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RESEARCH ARTICLE

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Detection of *Salmonella* in food samples by the combination of immunomagnetic separation and PCR assay

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Summary A combination of immunomagnetic separation and polymerase chain reaction (IMS-PCR) was used to detect *Salmonella* in food samples. Pre-enrichment of samples was combined with filtration through a membrane for the removal of food debris. The IMS-PCR assay combines selective extraction of bacteria by specific antibodies with primer specific PCR amplification that enables to detect *Salmonella* in non-fatty food samples in 24 h. In comparison with conventional cultural methods, the IMS-PCR is a rapid and specific method. Combined with filtration bags, it partially reduces the negative effects of the food matrix and allows the quick detection of *Salmonella* cells. The shortened protocols for *Salmonella* spp. detection described here can improve considerably current methodologies.

Key words *Salmonella* · Polymerase chain reaction (PCR) · Immunomagnetic separation · Food analysis · Pathogens detection

Introduction

Salmonella is a major foodborne pathogenic bacterium. The number of salmonellosis cases has increased significantly throughout the past decade in several European countries, including the Czech Republic. *Salmonella enteritidis* has become the most common cause of salmonellosis [14]. The inspection of food for the presence of *Salmonella* has become routine all over the world. Due to the low infective dose of *Salmonella*, methods for its detection are required to prove the presence of one cell in a defined food sample. Cultural methods for *Salmonella* detection involve a nonselective pre-enrichment, followed by selective enrichment and plating on selective and diagnostic agars. Suspect colonies are confirmed biochemically and serologically; the complete test requires three to four days to obtain a negative result and up to seven days to get a confirmed positive result [1]. A number of rapid methods for the detection of *Salmonella* in foods have been developed, including electrical techniques, immunoassays and nucleic acid probe analyses [2, 3, 10, 13]. However, there are still problems with their sensitivity and specificity. Analysis time depends on sensitivity of the detection system, and multiplication must typically result in a target cell concentration of 10^4–10^6 cells/ml to give a positive result. The polymerase chain reaction (PCR) is a sensitive, rapid technique, in which a few copies of target DNA can be amplified to a level detectable by gel electrophoresis. But PCR can be inhibited by several factors (e.g. food components, humic acid, urine, bile salts, etc.) [12, 15, 17]. The removal of inhibitory substances is a major step in the preparation of samples for PCR-based detection of food pathogens. Immunomagnetic separation (IMS) is a powerful tool to extract bacteria from food samples. Bacteria are specifically separated from the specimen, resulting in a useful sample for PCR with little or no nonspecific DNA or interfering factors [5].

This study was carried out to develop the rapid (in 24 h) detection of *Salmonella* in food samples. The presence of food debris after mechanical blending of the sample in the first nonselective pre-enrichment is often troublesome. Several ways to separate food particles from the medium with target organisms have been described [6, 9, 11, 12]. In this study, commercial filter bags have been tested. Our approach consisted of nonselective enrichment of food samples combined with filtration, concentration of target bacteria from samples, removal of inhibitory food debris by IMS and final specific detection by PCR.

Materials and methods

Microorganisms Strains of *Salmonella* were obtained from the Microbiology Department of the Bulovka Hospital, Prague, and from the State Health Institute, Prague. Other Enterobacteriaceae were obtained from the collection of microorganisms of the Institute of Chemical Technology, Prague. The organisms were grown on nutrient agar (NA, Difco) and subsequently cultured in buffered peptone water (BPW, Oxoid) at 37ºC with shaking at 100 rpm, for 18–24 h.

Estimation of the effective filtration time Minced meat was taken as a representative food. Samples (25 g) of minced meat and 225 ml BPW were incubated in a Stomacher filtration bag (A.E.S. Laboratoire, France) at 37ºC, 100 rpm. After 3, 5, 7, 9 and 24 h the number of microorganisms that passed through the membrane was estimated by plating on NA. The value was compared with blank, represented by the number of microorganisms grown in the bag without filtration membrane.

Filtration pre-enrichment of food samples 25 g of food sample and 225 ml BPW were incubated after homogenization in the Stomacher filtration bag at 37ºC, 100 rpm, for 16 h.

Immunomagnetic separation (IMS) of food samples During IMS, target bacteria from the pre-enriched sample are specifically caught onto magnetic beads coated with anti-*Salmonella* antibodies. This complex of bacteria and beads is separated using a magnet and washed several times to remove food debris and other microorganisms [18]. Standard procedure according to the Dynal Manual (Dynal, Norway) was followed throughout the separation of target bacteria from pre-enriched food samples.

IMS specificity test Different liquid cultures of Enterobacteriaceae were prepared by cultivating strains in 3 ml of BPW at 37ºC, 100 rpm, for 4 h. Different mixtures of *Salmonella enteritidis*, *Enterobacter cloacae*, *Escherichia coli*, *Citrobacter freundii* and *Klebsiella pneumoniae* were prepared and diluted to approximately 10^4 colony forming units (CFU)/ml. Standard protocol of IMS with anti-*Salmonella* Dynabeads (Dynal, Norway) was followed. After IMS the final samples were decimally diluted and plated on diagnostical media—brilliant green agar (BGA, Oxoid), Rambach agar (Merck) and Chromocult (Merck). Cultivation on various types of diagnostic media enabled the detection of the possible cross-reactions.

Polymerase chain reaction (PCR) Two pairs of oligonucleotide primers were prepared according to the sequences of the chromosomal *invA* and plasmid *spvC* genes [4]. The primer sequences are listed in Table 1. With these two PCR primers, either one amplicon (from the *invA* gene) or two amplicons (from the *invA* and *spvC* genes) were produced, depending on whether or not *Salmonella* contained a virulence plasmid. There are nearly 2200 *Salmonella* serovars, and all of those tested so far seem to contain *inv* genes, which enable the bacteria to invade celsl. There are six *Salmonella* serovars known to contain the virulence plasmid carrying *spvC* genes: *S. typhimurium*, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum* and *S. pullorum*. Except for *S. gallinarum* and *S. pullorum*, which are specific for fowl, the other serovars named here are common etiologic agents of enteritis in humans. Therefore, the appearance of at least one band, or two bands if there were a virulence plasmid, would indicate the presence of *Salmonella*.

Table 1 Synthetic oligonucleotides used as primers for PCR. All primers have 24 nucleotides

Primer	Sequence
spvC1 [a]	ACT CCT TGC ACA ACC AAA TGC GGA
spvC2 [a]	TGT CTC TGC ATT TCG CCA CCA TCA
invA1 [b]	ACA GTG CTC GTT TAC GAC CTG AAT
invA2 [b]	AGA CGA CTG GTA CTG ATC GAT AAT

[a]Primers for the *spvC* gene.
[b]Primers for the *invA* gene.

PCR mixture: 5 µl of 10× PCR amplification buffer (Promega), 1.5 mM $MgCl_2$, 200 µM (each) dNTP (Promega), 1 µM (each) of primer pairs, 1.25 U of Taq polymerase (Promega), 5 µl of bacterial culture, plus double-distilled water to make a total volume of 50 µl.

PCR cycle: The mixture was subjected to 30 PCR cycles in a Progene Thermal Cycler (Techne, England). The variables for the amplification cycles were as follows: denaturation for 30 s at 94ºC, annealing of primers for 30 s at 56ºC, primer extension for 2 min at 72ºC. Prior to the first cycle, the PCR mixture was incubated for 1 min at 94ºC. After the last cycle, the mixture was incubated for 10 min at 72ºC.

Detection of PCR products: amplified products were detected by 1% agarose gel electrophoresis pre-stained with ethidium bromide, at 100 V for 1 hour. A positive result (the *Salmonella* specific band) was indicated by a fluorescent band at the 244 base-pair level. If the target *Salmonella* had the virulence plasmid, a second band was detected at the 571 base-pair level.

Detection of *Salmonella* in food samples Food samples of egg melange, egg melange with sugar, dried eggs, minced meat and soft cheese were tested. From each of them, several samples (25 g) were taken. The samples of food were inoculated with different amounts of *Salmonella enteritidis* cells. The cell concentration was determined by the standard plate count technique using BGA. As a positive control, inoculated BPW without any food sample was used. Samples in BPW (225 ml) were homogenized in a Stomacher filtration bag and incubated at 37ºC, 100 rpm, for 16 h. After pre-enrichment of food samples, aliquots were taken for further

treatment by IMS and PCR. For immunomagnetic separation of the target cells, 1 ml of non-diluted and 10 times diluted pre-enriched samples were taken and treated according to the standard protocol of IMS. A final volume of 100 µl was obtained. Samples were incubated for 5 min at 95ºC, and 5 µl of each sample was taken for PCR for final detection of *Salmonella* in the food samples. All the food samples were also tested according to the protocol of a standard method [1].

Results

Estimation of the effective filtration time The efficiency of filtration was estimated by counting the bacteria that passed through the filter pores. During pre-enrichment of minced meat in the filtration bag, samples were taken and the CFU/ml was estimated. A control experiment was carried out under the same conditions but without filtration. Comparing the CFU/ml of samples with or without filtration over time, the cultivation time required for filtration pre-enrichment was estimated. The balance point was reached after 7 h (Table 2).

IMS Experiments to estimate the specificity of IMS were carried out with a mixture of *Salmonella enteritidis* plus one of the following species: *Enterobacter cloacae*, *Escherichia coli*, *Citrobacter freundii*, or *Klebsiella pneumoniae*. The CFU/ml was measured before and after IMS. Table 3 shows that there was a non-specific reaction only with *Klebsiella pneumoniae*, which reached two orders less than *Salmonella* cells in BGA, and one order less when grown in Chromocult. We also found that, in highly-concentrated bacterial suspensions with competitive microbiota over 10^7 cells/ml, the cross-reactions are much higher (data not shown).

IMS enables the removal of food debris and the concentration of target bacteria at the same time. One magnetic bead can entrap one or more bacterial cells that may be of various genera, if non-specific reaction occurs. Therefore, the number of CFU may differ from the number of cells in the tested sample.

Different diagnostic media were used for the determination of CFU/ml in samples after IMS to eliminate the influence of various stress factors on bacterial growth. The number of CFU/ml was found to be influenced by the medium used. Most notably, the growth of all samples on Rambach agar was lower in comparison to the other media (Table 3).

PCR To evaluate the specificity of the primers, 4 species of non-*Salmonella* bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Enterobacter cloacae*) and 17 known *Salmonella* serovars (*S. enteritidis*, *S. typhimurium*, *S. infantis*, *S. anatum*, *S. hadar*, *S. rissen*, *S. java*, *S. virchow*, *S. agona*, *S. montevideo*, *S. lichtfield*, *S. bareilly*, *S. albany*, *S. pomona*, *S. othmarschen*, *S. saintpaul* and *S. derby*) were tested. All non-*Salmonella* strains failed to produce any band, whereas all the *Salmonella* isolates produced the *inv*A amplicon, and serovars *S. enteritidis* and *S. typhimurium* produced an additional band—the *spv*C amplicon (Fig. 1).

Food samples The addition of *Salmonella* cells to the samples took place before homogenization. The detection

Table 2 Estimation of the effective filtration time

Time of cultivation (h)	Concentration of cells (CFU/ml)[a]		
	Blank[b]	Filter bag	FB/B[c]
3	2.0×10^4	7.1×10^3	36%
5	2.1×10^6	1.4×10^6	66%
7	8.5×10^7	7.9×10^7	93%
9	4.1×10^7	4.4×10^7	100%
24	1.3×10^9	1.2×10^9	93%

[a]Minced meat was taken as representative food sample.
[b]Blank: 25 g of minced meat + 225 ml BPW incubated in the bag without filtration membrane.
[c]FB/B: Percentage of efficiency (Filter bag/Blank × 100)

Table 3 Specificity of immunomagnetic separation (IMS) in mixed culture

Species	CFU/ml before IMS	CFU/100 µl[a] after IMS		
		BGA	Rambach	Chromocult
Salmonella enteritidis	8.0×10^4	4.2×10^4	3.6×10^4	9.7×10^4
Klebsiella pneumoniae	1.3×10^4	9.0×10^2	0	1.0×10^3
Salmonella enteritidis	8.0×10^4	5.7×10^4	3.0×10^3	1.1×10^5
Escherichia coli	5.0×10^4	0	0	0
Salmonella enteritidis	8.0×10^4	3.3×10^4	3.3×10^4	1.5×10^5
Citrobacter freundii	1.6×10^4	0	0	0
Salmonella enteritidis	8.0×10^4	6.9×10^4	3.1×10^4	1.2×10^5
Enterobacter cloacae	1.4×10^4	0	0	0

[a]According to standard Dynal protocol, during IMS the 10 times reduction of sample volume takes place (from 1 ml to 100 µl).

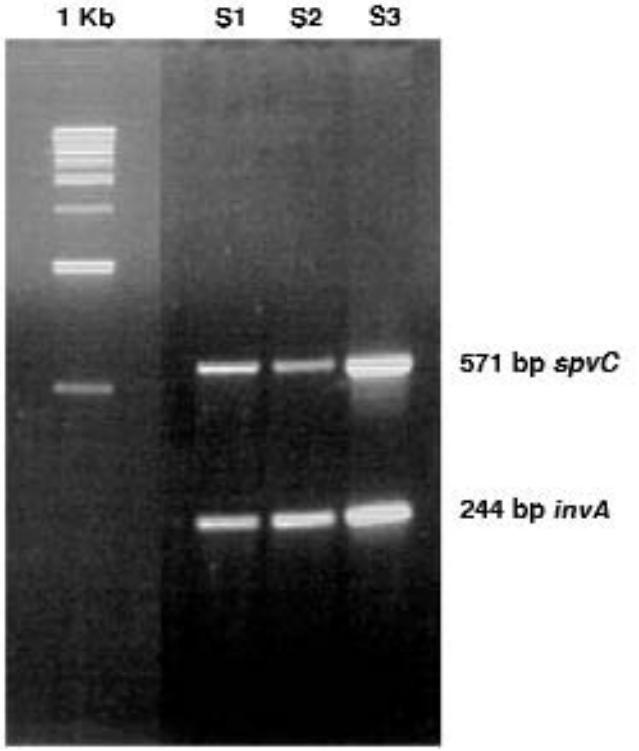

Fig. 1 PCR of *Salmonella enteritidis*. Samples S1, S2, S3 have increasing concentration of target cells

limit of *Salmonella* in the inoculated egg samples was very low (1–5 cells). Egg samples had been pasteurized before adding *Salmonella*, to ensure that there would be no problems with high-competitive microbiota. In addition, the low content of fat allows a very good yield of bacteria in the immunomagnetic separation.

The detection limit was compared between IMS and simple centrifugation of enriched samples. The results differed according to the ability of the method used to remove inhibiting factors from various food samples before PCR while retaining the required amount of target bacteria. The combination of IMS and PCR was suitable to detect *Salmonella* in egg samples, and the detection limit of 1–5 cell units per 25 g of sample was achieved. Centrifugation was more favorable for minced meat (detection limit: $1–5 \times 10^2$), the detection limit being ten times lower than in the case of IMS. IMS was less sensitive due to the presence of fat in the matrix of minced meat. The matrix of soft cheese contained both fat and inhibiting factors, which explains why the detection limit was the highest of all tested samples ($1–5 \times 10^3$ in the case of IMS, and $1–5 \times 10^4$ with centrifugation).

Discussion

Filtration pre-enrichment of food samples for 7 h (Table 2) was sufficient to reach the equilibrium between filtered and non-filtered samples. For practical reasons, a 16-hour (i.e. overnight) time of cultivation was employed. The usage of filtration bags instead of more conventional techniques provided final samples with significantly lower amounts of food debris. With smaller amounts of the food matrix present in the final liquid, samples were more easily tested and the results of these tests were more reliable.

Like other authors working with real food samples [7, 16], we had to overcome the adverse influence of contaminating bacteria on the sensitivity of the PCR assay. Using IMS, target bacteria were specifically separated from the competitive microbiota and the food matrix, and the inhibition of PCR by components of foods was overcome. Prior to natural samples, we tested designed mixtures of bacteria (Table 3). The non-specific cross-reaction appeared only with *Klebsiella pneumoniae*, which reached two orders of magnitude less than *Salmonella* cells. In highly-concentrated bacterial suspensions with more than 10^7 cells/ml of competitive microbiota, the cross-reactions are much higher (data not shown). Note also that problems do arise when using IMS with fatty food samples (minced meat) or with samples containing a high amount of other microorganisms.

The immunomagnetic separation and PCR assay combines selective extraction of bacteria by specific antibodies with primer-specific PCR amplification. PCR was the final step of specific detection. We used two pairs of primers in PCR assay. The advantage of multiplex PCR was that it could simultaneously identify the *Salmonella* strains which had a virulence plasmid, thus facilitating the search for specific etiologic *Salmonella* serovars. When using samples of soft cheese and minced meat, different sensitivities were obtained. Extracts from cheese are known to interfere with amplification, as was shown in earlier studies by Herman and de Ridder [8], Rossen et al. [15], and Wernars et al. [19]. Secondly, the high fat content in minced meat caused problems. IMS of these pre-enrichment samples failed, probably due to the high fat content that caused a loss in number of magnetic beads, which stuck to the food matrix and could not be separated by the magnetic field. Because of highly competitive microbiota, strong cross-reactions also occurred, which led to non-specific binding of other bacteria (especially Enterobacteriaceae) on the anti-*Salmonella* Dynabeads. These factors obviously were responsible for the decrease in sensitivity of the whole process.

In comparison with conventional cultural methods, the IMS-PCR is a rapid, specific method for the detection of *Salmonella* in foods that contain neither fat nor a high amount of other microorganisms. Combined with the use of filtration bags, it reduces the negative effects of food matrix and gives the ability to detect *Salmonella* cells within 24 h. The shortened protocols for *Salmonella* spp. detection described here can offer considerable improvement over current methodologies.

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