Analytical Letters, 39: 1611–1625, 2006

ISSN 0003-2719 print/1532-236X online
DOI: 10.1080/00032710600713354

Taylor & Francis
Taylor & Francis Group

Development of SYBR-Green Real-Time PCR and a Multichannel Electrochemical Immunosensor for Specific Detection of *Salmonella enterica*

E. Delibato
Centro Nazionale per la Qualità degli Alimenti e per i Rischi Alimentari, Istituto Superiore di Sanità, Rome, Italy

G. Volpe and D. Stangalini
Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata", Rome, Italy

D. De Medici
Centro Nazionale per la Qualità degli Alimenti e per i Rischi Alimentari, Istituto Superiore di Sanità, Rome, Italy

D. Moscone and G. Palleschi
Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata", Rome, Italy

Abstract: The objective of the present work was to develop and evaluate an SYBR Green real time polymerase chain reaction (PCR) method for the specific detection of *Salmonella* spp in broth cultures and meat samples experimentally contaminated. Also, a simple and rapid multichannel electrochemical immunosensor (MEI) for this pathogen is under study.

The PCR was carried out using primers *ttr6* and *ttr4* for the amplification of a highly conserved DNA region (*ttr* sequence) specific for all *Salmonella* serovars.

A boiling step, for the extraction of DNA, was combined with a real time PCR method based on the double-stranded DNA (dsDNA) binding dye SYBR

Received 15 December 2005; accepted 20 February 2006
Address correspondence to G. Palleschi, Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata", Via della Ricerca Scientifica 1, 00133 Rome, Italy. E-mail: giuseppe.palleschi@uniroma2.it

Green. The standard curve constructed using the mean threshold cycle and various concentrations of *S. enteritidis* (ranging fron 10^2 to 10^8 CFU mL^{-1}) showed good linearity ($R^2 = 0.999$) with the minimum level of detection of 10^2 CFU mL^{-1}. The experiments were conducted analyzing 30 *Salmonella* strains and 20 non-*Salmonella* strains. All *Salmonella* serotypes tested were *ttr*-positive and all other bacteria yielded no amplification products. The specificity of the reaction was confirmed by the melting temperature (T_m) of the amplicon obtained ($T_m = 80.1 \pm 0.1$).

To verify the effectiveness of the assay, experiments were conducted on experimentally contaminated samples, which were also analyzed for comparison by the standard cultural method.

A multichannel electrochemical immunosensor for detection of *Salmonella* also was developed. It consists of a disposable screen-printed sensor array, coupled with a multichannel pulse monitor, which was assembled as an immunosensor through the use of specific monoclonal (MAb) and polyclonal (PAb) antibodies in a sandwich format. The limit of detection was calculated to be 2×10^6 UFC mL^{-1} with a working range between 5×10^6 to 5×10^8 UFC mL^{-1} and a total analysis time of about 3 h. This immunoelectrochemical system is economical, rapid, and easy to use but it is still under development in order to improve its analytical performance.

Keywords: PCR real-time SYBR green, *Salmonella*, MEI

1. INTRODUCTION

In the last 20 years, *Salmonella enterica* has become the most common cause of food poisoning in the United States and in most European countries including Italy. Foods of animal origin are frequently implicated in human salmonellosis owing to a significant occurrence of *Salmonella* strains in animal. Although various control measures have been adopted along the food-production chain (Tirado et al. 2001), the microbiological testing of poultry products during production still plays a major role in preventing foodborne infection (Tietjen et al. 1995). According to European legislation *Salmonella* must be absent in an established amount of food products (1 g, 10 g, or 25 g, depending on the kind of the product). However, an important issue is that the standard cultural method for detecting *Salmonella* (ISO 6579: 2002) requires up to 5 days to produce results. Thus the development of rapid, cost-effective, and automated diagnostics for foodborne pathogens (*Salmonella* included) throughout the food chain continues to be a major concern for the industry and public health. To respond to these needs, the polymerase chain reaction (PCR) has became a powerful tool in microbiological diagnostics in the last decade (Sachse 2003).

An issue is that qualitative PCR includes a final confirmation phase, such as electrophoresis, which is time consuming, carries the risk of laboratory contaminations with nucleic acid due to post-PCR manipulation, and also requires the use of ethidium bromide that, being a potent mutagenic agent, is not desiderable for routine use (Singer et al. 1999).

In recent years, a method based on quantitative PCR with an automatic confirmation phase has been developed. This method, known as "real-time fluorogenic 5′ nuclease PCR," has been used to detect a number of pathogenic microorganisms in foods (Bassler et al. 1995; Vishnubhatla et al. 2000), including *Salmonella* (Hoorfar et al. 2000). The method uses the 5′nuclease activity of Taq DNA polymerase to hydrolyze an internal fluorogenic probe for monitoring the amplification of the DNA target. Its application then requires the availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily met. To overcome this limitation, the use of double-stranded DNA binding dye SYBR Green I was introduced for the detection of PCR products and allows real-time PCR to be applied without the need of probes linked to fluorescent molecules (De Medici et al. 2003).

In the absence of probes, the specificity of the reaction can be determined by the melting temperature (T_m) of the obtained amplicon. The T_m is defined as the temperature at which 50% of the DNA amplicon is in a double-stranded configuration. The T_m depends on various factors including: the amplicon length and the nucleotide sequence (Ririe et al. 1997).

The analysis of *Salmonella* has also been performed with other new technologies such as an ELISA-based method (Seo et al. 1999; Gehring et al. 1996). In this area, we recently (Croci et al. 2001) reported the development of an electrochemical ELISA for the detection of *Salmonella* in meat samples using a sandwich format and a conventional ELISA plate; after all immunological and enzymatic reactions are in place, the mixture contained in each well is injected into a flow injection analysis (FIA) system coupled to an electrochemical cell. Although the effectiveness of this system has been demonstrated (Croci et al. 2001, 2004) for the analysis of meat samples, a certain level of skill and quite complex instrumentation are required.

The objective of the present work was to develop and evaluate a real-time PCR for the specific detection of *Salmonella* spp in broth cultures and meat samples experimentally contaminated. A very highly conserved region of the genome of different *Salmonella* serovars was chosen in order to develop an SYBR Green real-time PCR coupled with melting curve analysis. The selected target region, the ttrRSBCA locus, has already been described by Hensel et al. (1999) and seems more specific than other published *Salmonella* target regions. This region was utilized in real-time PCR from Malorny et al. (2004) using the probe 5′ nuclease TaqMan instead of the SYBR-Green dye.

A simple and rapid multichannel electrochemical immunosensor for the detection of this pathogen was also developed. This system consists of an array of 96 screen-printed electrochemical sensors (SPEs), with a 96-well plate glued on. Each sensor is formed by a carbon working electrode and a silver pseudoreference electrode. We have immobilized specific antibodies against *Salmonella* onto the electrode surface, converting the sensors into immunosensors. This new method would represent an alternative approach

to the PCR that is cost effective and more suitable for routine analysis. Preliminary results for this approach are reported in the present work.

2. EXPERIMENTAL

2.1 Reagents and Materials

S. enteritidis (ATCC13076) and all reagents used for culture media were from Oxoid Ltd., Basingstoke, UK. The other *Salmonella* and non-*Salmonella* strains were supplied by the Pathogenic Enterobacteria Center (Istituto Superiore di Sanità, Rome, Italy).

The two primers of 23 bases for *Salmonella* spp, ttr6 (5′-CTCACCAGGA GATTACAACATGG-3′) and ttr4 (5′-AGCTCAGACCAAAAGTGACCA TC-3′) were purchased from M-Medical Genenco, Florence, Italy. SYBR Green I PCR Master mix (which contains *Taq* polymerase, deoxynucleoside triphosphate, reaction buffer, and $MgCl_2$) was from Applied Biosystems of Roche Molecular Systems, Inc., Branchburg, N.J.

DNase/RNase-free distilled water was from Sigma (St. Louis, MO, USA).

Mouse monoclonal antibodies against boiled *Salmonella* ($1\,mg\,mL^{-1}$) were purchased from Chemicon Inc. (Temecula CA); rabbit polyclonal antibodies-HRP ($1\,mg\,mL^{-1}$) against *Salmonella* were obtained from Biogenesis (UK) and affinity purified anti-mouse IgG (H + L) from Vector Laboratories Inc. (Burlingame, CA, USA); nonfat dry milk blotting grade was from Bio-Rad Laboratories (Hercules, CA, USA). 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB) and all other reagents of analytical-reagent grade were obtained from Sigma (St. Louis, MO, USA).

Meat samples (pork, chicken and beef) were purchased from local retail outlets.

2.2 Apparatus

All amplification reactions were performed with an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) using a 96-microwell plate (MicroAmp, Applied Biosystems).

The multichannel electrochemical system, which consists of a multichannel pulse monitor (Mod. AndCare 9600) and of disposable 96-well screen-printed carbon electrode plates, was from Alderon Biosciences Incorporated (AndCare-Electrochemical Sensing Technologies, http://www.andcare.com).

2.3 Preparation and Standardization of Bacterial Cultures

The preparation and standardization of bacteria cultures were described by Croci et al. (2001). After the mentioned procedures, the bacterial suspensions

(prepared in 0.9% NaCl solution) were frozen at −20°C up to 6 months (it was not tested beyond this period) and defrosted at the moment of the use. The bacterial suspensions were used to setup PCR and MEI methods and also for the experimental contamination of the meat samples.

2.4 Experimentally Spiked Sample

Samples of three kinds of meat (i.e., pork, chicken, and beef), which were confirmed to be *Salmonella* free by using the ISO (6579:2002) method, were divided into two aliquots: one aliquot was experimentally contaminated by spiking 25 g of product with 1 mL of different *Salmonella* serotype (*S. enteritidis*, *S. infantis*, *S. typhimurium*, and *S. derby*) suspensions (1–10 viable CFU mL^{-1}); the second aliquot was used as a negative control. Each aliquot was homogenized with 225 mL of pre-enrichment broth (buffered peptone water) in a stomacher for 1–2 min.

The samples were incubated at 37°C for 24 h (pre-enrichment phase) to allow bacterial growth. After incubation, they were analyzed with the ISO method and SYBR Green real-time I PCR coupled with melting curve analysis.

2.5 Extraction of Broth Cultures and Samples

For PCR, the extraction and purification of DNA was performed as follows:

1 mL of both the broth cultures and the pre-enriched samples was transferred to a micro-centrifuge tube with a capacity of 1.5 mL. The cell suspensions were centrifuged for 10 minutes at 14,000 *g*. The supernatant was discarded carefully. The pellet was re-suspended in 100 μL of DNase-RNase-free distilled water by vortex. The micro-centrifuge tube was incubated for 15 min at 100°C and immediately chilled on ice. It was centrifuged again for 5 minutes at 14,000 *g*. An aliquot of 5 μL of the supernatant was used as template DNA in the PCR reaction.

For the MEI test, the bacteria suspensions (10 mL) were centrifuged (10,000 *g* for 15 minutes) and the pellet was resuspended in 10 mL PBS and boiled for 10 minutes. After this step, up to 10 mL PBS was added. The scheme for the extractions is reported in Fig.1

2.6 PCR Amplification

All amplification reactions were performed in a total volume of 50 μL with an ABI Prism 7700 sequence detector using 96-well microwell plates. In each well, we placed 5 μL of purified DNA, 25 μL SYBR Green I PCR Master Mix, and 200 nM of each primer (ttr4 and ttr6). DNase-RNase-free distilled water was added to reach a total volume of 50 μL per well. The reaction

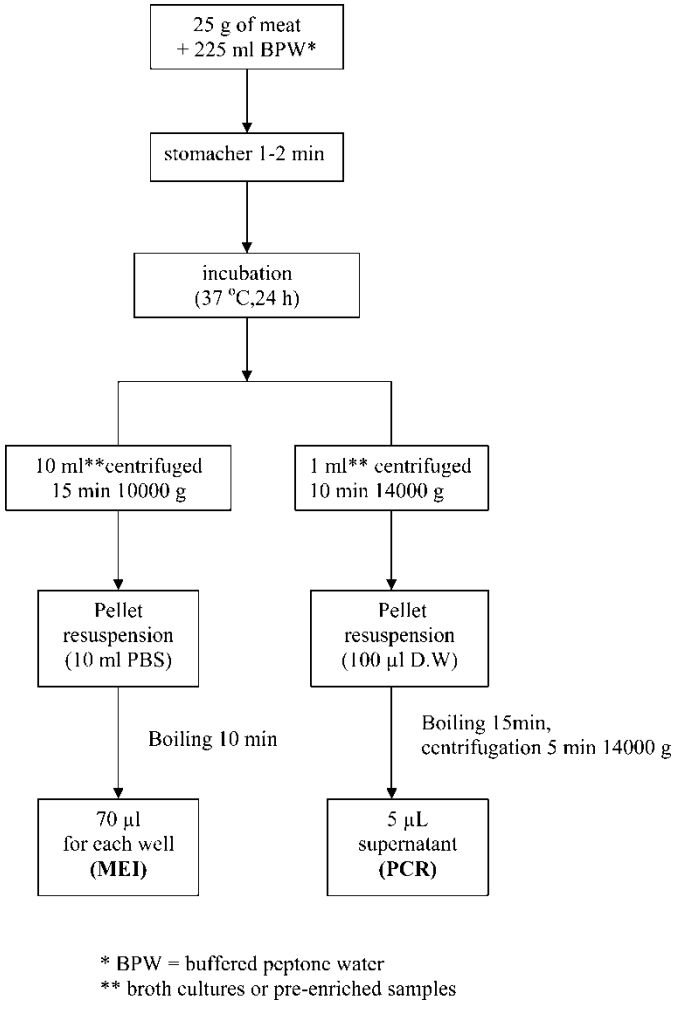

Figure 1. Extraction scheme of the pre-enriched samples and the broth cultures.

was run on-line at 95°C for 5 min, followed by 40 amplification cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 60 sec.

2.7 SYBR Green I Real-Time PCR and Melting Curve Analysis

In the SYBR Green I real-time PCR, the amplification of the DNA target is measured in terms of the increment in the quantity of fluorescence determined at the end of each amplification cycle. In brief, SYBR Green I binds to the minor groove of dsDNA, greatly enhancing the fluorescence. The cycle in

which there is a significant increase of the fluorescence is defined as the threshold cycle (C_T) (De Medici et al. 2003). The C_T is a measure of the quantity of target DNA, thus target DNA copy number and C_T values are inversely related.

The specificity of the reaction is verified by the melting temperature (T_m) of the amplification products immediately after the last reaction cycle. The T_m, which is specific for each amplicon, is determined during an additional phase of slow heating, from 60°C to 95° in 19 min 59 s. During this period, a rapid decrease in the fluorescence occurs due to the denaturation of the amplicons, such that single strands of DNA appear with the successive detachment of the SYBR Green I. The melting curve was visualized with the software Dissociation curve 1.0 provided with the ABI Prism 7700 system. The T_m is the temperature at which 50% of the amplicons are associated. The same software plots the negative derivative of fluorescence over temperature vs. temperature ($-dF/dT$ vs. T) and the maximum point of the obtained peak corresponds to the T_m.

2.8 MEI Procedure

This assay procedure was performed using anti-mouse IgG (Ab_2), mouse monoclonal anti-*Salmonella* antibodies (MAb), *Salmonella* standard solutions (Ag), polyclonal anti-*Salmonella* antibodies-horseradish peroxidase (HRP) conjugated (PAb-HRP), and microtiter plates (96 wells) having screen-printed electrodes localized on the bottom of each well.

The microtiter plate wells were precoated with a 10 μg/mL solution (70 μL per well) of anti-mouse IgG in 0.1 M carbonate buffer solution pH 9.6 (at 4°C, overnight) and then blocked [30 min at room temperature (RT)] with 3% dry milk. A MAb solution diluted 1:600 (70 μL per well) was then applied to the microtiter plate for 1 h at RT. The plates were then incubated (1 h at RT) with 70 μL of *Salmonella* standard solutions (ranging from 5×10^6–5×10^9 UFC mL^{-1}) added to triplicate wells and followed by addition of 70 μL of PAb-HRP (1:50 for 30 min at RT). Each solution, except the one used for pre-coating, was prepared in phosphate buffered saline solution, pH 7.4 (PBS). Between each step (precoating, coating, blocking, MAb/Ag binding, Ag/PAb-HRP binding) a three-cycle washing procedure using PBS containing 0.05% Tween 20 was adopted. Finally, a stock solution of TMB (20 mM) in DMSO was prepared and 70 μL of substrate solution (3×10^{-4} M TMB + 10^{-3} M H_2O_2 in 0.05 M citrate phosphate buffer pH = 5) were added to each well. After 2 minutes of incubation, the HRP activity was measured electrochemically using intermittent pulse amperometry (IPA). The IPA measurements involve a series of millisecond pulses of constant potential separated by longer periods when the electrode is disconnected from the potentiostat. A representation of the employed instrumentation is found in Fig. 2

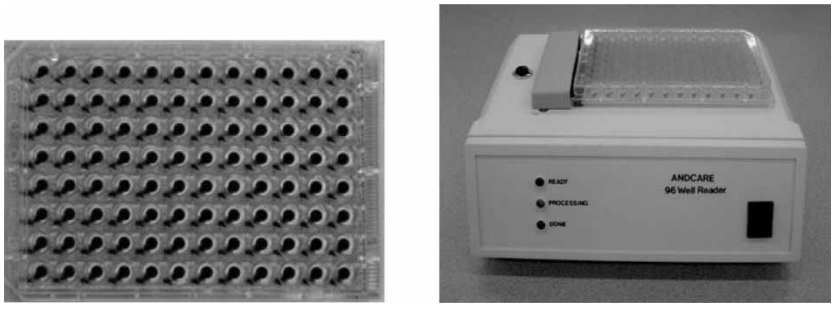

Figure 2. Representation of the instrumentation used for MEI test.

3. RESULTS AND DISCUSSION

3.1 SYBR Green I Real-Time PCR and Tm

The PCR was carried out in accord with Malorny et al. (2003) using primers *ttr6* and *ttr4* for the amplification of a highly conserved DNA region (*ttr* sequence) specific for all *Salmonella* serovars. This region encoded for tetrathionate reductase structural proteins. The ability to respire tetrathionate is likely to be significant within the life cycle of *Salmonella* spp. (Hensel et al. 1999). The *ttr* genes should be genetically stable in all *Salmonella* strains.

To determine the optimal concentration of primers, preliminary tests were performed using equimolar primer concentrations (100 nM, 200 nM, and 300 nM). The concentration chosen for the experiment was 200 nM because it provided the lowest C_T and no improvement in the analytical performance was found among the different concentrations tested (data not show).

In Fig. 3 the C_T is reported for decreasing *Salmonella* concentrations vs. the corresponding fluorescent signals. The standard curve constructed by plotting the mean C_T (n = 3) vs. logarithmic concentrations of *S. enteritidis* (ranging from 10^2 to 10^8 CFU mL^{-1}) showed a good linearity ($R^2 = 0{,}999$) (Fig. 4). It can be observed that the C_T and *Salmonella* concentrations are inversely related. The detection limit of the reaction was 10^2 CFU/mL. Standard regression analyses of the linear portion of the curve gave a slope of -3.35. In a 100% effective PCR (i.e., the DNA doubles in each cycle) the slope should be -3.32, and thus our real time PCR appeared close to the 100% efficient criterion.

The next experiments were conducted analyzing 30 *Salmonella* strains and 20 non-salmonella strains. All *Salmonella* serotypes tested were found to be *ttr* positive while all other bacteria yielded no amplification products. In addition, specific PCR products were identified by melting curve analysis and a reproducible melting temperature of 80.1 ± 0.1 was observed for all *Salmonella* serotypes (Fig. 5). Negative controls and non-*Salmonella* strains

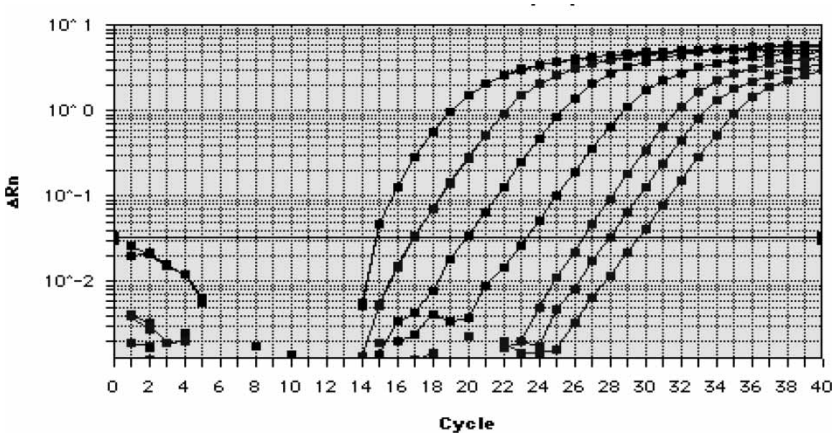

Figure 3. Threshold cycles (C_T) of decreased *Salmonella* concentrations *versus* the corresponding fluorescent signals.

did not show peaks, confirming the specificity of the method (Table 1 and Table 2).

Finally, no false positive or false negative results were obtained when analyzing meat samples spiked with 1–10 CFU of different *Salmonella* serotypes, after 24 h of pre-enrichment. However, there was a difference observed between the C_T in the various matrices: spiked pork samples resulted as positive after C_T 18.1–19.3, beef samples after C_T 22.3–23.3, and poultry samples after C_T 24.8–25.4 (Table 3). The results indicated that *Salmonella* strains grown differently in pre-enrichment broths derived from different food matrixes. We previously described this phenomenon. (Croci et al. 2001).

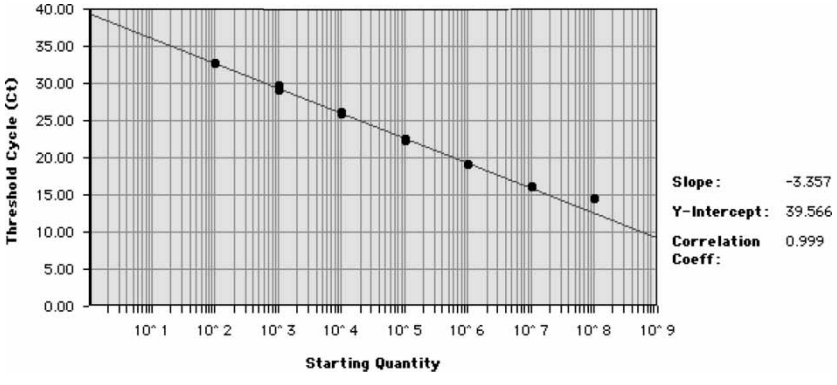

Figure 4. PCR calibration curve obtained by plotting logarithmic *Salmonella* concentrations vs C_T.

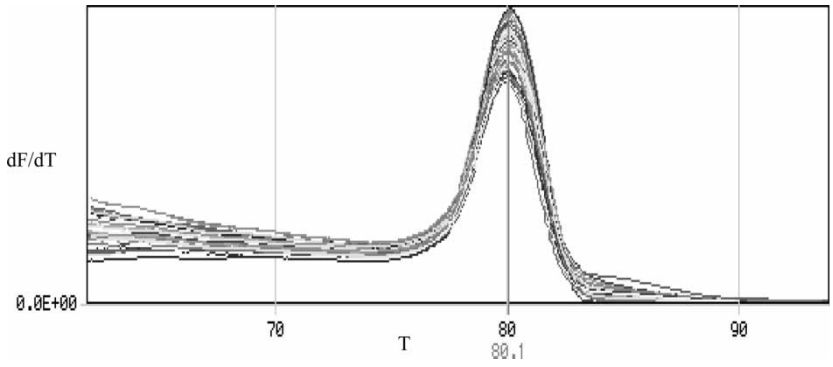

Figure 5. Melting curve of *Salmonella* spp. after 40 cycles of amplification.

Table 1. *Salmonella* spp. used for PCR to assess primers specificity

Salmonella strains	No. of strains	Result
Enteritidis	5	+
Typhimurium	4	+
Derby	1	+
Infantis	1	+
Agona	1	+
Newport	1	+
Give	1	+
Gallinarum pullorum	1	+
Abortus equi	1	+
London	1	+
Anatum	1	+
Panama	1	+
Saintpaul	1	+
Bredeney	1	+
Livingstone	1	+
Montevideo	1	+
Cholerasuis	1	+
Kottbus	1	+
S. havana	1	+
S. napoli	1	+
S. muenchen	1	+
S. manhattan	1	+
S. ruirus	1	+

Table 2. Non-*Salmonella* strains used for PCR to assess primers specificity

Bacteria	No. of strains	Result
Aeromonas hydrophila	1	−
Campylobacter coli	1	−
Campylobacter jejuni	1	−
Citrobacter freundii	1	−
Enterobacter agglomerans	1	−
Enterobacter cloacae	1	−
Enterobacter faecalis	1	−
Escherichia coli	1	−
Klebsiella pneumonite	1	−
Listeria innocua	1	−
Listeria ivanovi	1	−
Listeria monocytogenes	1	−
Morganella morganii	1	−
Proteus vulgaris	1	−
Pseudomonas aureuginosa	1	−
Pseudomonas fluorescens	1	−
Serratia marcescens	1	−
Staphylococcus aureus	1	−
Staphylococcus epidermidis	1	−
Yersinia enterocolitica	1	−

Table 3. C_T obtained from different food matrices Experimentally contaminated with 1–10 CFU mL^{-1} of *Salmonella* and incubated for 24 hours at 37°C

Meat samples	*Salmonella enterica* serotypes	SYBR Green I real time PCR	
		Mean Ct ± SD[a]	T_m (°C)
Pork	*S. enteritidis*	19.4	80.1
	S. derby	18.3	80.1
	S. infantis	18.1	80.2
	S. typhimurium	19.3	80.1
Beef	*S. enteritidis*	22.3	80.0
	S. derby	22.2	80.1
	S. infantis	22.5	80.0
	S. typhimurium	23.3	80.1
Chicken	*S. enteritidis*	24.8	80.0
	S. derby	24.8	80.1
	S. infantis	25.4	80.0
	S. typhimurium	25.0	80.1

[a]The SD of all experiments was lower that 0.1.

3.2 MEI

The multichannel electrochemical sensors array is a device commercialized by Alderon as an economical and easy to use electrochemical systems for nucleic acid detection, enabling ultrasensitive quantification of DNA and RNA directly or after PCR or reverse transcription-PCR amplification of target sequences, which are detected with an enzyme-linked assay.

The only paper available in the literature is, to the best of our knowledgement, the one published by Wojciechowski et al. (1999) illustrating the previous applications. In our work the system has been, for the first time, assembled as multichannel electrochemical immunosensors. Its represents a continuation of our previous studies in which a conventional ELISA plate, coupled both with a FIA system and an electrochemical cell, was developed and employed successfully for the detection of different *Salmonella* serovars in meat samples (Croci et al. 2001). However, this system has two drawbacks: it requires the use of an instrumentation not quite manageable and the electrochemical measurements are time consuming because it is necessary to inject one standard or sample at a time into the FIA stream.

For this application, we decided to assemble an ELISA using the same bioreagents on the commercial 96-wells screen-printed electrode plate, which was then read with a multichannel electrochemical pulse monitor for a multisamples' analysis. The aim was to develop a simpler and more rapid screening method that is suitable for routine analysis of *Salmonella*.

During the optimization of the assay, it was found that the use of a precoating solution of anti-mouse IgG on the surface of the SPEs resulted in an enhancement of the interaction between monoclonal antibodies and *Salmonella*. Thus, after precoating with IgG, different dilutions of MAb and PAb-HRP were tested in order to obtain, in a short time (about 3 h), the best conditions for the *Salmonella* assay (data not shown).

A calibration curve for *S. enteritidis* under the optimized experimental conditions is reported in Fig. 6.

Detection limit (LOD), defined as the concentration corresponding to the f(x) value obtained by adding 3 standard deviations of the zero point to the mean of the zero standard measurement (mean value + 3s), was found to be 2×10^6 UFC mL^{-1}.

The following four parameter logistic equation was used to fit the experimental data:

$$f(x) = \frac{a - d}{1 + (x/c)^b} + d \tag{1}$$

where a and d are the asymptotic maximum and minimum values, respectively; c is the $\times$ value at the inflection point, and b is the slope (Fare et al. 1996).

Experiments performed to verify the selectivity of the antibodies used, towards different *Salmonella* serotypes and their noncross-reactivity with

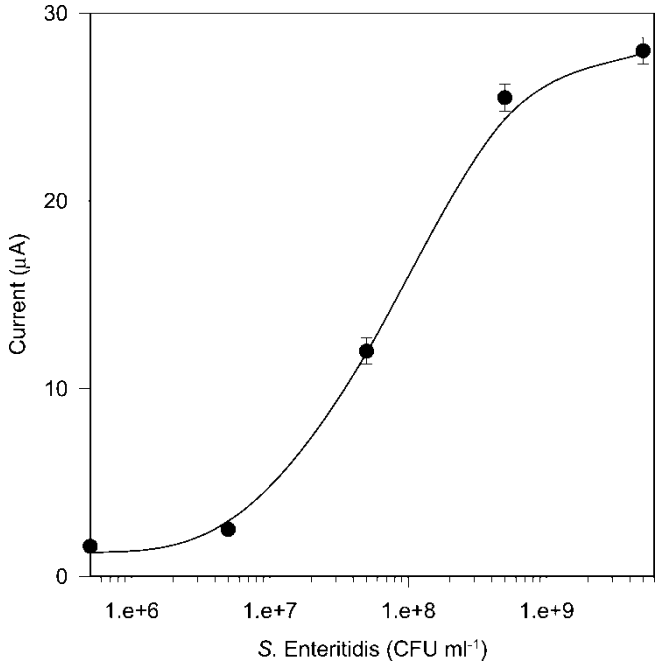

Figure 6. MEI calibration curve of *S. enteritidis* obtained in optimize experimental conditions.

other bacteria commonly present in foods, has been already demonstrated (Croci et al. 2001).

Preliminary test on food samples experimentally contaminated with 1–10 UFC mL^{-1} were carried out. Although this system was very rapid and easy to use for the analysis of *Salmonella* in food (after 24 h of pre-enrichment), it is still in study in order to improve the detection limit and then to reduce the length of the pre-enrichment phase.

4. CONCLUSIONS

Two innovative techniques for the detection of *Salmonella* spp are reported in this work: a SYBR Green real time PCR and a multichannel electrochemical immunosensor.

Both tests were found to be rapid, specific, and allow simultaneous analysis of numerous samples, a characteristic that is particularly useful for both monitoring and control as part of the application of (HACCP) hazard analysis and critical control points (HACCP) in the food production industry.

For future work the following experiments will be carried out: improvement of detection limit for the electrochemical system by using

avidine-biotine complex and/or immunomagnetic beads; new analysis of food samples experimentally contaminated with 1–10 CFU mL^{-1} to establish the minimum pre-enrichment time to revel this *Salmonella* concentration with both proposed methods. The pre-enrichment phase is typically requests to allow the growth of viable cells, overcoming the problem of the inability of PCR and ELISA based methods, to distinguish between livings and dead cells (Rahn et al. 1992).

Finally, both methods (PCR and MEI) will be employed for the analysis of food samples naturally contaminated.

REFERENCES

Bassler, H.A., Flood, S.J., Livak, K.J., Marmaro, J., Knorr, R., and Batt, C.A. 1995. Use of a fluorogenic probe in a Pcr-based assay for the detection of Listeria monocytogenes. *Appl. Environ. Microbiol.*, 61: 3724–3728.

Croci, L., Delibato, E., Volpe, G., and Palleschi 2001. A rapid electrochemical ELISA for the detection of *Salmonella* in meta samples. *Anal. Lett.*, 34 (15): 2597–2607.

Croci, L., Delibato, E., Volpe, G., De Medici, D., and Palleschi, G. 2004. Comparison of Pcr, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *salmonella* in meat products. *Appl. Environ. Microbiol.*, 70: 1393–1396.

De Medici, D., Croci, L., Delibato, E., Di Pasquale, S., Filetici, E., and Toti, L. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time pcr to detect *Salmonella enterica* serotype enteritidis in poultry. *Applied & Environmental Microbiology*, 69: 3456–3461.

Fare, T.L., Sandberg, R.G., and Herzog, D.P. 1996. Considerations in Immunoassay calibration. In *Enviromental Immunochemical Methods: Perspectives and Applications*. Vanehmon, J.H. and Gerlach, G.L. (eds.), American Chemical Society: Washington, DC, 240–253.

Fukushima, H., Tsunomori, Y., and Seki, R. 2003. Duplex real-time Sybr Green Pcr assays for detection of 17 species of food- or waterborne pathogens in stools. *J. Clin. Microbiol*. 41: 5134–5146.

Gehring, A.G., Crawford, C.G., Mazenko, R.S., Van Houten, L.J., and Brewster, J.D. 1996. Enzyme-linked immnunomagnetic electrochemical detection of *Salmonella typhimurium*. *J. Immunol. Methods*, 195: 15–25.

Hensel, M., Hinsley, A.P., Nikolaus, T., Sawers, G., and Berks, B.C. 1999. The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. *Mol. Microbiol.*, 32: 275–287.

Hoorfar, J., Ahrens, P., and Radstrom, P. 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. *J. Clin. Microbiol*, 38 (9): 3429–3435.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., and Helmuth, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in Food. *Appl. Environ. Microbiol.*, 70: 7046–7052.

Moen, E.M., Sleboda, J., and Grinde, B. 2002. Real-time Pcr methods for independent quantitation of Ttv and Tlmv. *J. Virol. Methods*, 104: 59–67.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtiss, R., 3rd, and Gyles, C.L. 1992. Amplification of an inva gene sequence of

Salmonella typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes*. 6: 271–279.

Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.*, 245: 154–160.

Sachse, K. 2003. Specificity and performance of diagnostic PCR assays. *Methods Mol. Biol.*, 216: 3–29.

Seo, K.H., Brackett, R.E., Hartman, N.F., and Campbell, D.P. 1999. Development of a rapid response biosensor for detection *Salmonella typhimurium*. *J. Food Prot.*, 62: 431–437.

Singer, V.L., Lawlor, T.E., and Yoe, S. 1999. Comparison of SYBR Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in salmonella/mammalian microsome reverse mutation assay. *Mutat. Res.*, 439: 37–47.

Tietjen, M. and Fung, D.Y. 1995. *Salmonella* and Food Safety. *Crit. Rev. Microbiol.*, 21: 53–83.

Tirado, C. and Schmidt, K. 2001. WHO surveillance programme for control of foodborne infections and intoxications: preliminary results and trends across greater Europe. *World Health Organization. J. Infect.*, 43: 80–84.

Vishnubhatla, A., Fung, D.Y., Oberst, R.D., Hays, M.P., Nagaraja, T.G., and Flood, S.J. 2000. Rapid 5′ nuclease (Taqman) assay for detection of virulent strains of Yersinia enterocolitica. *Appl. Environ. Microbiol.*, 66: 4131–4135.

Wojciechowski, M., Sundseth, R., Moreno, M., and Henkens, R. 1999. Multichannel electrochemical detection system for quantitative monitoring of PCR amplification. *Clin. Chem.*, 45 (9): 1690–1693.