DETECTION OF LISTERIA MONOCYTOGENES IN FOOD PRODUCTS USING A POLYMERASE CHAIN REACTION-BASED METHOD IN COMBINATION WITH A STANDARD REFERENCE ENRICHMENT STEP

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Abstract

Listeria monocytogenes remains one of the many foodborne pathogens that require continuous monitoring. The detection and tracing methods are quickly developed for a better improvement and a quicker identification of this foodborne pathogen that may be present in food samples.
A total number of samples consisting of different types of food products were collected from local markets in Bucharest, subjected to enrichment steps (according to EN ISO 11290-1 and ISO 11560) and further on to a PCR analysis.
This resulted in eight samples with positive results, among them goat cheese, smoked fish, sausages and raw chicken meat. The identification was possible and quick, no matter the bacterial load of the samples, due to the existence of a virulence gene pertaining to the internalin gene family: InlB, which is specific to L. monocytogenes. This method ensures a high sensitivity and specificity in a very short period of time, reducing the work time for this analysis with five days concerning the negative results and seven days for the positive ones.

Key words: *food products, Listeria monocytogenes, PCR-based methods, standard enrichment.*

INTRODUCTION

Listeria monocytogenes is a pathogenic bacterial species that causes illness but without a high prevalence and low mortality rates, like other foodborne pathogens (Kaclikova et al., 2003). It frequently contaminates food products, particularly cheese and ready-to-eat meat-containing products (Farber and Peterking, 1991). Due to the necessary period of 7-10 days for a

thorough examination considering the detection and tracing *L. monocytogenes* (EN ISO 11290-1 for food and animal feeding stuff and ISO 10560 for milk and milk products) (Kaclikova et al., 2003), new methods emerge, in a trial to shorten the period of analysis.
Growth rates during enrichment vary among *Listeria* species due to interactions with food matrices, production of inhibitors by the organism (monocins, bacteriophages) or the competing background microflora (Besse et al., 2005, Curtis and Lee, 1995, Jasson et al., 2009).
When using nucleic acid amplification based methods, the recovery of *L. monocytogenes* is problematic if food samples containg accompanying non-pathogenic *Listeria* spp. (Aznar and Alarcon, 2002; Churchill et al., 2006). Polymerase chain reaction (PCR) presents a great potential to speed-up the detection process, especially for *L. monocytogenes* (Olsen, 2000). However, nowadays this method is used frequently on highly contaminated samples (Bansal et al., 1996), therefore comparable methods to the standard ones were developed in order to shorten the analysis time, one of them including a three days PCR-based method (Kaclikova et al., 2003). The inclusion of a non-selective post-enrichmnt step to obtain a useful detection limit for PCR ($\geq 10^4$ CFU/mL of *L. monocytogenes*) was used to improve the sensitivity of the PCR assay. Also the targeting of *inlB*, which is 100 % specific for this bacterial species facilitates the detection of 10^0 CFU in a sample. The presence of internalin genes, associated with the virulence cycle of *L. monocytogenes* represents a valuable indicator for their virulence potential (Barocci et al., 2007; Liu et al., 2007).
This paper aims to use this shortened PCR-based method for the detection and tracing of *L. monocytogenes* in several food products.

MATERIALS AND METHODS

Food samples. A total number of 90 samples consisting of smoked fish (10), goat cheese (10), sausages (10), tuna salad (10), ground chicken meat (10), ready-to-eat hamburgers (10), pastry products containing meat (10), mayonnaise salad (10) and pepperoni pizza (10) were obtained from local supermarkets. For the determination of the detection limit, 10 g of food samples were homogenized in 90 ml of half Fraser or *Listeria* enrichment broth (AES Chemunex) using a stomacher for homogenization. Further on, they were inoculated with decimal dilutions (10^1-10^2 CFU/sample) obtained from a culture of *L. monocytogenes* prepared in brain heart infusion broth (AES Chemunex), at 37^oC, for 24-48 h.

The enrichment. The food samples quantities of 10 g were homogenized in 90 ml of Fraser broth and incubated for 24h at 30^{o}C. A volume of 0.1 ml of the primary-enriched sample was introduced in 10 ml of Fraser broth and incubated 24 h at 37^{o}C. A volume of 0.1 ml of the secondary-enriched sample was introduced in 10 ml of BHI broth and incubated for 5 h at 37^{o}C (EN ISO 11290-1).
DNA extraction. For this step, a volume of 1 ml of the enriched sample was introduced to centrifuge at 13000g for 10 min. Afterwards, the sediment was subjected to washing with 0.85% NaCl, and re-suspended in 200 µl of buffer, that contained 20 mM Tris-HCl (pH 8.0) and 50 mM KCl. This mix was incubated at 95^{o}C for 25 minutes. The sample was afterwards centrifuged at 13000g for 3 minutes, and the supernatant was further on used.
Internal control preparation. For the internal control preparation, DNA of *Enterococcus faecalis* was used, by the method described by Pangallo et al. (2001a). The DNA was amplified using PCR, with primers inlB-L and inlB-R (Pangallo et al., 2001b) and a fragment of 400 bp was selected.
PCR. A quantity of 25 µl of mixture for the reaction, containing 200 µM of dNTP, 250 nM of each primer (inlB-L: ctggaaagtttgtatttgggaaa, inlB-R: tttcataatcgccatcatcact; Roche), 1.5 U of *Taq* DNA polymerase, 2.5 µl of the buffer supplied with the polymerase, 2.5 µl internal control and 2.5 µl of the sample lysate. The reaction was performed in a Roche Light Cycler 2.0 (Roche) using an amplification program consisting of initial denaturation at 94^{o}C for 2 min, 35 cycles with a denaturation at 94^{o}C for 45 s, annealing at 60^{o}C for 45 s and polymerization at 72^{o}C for 90s, followed by the final polymerization at 72^{o}C, for 8 min.
Detection of the amplification product. After the PCR, the LightCycler includes a step that replaces the use of electrophoresis, but instead uses the UV-light in toder to identify the amplified product, with a molecular weight control of 100 bp analyzed at the same time with the samples.
Interpretation of the PCR results. The presence of fragments consisting of DNA with less than 400 bp was interpreted as positive result, while those with fragments of approx. 400 bp were interpreted as positive results.

RESULTS AND DISCUSSION

PCR-based methods are considered very reliable and having a great potential for fulfilling the requirements fast enough, while being specific and sensitive for the detection, even though they require a specific prior

preparation step for the samples. In this case, we used the enrichment step described in EN ISO 11290-1 and ISO 10560, while the last steps were performed using the PCR-based method. This approach has been previously studied by Kaclikova et al. (2003), proving its efficiency. The results were positive for the samples artificially contaminated by $\geq 10^0$ CFU for 10 g of *L. monocytogenes*. However, among the 90 samples studied only eight samples were positive for the presence of the foodborne pathogen (table 1).

Table 1. Results of the analysis of *L. monocytogenes* in naturally contaminated food samples by PCR-based methods

Sample	Number of positives/number of analyzed samples
Tuna salad	0/10
Goat cheese	1/10
Smoked fish	1/10
Sausages	4/10
Ready-to-eat hamburgers	0/10
Pastry containing meat	0/10
Raw chicken meat	2/10
Mayonnaise salad	0/10
Pepperoni pizza	0/10

The used method was advantageous due to the wider detection window. This facilitates the detection of 10^0 CFU of *L. monocytogenes* per sample, without a danger of false positivity when detection of dead cells may happen. This is due to the effective enrichment step, performed as required in EN ISO 11290-1 and ISO 10560. Also, the dilution of the food samples by a factor of 10^5 was an effective method for the high sensitivity. The cell lysis step was done shortly after the enrichment step, the latter ensuring an exponentially growing cells that can be easy to lyse.

CONCLUSIONS

The presented PCR-based method combined with a selective enrichment proved to be very effective and the necessary period was therefore reduced from 7-10 days to only three. The results are considered equivalent to the reference methods (EN ISO 11290-1 and ISO 10560) as stated by Kaclikova et al. (2003). Dead cells were not detected by this method due to the multiple dilutions performed on each sample. However, the application of this combination between reference enrichment step and PCR-based method reduced with five days the period in case of the negative results and with seven days in case of positive results, as confirmation is not needed anymore. This study consisted also as a confirmation of the use of this sample as a faster alternative for the detection of *L. monocytogenes* in food samples of different types.

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