

Evaluation of immunomagnetic separation and polymerase chain reaction for culture-independent detection of *Listeria monocytogenes* low numbers in cheese

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Summary

Listeria monocytogenes continues to be a major food-borne bacterial pathogen. Therefore, an improvement of rapid and reliable methods for its detection in food is still required. In our study, a potential of immunomagnetic separation (IMS) for culture-independent determination of low numbers of *L. monocytogenes* in cheese was evaluated. IMS using Dynabeads anti-*Listeria* (Thermo Fischer Scientific, Waltham, Massachusetts, USA) followed by simple thermal cell lysis and specific real-time polymerase chain reaction (PCR) was applied to various steamed cheeses artificially contaminated with *L. monocytogenes* strains. The described protocol was applied to six smoked and non-smoked steamed cheese products artificially contaminated at *L. monocytogenes* calculated levels ranging from 8.8×10^4 CFU·g⁻¹ to 2.2×10^1 CFU·g⁻¹. Results were obtained in five hours with PCR detection limit of *L. monocytogenes* numbers $(3.8\text{--}7.4) \times 10^2$ CFU·g⁻¹ as estimated in individual artificially contaminated test portions. An additional step of 5-fold concentration of the filtered sample homogenate by centrifugation prior to IMS improved the detection to the level of $(1.6\text{--}2.0) \times 10^2$ CFU·g⁻¹ with no negative effect due to relatively higher content of natural microflora in cheese made from raw milk. The evaluated procedure proved to be a fast method for *L. monocytogenes* low numbers detection in cheese.

Keywords

Listeria monocytogenes; cheese; immunomagnetic separation; polymerase chain reaction; culture-independent detection

Listeria monocytogenes is a food-borne pathogen ranking among major health issues due to its high mortality rate [1]. Significant numbers of listeriosis food-borne outbreaks have been associated with the consumption of ready-to-eat foods, contaminated with the pathogen [2]. In European Union, the number of reported confirmed listeriosis cases further increased as 5146 food- and waterborne outbreaks were reported in 2018 [3].

L. monocytogenes was isolated from numerous ready-to-eat food products of animal and plant origin, such as meat, fish or delicatessen products, cheese and vegetables [4]. In particular, soft cheeses made from unpasteurized milk represent products of high risk regarding potential *L. monocytogenes* contamination [5, 6].

Smoked or non-smoked steamed cheeses are traditional Slovak semi-soft unripe cheeses that are made from unpasteurized or pasteurized ewes'

or cows' milk either directly at sheep farms or chalets or in cheese-making factories.

As a food safety criterion for *L. monocytogenes*, the limit of 100 CFU·g⁻¹ is set for ready-to-eat food products placed on the market during their shelf-life for the products able or unable to support the growth of *L. monocytogenes* [7], including soft and semi-soft cheeses. A reference analytical method associated with the criterion is given, however, the use of rapid alternative analytical methods validated against reference method is acceptable. Much effort has been done to improve the methods for quantitative detection of *L. monocytogenes*, particularly of low numbers, including several proposed alternative methods based on molecular and bacterial cell-concentration techniques [8].

Culture-independent alternative approaches based on molecular identification using polymerase chain reaction (PCR) require upstream

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procedures of appropriate sample treatment, separation or concentration of the analysed target followed by DNA extraction [9]. Cheeses are complex and heterogeneous food matrices containing various potential inhibitory compounds, which could compromise DNA analysis [10]. Since microorganisms are not uniformly distributed in the matrix, efficient homogenization is a very important first step [11]. A homogenized food sample can be subsequently subjected to a separation procedure, such as filtration, centrifugation or specific immunoseparation.

Immunomagnetic separation (IMS) presents a separation procedure suitable for the purpose. The simplest DNA extraction protocol of “boiling” is restricted to bacterial cell lysis. It is largely used for DNA extraction from pure cultures or enriched microflora for immediate downstream analysis [12]. This protocol should be successfully applicable to cells separated using IMS, as most food matrix components with a potential inhibitory effect should be eliminated.

Numerous studies on application of IMS are available in the field of detection of food bacterial pathogens. However, most of them are based on enrichment of the target microorganism(s). This approach, depending on culture, can provide sufficiently sensitive results with a detection limit of 1–10 CFU in the analysed sample [13, 14], but with the results available not sooner than in 2–3 days. However, the application of IMS for culture-independent food-borne bacteria detection is still studied only rarely [15, 16] and, to our best knowledge, no published study is focused to cheese products.

The aim of our study was to evaluate the potential of a commercial IMS system Dynabeads anti-*Listeria* (Thermo Fischer Scientific, Waltham, Massachusetts, USA) when used as a single step for cell concentration of *L. monocytogenes* from diluted, homogenized and filtered samples of artificially contaminated traditional steamed cheese products produced in Slovakia. Simple thermal lysis of *L. monocytogenes* cells attached to immunomagnetic beads and species-specific real-time PCR analysis for culture-independent *L. monocytogenes* low number determination was applied on series of artificially contaminated samples.

MATERIALS AND METHODS

Bacterial strains

Listeria monocytogenes strain NCTC 11944 serogroup IVb (National Collection of Type Cultures, London, United Kingdom), *L. monocytogenes* LM 67/3 serogroup IIa and *L. monocytogenes* LM 87/13/2 serogroup IIb [17] isolated from cheese and cheese production environment in our laboratory in a mixture (1:1:1) were used for artificial contamination of cheese samples.

L. ivanovii CCM 5884 and *L. innocua* CCM 4030 (Czech Collection of Microorganisms, Brno, Czech Republic) were used to prepare mixtures (1:1, 10:1 and 100:1) with *L. monocytogenes* to evaluate *L. monocytogenes* detection in the presence of non-*monocytogenes* *Listeria* strains.

The strains were grown in Tryptone Soya Broth (TSB) medium (Merck, Darmstadt, Germany) at 37 °C and maintained on Tryptone Soya Agar (Merck) at 4 °C and in 20% glycerol solution at –18 °C. Cell suspensions for artificial contamination were prepared from mixed overnight cultures of $(8.8 \pm 0.6) \times 10^8$ CFU·g⁻¹.

Food samples

Six types of steamed, non-smoked and smoked cheese products were used in the study. The two products made from pasteurized cows' milk (products 1 and 2) were obtained from shops in Slovakia. Three products made from raw ewes' milk (products 3–5) were obtained from a local market-place in Bratislava, Slovakia. One product made from unpasteurized ewes' milk (product 6) was obtained from a sheep farm retail (Chalet Pružina, Pružina, Slovakia). Characteristics of the cheese products are summarized in Tab. 1. The limit of 100 CFU·g⁻¹ *L. monocytogenes* is set for the category by Regulation No. 2073/2005 [7] that also includes the cheeses analysed in our study.

Microbiological analysis of cheese samples

Microbiological analysis of cheeses was performed by total aerobic counts [18] and coliforms enumeration [19]. For *L. monocytogenes* detection after enrichment and for direct quantification, the methods according to ISO 11290 [20, 21] with PCR identification of typical *L. monocytogenes* colonies were used.

PCR detection of *L. monocytogenes*

TaqMan real-time PCR with primers LMrt3F (caa agc gag aat gtg gct ata aat ga) and LMrt3R (taa ttg ccg ctg cgc tat ccg) and with a probe list-P (FAM-cct gga tga cga cgc tcc act tg-BHQ) targeting *actA* gene [22] was applied to the detection of *L. monocytogenes* in cell lysates from enriched cheese samples [20] subjected to analysis prior to the artificial contamination. The same real-time PCR procedure was used for the culture-independent detection of *L. monocytogenes* in artificially contaminated samples after IMS.

Tab. 1. Characteristics of steamed cheeses used in the study.

Cheese	Milk treatment	Composition				
		Dry matter [%]	Fat in dry matter [%]	Fat [g]	Proteins [g]	Carbohydrates [g]
Product 1	Cows' pasteurized unsmoked	≥ 47	≥ 35	19.9	24.2	1.6
Product 2	Cows' pasteurized smoked	≥ 48	≥ 35	19.0	24.4	2.0
Product 3	Ewes' raw unsmoked	≥ 40	≥ 25	24.0	21.0	0.8
Product 4	Ewes' raw unsmoked	ns	≥ 45	25.0	19.7	0.8
Product 5	Ewes' raw smoked	ns	≥ 45	30.0	23.1	1.8
Product 6	Ewes' raw unsmoked	≥ 46	45	ns	ns	ns

The values indicated on the product packaging are given; values of fats, proteins and carbohydrates are expressed per 100 g. ns – not specified.

Preparation of artificially contaminated samples

Artificial contamination of cheese samples was carried out using target dilutions prepared from a mixture (1:1:1) of three *L. monocytogenes* strains. Individual strain cultures were prepared by 18 h culture in TSB at 37 °C. Final concentration of $(8.8 \pm 0.6) \times 10^8$ CFU·ml⁻¹ in a mixture of *L. monocytogenes* strains was determined from appropriate decimal dilutions by plate-count technique on Agar Listeria according to Ottaviani and Agosti (ALOA, Merck). Two 10 g test portions from each of six different cheese samples were used to prepare artificially contaminated samples at each contamination level. Target artificial contaminations were prepared by adding 1 ml of one order higher concentration of *L. monocytogenes* mixed culture to obtain the required calculated final content of *L. monocytogenes* cells per gram of sample at seven contamination levels (8.8×10^4 , 8.8×10^3 , 2.2×10^3 , 8.8×10^2 , 4.4×10^2 , 2.2×10^2 and 8.8×10^1 CFU·g⁻¹). Each artificially contaminated 10 g test portion was diluted with 89 ml of 8.5 g·l⁻¹ NaCl solution to obtain the first decimal dilution and homogenized in 400 ml sterile filter bags (Interscience, Saint-Nom-la-Bretèche, France) using Stomacher 400 (Seward, Worthing, United Kingdom). Portions of 10 ml of filtered homogenates were subjected to subsequent immunomagnetic separation. *L. monocytogenes* counts of prepared artificially contaminated test portions were estimated on ALOA according to EN ISO 11270-2 [21].

Immunomagnetic separation

Procedure of IMS using Dynabeads anti-Listeria performed according to the manufacturer's instructions was used as a reference method. Briefly, 10 ml of each filtered homogenized artificially contaminated samples were transferred to 15-ml

conical plastic tubes (Sarstedt, Nümbrecht, Germany) and incubated at gentle shaking in Multi-Rotator PTR-60 Grant-bio (Grant Instruments, Shepreth, United Kingdom) with 200 µl magnetic Dynabeads anti-Listeria coated with antibodies to specifically bind *Listeria* cells and to form a complex. Cell-bead complexes were subsequently separated and isolated from the sample matrix using a magnetic particle concentrator DynaMag-15 (Invitrogen, Carlsbad, California, USA).

Preparation of template DNA samples and real-time PCR analysis

Magnetically separated beads with captured *L. monocytogenes* cells were re-suspended in a lysis solution (20 mmol·l⁻¹ Tris-HCl, 2 mmol·l⁻¹ EDTA, 12 g·l⁻¹ Triton X-100 (Merck)) to a final volume of 1 ml and immediately subjected to DNA extraction using thermal lysis (95 °C for 20 min with thorough mixing every 5 min) to prepare 100 µl of a template DNA sample solution. DNA sample solutions were subjected to *L. monocytogenes*-specific TaqMan real-time PCR analysis [22].

Optimization of IMS procedure

Two sample volumes (5 ml and 10 ml) in combination with three different volumes of Dynabeads anti-Listeria (50, 100 and 200 µl) subjected to four different periods of incubation (15, 20, 30 and 40 min) were applied and evaluated for capture efficiency based on the resulting threshold cycle (*c_T*) values of real-time PCR analyses. The procedure was previously performed separately with three *L. monocytogenes* strains of different serotypes, which were then used for artificial contamination as a mixture. Additional concentration step of 5 × 10 ml filtered homogenates by centrifugation for 5 min at 2175 ×g (Sorvall H1000B rotor equipped with a 00884 adapter; DuPont Instru-

ments, Newtown, Connecticut, USA) to prepare 10 ml (final volume) subsequently subjected to IMS was also applied in an effort to improve the detection of *L. monocytogenes* low numbers.

RESULTS AND DISCUSSION

Microbiological and PCR analysis of cheese samples

Microbiological analysis of six different steamed cheese products were represented by total aerobic counts, counts of coliforms and counts of *E. coli*. Detection and counts of *L. monocytogenes* were determined by standard culture-based procedures and TaqMan real-time PCR. Results are summarized in Tab. 2. All cheese samples were identified to be free from *L. monocytogenes* based on culture and PCR detection as well. Total aerobic counts in cheese were in the range from 10^2 CFU·g⁻¹ to 10^7 CFU·g⁻¹ with relatively higher counts found in cheese products made from unpasteurized ewes' milk (products 3–6). Three of them contained more than 10^2 CFU·g⁻¹ of coliforms including *E. coli* (products 4–6).

Optimization of IMS procedure

Three concentrations of *L. monocytogenes* mixed culture (8.2×10^4 , 8.2×10^3 and 8.2×10^2 CFU·ml⁻¹) were used to optimize the IMS procedure based on evaluation of capture efficiency using PCR analysis. The evaluation was based on the results of PCR for IMS procedure using different ratios of sample homogenate and bead suspension volumes, and using different incubation periods (data not shown). The procedure recommended by the manufacturer was used as a reference. No significant differences in low numbers detection were found when the volume of

Dynabeads anti-*Listeria* suspension recommended by the manufacturer was reduced to 50 %. However, extending the incubation period to 20, 30 and 40 min resulted in increasing *L. monocytogenes* capture efficiency (reflected by lower c_T values), particularly for the initial concentration of 8.2×10^2 CFU·ml⁻¹. The IMS procedure at optimized conditions, i.e. using 10 ml filtered sample homogenate, 100 μ l Dynabeads anti-*Listeria* suspension and 40 min incubation period, was used in further analyses to evaluate the performance of the method with artificially contaminated cheese samples.

Our results were similar to those obtained previously when different immunoreaction times (10–40 min) were applied to achieve optimal conjugation of three concentrations of *L. monocytogenes* (10^2 , 10^4 and 10^6 CFU·ml⁻¹) to Dynabeads anti-*Listeria* [23]. In that study, no significant differences were found for higher cell concentrations but, for 10^2 CFU·ml⁻¹, the recovery significantly increased with the immunoreaction time.

Analysis of artificially contaminated cheese samples

Results of *L. monocytogenes* numbers determination in artificially contaminated cheese samples based on PCR analyses are summarized in Tab. 3. Results were obtained in five hours with positive *L. monocytogenes* low number detection of $(4.4\text{--}8.8) \times 10^2$ CFU·g⁻¹ (as calculated). When the estimated *L. monocytogenes* counts for individual test portions on ALOA were taken to account, the resulting value was $(3.8\text{--}7.4) \times 10^2$ CFU·g⁻¹.

In an effort to improve the detection of *L. monocytogenes* low numbers, concentration of 50 ml of filtered homogenates by centrifugation to a final volume of 10 ml was also applied. This additional step of 5-fold concentration of the fil-

Tab. 2. Results of analysis of *L. monocytogenes* in cheese products prior to artificial contamination.

	Product 1	Product 2	Product 3	Product 4	Product 5	Product 6
Total aerobic plate counts [CFU·g ⁻¹]	4.5×10^2	2.2×10^2	9.8×10^6	2.9×10^7	1.2×10^7	7.3×10^7
Coliform counts [CFU·g ⁻¹]	< 50	< 50	4.6×10^2	4.9×10^5	3.8×10^5	7.5×10^4
<i>E. coli</i> counts [CFU·g ⁻¹]	< 50	< 50	< 50	6.8×10^3	1.3×10^3	3.4×10^4
<i>L. monocytogenes</i> counts [CFU·g ⁻¹]	< 50	< 50	< 50	< 50	< 50	< 50
<i>L. monocytogenes</i> detection	nd	nd	nd	nd	nd	nd
<i>L. monocytogenes</i> real-time PCR	nd	nd	nd	nd	nd	nd

L. monocytogenes counts were estimated according to EN ISO 11290-2:2017 [22], *L. monocytogenes* detection was performed in samples of 25 g according to EN ISO 11290-1:2017 [21]; *L. monocytogenes* real-time PCR detection was performed using 50 cycles.

nd – not detected.

tered sample homogenate prior to IMS improved the detection limit to $(1.6\text{--}2.0) \times 10^2$ CFU·g⁻¹ for all six analysed cheese products regardless of the relatively higher contents of natural microflora in cheese products made from unpasteurized ewes' milk. However, the resulting values were still above the required limit of 100 CFU·g⁻¹. The successive lower artificial contamination level of estimated *L. monocytogenes* numbers in test portions within the range of $(7.8\text{--}9.1) \times 10^1$ CFU·g⁻¹ was

Tab. 3. *L. monocytogenes* detection in artificially contaminated cheese products after immunomagnetic separation.

Cheese	<i>Listeria monocytogenes</i> [CFU·g ⁻¹]	Procedure A		Procedure B	
		PCR	ALOA	PCR	ALOA
Product 1	6.8×10^2	+	+	+	+
	3.6×10^2	–	–	+	+
	1.9×10^2	–	–	+	+
	7.8×10^1	–	–	–	–
Product 2	7.1×10^2	+	+	+	+
	3.8×10^2	+	+	+	+
	2.0×10^2	–	–	+	+
	8.8×10^1	–	–	–	–
Product 3	7.6×10^2	+	+	+	+
	3.8×10^2	+	+	+	+
	1.8×10^2	–	–	+	+
	9.1×10^1	–	–	+	–
Product 4	7.1×10^2	+	+	+	+
	4.0×10^2	+	+	+	+
	2.0×10^2	–	–	+	+
	7.8×10^1	–	–	–	–
Product 5	7.4×10^2	+	+	+	+
	4.1×10^2	–	–	+	+
	1.9×10^2	–	–	+	–
	8.2×10^1	–	–	–	–
Product 6	7.1×10^2	+	+	+	+
	3.8×10^2	–	+	+	+
	1.6×10^2	–	–	+	–
	8.4×10^1	–	–	–	–

Contamination levels (calculated as 8.8×10^2 , 4.4×10^2 , 2.2×10^2 and 8.8×10^1 CFU·g⁻¹ *L. monocytogenes*) were estimated in corresponding test portions of individual samples according to EN ISO 11290-2 [22] in ranges of $(6.8\text{--}7.6) \times 10^2$, $(3.6\text{--}4.1) \times 10^2$, $(1.6\text{--}2.0) \times 10^2$ and $(7.8\text{--}9.1) \times 10^1$ CFU·g⁻¹. Procedure A – 10 ml of the filtered homogenate was subjected to immunomagnetic separation.

Procedure B – 50 ml of the filtered homogenate concentrated by centrifugation with the sediment re-suspended in 10 ml and subjected to immunomagnetic separation.

ALOA – Agar *Listeria* according to Ottaviani and Agosti plating for *L. monocytogenes* detection in 100 µl of suspension after immunomagnetic separation.

detected in one out of twelve analysed test portions and it was considered to be a random result.

The results based on PCR detection of *L. monocytogenes* low numbers after IMS were compared with the results of the detection on ALOA. The limit of microbiological detection after IMS was higher for some cheese samples (Tab. 3), probably because the production of aggregates of Dynabeads with *Listeria* cells may lead to reduction of numbers of colonies formed on selective media. It has to be stressed that microbiological detection required by 2–3 days more than PCR detection for confirmation of positive results.

To check the limitation for *L. monocytogenes* low numbers detection in the presence of other *Listeria* sp., mixtures with *L. ivanovii* and *L. innocua* were prepared and analysed. The presence of *L. ivanovii* and *L. innocua* did not affect the detection limit of *L. monocytogenes* up to the ratio of 1:100. This observation was unexpected, since Dynabeads anti-*Listeria* utilize antibodies that are not specific exclusively for *L. monocytogenes*. According to the manufacturer's declaration, Dynabeads anti-*Listeria* react with all *Listeria monocytogenes* serotypes but show a reduced reaction with all other *Listeria* species. Although this information is not supported by any evidence, our results (data not presented) showed no effect of *L. ivanovii* and *L. innocua* on *L. monocytogenes* low numbers detection in mixed cultures in a ratio range from 1:1 to 1:100.

Dynabeads anti-*Listeria* were designed for rapid isolation and concentration of *Listeria* cells from enriched samples by IMS. The application of IMS for culture-independent food-borne bacteria detection is still studied rarely. Recently, large-volume (10 ml) IMS combined with PCR for rapid detection of *L. monocytogenes* without further enrichment was successfully used, with the detection limit of 10 CFU·g⁻¹ in lettuce [15] or 8×10^1 CFU·ml⁻¹ in artificially contaminated pasteurized milk [16]. On the other hand, the rapid detection protocol for *L. monocytogenes* in soybean sprouts that was based on IMS with Dynabeads anti-*Listeria* followed by DNA extraction and real-time PCR targeting *hly* gene [23] demonstrated a comparatively higher detection limit of 10⁴ CFU·g⁻¹ in artificially contaminated soybean sprout samples.

When discussing the results of this study, it has to be taken into account that cheeses are “difficult” matrices regarding DNA-based analysis. In a previous study, when direct detection of *L. monocytogenes* in yoghurt and cheese was attempted using a combined bacterial concentration-PCR approach without prior cultural enrich-

ment, a detection limit of 10^3 CFU·g⁻¹ was determined for *L. monocytogenes* for both types of dairy products [24]. In this respect, our study presents a certain step ahead towards a rapid quantitative method for low numbers of *L. monocytogenes* in cheeses that would comply with all legal requirements [7].

CONCLUSIONS

Results of this study demonstrated that the described procedure facilitated detection of *L. monocytogenes* low numbers at the level of $(1.6\text{--}2.0) \times 10^2$ CFU·g⁻¹ in various steamed cheese products. Culture-independent detection of food-borne bacterial pathogens based on the sample preparation using filter homogenization, concentration and large-volume immunomagnetic separation, followed by the simplest and low-cost DNA extraction method and a target-specific PCR have a potential to obtain the results on *L. monocytogenes* low number detection in five hours. The method has a potential to be adapted and used for other bacterial pathogens and food matrices.

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