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Comparison of PCR, Electrochemical Enzyme-Linked Immunosorbent Assays, and the Standard Culture Method for Detecting *Salmonella* in Meat Products

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An electrochemical enzyme-linked immunosorbent assay (ELISA) coupled with flow injection analysis (ELISA-FIA) and a PCR-based method using ST11 and ST15 primers for detecting salmonellae in meat were evaluated in comparison with the International Organization for Standardization (ISO) culture method. The methods were applied to experimentally contaminated and naturally contaminated meat samples. The results showed that both ELISA-FIA and PCR allowed detection of salmonella in a product contaminated with a low number of the microorganisms (1 to 10 salmonellae/25 g) after only 5 h of incubation of preenrichment broth, and they were just as effective as the ISO method.

The incidence of foodborne infection by *Salmonella* continues to be an important problem in the United States (16) and in the European Community (4, 16).

Raw and undercooked meat, eggs, milk, and especially poultry are the most commonly implicated vehicles of *Salmonella* spp. infection (10, 15, 16, 20, 24). For foods to be considered "salmonella free," European legislation requires that from 1 to 25 g be tested, depending on the type of food. The standard culture method (ISO 6579/2002) for detection of salmonella in food is rather sensitive and quite inexpensive, but it requires 4 to 5 days to generate results.

Given that efforts to prevent salmonellosis must be applied at all phases of the food production chain, based on the Hazard Analysis Critical Control Point (HACCP) system (23), the methods for detecting salmonella need to be more rapid than the ISO method. To this end, new technologies have been used to develop rapid methods. Specifically, a number of antibody-antigen-based methods have been developed to detect *Salmonella* spp. or specific serotypes in a variety of food (3, 8, 13, 18, 21, 25). Other authors developed immunoelectrochemical assays or piezoelectric flow injection analysis (FIA) biosensors to detect *Salmonella enterica* serotype Typhimurium (2, 7, 26). However, although these biosensors have the potential to provide direct label-free detection of bacteria, these piezoelectric FIA systems have a low level of sensitivity, and neither has been applied to food samples.

Methods based on PCR for the detection of salmonella in food have also been developed (12, 17, 19).

All of the above methods require that a preenrichment step be performed (5, 9, 14, 18, 22).

The objective of the present study was to evaluate two rapid techniques for detection of salmonella: an electrochemical enzyme-linked immunosorbent assay (ELISA) coupled with FIA (ELISA-FIA) (6) and a PCR method using ST11 and ST15 primers (1).

The experiments were first performed with experimentally contaminated samples to reduce and optimize the preenrichment times; the two methods were then applied to naturally contaminated meat samples, and the results were compared to those obtained with the International Organization for Standardization (ISO) method.

MATERIALS AND METHODS

The experiments were performed on the following: (i) experimentally contaminated meat samples, to establish the minimum preenrichment incubation time for detection of salmonella by means of ELISA-FIA and PCR methods; and (ii) naturally contaminated meat samples purchased from local retail outlets, to evaluate the two rapid methods in comparison with the ISO method.

Bacterial strain. *S. enterica* serotype Enteritidis ATCC 13076 was used. The strain, grown in tryptone soy broth (Oxoid LTD, Basingstoke, Hampshire, United Kingdom) at 37°C for 24 h, was washed three times by means of centrifugation (8,000 × *g* for 8 min) in a 0.8% NaCl solution. The microbial suspensions were standardized by turbidimetry (40% transmittance at 540 nm, Spectronic 20 turbidimeter; Bausch & Lomb, Rochester, N.Y.). A parallel count of serotype Enteritidis on tryptone soy agar yielded about 10^8 CFU/ml.

Food samples. (i) Experimentally contaminated samples. Samples of three kinds of meat (i.e., pork, chicken, and beef), which had been confirmed to be salmonella free by using the ISO method, were divided into two aliquots: one aliquot was experimentally contaminated by spiking 25 g of product with 1 ml of serotype Enteritidis suspension (1 to 10 CFU/ml); the second aliquot was used as a negative control. The two aliquots were analyzed with the ELISA-FIA and PCR methods.

(ii) Naturally contaminated samples. Thirty samples of the various edible parts of pork, chicken, and beef were purchased from local retail outlets and analyzed with ELISA-FIA, PCR, and the ISO method.

Preparation of the samples. All of the samples were treated as follows: 25 g was homogenized with 225 ml of preenrichment broth (buffered peptone water; Oxoid) in a stomacher for 1 to 2 min and incubated at 37°C. The aliquots (i.e., 15 ml each) were collected at 0, 2, 4, 5, 6, 8, and 24 h during incubation, filtered through gauze, and subjected to different methods of analysis.

For the ISO method, applied on the naturally contaminated samples, only the aliquots of the preenrichment broth collected after 24 h of incubation were used.

Standard culture method. The detection of salmonella in meat samples was done according to the ISO 6579/2002 method. The presumptive salmonella colonies were serologically typed using commercial sera (Statens Serum Institut, Copenhagen, Denmark).

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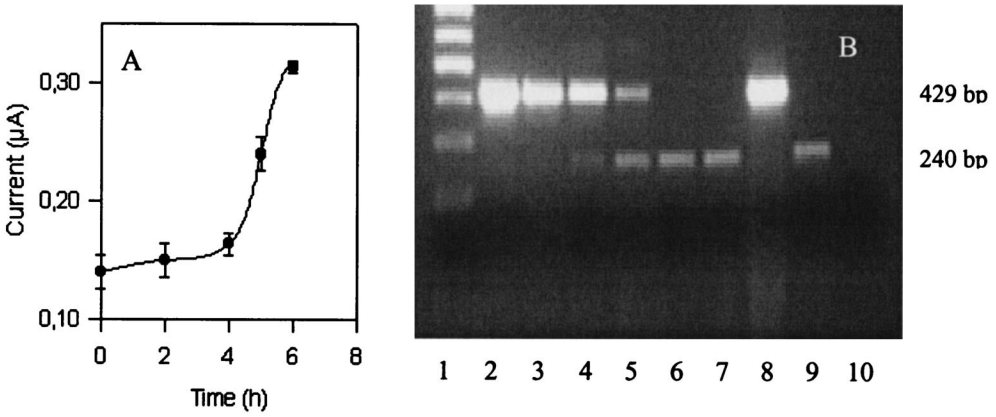

FIG. 1. Naturally contaminated sample underwent ELISA-FIA (A) and PCR (B) testing after different preenrichment times.(A) The bars represent the standard deviation obtained for the results of three replicates. (B) Lane 1, 100-bp DNA Ladder Plus marker (M-Medical Genenco); lane 2, preenrichment broth aliquot collected after 8 h of incubation; lane 3, preenrichment broth aliquot collected after 6 h of incubation; lane 4, preenrichment broth aliquot collected after 5 h of incubation; lane 5, preenrichment broth aliquot collected after 4 h of incubation; lane 6, preenrichment broth aliquot collected after 2 h of incubation; lane 7, preenrichment broth aliquot collected before incubation (time zero); lane 8, positive control; lane 9, internal control; lane 10, negative control.

Sandwich ELISA with electrochemical detection. (i) Sample extraction. Ten milliliters of filtered preenrichment broth was centrifuged for 15 min at 3,000 rpm (Centrifuge PK121R; ALC International S.r.l., Cologno Honzese, Italy). The pellet was resuspended in a final volume of 10 ml with phosphate-buffered saline (Oxoid) and boiled for 3 min; the volume was adjusted to the initial value (i.e., 10 ml) with phosphate-buffered saline.

(ii) Sandwich ELISA. The test was performed as previously described (6), using anti-mouse immunoglobulin G (H+L) (Vector Laboratories, Inc., Burlingame, Calif.), mouse monoclonal antibodies (Chemicon, Inc., Temecula, Calif.), the sample, and polyclonal antibodies conjugated with horseradish peroxidase (Biogenesis, Poole, United Kingdom). The mixture contained in each well was injected into the FIA system for electrochemical detection, described below.

(iii) Flow injection analysis. For electrochemical detection, a thin-layer transducer cell for liquid chromatography-electrochemistry from BioAnalytical Systems (West Lafayette, Ind.) was used (6).

Citrate-phosphate buffer (0.1 mol liter^{-1}, pH 5.0) was pumped with a Minipuls 3 peristaltic pump (Gilson, Inc., Middletown, Wis.) through the electrochemical cell until a constant baseline current was reached. The solutions to be analyzed were then injected into the flow stream (FIA model 7125 HPLC; Rheodyne) via the automatic valve loop (20 μl) (Supelco, Bellefonte, Pa.), and a transient current variation was measured with a Metrohm (Herisau, Switzerland) 641 VA detector and recorded with a model 868 Amel (Milan, Italy) recorder. A value of the current output of $\leq 0.16 \pm 0.012$ μA indicated the absence of salmonella on the sample, and a value of >0.21 μA was considered positive for salmonella. This threshold limit, calculated by adding four times the standard deviation to the mean value of different negative samples, has been determined in a preliminary study (data not shown). Nonetheless, it is best to always test aliquots taken both at 0 h and after 5 h of incubation of preenrichment broth to point out the difference of the current signal.

PCR assay. (i) DNA extraction. One milliliter of filtered preenrichment broth was centrifuged for 15 min at 14,000 rpm. The pellet was resuspended in a final volume of 50 μl with DNase-RNase-free distilled water (Sigma) and boiled for 10 min. The suspension was centrifuged again at 14,000 rpm for 2 min, and 5 μl of the supernatant was used for PCR (http://www.pcr.dk/DNA-purification.htm), using the primers ST11 (5′AGCCAACCATTGCTAAATTGGCGCA3′) and ST15 (5′GGTAGAAATTCCCAGCGGGTACTG 3′), previously shown to be 100% specific for *Salmonella* (1, 19).

(ii) Internal control construction. An aliquot (5 μl) of the DNA, extracted from 1 ml of the broth culture of serotype Enteritidis (10^8 CFU/ml) was used for the construction of the internal control (IC) (240 bp), using the primers ST15 and IS-ST11 (5′GCCTGCAAGTAGCCAACCATTGCTAAATTGGCGCATGCA CCAGACTCCCCTTTG 3′) (a recombinant primer) (M-Medical Genenco, Florence, Italy) (17).

Five microliters of DNA was transferred to the PCR mixture, and the PCR conditions specified below were used. Experiments were then conducted to balance the PCR coamplification so that the 240-bp amplicon was always present in the salmonella-negative samples and present or absent in the salmonella-positive samples, depending on the salmonella concentration. For this purpose, 10-fold dilutions of IC (80 μg/μl to 80 fg/μl) were tested in the presence of different concentrations of a broth culture of serotype Enteritidis (10^2 to 10^7 CFU/ml). The chosen IC concentration was evaluated using experimentally contaminated samples.

(iii) Coamplification of salmonella DNA fragment and internal control DNA. Five microliters of IC and 5 μl of extracted samples were transferred to a tube containing 50 μl of a mixture of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM (each) deoxynucleoside triphosphates, 1 μM (each) primer (ST11 and ST15), and 2.5 U of *Taq* polymerase (Applied Biosystems by Roche Molecular Systems, Inc., Branchburg, N.J.). A 30-cycle PCR was carried out using the following conditions: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 2 min; in the last cycle, the elongation step lasted 10 min.

The products were visualized by agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, Calif.).

Statistical evaluation. The statistical analysis was performed using the McNemar χ^2 *test*.

A χ^2 value of >3.84 indicates significance at the 0.05 level.

The sensitivity was calculated in accordance with the method of Ilstrup (11).

RESULTS AND DISCUSSION

The experiments carried out on experimentally contaminated samples showed that both the ELISA-FIA and PCR methods were able to detect salmonella, even in poorly contaminated products (1 to 10 salmonellae/25 g), after only 5 h of incubation of the preenrichment broth (data not shown). Such preenrichment time, in fact, allows the target salmonellae to multiply until reaching a detectable concentration by the two methods, i.e., 5×10^3 cells/g for the ELISA-FIA (6) and, in our experimental conditions, 10^3 cells/g for the PCR method. Moreover, after the preenrichment step, the number of dead cells becomes negligible, thus overcoming the problem of the inability of ELISA-FIA and PCR to distinguish between living and dead organisms.

With regard to the PCR method specifically, the preliminary tests showed that the IC concentration of 80 fg/μl was the most appropriate for all three types of meat used (data not shown). Using this concentration, the IC 240-bp fragment was visible in

TABLE 1. Meat samples positive for salmonella by ELISA-FIA, PCR, and the ISO method and salmonella serotypes

Sample	Presence of salmonella[a]					*S. enterica* serotype
	ELISA-FIA		PCR		ISO	
	4 h	5 h	4 h	5 h	24 h	
Chine of pork	−	+	−	+	+	Typhimurium
Sausage of pork	−	−	−	−	−	
Leg of chicken	+	+	+	+	+	Enteritidis
Ground beef	−	+	−	+	+	London
Breast of chicken	−	−	−	−	−	
Breast of chicken	−	−	−	−	−	
Pork side	−	+	−	+	+	Derby
Leg of chicken	−	+	−	+	+	Give
Pork side	−	+	−	+	+	Derby
Breast of chicken	−	+	+	+	+	Give
Sausage of pork	−	+	+	+	+	Panama
Ground beef	−	−	−	−	−	
Giblets of fowl	−	+	+	+	+	Enteritidis
Sausage	−	−	−	−	−	
Breast of chicken	−	+	+	+	+	Give
Chine of pork	−	−	−	−	−	
Ground pork	−	−	−	−	−	
Ground pork	−	+	−	+	+	Typhimurium
Breast of chicken	−	+	−	+	+	Give
Pork Side	−	+	−	+	+	Anatum
Breast of chicken	−	+	+	+	+	Enteritidis
Ground pork	−	−	−	−	−	
Leg of chicken	−	−	−	−	−	
Sausage	−	−	−	−	−	
Breast of chicken	−	−	−	−	−	
Chine of pork	−	+	−	+	+	Panama
Ground pork	−	+	−	+	+	Anatum
Beef hamburger	−	+	+	+	+	London
Pork chop	−	+	−	+	+	Typhimurium
Ground chicken	−	+	+	+	+	Newrochelle

[a] Results for different preenrichment culture medium incubation times are given.

presence of 10^2 CFU of salmonella/ml, whereas the 429-bp target fragment of salmonella was not yet visible. Both fragments were instead visible using 10^3 CFU of salmonella/ml. The IC fragment was absent in the presence of salmonella concentrations higher than 10^3 CFU/ml. These results are consistent with those of the analyses performed on experimentally contaminated samples (data not shown). The suitability of the IC concentration was also confirmed by the results on naturally contaminated samples: the IC 240-bp fragment was visible using the aliquots of the preenrichment broth taken after 0 and 2 h of incubation, when the 429-bp fragment was not yet visible (Fig. 1B, lanes 6 and 7). Using the aliquots taken after 4 h of incubation, only IC fragment was visible, but sometimes both fragments were present (Fig. 1B, lane 5). In the aliquots taken after 5, 6, and 8 h, only the 429-bp fragment was present (Fig. 1B, lanes 4, 3, and 2).

In Table 1, the results of the analyses performed on naturally contaminated samples are reported. Of the 30 samples analyzed, 19 tested positive for salmonella after 5 h of incubation for both rapid methods.

In some cases, PCR was able to identify positive samples after only 4 h of incubation, showing a higher level of sensitivity than ELISA-FIA (0.37 versus 0.05, respectively), although not an adequate level.

In Fig. 1, one sample testing positive by both the ELISA-FIA and PCR methods is shown.

The ISO method confirmed the presence of salmonella in all 19 of the samples, while the other samples were negative. The serological tests showed the presence of various salmonella serotypes (Table 1).

When the analyses were performed after 4 h of preenrichment incubation, the statistical analysis revealed a significant difference between the PCR and ELISA-FIA results ($\chi^2 = 5.14$; $P < 0.05$) and between these methods and the ISO results (ISO versus PCR, $\chi^2 = 9.09$ and $P < 0.05$; ISO versus ELISA-FIA, $\chi^2 = 16.05$ and $P < 0.05$). After 5 h of incubation, with respect to the limited number of analyzed samples, no differences were observed between the two methods or between these two methods and the ISO method (100% relative accuracy).

Thus, both tests are rapid, efficient, and allow simultaneous analysis of numerous samples, characteristics particularly useful for both monitoring and control as part of the application of HACCP in the food production industry. These methods, in fact, are easy to perform and less expensive than the available commercial methods; moreover, for PCR, the use of IC avoids false-negative results due the presence of PCR inhibitors in the sample matrix. Finally, given that these methods produce similar results, the person in charge of HACCP application is free to choose a method based on the specific resources available and the qualifications of the personnel.

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