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Non-Specific Reactions during Immunomagnetic Separation of *Listeria***

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Summary

Problems occurring during the immunomagnetic separation (IMS) of *Listeria* using immunomagnetic particles Dynabeads® anti-*Listeria* (Dynal Biotech, Norway) were specified. Characteristics of these particles were compared with anti-*Listeria* spp. magnetite particles (Quantum Magnetics, USA). Pure cultures of *Listeria innocua*, *Arthrobacter* spp., *Bacillus subtilis*, *Citrobacter braakii*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae* and *Staphylococcus aureus* were used to evaluate non-specific reactions during IMS. Gram-positive microorganisms, especially *Staphylococcus aureus* and *Arthrobacter* spp., were found to be responsible for non-specific reactions in most cases. The capacity of Dynabeads® anti-*Listeria* particles was determined to be about 10 % of the initial pure cultures of *Listeria* spp., after 10 min of incubation. Non-specific reactions during IMS of *Listeria* were examined on the artificially inoculated food samples in which Gram-positive bacteria showed the highest percentage of capture. Influence of washing in two buffers was also studied.

Key words: immunomagnetic separation, immunomagnetic particles, non-specific reactions, *Listeria*

Introduction

Immunomagnetic separation represents a feasible method for rapid isolation and concentration of *Listeria* cells. In principle, a specific bond is formed between microorganisms and a magnetic support on which the specific antibody against selected surface structures of microbial cells is immobilized. To separate paramagnetic particles with linked bacterial cells from solutions, a magnetic separator with permanent magnet is used. The recovery rate of IMS is influenced by incubation time of immunomagnetic particles and by microbial cell density. The longer the incubation period the more preferable recovery rate has been obtained by several authors (*1–3*). As a consequence of a long incubation period, however, non-specific reactions of accompanying organisms occurred. Generally, the addition of detergents partially decreased the rate of non-specific reactions (*1,2*). Paramagnetic particles to enrich and concentrate *Listeria* cells have been developed by several companies. Dynabeads® anti-*Listeria* (Dynal Biotech, Norway) contains polyclonal antibodies against *Listeria* cells covalently linked to superparamagnetic polystyrene particles (*4*). Anti-*Listeria* spp. magnetite particles (Quantum Magnetics, USA) re-

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present immunomagnetic particles based on the same principle. ListerTest (Vicam, Watertown, USA), or known as Listerscreen in France, is a newly developed IMS set for the isolation of *Listeria* from food and environment (*5*).

The aim of this study was to find which of the microorganisms occurring together with *Listeria* species in real samples may induce non-specific reactions during IMS and to compare two kinds of immunomagnetic particles.

Materials and Methods

The following immunomagnetic particles (IMP) were used: Dynabeads® anti-*Listeria* (2.8 μm diameter, Dynal Biotech, Norway), anti-*Listeria* spp. magnetite particles (250 nm diameter, Quantum Magnetics, USA) using 3 in 1 MPS magnetic separator (CPG Inc., USA).

Bacterial strains

Listeria innocua CCM 4030, *Bacillus subtilis* CCM 2216, *Citrobacter braakii* CCM 158, *Enterobacter aerogenes* CCM 2531, *Escherichia coli* CCM 3954 and *Staphylococcus aureus* CCM 4223 were obtained from Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). One strain of *Arthrobacter* species was isolated in our laboratory and one strain of *Enterobacter cloacae* was purchased from Food Research Institute Bratislava, Slovakia. Bacterial strains were cultured on nutrient agar no. 2 (HiMedia, India), except *L. innocua* and *S. aureus*, which were cultured on blood agar base supplemented with defibrinated sheep´s blood (Oxoid, UK), at their optimal temperatures. Stock cultures of all the bacterial strains were maintained in refrigerator at 8 °C and were transferred at least once a month.

Cultivation media

The following agar plates were used: Oxford (HiMedia, India), PALCAM (Oxoid, UK), Baird-Parker (IMUNA, Slovakia), blood agar and nutrient agar no. 2 (both Oxoid, UK), tryptic soy yeast extract agar – TSYEA (HiMedia, India), crystal violet, neutral red and bile agar – VRB (Merck, Germany), and brain heart infusion agar – BHI (Oxoid, UK). Enrichment media were the following: *Listeria* enrichment broth (LEB, Oxoid, UK) with the supplement SR141 (Oxoid, UK), *Listeria* enrichment broth base (UVM, Oxoid, UK) using SR142 supplement, and Fraser broth base (HiMedia, India) with the addition of Fraser *Listeria* Supplement FD 064 (HiMedia, India). All the media were prepared according to manufacturers´ instruction. Selective supplements were added aseptically to nutrient base after autoclaving (121 °C, 15 min) and cooling to 45–50 °C. PBS Tween buffer and PBS buffer supplemented with 5.0 % defatted milk powder was used as a washing solution.

Food samples

Ripened soft cheeses Romadúr and Olomoucké tvarůžky, liver paté with mushrooms, and frozen vegetable mixture were obtained from local markets.

Immunomagnetic separation

After appropriate incubation of one-species culture, two-species culture or processed sample, 1-mL aliquot of bacterial suspension was transferred to Eppendorf tubes containing immunomagnetic particles (20 μL). Eppendorf tubes were tightly closed and incubated by stirring gently for 10 min, and then 3-min separation in magnetic separator was applied. Supernatant was removed and particles were washed with 1 mL of PBS Tween buffer. Another magnetic separation step followed. This procedure was repeated three or five times in the case of food sample. The resulted IMP-cell complex was resuspended in 100 μL of PBS Tween buffer and 50 μL--aliquot was inoculated onto appropriate agar plates.

Verification of the formation of non-specific reactions at IMS

All the tested strains were selected as representative bacteria accompanying *Listeria* spp. in real samples (see Table 1). Suspensions of reference bacterial strains *Escherichia coli*, *Citrobacter braakii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Bacillus subtilis*, *Staphylococcus aureus*, *Arthrobacter* spp. alone and in coculture with *Listeria innocua* were prepared in sterile physiological water according to McFarland turbidimetric standards. A 10-fold serial dilution was made giving initial concentration of 10^4 CFU/mL. One-millilitre aliquot of each bacterial suspension was mixed up with 20 μL of IMP and separated immunomagneticaly, and 100 μL of the obtained suspension was surface streaked onto Oxford and PALCAM selective agar plates. To find capture of individual pure cultures on IMP, other media mentioned above were also applied. Typical colonies of target bacterial strain were checked after 24–96 h of incubation at 37 °C, or at 30 °C in the case of *Listeria innocua* and *Arthrobacter* spp. coculture.

Table 1. Accompanying organisms of the food samples tested causing non-specific reactions during IMS of *Listeria innocua*

Sample	Organism causing non-specific reaction
Ripened soft cheese Romadúr	*Staphylococcus*, *Proteus vulgaris*
Frozen vegetable mixture	Yeasts, *Citrobacter* spp.
Liver paté with mushrooms	*Staphylococcus* spp., *Bacillus* spp., *E. coli*, *Enterobacter* spp.
Ripened soft cheese Olomoucké tvarůžky	*Arthrobacter* spp., *Staphylococcus* spp., yeasts

The food sample (25 g) was homogenized in 225 mL LEB and Fraser selective enrichment broth with half a dose of antibiotics in respect to the food type (*6*,*7*). In addition, the food homogenate was artificially contaminated with *Listeria innocua* suspension of proper concentration (1 mL) to obtain final densities of 100, 10, and 1 CFU per 25 g of the sample. After 24 h of incubation at 30 °C, 1-mL aliquot of enriched food homogenate was removed for immunomagnetic separation followed by the incubation of separated *Listeria* cells on selective agar plates (Oxford, PALCAM) at 37 °C for 24–72 h.

Comparison among the tested IMP

Differences occurring during the immunomagnetic separation process are listed in Table 2. PBS buffer supplemented with 5 % defatted milk powder was also tested in the present study as it is a recommended IMS using anti-*Listeria* spp. magnetite particles.

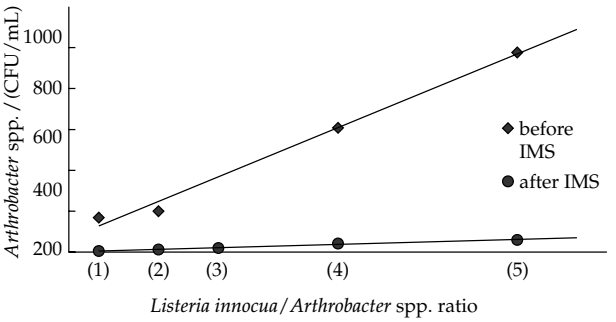
Table 2. Procedure differences using two types of IMPs

Immunomagnetic particles, manufacturer	*t* (incubation) min	*t* (separation) min	Number of buffer washings
Dynabeads® anti-*Listeria*, Dynal	10	3	3
Anti-*Listeria* magnetite, Quantum Magnetics	15	5	3

Results and Discussion

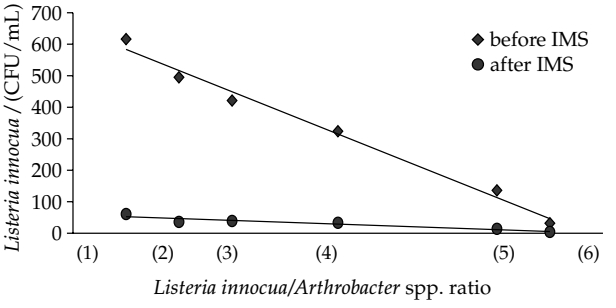
Non-specific reactions

Immunomagnetic separation of bacterial strain in monoculture and coculture with *L. innocua* in ratio of $5{\cdot}10^3/5{\cdot}10^3$, as well as in $3{\cdot}10^3/7{\cdot}10^3$, $2{\cdot}10^3/8{\cdot}10^3$ and $1{\cdot}10^3/9{\cdot}10^3$ CFU/mL was examined. *E. coli* was frequently captured by Dynabeads® anti-*Listeria* particles. Nevertheless, *Listeria innocua* was always successfully isolated from the coculture with *E. coli*. VRB agar plates were used to enumerate *E. coli* after IMS due to their inability to grow on *Listeria* selective agar plates. The same plates were also successfully used to isolate *Citrobacter braakii*, which was occasionally found to be captured by immunomagnetic particles. Thus, both *E. coli* and *C. braakii* could not cause any difficulties during IMS of *Listeria*. *Listeria* antibodies tied up on Dynabeads® anti-*Listeria* particles did not react with surface molecular structure contained in *Enterobacter aerogenes* and *E. cloacae* cells. *Bacillus subtilis* was rarely isolated from broths using Dynabeads® anti-*Listeria* particles. Since this strain did not grow on *Listeria* selective agar plates, *L. innocua* was clearly distinguished from *Bacillus subtilis* in coculture. Pronounced non-specific reaction during IMS occurred using *Staphylococcus aureus* and *Arthrobacter* spp. reference strain in coculture (Figs. 1 and 2). There is a high percentage of *S. aureus* capture using Dynabeads® anti-*Listeria* particles. Higher percentage of capture was also observed in pure culture of *Staphylococcus aureus* too (Table 3). *S. aureus* grew and formed yellow, greyish colonies after 2–3 days of incubation on Oxford agar plate. Poor growth was also observed on PALCAM agar plates. Despite these facts, typical colonies of *Listeria* should be clearly distinguished from those of *S. aureus* due to the presence of enzyme hydrolysing aesculin incorporated into agar plates as a selective supplement. A number of *S. aureus* colonies was determined after incubation on Baird-Parker selective agar plates in which black colonies with clear zone are formed (*L. innocua* failed to grow). It is not clear if accompanying bacterial cells are able to attach *Listeria* specific antibodies or to physically adsorb on the surface of IMP. Moreover, the question why *S. aureus* cells were not buffer washed before IMS also remained.



(1) $1{\cdot}10^3/9{\cdot}10^3$, **(2)** $2{\cdot}10^3/8{\cdot}10^3$, **(3)** $3{\cdot}10^3/7{\cdot}10^3$, **(4)** $1{\cdot}10^3/1{\cdot}10^3$, **(5)** $8{\cdot}10^3/2{\cdot}10^3$ (CFU/mL) / (CFU/mL)

Fig. 1. Colony count of *Arthrobacter* spp. before and after IMS in relation to *Arthrobacter* spp./*L. innocua* inoculum ratio using Dynabeads® anti-*Listeria* particles



(1) $1{\cdot}10^3/9{\cdot}10^3$, **(2)** $2{\cdot}10^3/8{\cdot}10^3$, **(3)** $3{\cdot}10^3/7{\cdot}10^3$, **(4)** $1{\cdot}10^3/1{\cdot}10^3$, **(5)** $8{\cdot}10^3/2{\cdot}10^3$, **(6)** $9{\cdot}10^3/1{\cdot}10^3$(CFU/mL)/(CFU/mL)

Fig. 2. Colony count of *Listeria innocua* before and after IMS in relation to *Arthrobacter* spp./*L. innocua* inoculum ratio using Dynabeads® anti-*Listeria* particles

Arthrobacter spp. was frequently captured by Dynabeads® anti-*Listeria* particles. The growth of these Gram-positive rods is poor on Oxford agar plates, whereas in PALCAM agar plates it is very good. *Arthrobacter* spp. may clearly be distinguished as they form yellow colonies (with 3 days of cultivation), later (after 5 days) having irregular, rough and elevated surface without aes-

Table 3. Immunomagnetic separation of reference strains using Dynabeads® anti-*Listeria* particles

Initial colony count (Colony count after IMS[1]) (CFU/mL)			Recovery rate %		
L.i.[1]	*S.a.*[2]	*A.*[3]	*L.i.*	*S.a.*	*A.*
5840 (200)	1400 (140)	4300 (580)	3.4	10.0	13.5
2200 (110)	1370 (90)	3100 (490)	5.0	6.6	15.8
370 (20)	1030 (230)	1120 (200)	5.4	22.3	17.9
110 (10)	1100 (180)	250 (40)	9.1	17.8	16.0

L.i. – *Listeria innocua*, *S.a.* – *Staphylococcus aureus*, *A.* – *Arthrobacter* spp.

[1]cultivated on PALCAM agar plates; [2]cultivated on Baird-Parker selective agar plates; [3]cultivated on TSYEA selective agar plates

culin hydrolysis. The mode of attachment to *Listeria* specific antibodies was not precisely explained as it was beyond the focus of the current study. Morphological similarity of both *Listeria* and *Arthrobacter* species could be a presumable elucidation. As observed in further experiments, non-specific reactions are much higher in concentrated bacteria suspensions where competitive microflora is higher than 10^5 cells/mL. Isolation of *Listeria* from such matrices is irregular and accidental. Thus, it is impossible to state unambiguously which concentration level of accompanying microflora could disable the detection of *Listeria* using IMS procedure by the selective plating.

Capacity of Dynabeads® anti-Listeria IMPs

Immunomagnetic separation was applied using different inoculum levels of *L. innocua*. After 10 min of incubation, magnetic particles were able to capture less than 10 % of initial inoculum level (Table 3). Our results are in agreement with those obtained by Kuhn and Goebel (*5*). They reported 10 % of *Listeria monocytogenes* cells bound into magnetic particles coated with specific antibodies after 10 min of incubation. Almost 80 % of bound *Listeria* cells were determined after 100 min of incubation in this experiment. However, as a consequence of long incubation period, a high percentage of non-specific reactions may occur (*2*). Kuhn and Goebel (*5*) reached significantly higher percentage of bound cells using Lister Test™ VICAM, combining non-specific and specific antibodies against *Listeria*.

IMS of artificially contaminated food samples

The food samples (ripened soft cheeses, liver paté with mushrooms, and frozen vegetable mixture) were artificially contaminated with *Listeria innocua*, which was successfully isolated after 24 h of incubation in LEB and Fraser enrichment broth with half a dose of antibiotics supplement (Table 4). The initial level of inoculum ranged from 1 to 100 CFU/25 g.

Immunomagnetic separation procedure is very sensitive to the presence of fat or other scraps, which may hinder proper fixation of magnetic particles to the magnet. Therefore, pipetting must be done with proper attention. As mentioned above, the fat interference may partially be eliminated by the addition of Tween 20 to the sample (*8*). To remove the scraps, both filtration and centrifugation techniques may also be applied (*9*). Even though specific antibodies against *Listeria* are bound to magnetic particles, a frequent capture of accompanying microflora from real food samples was detected. Quintuple buffer washing was found to be suitable for elimination of non-specific reactions in comparison with triple one. From microflora present in real samples (Table 1), the most frequent non-specific reactions occurred in case of staphylococci and *Arthrobacter* spp.

Table 4. Detection of *Listeria innocua* after 24 h of enrichment followed by IMS using Dynabeads® anti-*Listeria* particles

Food sample	Enrichment medium	Initial level of *Listeria innocua* count / (CFU/25 g)		
		100	10	1
Romadúr cheese	LEB	+	+	+
Olomoucké tvarůžky cheese	LEB	+	+	+
Vegetable mixture	Fraser ½	+	+	+
Liver paté	Fraser ½	+	+	+

Fraser ½ – Fraser enrichment broth with half a recommended dose of antibiotics; LEB – *Listeria* enrichment broth; + means a successful detection of *Listeria* cells after IMS

Washing buffers comparison

To reduce non-specific reactions of accompanying organisms during IMS of *Listeria*, PBS buffer supplemented with either 1 % casein or 5 % defatted milk powder is recommended when using anti-*Listeria* spp. magnetite particles. A 15-minute incubation of IMPs together with bacterial cells and a 5-minute magnetite separation are also advised by the manufacturer. Since the results of our experiment did not show any differences between the buffers prepared according to the manufacturer´s recommendation and commonly used PBS Tween buffer during IMS, the latter was employed in this study (Table 5). Magnetite separation period should be extended from 5 to 10 min due to the low diameter of magnetic particles (250 nm), which may float for a longer time in suspension compared to those of wider diameter. Therefore, the extended time of magnetic separation

Table 5. Comparison of two buffer solutions used in immunomagnetic separation procedure

Organism	IMP, manufacturer	Buffer solution	Growth	
			PALCAM	Oxford
Listeria innocua	anti-*Listeria*, Dynal	PBS Tween	+	+
	anti-*Listeria*, Quantum Magnetics	PBS Tween	+	+
		PBS 5 % DMP	+	+
Listeria innocua/ *Staphylococcus aureus*	anti-*Listeria*, Dynal	PBS Tween	+/p	+/+
	anti-*Listeria*, Quantum Magnetics	PBS Tween	+/p	+/+
		PBS 5 % DMP	+/p	+/+
Listeria innocua/ *Arthrobacter* spp.	anti-*Listeria*, Dynal	PBS Tween	+/+	+/+
	anti-*Listeria*, Quantum Magnetics	PBS Tween	+/+	+/+
		PBS 5 % DMP	+/+	+/+

+ growth; p – poor growth; 5 % DMP – 5 % defatted milk powder

should be applied to avoid the loss of IMPs during separation procedure.

Based on the results obtained in this study, it could be stated that anti-*Listeria* magnetite and Dynabeads® anti-*Listeria* particles have similar properties concerning non-specific reaction occurrence. Due to the low diameter of anti-*Listeria* spp. magnetite particles, the magnetite separation time was prolonged and handling practice was also difficult in comparison with Dynabeads® anti-*Listeria* ones (2.8 μm).

Conclusion

Members of genera *Staphylococcus* and *Arthrobacter* were frequently found to be responsible for non-specific reactions which occurred during IMS of *L. innocua*. Although the recovery rate of bacterial cells increased with the increase of incubation time of IMPs (ranged 10 to 60 min), a high degree of non-specific reactions may occur. Ten percent of initial *Listeria* cells were recovered after 10 min of incubation using Dynabeads® anti-*Listeria* particles. Although different buffer solutions were tested, immunomagnetic particles used in this study were found to be suitable for isolation and concentration of *Listeria* cells. In real samples with customary level of contamination of competitive microflora, it was always possible to detect *Listeria* by using IMS.

Acknowledgement

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Nespecifične reakcije tijekom imunomagnetskog izdvajanja *Listeria*

Sažetak

Uočeni su problemi tijekom imunomagnetskog izdvajanja bakterije roda *Listeria* korištenjem imunomagnetskih čestica Dynabeads® anti-*Listeria* (Dynal Biotech, Norway). Osobine tih čestica uspoređene su s magnetitnim česticama anti-*Listeria* spp. (Quantum Magnetics, USA). Da bi se procijenile nespecifične reakcije tijekom imunomagnetskog izdvajanja, upotrijebljene su čiste kulture *Listeria innocua*, *Arthrobacter* spp., *Bacillus subtilis*, *Citrobacter braakii*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae* i *Staphylococcus aureus*. Gram-pozitivni mikroorganizmi, osobito *Staphylococcus aureus* i *Arthrobacter* spp., najčešće su bili odgovorni za nespecifične reakcije. Utvrđeno je da kapacitet čestica Dynabeads® anti-*Listeria* iznosi oko 10 % od početne čiste kulture *Listeria* spp. nakon 10 min inkubacije. Nespecifične reakcije tijekom imunomagnetskog izdvajanja *Listeria* ispitane su na umjetno inokuliranim uzorcima hrane u kojima su Gram-pozitivne bakterije imale najveći postotak vezanja. Ispitan je i utjecaj ponovljenog pranja s dvama puferima.