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ARTICLE

Development and Application of an Electrochemical Plate Coupled with Immunomagnetic Beads (ELIME) Array for *Salmonella enterica* Detection in Meat Samples

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Salmonella is one of the main organisms causing outbreaks of foodborne illness, and meat is one of the major vehicles of salmonellosis throughout the world. A novel analytical immunosensor array, based on a 96-well electrochemical plate coupled with immunomagnetic beads (ELIME array), is proposed for the detection of *Salmonella* in meat samples. After an optimization study, using *Salmonella enterica* serotype Enteritidis as reference antigen, the ability of the method to interact with a large number of *Salmonella* serovars commonly present in food was evaluated. The assay was then used to analyze samples of pork, chicken, beef, and turkey experimentally inoculated with *Salmonella* as well as real samples. The results were compared with those from the International Standard of Organization (ISO) culture method. The comparison showed that the ELIME array is able to detect a low number of *Salmonella* cells (1−10 CFU per 25 g) after only 6 h of incubation in a pre-enrichment broth. The investigation revealed a very good agreement between culture and ELIME array methods for meat samples, reducing the time for performing the analysis and obtaining the results quickly.

KEYWORDS: *Salmonella*; meat samples; ELIME array; immunomagnetic beads

INTRODUCTION

Foodborne pathogens have been shown to have caused food contamination at every stage of food production, processing, and distribution. *Salmonella* is one of the main organisms causing the outbreaks of foodborne illnesses, and meat is one of the major vehicles of salmonellosis throughout the world (*1*).

According to European Criteria (*2*), *Salmonella* must be absent in an established amount of a given food product (10 or 25 g, depending on the kind of the product). However, the standard culture method for detecting *Salmonella* requires up to 5 days to produce results; this includes stages of pre-enrichment, selective enrichment, isolation in selective agars, biochemical characterization of suspected colonies, and final serological confirmation (*3*). In this context the development of rapid, cost-effective, and automated methods for the determination of foodborne pathogens (*Salmonella* included), integrated with preventive strategies such as Hazard Analysis Critical Control Points (HACCP), could significantly improve safety throughout the food chain (*4*). To respond to this need, several methods, mainly involving Polymerase Chain Reaction (PCR) and immunoassay (*5*), have been developed to detect the presence of *Salmonella* in foods.

PCR requires an expensive and sophisticated instrument, whereas, in contrast, most of the immunochemical methods employ a simpler apparatus and unspecialized staff. Several PCR-based methods for the detection of *Salmonella* in foodstuffs have been published; most of these methods operate with pre-enrichment periods of 18−24 h followed by DNA extraction, amplification, and detection of the PCR products (*6*−*11*). Some authors have reported reduced pre-enrichment times of 6−12 h for the detection of *Salmonella* by PCR (*12*−*14*). However, the samples analyzed after a pre-enrichment time of 6 h (*12*, *13*) had been inoculated with at least 1 CFU/g and not per 25 g as requested by the legislative demand.

In recent years, several immunoassays (coupled with spectrophotometric, fluorometric, and SPR detectors) have been developed for *Salmonella* analysis, but only a few applications in food have been reported (*15*−*18*). Furthermore, where these applications were made, the pre-enrichment phase was often followed by a selective enrichment step (*17*, *18*). Within the group of immunological methods reported, our research group has developed an electrochemical ELISA for the detection of *Salmonella* in meat samples using a sandwich assay format and a conventional microplate. Following completion of all immunological steps and enzymatic reactions, the mixture contained in each well was injected into a flow injection analysis (FIA) system coupled to an electrochemical cell (*19*, *20*). Although the effectiveness of this system has been demonstrated for the analysis of meat samples after 5 h of pre-enrichment, it made use of a somewhat cumbersome apparatus and the electrochemical measurements

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were time-consuming because it is necessary to inject single standards or samples sequentially into the FIA stream. This method requires a total analysis time of 5 h for the pre-enrichment phase, 6.5 h for immunological and enzymatic reactions, and 2 min for each electrochemical measurement.

To overcome these drawbacks, more recently we (*21*) have developed a simple and rapid multichannel electrochemical immunosensor (MEI) system for the detection of *Salmonella enterica*. It employs a sensor array consisting of a disposable 96-well plate, the bottom of which has been modified with an array of 96 screen-printed sensors, and the measuring procedure is carried out using a multichannel pulse monitor. This device, assembled as an immunosensor array for *Salmonella* detection based on a sandwich format, provided numerous advantages such as rapidity of analysis and the possibility to carry out the calibration and the analysis of several samples (in replicate) at the same time. However, in this formulation the system was not very sensitive, so it was applied to meat samples experimentally inoculated with *Salmonella* Enteritidis (1−10 cells per 25 g) after 24 h of pre-enrichment.

The aim of this work is the development of an enzyme-linked immunomagnetic−electrochemical array (ELIME array) for the simple and rapid detection of *Salmonella* in meat samples. To improve the sensitivity of the MEI system (*21*) and thus reduce the time of the pre-enrichment phase, immunomagnetic beads (IMBs, in a sandwich format) were coupled with the disposable 96-well electrochemical plate. The strategy of using IMBs derives from our previous work, where this type of support was successfully coupled with single SPEs for the rapid and sensitive detection of *S. aureus* (*22*).

After an optimization study, the developed method was applied to experimentally inoculated meat samples to reduce and establish the minimum pre-enrichment time; then it was applied to real samples, and the results were compared to those obtained with the ISO method.

The ELIME array is a simple and fast system (total analysis time of 6 h for the pre-enrichment phase, 2 h for immunological and enzymatic reactions, and 1 min for all 96 electrochemical measurements) to detect *Salmonella* in food and offers the possibility of combining a high sensitivity with the favorable high-throughput characteristics of conventional ELISA procedures.

MATERIALS AND METHODS

Reagents and Materials. Mouse monoclonal antibodies against boiled *Salmonella* (1 mg/mL) were purchased from Chemicon Inc. (Temecula, CA); rabbit polyclonal antibodies−HRP (1 mg/mL) against *Salmonella* were obtained from Biogenesis (U.K.), and nonfat dry milk blotting grade was from Bio-Rad Laboratories (Hercules, CA). 3,3′,5,5′-Tetramethylbenzidine substrate supersensitive solution and all other reagents of analytical reagent grade were obtained from Sigma (St. Louis, MO).

Dynadeads Pan Mouse IgG (supplied as a suspension containing 4×10^8 beads/mL in phosphate -buffered saline, pH 7.4), a rotation device (Dynal sample mixer), and a magnetic particle concentrator (Dynal MPC) were from Dynal, Lake Success, NY.

Blank meat samples (pork, chicken, beef, and turkey), used for experimental inoculation, were purchased from local retail outlets, whereas real samples were obtained from official control performed by the Department of Veterinary Public Health and Food Safety, Microbiological Foodborne Hazard Unit, Istituto Superiore di Sanità.

Apparatus. The 96-well screen-printed microplates were obtained from Alderon Biosciences Inc. (Durham, NC). A working graphite electrode (∅ 3 mm) with a silver pseudoreference electrode, screen-printed on a 0.5 mm plastic support, formed the two-electrode system placed on the bottom of each well. The 96-well microplate is connected to the electrochemical reader through a 56 dual position card edge connector. Neodymium magnets, 4 × 3 mm, from Italfit Magneti (Fagagna-Udine, Italy) were used to construct a custom-designed support placed underneath the microplate.

The electrochemical detection was carried out using a 96-well microplate reader (AndCare 9600), which operates using intermittent pulse amperometry. This technique involves a series of millisecond pulses of the same potential applied individually to each of the 96 sensing electrodes. All IPA measurements were carried out at an applied potential of −100 mV with a pulse width of 10 ms and a selected frequency of 5 Hz. The results were recorded on a PC using dedicated software.

Bacteria Strains, Preparation and Standardization of Cultures. *Salmonella* Enteritidis (ATCC13076) and all reagents used for culture media were from Oxoid Ltd., Basingstoke, U.K. The other *Salmonella* strains were supplied by the Pathogenic Enterobacteria Center (Istituto Superiore di Sanità, Rome, Italy).

The preparation and standardization of bacteria cultures have already been described by Croci et al. (*19*). After preparation, 10 mL of the standardized broth cultures (in 0.9% NaCl solution) was centrifuged at 3000 rpm for 15 min, and the pellets were resuspended in phosphate-buffered saline (PBS) and boiled for 3 min. Finally, PBS was added up to 10 mL, and the solutions were frozen at −20 °C and defrosted before use. The bacterial suspensions were used to set up the ELIME array method and also for the experimental contamination of the meat samples.

Meat Sample Preparation. Experimentally inoculated samples of four kinds of meat, which were confirmed to be *Salmonella* free by using the ISO 6579:2002 method, were divided into two aliquots: one aliquot was experimentally inoculated by spiking 25 g of product with 1 mL of *Salmonella* Enteritidis suspension (1−10 viable CFU/mL); the second aliquot was used as a negative control. Each aliquot was homogenized with 225 mL of buffered peptone water (BPW, as pre-enrichment broth) in a stomacher for 1−2 min and incubated at 37 °C. For ELIME array analysis, aliquots (10 mL each) were collected at 0, 2, 3, 4, 5, 6, 8, and 10 h during incubation, filtered through gauze, and centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the pellet was resuspended in a final volume of 10 mL with PBS and boiled for 3 min; prior to the analysis, the volume was adjusted to the initial value with PBS.

The standard culture method was carried out in parallel using aliquots taken only after 20 h of incubation in accordance with the established procedure.

Real Samples. Thirteen real samples (not experimentally inoculated) of the various edible parts of pork, chicken, beef, and turkey were homogenized with 225 mL of BPW (in a stomacher for 1−2 min) and incubated for 6 h and 20 h (at 37 °C) for the ELIME array and the ISO method, respectively. After incubation, the same procedure, reported for experimentally inoculated samples, was used.

Standard Culture Method. The detection of *Salmonella* in meat samples was performed according to the ISO method. The presumptive *Salmonella* colonies were serologically typed using commercial sera (Statens Serum Institute, Copenhagen, Denmark).

ELIME Array. The ELIME array method employs a preliminary washing step, then an immunoassay procedure, followed by electrochemical detection. The immnomagnetic beads, Pan Mouse IgG (immunoglobulin G), employed in this work are precoated with monoclonal antibody specific for the constant fragment (Fc) of all mouse IgG.

Salmonella enterica serotype Enteritidis was used as reference antigen, whereas other *Salmonella* serotypes (**Table 1**) were tested to evaluate the cross-reactivity.

Washing Procedure. (1) The Dynabeads Pan Mouse IgG (suspension containing 4×10^8 beads/mL in PBS, pH 7.4) were resuspended thoroughly in the vial; (2) 10 μL of beads was transferred into a 2 mL Eppendorf tube (for each standard or sample to analyze); (3) the beads were quickly washed three times with 1 mL of buffer prepared by dissolving 1% (w/v) BSA in PBS (pH 7.4).

Between each washing step, the Eppendorf tubes were placed in the Dynal MPC for 2 min and the liquid was pipetted off.

Immunoassay Procedure. Dynabeads were then used as a solid phase in a sandwich immunoassay using mouse monoclonal antibodies (mAb) against boiled *Salmonella* and rabbit polyclonal antibody−horseradish peroxidase (PAb−HRP) against *Salmonella*. The procedure adopted for the construction of the *Salmonella* calibration curve was as follows: (1) block the surface of the beads with 1 mL of 1% dry milk

suspension for 30 min (to avoid nonspecific adsorption of the reagents); (2) add 400 μL of a mAb solution diluted 1:1000 and incubate for 30 min; (3) add 400 μL of *Salmonella* standard solutions (0, 10^3−10^9 CFU/mL) or treated samples and incubate for 30 min; (4) add 400 μL of PAb−HRP solution diluted 1:100 and incubate for 30 min; (5) quickly wash the particles two times with 1 mL of PBS (pH 7.4) + 0.05% (w/v) Tween 20 and one time with PBS only.

The solutions for steps 1−4 were prepared in PBS, and the incubation was performed with slow tilt rotation using the Dynal sample mixer. At the end of each step, the tubes were place in the Dynal MPC for 2 min and the liquid was pipetted off. Finally, the immunomagnetic beads (IMBs) were resuspended in 100 μL of PBS.

Electrochemical Measurement. Ninety-six electrochemical measurements were made simultaneously by localizing 20 μL of the IMBs suspensions onto the surface of each SPE of the 96-well microplate with the aid of a support including 96 neodymium magnets placed underneath. The sandwich complex (immobilized onto the IMBs surface) was revealed by adding (in each well) 70 μL of 3,3′,5,5′-tetramethylbenzidine substrate supersensitive solution. After 3 min of incubation, the HRP activity was measured electrochemically using the IPA technique. Each standard/sample was tested in triplicate. A representation of the instrumentation employed is found in **Figure 1**.

RESULTS AND DISCUSSION

This paper represents a continuation of our previous studies in which a simple and rapid multichannel electrochemical immunosensor for detection of *Salmonella enterica* was developed. The MEI system, where the immunological chain (sandwich) took place directly on the SPE surface of the 96-well microplate, has been proven to be not very sensitive and, for this reason, was applied only to meat samples experimentally inoculated with *Salmonella* Enteritidis (1−10 cells/25 g) after 20 h of pre-enrichment (*21*).

In the present work, the 96-well electrochemical plate was coupled with immunomagnetic beads in an attempt to improve the sensitivity and rapidity (by reducing the pre-enrichment phase) of the assay but preserving the possibility of analyzing standard solutions and several samples (in replicate) at the same time.

Table 1. Cross-Reactivity (CR) Study Performed with the ELIME Array Method

Salmonella enterica serovar	CR %	*Salmonella enterica* serovar	CR %
Enteritidis	100	Infantis	70
London	67	Rissen	70
Derby	106	Give	82
Typhimurium	106	Panama	78
Anatum	97	Newport	85

The assay was developed using *Salmonella* Enteritidis as reference antigen. To establish the best conditions for the sandwich ELISA, different dilutions of specific antibodies (mAb and PAb) were tested using a fixed concentration of *Salmonella* Enteritidis (2×10^6 CFU/mL). The experimental results (data not shown) demonstrated that the selected dilutions, reported under ELIME Array, gave the maximum electrochemical signals, whereas no further current increases were observed for the higher concentrations tested.

Once the studies to optimize antibody concentrations and other parameters such as incubation time and the blocking agent (necessary to reduce nonspecific adsorption onto the surface of the coated beads) had been completed, a calibration curve for *Salmonella* Enteritidis was constructed (**Figure 2**).

The experimental data were fitted using a "nonlinear four-parameter logistic calibration plot" (*23*) as in the equation

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

in which a and d are the asymptotic maximum and minimum values, c is the value of x at the inflection point, and b is the slope.

The detection limit (LOD), defined as the concentration corresponding to the $f(x)$ value obtained by adding three standard deviations (sd) of the zero point (blank solution = without *Salmonella*) from the mean of the blank solution measurements (mean value + 3 sd) was determined to be 3×10^3 CFU/mL. The value for the sensitivity, calculated as the amount of *Salmonella* Enteritidis needed to produce a 50% increase in the signal, was 4×10^5 CFU/mL.

Thus, using IMBs coupled with the electrochemical microplate, a significant enhancement in terms of detection limit and sensitivity was observed by comparing these results with those obtained in our previous work (with a LOD = 2×10^6 CFU/mL and a sensitivity = 7×10^7 CFU/mL), where the reactions of the immunological chain took place directly on the SPE surface of the 96-well microplate (*21*).

Although we have already demonstrated (*19*) the capacity of the employed antibodies to interact with different *Salmonella*

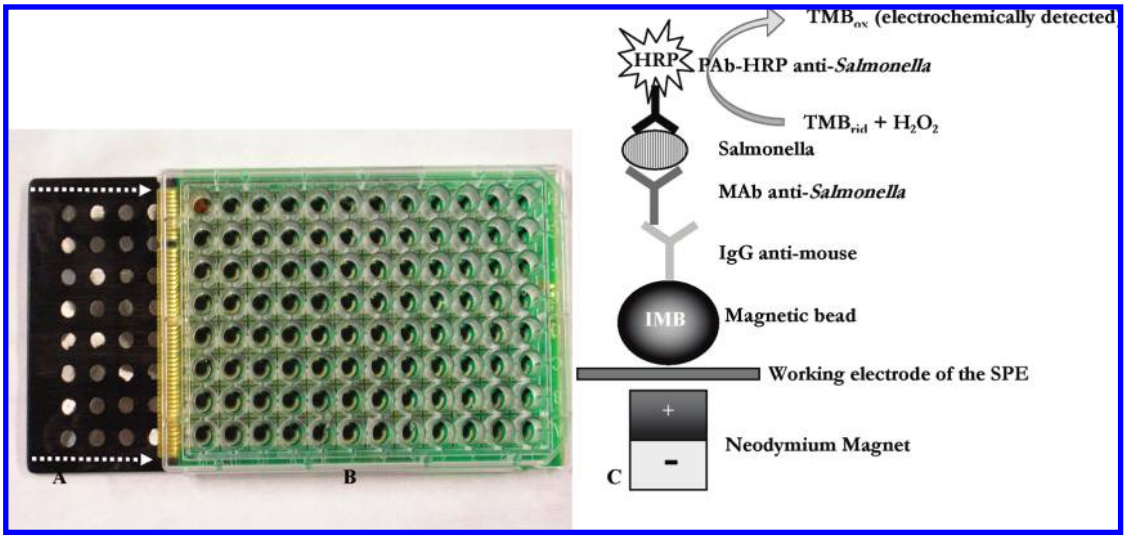

Figure 1. Picture of the ELIME array: (**A**) specially designed magnetic support including 96 neodymium magnets placed underneath the microplate; (**B**) 96-well screen-printed microplate; (**C**) schematic representation of the immunological chain onto the surface of the magnetic beads localized on the screen-printed working electrode.

serotypes (*Salmonella* Enteritidis, *Salmonella* Typhymurium, *Salmonella* Derby) and their non-cross-reactivity with other bacteria commonly present in food, in this work we have extended the experiments to a broader range of *Salmonella* serovars (*Salmonella* Enteritidis, *Salmonella* Derby, *Salmonella* Typhimurium, *Salmonella* London, *Salmonella* Anatum, *Salmonella* Infantis, *Salmonella* Rissen, *Salmonella* Give, *Salmonella* Panama, *Salmonella* Newport), all belonging to the *S. enterica* species, which is the most common cause of foodborne illness. For this purpose, a standard curve for each *Salmonella* serotype, mentioned above, was generated. The ability of the method to detect different *Salmonella* strains was expressed as cross-reactivity (CR, %) and calculated as $100x/y$, were x is the amount of *Salmonella* Enteritidis and y is the amount of the other *Salmonella* serotypes required to produce a signal increase of 50%. Results of this study are reported in **Table 1**.

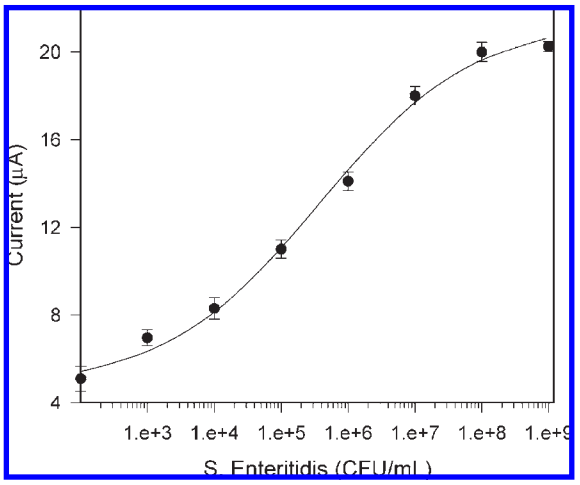

Figure 2. Calibration curves of *Salmonella* Enteritidis obtained in optimized experimental conditions.

Meat Sample Analysis. After optimization of all analytical parameters, the ELIME array was applied to blank meat samples (pork, chicken, beef, and turkey resulted negative by the microbiological test) experimentally inoculated with 1−10 cells per 25 g. Then, during the pre-enrichment phase (first step of the cultural official method, which allows the resuscitation and multiplication of sublethally damaged *Salmonella* cells) samples were collected at different time intervals and treated as reported under Materials and Methods. These tests were carried out to establish the minimum incubation time necessary for the multiplication of *Salmonella* cells until a concentration detectable by the ELIME array method was reached.

The results obtained when meat samples experimentally inoculated with *Salmonella* Enteritidis (1−10 viable cells per 25 g) were tested are shown in **Figure 3**. Only 4 of the 20 samples tested, one for each kind of meat, are shown. All samples were analyzed

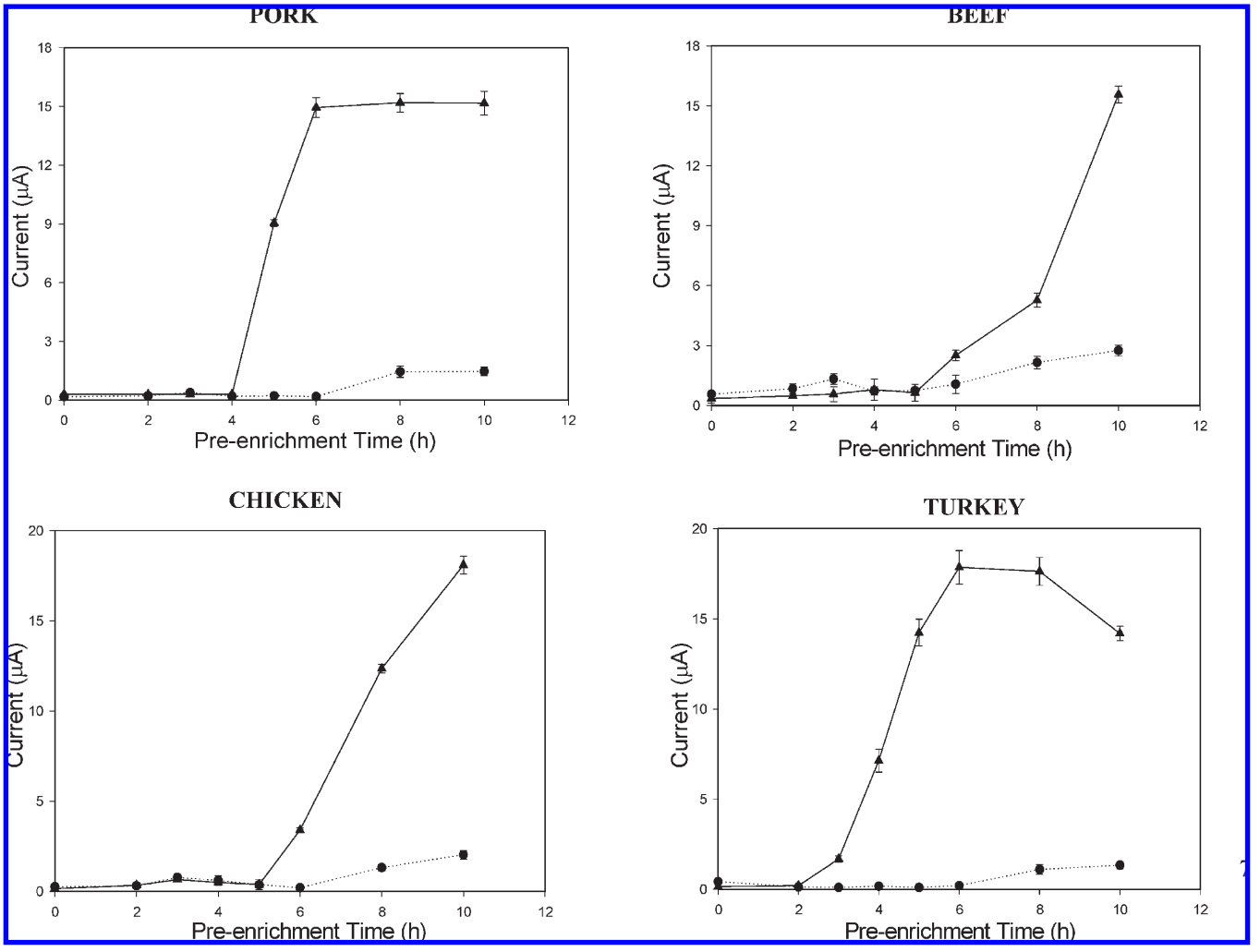

Figure 3. Meat samples taken at different pre-enrichment times, analyzed by ELIME array: (▲) samples experimentally inoculated with *Salmonella* Enteritidis (1−10 CFU/25 g); (●) same samples not inoculated.

Table 2. Analysis of Meat Samples Using ELIME Array and ISO Method

sample	ELIME array	culture method ISO 6579: 2002	agglutination method
pork	+	+	*Salmonella* Typhimurium
pork	−	−	
pork	+	+	*Salmonella* London
turkey	−	−	
turkey	−	−	
turkey	−	−	
chicken	−	−	
chicken	−	−	
chicken	+	+	*Salmonella* Give
chicken	−	−	
beef	−	−	
beef	−	−	
beef	−	−	

in triplicate for 3 days ($n = 9$). As can be seen, the minimum pre-enrichment time was variable (a phenomenon already observed in our previous work), and this was probably due to different concentration of competitor organisms naturally present in meat samples (*19*). However, 6 h of pre-enrichment incubation was sufficient to reveal the presence of *Salmonella* by ELIME array. The classic culture method, which was carried out in parallel on only the aliquots taken after 20 h of pre-enrichment, confirmed the positivity of all samples.

Finally, 13 real meat samples were analyzed, and only 3 of these samples resulted positive for *Salmonella* with both the classic culture method and the ELIME array (**Table 2**). Moreover, using the slide-agglutination method (performed after the ISO method), the three different strains of *Salmonella* were identified as *Salmonella* Typhimurium, *Salmonella* Give, and *Salmonella* London. The isolation of *Salmonella* Typhimurium, *Salmonella* Give, and *Salmonella* London from these samples demonstrates the effectiveness of the ELIME array method in revealing different *Salmonella* serotypes in meat samples as well as in standard cultures.

It is important to stress that the culture method to detect *Salmonella* in food takes more than 5 days. Because the control of salmonellosis requires action at all levels of the transmission chain according to the application of the HACCP system, several methods based on both PCR and antigen−antibody reaction have been developed to reduce the analysis time. However, the applications of immunological methods to *Salmonella* food analysis have been very poor. Even when experimentation in food is carried out, the pre-enrichment phase often needs to be followed by a selective enrichment step.

The ELIME array offers the possibility to both avoid the selective enrichment step and reduce the pre-enrichment phase. This method could also be an alternative for the detection of *Salmonella* in other kinds of food such as raw fruits and vegetables, recently implicated in outbreaks of salmonellosis in the United States and European Union.

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