have also been used in milk samples (Omiccioli et al. 2009).

In this study we developed a two-target real-time PCR assay based on two previously described TaqMan® systems for the detection of *Salmonella* spp. (Calvó et al. 2008) and *L. monocytogenes* (Rodriguez-Lazaro et al. 2005) in foods, also including an internal amplification control (IAC) to discard false negative results. The method was validated on a large variety of food matrices, using the enrichment procedure approved by the ISO standard methods (Anonymous 1998, 2002), and a simple DNA preparation method. Results could be obtained in less than 2 working days (<30 h). The detection limit was determined in a range of naturally and artificially contaminated samples, including fresh products as well as ready to eat cooked dishes. Finally, the accuracy of the method was further evaluated by comparing the results with those obtained by the standard methods in a series of routine samples. To our knowledge, this is the first reliable multiplex real-time PCR to simultaneously analyze *Salmonella* spp. and *L. monocytogenes* allowing a fast DNA extraction procedure, based on TaqMan® technology that can be applied in a large variety of foodstuffs.

Materials and Methods

Bacterial Strains and Culture Conditions

80 bacterial strains were selected to test both the inclusivity and the exclusivity of the PCR based assay. These strains were either purchased at the Spanish Type Culture Collection (CECT), at the American Type Culture Collection ATCC), at the National Collection of Industrial, Food and Marine Bacteria (NCIMB) or at the German Collection of Microorganisms and Cell Cultures (DSMZ). Other strains were isolated from distinct environmental sources at the University of Barcelona, at the University of Girona, at the Centre de Recerca en Sanitat Animal (CReSA) in Barcelona, or at the ITACyL (Table 1).

All bacterial strains were grown at 37 °C for 24 h in Brain Heart Infusion (BHA) agar.

Food Sample Analysis

Salmonella food enrichments were performed by homogenizing 25 g of food sample in 225 ml of buffered peptone water (BPW) and incubating at 37 °C for 18 h. *L. monocytogenes* enrichments were carried out homogenizing 25 g of food sample in 225 ml of Half Fraser Broth (HF) and incubated at 37 °C for 24 h. BPW and HF were prepared according to the manufacturer's indications (Biokar Diagnostics, Allonne, France). After 18 h at 37 °C, enrichments in BPW were maintained at 4 °C for 6 h.

Samples were artificially spiked following the procedures described in EN ISO16140:2003 (Anonymous 2003), and treated as indicated in UNE-EN ISO 11290-2:1998 and ISO 6579:2002. Briefly, samples were inoculated before homogenization in BPW or HF by adding known concentrations of *Salmonella* and *L. monocytogenes* cells from pure cultures in Brain Heart Infusion (BHI) medium as estimated by spectrophotometric measures. The concentration in the cell suspensions used for artificial inoculation was confirmed by plate counts on BHA kept at 37 °C overnight.

Analysis of Naturally Contaminated Food Samples

A total of 126 samples were analyzed in parallel by the classical culture methods described by the UNE-EN ISO 11290-2:1998 and ISO 6579:2002 to compare the results obtained using the multiplex real-time PCR method designed.

For *Salmonella* spp., the samples consisted of 72 BPW enrichments from diverse foods, including ten meat matrices (five beef hamburgers, four meat products, one turkey meat), 14 fish matrices (ten shellfish, three salmon, one fried batter coated fish), 18 fruit and vegetable matrices (six lettuce, one carrot salad, one vegetable purée, one sweet corn, one stewed lentils, one nutmeg, one flour, six horchata), dairy products (three samples of raw milk, five Roquefort cheese, one *fromage fraise*), two hard-boiled eggs, one omelet, one egg in béchamel sauce, eight lasagna and one chocolate bar.

Table 1 Strains used in this study and their sources

Organism	Strain	Source of isolation	qPCR *bipA/hly*
Arcobacter nitrofigilis	DSM7299		–/–
Bacillus cereus		Soil, Girona	–/–
Bacillus megaterium		Soil, Girona	–/–
Bacillus sp.	CECT 40		–/–
Bacillus subtilis		Soil, Girona	–/–
Campylobacter coli		Poultry, Barcelona	–/–
Campylobacter jejuni	CRESA 599	Poultry, Barcelona	–/–
Campylobacter lari		Poultry, Barcelona	–/–
Citrobacter freundii	CECT 401		–/–
Enterobacter aerogenes	CECT 684T		–/–
Enterobacter asburiae	ATCC 35953		–/–
Enterobacter cancerogenus	ATCC 33241		–/–
Enterobacter cloacae	ATCC 13047		–/–
Cronobacter sakazakii	DSM 4485		–/–
	ATCC 51329		–/–
	CECT 4078T		–/–
	CECT 857T		–/–
Enterobacter cowanii	DSM 18146		–/–
Enterobacter kobei	DSM 13645		–/–
Enterobacter ludwigii	DSM 16688		–/–
Enterobacter pyrinus	DSM 12410		–/–
Enterobacter radicincitans	DSM 16656		–/–
Enterobacter turicensis	DSM 18397		–/–
Enterococcus faecalis	CECT 481		–/–
Enterococcus faecium		Poultry, Barcelona	–/–
Enterococcus gallinarum		Poultry, Barcelona	–/–
Escherichia coli	CECT 101		–/–
	CECT 105		–/–
	CECT 12242		–/–
Listeria grayii	CECT 942		–/–
	CECT 931		–/–
	CECT 4181		–/–
Listeria inocua	CECT 910		–/–
	CECT 4030		–/–
	CECT 910		–/–
Listeria ivanovii	CECT 913		–/–
Listeria monocytogenes	CECT 933		–/+
	CECT 937		–/+
	CECT 934		–/+
	CECT 4031		–/+
	CECT 935		–/+
	CECT 938		–/+
	CECT 936		–/+
	CECT 940		–/+
	CECT 932		–/+
	CECT 4032		–/+
	CECT 911		–/+
Listeria seeligeri	CECT 917		–/+
	CECT 939		–/–

Table 1 (continued)

Organism	Strain	Source of isolation	qPCR *bipA/hly*
	CECT 941		–/–
Listeria welshimeri	CECT 919		–/–
Methylophilus methylotrophus	DSM 46235		–/–
Micrococcus luteus	CECT 241		–/–
Nitrosospira multiformis	NCIMB 11849		–/–
Paenibacillus polymyxa		Food, Girona	–/–
Proteus mirabilis	CECT 170		–/–
Pseudomonas aeruginosa	CECT 532		–/–
Pseudomonas fluorescens	CECT 378		–/–
Pseudomonas mendocina		Food, Girona	–/–
Pseudomonas putida	CECT 324		–/–
Salmonella choleraesuis	CECT 4182		+/–
	CECT 4153		+/–
Salmonella choleraesuis ssp. *choleraesuis*	CECT 4000		+/–
	CECT 4139		+/–
	CECT 4151		+/–
	CECT 4731		+/–
	CECT 409		+/–
	CECT 4152		+/–
	CECT 4155		+/–
Salmonella enterica ssp. *Arizonae*	CECT 4395		+/–
Salmonella typhimurium LT2		Food, Girona	+/–
Salmonella typhimurium	CECT 4296		+/–
Serratia liquefaciens	DSM 30125		–/–
Serratia marcescens	CRESA 441.7		–/–
Shigella sonnei		Food, Girona	–/–
Shigella spp,	CRESA GL17		–/–
Staphylococcus aureus	CRESA FM4		–/–
Streptococcus pneumoniae	CECT 933		–/–
Streptococcus pyogenes	CECT 598		–/–
Streptococcus salivarius	CECT 805		–/–

CECT Spanish Type Culture Collection, *DSM* German Collection of Microorganisms and Cell Cultures, *ATCC* American Type Culture Collection, *CReSA* Centre de Recerca en Sanitat Animal, Barcelona, *NCIMB* National Collection of Industrial, Food and Marine Bacteria

The ability to detect *L. monocytogenes* was tested in 54 HF food enrichments of one chocolate bar, 14 dairy products (seven Roquefort cheese, two soft cheese, two dairy products, one pudding, one pasteurized milk, one raw milk), 13 vegetable matrices (seven lettuce, one vegetable stew, two ready-to-eat mixed salads, one vegetable purée, one lentil stew, one dehydrated parsley), ten fish matrices (eight salmon, one shark, one halibut), seven meat matrices (one minced veal meat, one hot dog, one pork pate, three cured pork sausages, one beef hamburger), one ready-to-eat cooked dish, 14 lasagna and one cake.

Specificity (number of negative results properly assigned), efficiency (percentage of samples rightfully assigned) and sensitivity (percentage of positive results correctly assigned) of the new multiplex real-time PCR were measured against the reference method, according to the calculations described in the EN ISO16140:2003.

DNA Purification

Bacterial Cultures

Isolated colonies from bacterial strains were resuspended in sterile BHI medium, and DNA was extracted and purified using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) as specified by the manufacturer.

Food Samples

In order to obtain comparable results for the validation against the reference test method, 1-ml aliquots of the enrichments in BPW or HF from naturally contaminated food samples were extracted using the same kit mentioned above.

For the rest of food enrichments used to calculate the detection limit, DNA extraction was carried out using the DNAready® Lysis Buffer (Microbial SL, Girona, Spain). In this case, mixtures containing 900 µl of each BPW and HF enrichment were mixed and centrifuged during 5 min at 8,000× *g*. After pellet resuspension in 200 µl of lysis buffer, samples were incubated 30 min at 56 °C, 10 min at 95 °C and finally 5 min at −20 °C.

DNA concentration was determined using a Qubit™ Quantitation Platform (Invitrogen, Carlsbad, CA, USA). The number of genome equivalents in DNA extractions was calculated based on the genome size of *Salmonella enterica* ssp. *enterica serovar Choleraesuis str. SC-B67* genome (4.94 Mb) (Chiu et al. 2005) and *L. monocytogenes* EGD-e (2.94 Mb) (Glaser et al. 2001), and considering that the average base pair weight is 652 g/mol.

Multiplex Design and Real-time PCR Conditions

Two previously designed and validated sets of primers and probe specific for *Salmonella* spp. (Calvó et al. 2008) and *L. monocytogenes* (Rodriguez-Lazaro et al. 2005) were used to compose the multiplex assay. Primers and probe for the detection of *Salmonella* were complementary to a region of the *bipA* gene, whereas the primers and probe to detect *L. monocytogenes* targeted a region of the *hly* gene.

All primers and probes combinations were evaluated with the NetPrimer software (PREMIER Biosoft International, Palo Alto, CA, USA) for the formation of primer–dimer structures and to discard putative interactions between the two detection sets.

A 65-bp IAC system previously used in the real-time PCR system for the detection of *Salmonella* spp. (Calvó et al. 2008) was also incorporated.

Probe labeling was modified in order to allow the simultaneous multiplex assay. The probe used to detect *L. monocytogenes* was labelled with the fluorochrome 6-FAM, whereas the *Salmonella* specific probe was labelled with JOE, and the IAC probe with CY5. Black hole quenchers were used instead of TAMRA to minimize background fluorescence. BHQ1 was used in combination with FAM and JOE, whereas BHQ3 was used with CY5.

Real-time PCR was carried out in 20-µl (total volume) reaction mixtures using an Mx3005P™ system (Stratagene, Santa Clara, CA, USA). Positive and non-template control reactions were included in all tests. The IAC was used at an optimal concentration of 10^5 copies per reaction. PCR conditions were 95 °C for 10 min, and 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Concentration of the reaction mixture components was maintained as previously described (Calvó et al. 2008; Rodriguez-Lazaro et al. 2005), only increasing the number of copies of IAC DNA to 10^5 copies per reaction. Fluorescence values in PCR reactions were analyzed using the Mx3005P™ QPCR Systems software (Stratagene, Santa Clara, CA, USA).

Determination of the Linearity and Efficiency of the Reaction

Purified DNA from *Salmonella choleraesuis* CECT 4139 and *L. monocytogenes* CECT 933 were used to determine the linearity and the efficiency of the PCR assay. This was done for each targeted pathogen separately, and also using mixed DNA extracts. Tenfold dilutions of each DNA were tested within a concentration range of 10^1 to 10^7 genomic copies per reaction, with ten replicates, and in the presence of 10^5 copies of the IAC. Threshold cycle (Ct) values for positive reactions ranged from 17 to 35. Linearity for each strain was assessed by plotting the obtained threshold cycle (Ct) against the logarithm of the number of genomes in the reaction. The efficiency of the real-time PCR assay was calculated for each strain using the formula: $\text{Efficiency} = \left[10^{(-1/\text{slope})}\right] - 1$.

In addition, to test the robustness of the amplification limit of the multiplex assay and discard the possible interference in the detection of the two targeted pathogens by competence, equal (ranging from 10^1 to 10^7) as well as variable (testing up to differences of 4 log units) proportions of both targeted DNAs were mixed in PCR reactions and assayed with ten replicates.

Detection Limit of the Assay

Twenty-two different matrices, consisting of crab salad, pasteurized milk, smoked salmon, fresh eggs, veal hamburger, black olives, avocado, guacamole, boiled sausage, ready to eat salad, and a series of cooked dishes (Bolognese lasagna, vegetable lasagna, chicken lasagna, Bolognese macaroni, four-cheese tortellini, noodles with seafood, seafood casserole, hake *marinière*, veal and mushroom stew, meatballs with smashed potatoes, chicken curry with rice, and grilled chicken), were used per duplicate to calculate the detection limit of the multiplex real-time PCR method. Food types were checked for the absence of naturally occurring *Salmonella* spp. and *L. monocytogenes* and then inoculated with both pathogens at diverse levels. Every food type was inoculated with 5, 10 and 100 CFU/25 g prior to homogenization. Bacterial enumeration was carried out by plate count on BHA after food inoculation.

For each food type, eight analytical portions of 25 g (corresponding to inocula of 0, 5, 10 and 100 CFU/25 g of *Salmonella* or *L. monocytogenes*) were enriched in BPW or HF, respectively, and subjected to DNA extraction as previously described. After the required incubation time, nine hundred μl aliquots of enrichments in BPW and HF of the same food type were mixed in a single 2-ml micro-centrifuge tube, testing all possible inoculum combinations of *Salmonella* and *L. monocytogenes*, and DNA extractions were performed from the mixtures prior to real-time PCR analysis.

Results

Inclusivity and Exclusivity Tests

Inclusivity and exclusivity tests were performed with a total of 80 strains (Table 1). All 13 tested strains of *Salmonella* spp. and all 11 tested strains of *L. monocytogenes* were correctly identified, whereas the rest of tested strains yielded a negative result (Table 1). In all PCR reactions, Ct values for the IAC ranged from 27 to 29, which corroborated that no problems of inhibition occurred.

Linearity and Efficiency of the Reaction

Genomic DNA of *S. choleraesuis* CECT 4139 and *L. monocytogenes* CECT933 were used as a template for linearity and efficiency tests. The *bipA*- and *hly*-based PCR presented an amplification limit of 10^2 genomes per reaction for each target bacteria regardless of the presence and concentration of competitor target. Besides, the efficiency of the reaction was above 97% in both cases.

Validation

Two batches of 72 and 54 diverse food samples were tested in parallel by the *bipA* and *hly*-based multiplex real-time PCR and the UNE-EN ISO 6579:2002 and 11290-2:1998 methods, respectively. In two food samples, raw milk and vegetable purée, a delay of the IAC signal was observed, indicating PCR inhibition. To avoid PCR inhibitions, 1:10 dilutions were used in these samples to obtain reliable PCR results. The comparison of the obtained results (Table 2) allowed the calculation of sensitivity, specificity, and efficiency of the newly developed multiplex real-time PCR.

Considering as a reference the results obtained through standard methods for *Salmonella* spp. and *L. monocytogenes*, the new multiplex PCR-based system showed a percentage of positive results correctly assigned (sensitivity) of 100% and 94.1%, respectively. The specificity, which is the number of negative results properly assigned by the PCR method, resulted in a 95.2% and 94.6%. Finally, the efficiency of the multiplex PCR calculated as the percentage of samples rightfully assigned, was of 97.2% and 94.4%.

Table 2 Presence of *Salmonella* and *Listeria monocytogenes* in naturally contaminated food determined by multiplex PCR and ISO methods

		UNE-EN ISO 6579:2002		
		Presence	Absence	
bipA/hly-based real time PCR	+/+			
	+/−	30	2	32
	−/+			
	−/−	0	40	40
		30	42	72
		UNE-EN ISO 11290-2:1998		
		Presence	Absence	
BipA/hly-based real time PCR	+/+			
	+/−			
	−/+	16	2	18
	−/−	1	35	36
		17	37	54

Detection Limit of the Assay

The detection limit of the multiplex real-time PCR-based method was found to be of 5 CFU/25 g in all samples, although in eggs and smoked salmon only 100 CFU/25 g of *L. monocytogenes* could be detected, and it was impossible to obtain a positive PCR signal for *Salmonella* spp. in ready to eat mixed salad. Negative results were confirmed by single PCR and testing the enrichments separately.

Discussion

In this study, two pre-established real-time PCR based detection systems were combined to obtain a tool enabling the simultaneous analysis of *Salmonella* spp. and *L. monocytogenes* in a sample. The combination of the previous individual reaction set up allowed the co-amplification of down to 10^2 genome copies per reaction for each target when either equal or variable amounts (up to 4 log units of difference) of *Salmonella* and *Listeria* DNA were mixed without interference in efficiency or specificity. Therefore, amplification of multiple targets did not compromise the performance of the individual assays in terms of selectivity or amplification yield.

Two distinct extraction methods, one involving column-based purification and a simple lysis protocol, were employed for sample analysis. The NucleoSpin® Tissue kit commercial method was used in order to compare results with previous studies on PCR detection of *Salmo-*

nella and *L. monocytogenes* in foodstuffs. The lysis method was newly applied in this work and after proving its efficiency and suitability for an accurate detection, it is proposed for further routine testing, so that one single extraction procedure could be used, and both samples could be analysed together once they have been enriched per separate.

The accuracy of the analysis was verified in 22 food types artificially spiked with *Salmonella* spp. and *L. monocytogenes*. Results of multiplex real-time PCR were in agreement with single PCR tests in all cases and allowed the detection of initial contamination levels of 5 CFU/25 g of food, which is in accordance with the detection limits established in previous studies for *L. monocytogenes* (1–5 CFU/25 g or ml in soft cheese, meat, milk, vegetables and fish) (O'Grady et al. 2009), *Salmonella* (2.5 CFU/25 g in salmon and minced meat, 5 CFU/25 g in chicken meat and 25 ml of raw milk) (Hein et al. 2006), or other multiplex PCR systems (simultaneous detection of 3 and 4 CFU/g of *L. monocytogenes* and *Salmonella* in raw sausage meat, 5 CFU/25 g of *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7 in 40 food types, and 1 CFU/25 g in ground pork samples) (Kawasaki et al. 2009, 2010; Wang et al. 2004).

In our case, in eggs and salmon the detection limit for *Listeria* was 100 CFU/25 g, whereas none of the contamination levels of *Salmonella* gave positive results in ready-to-eat salads. The lack of detection of *Salmonella* or *L. monocytogenes* in such food samples may probably be attributed to a poor bacterial recovery in the enrichments, since IAC Ct values and single PCR tests discarded PCR inhibition problems or the loss of one target over the other. The interference of strong competent microbiota, or distinct food specific characteristics (pH, salinity, carbohydrate content, aw, preservatives, or bacterial metabolites) (Bhagwat 2003; Kim and Bhunia 2008) may explain the limited bacterial growth in certain foods. In order to solve these problems, standard culture methods describe alternative protocols, which in the case of *Listeria* include modifications such as the extension of the enrichment step to 48 h, a secondary enrichment step in Fraser medium, or the incubation at 30 °C during the initial hours of the incubation (O'Grady et al. 2008, 2009; Aznar and Alarcón 2003).

One of the aspects that should be addressed for multiple pathogen detection is the optimization of a single enrichment step enabling the simultaneous recovery of distinct species. Several attempts have already been made to obtain recovery of multiple pathogens concurrently (Omiccioli et al. 2009; Kim and Bhunia 2008; Kobayashi et al. 2009) through the development or improvement of single enrichment broths. Such advances represent a promising alternative for future food quality analysis. However, such formula still need to be tested in all kind of foods, since *Salmonella* generally exhibits higher growth rates in single food enrichments, which may lead to a predominance over *Listeria* (Jofré et al. 2005; Bailey and Cox 1992). Also, in meat and salami *L. monocytogenes* could not be detected by PCR in enrichments in the presence of large numbers of other microbes (O'Grady et al. 2009). Consequently, in our case each sample was separately enriched in BPW and HF to avoid biases in the detection due to unequal growth rates. Despite this separate enrichment for each pathogen, just a single fast DNA purification step and a single PCR reaction were needed for one food sample, thus reducing costs, effort and time of analysis. Nevertheless, in the future as single enrichment broths become further developed and tested with more diverse food matrices, it would be of great interest to use them in combination with the *bipA-hly*-PCR-based detection method.

Finally, the results of the multiplex real-time PCR assay showed good fit with those obtained by the standard methods ISO 6579:2002 and 11290-2.1998, demonstrating its accuracy to detect the presence of *Salmonella* spp. and *L. monocytogenes* in foods.

Hence, the reliability of the analysis performed demonstrates its high potential to be readily used as a food safety test in control laboratories. It would be of special interest to assure the safety of products with a very limited shelf life such as cooked or fresh ready to eat dishes where the delivery of results needs to be as fast as possible. This multiplex real-time PCR approach implies shortening the turnaround time to results from up to 7 to less than 2 working days, ensuring the detection of minimum initial contamination levels of both *Salmonella* spp. and *L. monocytogenes*. Moreover, the test is suitable for additional diagnostic applications, as a preliminary screening test, or to confirm isolated colonies.

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References

Anonymous (1998) Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of *Listeria monocytogenes*—Part 2: enumeration method. ISO 1290-2. International Organization for Standardization, Geneva

Anonymous (2001) Report on trends and sources of zoonotic agents in the European Union and Norway. European Food Safety Authority (EFSA)

Anonymous (2002) Microbiology of food and animal feeding stuffs—horizontal method for the detection of Salmonella spp. ISO 6579:2002. International Organization for Standardization, Geneva

Anonymous (2003) Microbiology of food and animal feeding stuffs. Protocol for the validation of alternative methods. ISO 16140: 2003. International Organization for Standardization, Geneva
Aznar R, Alarcón B (2003) J Appl Microbiol 95:958
Bailey JS, Cox NA (1992) J Food Prot 55:256
Bhagwat A (2003) Int J Food Microbiol 84:217
Calvó L, Martínez-Planells A, Pardos-Bosch J, Garcia-Gil LJ (2008) Food Anal Methods 1:236
Chiu Ch, Tang P, Chu C, Hu S et al (2005) Nucleic Acids Res 33:1690
Elizaquível P, Aznar R (2008) Food Microbiol 25:705
Germini A, Masola A, Carnevali P, Marchelli R (2009) Food Control 20:733
Glaser P, Frangeul L, Buchrieser C et al (2001) Science 294(5543):849
Hein I, Flekna G, Krassnig M, Wagner M (2006) J Microbiol Methods 66(3):538
Hoorfar J (1999) ASM News 65:799
Jofré A, Martina B, Garriga A, Hugasa M, Pla M, Rodríguez-Lázaro D, Aymerich T (2005) Food Microbiol 22:109
Jothikumar N, Wang X, Griffiths M (2003) J Food Prot 66:2141
Kawasaki S, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S (2005) J Food Prot 68:551
Kawasaki S, Fratamico PM, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S (2009) Foodborne Pathog Dis 6:81
Kawasaki S, Fratamico PM, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S (2010) Foodborne Pathog Dis 7(5):549
Kim H, Bhunia AK (2008) Appl Environ Microbiol 74:4853
Kobayashi H, Kubota J, Fujihara K et al (2009) FSTR 15:427
Malorny B, Hoorfar J, Bunge C, Helmuth R (2003) Appl Environ Microbiol 69:290
Mead PS, Slutsker L, Dietz V et al (1999) Emerg Infect Dis 5:607
Nguyen LT, Gillespie BE, Nam HM, Murinda SE, Oliver SP (2004) Foodborne Pathog Dis 1:231
O'Grady J, Sedano-Balbas S, Maher M, Smith T, Barry T (2008) Food Microbiol 25:75
O'Grady J, Ruttledge M, Sedano-Balbas S, Smith TJ, Barry T, Maher M (2009) Food Microbiol 26:4
Omiccioli E, Amagliani G, Brandi G, Magnani M (2009) Food Microbiol 26:615
Rijpens NP, Herman LM (2002) J AOAC Int 85:984
Rodriguez-Lazaro D, Jofre A, Aymerich T, Hugas M, Pla M (2004) Appl Environ Microbiol 70:6299
Rodriguez-Lazaro D, Pla M, Scortti M, Monzo HJ, Vazquez-Boland JA (2005) Appl Environ Microbiol 71:9008
Rudi K, Naterstad K, Drømtorp SM, Holo H (2005) Lett Appl Microbiol 40:301
Somer L, Kashi Y (2003) J Food Prot 66:1658
Tirado C, Schmidt K (2001) J Infect 43:80
Wang X, Jothikumar N, Griffiths MW (2004) J Food Prot 67:189
Wang L, Li Y, Mustaphai A (2007) J Food Prot 70:1366