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DETECTION OF *SALMONELLA* SP IN CHICKEN CUTS USING IMMUNOMAGNETIC SEPARATION

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ABSTRACT

The immunomagnetic separation (IMS) is a technique that has been used to increase sensitivity and specificity and to decrease the time required for detection of *Salmonella* in foods through different methodologies. In this work we report on the development of a method for detection of *Salmonella* in chicken cuts using in house antibody-sensitized microspheres associated to conventional plating in selective agar (IMS-plating). First, protein A-coated microspheres were sensitized with polyclonal antibodies against lipopolysacharide and flagella from salmonellae and used to standardize a procedure for capturing *Salmonella* Enteritidis from pure cultures and detection in selective agar. Subsequently, samples of chicken meat experimentally contaminated with *S.* Enteritidis were analyzed immediately after contamination and after 24h of refrigeration using three enrichment protocols. The detection limit of the IMS-plating procedure after standardization with pure culture was about 2x10 CFU/mL. The protocol using non-selective enrichment for 6-8h, selective enrichment for 16-18h and a post-enrichment for 4h gave the best results of *S.* Enteritidis detection by IMS-plating in experimentally contaminated meat. IMS-plating using this protocol was compared to the standard culture method for salmonellae detection in naturally contaminated chicken cuts and yielded 100% sensitivity and 94% specificity. The method developed using in house prepared magnetic microespheres for IMS and plating in selective agar was able to diminish by at least one day the time required for detection of *Salmonella* in chicken products by the conventional culture method.

Key words: *Salmonella*, immunomagnetic separation, rapid methods.

INTRODUCTION

Salmonella still is the leading cause of foodborne infections all over the world (5). Transmission of *Salmonella* to humans is usually by consumption of undercooked meat, milk, eggs and other cross-contaminated foods, such as vegetables, that are eaten without cooking. Animal food products are the major responsible for the large distribution of salmonellae and all the subsequent problems. Meat products predominate among the animal foods considered source of salmonellae, especially those obtained from poultry (12,16).

Conventional method used for *Salmonella* detection is time-consuming, requiring 4 days for confirming negative results and 5-7 days for positive results (6). The long time for final results is due to various incubation periods used in this method for pre-enrichment, selective enrichment, colony isolation in selective and differential agar and biochemical and serological confirmation. To overcome this disadvantage, several rapid methods for the detection of *Salmonella* in foods have been developed to decrease the detection time. The immunomagnetic separation (IMS), that uses magnetic polystyrene microspheres coated with anti-*Salmonella* specific antibodies to capture the

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target cells, is a technique that has been proposed to replace or to improve the performance of the conventional selective enrichment of *Salmonella* (4,7,11).

In this work the IMS was used to improve sensitivity and specificity and to decrease detection time of *Salmonella* in chicken cuts by the conventional culture method. To reach these objectives an in-house capture reagent was first prepared with commercially available protein A-coated magnetic polystyrene microspheres and anti-*Salmonella* polyclonal antibodies. After, the reagent was used to develop a method that combines IMS and conventional selective plating (IMS-plating) for detection of *Salmonella* in chicken products experimentally contamined. Finally, the performance of the IMS-plating method for detecting *Salmonella* in chicken products naturally contaminated was evaluated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Salmonella enterica subsp *enterica* serovar Enteritidis (*S.* Enteritidis) and *Escherichia coli* were obtained from the culture collection of FIOCRUZ (Rio de Janeiro, Brazil) and were grown in brain heart infusion broth (BHI, Difco, Detroit, USA) at 37ºC for 24h.

Magnetic microspheres and antibodies

Protein A-covered magnetic polystyrene microspheres with 0.86 µm diameter in 1% solids suspension (Bangs Laboratories, Fishers, IN, USA) were used. Polyvalent somatic and flagellar anti-*Salmonella* sera (PROBAC, São Paulo, Brazil) containing antibodies, respectively, against a common structural O antigen of groups A, B, C, D, E and against H antigens a, b, c, d, i, 1, 2, 5, were used. The immunoglobulin G (IgG) from both sera was purified by affinity chromatography with protein A-Sepharose 4B resin, concentrated in a PEG 20% solution and dialyzed against 50 mM sodium borate (pH 8.2). Purity of IgG preparations was evaluated by SDS-PAGE and protein concentration determined by UV at 280 nm using 1.35 absorbance units as extinction coefficient (1 mg/mL) (7).

Sensitization of microspheres with polyclonal antibodies

Magnetic microspheres at a concentration of 1 mg/mL were washed twice with borate buffer (50 mM, pH 8,2) using a magnet (MPC-S, Dynal, Norway). After washing, microspheres were suspended in one volume of borate buffer containing 0.6 mg/mL of purified IgG and incubated at 4ºC for 16-18 hours. Sensitized microspheres were washed three times with borate buffer, suspended at 1% solids in stock buffer (borate buffer 10 mM, pH 8.5 + 0.01% BSA + 0.05% Tween 20 + 10 mM EDTA + 0.1% NaN_3) and stored at 4ºC (2). The microspheres were six fold diluted in stock buffer before using in IMS. Efficiency of IgG binding was determined indirectly from the amount remaining in the supernatant after the sensitization procedure. Sensitized microspheres were further observed under microscope to investigate the degree of auto-agglutination and the agglutination behaviour with *S.* Enteritidis and *E. coli* cells. Batchs of microspheres with IgG reactive against somatic and flagella antigens were prepared separately.

Standardization of IMS using *Salmonella* in pure culture

S. Enteritidis was grown in BHI (Difco) at 37ºC for 24h and ten fold serial diluted to 10^{-9} with 0.1% peptone water. Aliquots (1mL) of each dilution were incubated separately with different volumes (5, 10 e 20 µL) of each type of microsphere in eppendorf tubes at room temperature for 15 minutes with constant agitation. The microspheres were exposed for 3-5 minutes to a magnetic concentrator (MPC-S, Dynal), the supernatant was removed by aspiration and the microspheres-antigen complex was suspended in 1mL of phosphate-buffered saline with 0,05% de Tween 20 (PBST, 0.15M sodium chloride, 0.01M sodium phosphate, pH 7.4). After repeating this washing procedure three times the microspheres were suspended in 20 µL of sterile PBS, streaked onto XLD and incubated at 37ºC for 24h to observe growth of typical colonies. The experiment was carried out in triplicate with each volume of microsphere tested. The actual number of *Salmonella* in BHI culture was determined by plating decimal dilutions onto plate count agar plates (PCA, Merck, Darmstadt, Germany).

Salmonella enrichment protocol

To select an enrichment protocol (EP) for providing an adequate number of *Salmonella* in chicken meat for detection by IMS-plating, samples of ground meat (25g) were inoculated with *S.* Enteritidis at different levels (1-10, 10-100, 100-1000 CFU), estimated by plating in PCA as above, and submitted to the following EP immediately after contamination and after 24h of refrigeration at 4ºC: (EP1) non-selective enrichment in lactose broth for 16-18h at 37ºC; (EP2) lactose broth for 6-8h at 37ºC, followed by selective enrichment in tetrathionate broth (TT) and Rappaport Vassiliadis broth (RV) for 16-18h at 42ºC; (EP3) lactose broth for 6-8h at 37ºC, followed by selective enrichment in TT broth and RV broth for 16-18h at 42ºC and a post-enrichment in BHI for 4h at 37ºC. At the end of each EP, 20 µL of mixed microspheres against somatic and flagellar *Salmonella* antigens was added to 1 mL of enrichment broth for IMS and plating as described before (item 4). Suspected salmonellae colonies growing onto selective agar were confirmed by biochemical and serological tests. The experiment was repeated three times with each EP and contamination level. A non-inoculated sample of the chicken meat was included in each repeat to certify that the meat was free from naturally occurring *Salmonella*.

Evaluation of IMS-plating for detection of *Salmonella* in naturally contaminated chicken cuts.

This experiment was performed with 80 chicken cuts (upper thigh, drummette, ground meat and liver) obtained in packages of approximately 0.3Kg from local supermarkets. Sampling of upper thighs and drummettes was made by rinsing the entire package content with 300 mL of lactose broth (CL, Merck, Darmstadt, Germany) in sterile plastic bags, while for ground meat and liver 25g samples were weighed in sterile plastic bags and homogenized with 225 mL of CL in a Stomacher® (Thetford, Norfolk, UK). Following the sampling procedure the samples of chicken cuts were submitted to EP3 for detection of salmonellae by IMS and plating onto XLD and BPLS agar with novobiocin (10 µg/mL) and, for comparison purposes, to conventional enrichment for detection by the standard culture method (6).

Sensitivity and specificity analysis

The results obtained in this experiment were used to calculate the sensitivity (positive samples in the IMS also positive in the conventional methodology) and specificity (negative samples in the IMS also negative in the conventional methodology).

RESULTS AND DISCUSSION

Microspheres were successfully sensitized with affinity purified polyvalent IgG against somatic and flagella antigens. Approximately 50% of the IgG used for sensitization remained bound to microspheres, and a scanty auto-agglutination and specific agglutination with *S.* Enteritidis cells and no agglutination with *Escherichia coli* cells were observed (data not shown).

An experiment to determine the adequate volume of sensitized microspheres for use in IMS was carried out using a pure culture of *S.* Enteritidis (Table 1). The two types of microspheres were tested, using three different volumes, and the detection limit of the IMS procedure was approximately 20 UFC/mL for both. This detection limit was similar to that found in other IMS studies with *Salmonella* (11) and other bacteria such as *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* (1) and was better than the 10^3 UFC/mL found for *Campylobacter jejuni* (17).

A direct relationship between the quantity of microspheres used for IMS and the detection limit was not observed. The results have shown that even in the smallest volume (5 µL) used there was an adequate amount of microspheres for capturing *S.* Enteritidis from a pure culture suspended in peptone water. However, this amount could not be sufficient when the target organism is to be separated from enrichment broth containing natural flora and food constituents (4,10). Polyvalent antibodies used in microsphere sensitization, specially the anti-LPS antibodies, can cross-react with other enterobacteria from chicken meat thus lowering the number of sites available for *Salmonella* binding. For this reason, and to increase the panel of antibodies capable of binding *Salmonella* antigens, a decision was made to use in IMS a mixture of equal parts (10 µL) of the microspheres sensitized with IgG against somatic or flagella antigens.

Table 1. Performance of different volumes of microspheres sensitized with somatic and flagellar antibodies in the recovery of *Salmonella* Enteritidis from pure cultures.

Dilutions	Microsphere volumes					
	5 µL		10 µL		20 µL	
	S	F	S	F	S	F
10^{-3}	**3/3**[a]	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
10^{-4}	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
10^{-5}	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
10^{-6}	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
10^{-7}	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
10^{-8}	**2/3**	**3/3**	**3/3**	**2/3**	**3/3**	**2/3**
10^{-9}	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**

[a] positive recovery /repeats; S: somatic antibodies; F: flagellar antibodies; Initial count: 2 x 10^9 CFU/mL.

Performance of different EP associated to IMS-plating for detection of *S.* Enteritidis in samples of chicken meat experimentally contaminated is shown in Table 2. Three independent experiments were carried out using three levels of

Table 2. Performance of the different enrichment protocols[a] used for detection of *Salmonella* Enteritidis in chicken meat experimentally contaminated.

Contamination level (CFU/25g)	Immediately after contamination				After 24h of refrigeration			
	EP1	EP2		EP3	EP1	EP2		EP3
		RV[b]	**TT**[b]			**RV**	**TT**	
0-10	1/3[c]	2/3	2/3	3/3	0/3	0/3	0/3	2/3
10-100	3/3	2/3	3/3	3/3	0/3	1/3	0/3	1/3
100-1000	2/3	2/3	3/3	3/3	0/3	1/3	0/3	1/3

[a] EP1 (Enrichment Protocol 1) = lactose broth (16-18 h), IMS and plating; EP2 (Enrichment Protocol 2) = lactose broth (6-8h), selective enrichment in TT and RV (16-18h), IMS in TT and RV and plating; EP3 (Enrichment Protocol 3) = lactose broth(6-8h), selective enrichment in TT and RV (16-18h), post-enrichment of TT+RV in BHI (4h), IMS and plating.

[b] RV=Rappaport-Vassiliadis broth; TT=Tetrathionate broth

[c] Positive samples /repeats.

S. Enteritidis contamination and samples were analysed immediately after contamination and after 24h of refrigeration. *S.* Enteritidis was detected immediately after meat contamination, in every level of contamination, by the enrichment protocols tested. However, after 24h of refrigeration only EP3 was able to detect *S.* Enteritidis at the lowest level of contamination tested (1-10 CFU/25g), while EP1 and EP2 yielded unsatisfactory results. Since the best result was obtained with EP3, this protocol was chosen for association with IMS-plating in the study of *Salmonella* detection in naturally contaminated chicken cuts.

Our attempt to substitute selective enrichment by using IMS directly in the non-selective enrichment broth (EP1) was not successful. Previous attempts of reducing analysis time of conventional *Salmonella* detection methodology by using IMS after the non-selective enrichment of several types of foods were also unsuccessful (10,14). These results point out the importance of the enrichment step in novel methodologies for detection of salmonellae in foods. Besides being present in a few number, *Salmonella* cells can be injuried during storage or processing of foods so that they will need an incubation time in a rich liquid broth to recover the capacity to grow onto selective agar plates. A study by Skjerve and Olsvik (15), suggest that at least 500 *Salmonella* cells must be originally present in a 25g sample of food to obtain an isolated colony in selective agar after IMS and plating directly from non-selective enrichment broth.

Another factor influencing growth of isolated *Salmonella* colonies is the presence in the enrichment broth of cross-reacting mucoid bacteria that bind to microspheres coated with polyclonal antibodies. Cudjoe and Krona (3), reported that growth of mucoid colonies of *Proteus* spp and coliforms such as *Escherichia coli*, *Klebsiella aerogenes* and *Enterobacter* spp, impairs isolation of salmonellae in selective agar after IMS. The same problem came out in this work and was resolved in part by adding novobiocin to plating media as suggested by others (11). Even with the antibiotic in it the selective agar still allowed growth of suspect colonies of *Salmonella* that were not confirmed in the biochemical and serological testing.

IMS-plating detected *Salmonella* in 7 out of 80 (8.7%) chicken cuts, while conventional methodology detected the bacterium in only 2 (2.5%) (Table 3). The two samples detected by conventional methodology were also detected by IMS-plating. Positive *Salmonella* isolates were subsequently serotyped by FIOCRUZ (Rio de Janeiro, Brazil). Six isolates (85.7%) were serotyped as *Salmonella* Enteritidis and one (14.3%) was *Salmonella* Derby.

The good performance of the IMS-plating method is consistent with previous results, which found IMS associated to plating on selective agar better than conventional culture method for salmonellae detection (3,8,9,15). In contrast, other studies have reported a poor performance of the method that combines IMS with plating on selective agar for *Salmonella* detection in chicken samples (13,14). The use of different enrichment protocols before IMS can be the cause of such contrasting results, since it is well known that selective agents of enrichment broths and incubation time and temperature are factors that affect decisively the analysis outcome (6).

Table 3. Comparison of IMS-plating with conventional methodology for *Salmonella* detection in chicken cuts.

Chicken cuts	IMS-plating	Conventional methodology
Upper thighs	0/20*	0/20
Drummettes	0/20	0/20
Liver	0/20	0/20
Ground meat	7/20	2/20
Total	**7/80**	**2/80**

*Positive samples/Tested samples.

The IMS-plating method developed in this study did not allow bypassing the biochemical and serological confirmation steps of conventional methodology because several of the colonies growing on agar were not confirmed as *Salmonella*. Among the 80 samples of chicken products analyzed about 90% (72/80) and 28% (22/80) presented suspected colonies on selective agar in the conventional methodology and IMS-plating method, respectively. In the latter method isolates from only 7 samples were confirmed as *Salmonella*, suggesting that the antibodies used for capture were not specific and that novobiocin used on selective agar to inhibit *Proteus* sp was not efficient to inhibit other enterobacteria. Even though our IMS-plating method did not avoid confirmation of suspected colonies it still allows reducing detection time by 24h compared to conventional methodology, a result also found by others (3,10). Associating IMS to rapid and sensitive methods such as ELISA is the preferred approach to reduce *Salmonella* detection time while increasing specificity. Recently, an ELISA performed after an enrichment protocol similar to that used in the present study (EP3) was able to detect *Salmonella* in chicken meat within 27 hours (11).

RESUMO

Detecção de *salmonella* sp em cortes de frango usando separação imunomagnética

A separação imunomagnética (IMS) é uma técnica que tem sido associada a diferentes métodos de detecção de *Salmonella* em alimentos para aumentar a sensibilidade e a especificidade e diminuir o tempo de análise. Neste trabalho é comunicada a obtenção de microesferas magnéticas sensibilizadas com anticorpos anti-*Salmonella* e seu uso em associação com a

metodologia de cultivo convencional para desenvolver um método de detecção de salmonelas em cortes de frango (IMS-plaqueamento). Inicialmente, microesferas cobertas com proteína A foram sensibilizadas com anticorpos policlonais contra lipopolissacarídeo e flagelo de salmonelas e usadas na padronização de um procedimento que captura *Salmonella* Enteritidis em cultivo puro e faz posterior detecção em ágar seletivo. A seguir, amostras de carne de frango experimentalmente contaminadas com *S.* Enteritidis foram analisadas imediatamente após a contaminação e após 24h de refrigeração utilizando três protocolos de enriquecimento. O limite de detecção foi cerca de 2x10 UFC/mL. O protocolo que incluiu enriquecimento não-seletivo por 6-8h, enriquecimento seletivo por 16-18h e pós-enriquecimento por 4h foi o que proporcionou melhor resultado na detecção de *S.* Enteritidis em carne de frango experimentalmente contaminada. Este protocolo foi comparado à metodologia convencional em estudo de detecção de salmonelas em cortes de frango naturalmente contaminados e obteve 100% de sensibilidade e 94% de especificidade. O método desenvolvido foi capaz de diminuir em pelo menos um dia o tempo de detecção de salmonelas em cortes de frango pela metodologia convencional.

Palavras-chave: *Salmonella*, separação imunomagnética, métodos rápidos.

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