PCR Detection of *Listeria monocytogenes* in Milk and Milk Products and Differentiation of Suspect Isolates

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Abstract

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The main goal of our work was to solve the problems of normative diagnostic method for the detection of *Listeria monocytogenes* in foods by STN ISO 10560 (1999), i.e. to shorten the long time of analysis (5-10 days) and differentiate the non-haemolysed strains of *Listeria monocytogenes* from non-pathogenic *Listeria innocua* yielding the same results of biochemical and serological typing (serotype 4ab). PCR was employed to confirm the method for testing 60 suspect isolates from other laboratories of the Slovak Republic.

We verified the detection of *Listeria monocytogenes* in milk and dairy products by nested PCR. The objective of this experiment was to analyze 100 various samples by traditional cultivation method and compare the results with those yielded by PCR. The results were in good agreement; all 18 positive and 72 negative samples were detected by both methods. However, the PCR method yields results within 2 days. This makes diagnosis in food control laboratories much more efficient.

For PCR detection, two pairs of primers (PRFA 1 and 2, LIP 1 and 2) were used with affinity to *prfA* gene involved in the regulation of listeriolysin synthesis. The size of the PCR product was 1060 bp fragment in a first step of PCR and 273 bp fragment in nested PCR. The sensitivity and reliability of PCR was comparable with conventional methods. The PCR method solved the problem of interpretation of classical biochemical and serological typing in the only one step without the necessity of using additional examinations.

Furthermore, we found that in most of the strains isolated from foods with a biochemical profile of non-haemolytic *Listeria monocytogenes* on blood agar serotype 4ab were not *Listeria monocytogenes* but a non-pathogenic *Listeria innocua*. This finding is very important from the point of view of food evaluation according to the STN ISO 10560 (1999).

Diagnostic methods, food, Listeria innocua, non-haemolysed strains of Listeria monocytogenes, prfA gene

Bacteria of the genus *Listeria* are widely distributed in the environment and they frequently contaminate food. *Listeria monocytogenes* is a pathogenic species causing listeriosis, increasing incidence of which has been reported in the last two decades. Food products most frequently associated with listeriosis are soft cheeses, particularly those made from unpasteurized milk, and ready-to-eat meat-containing food products (Farber and Peterkin 1991; Goulet et al. 1993; Kozák et al. 1996; Kacliková et al. 2001).

Listeria monocytogenes is an important food-borne pathogen, associated with septicemia, meningoencephalitis and abortion in humans and animals, primarily affecting pregnant, new-born, and immunocompromised individuals. The infectious dose is very high (1000 per g). The incidence of human listeriosis has been growing in the last few years, whereby most cases are sporadic but some are occasionally also epidemic (Schuchat et al. 1991).

In 1999, State Veterinary Institutes in the Slovak Republic have registered an occurrence of the strain *Listeria monocytogenes.* A total of 6247 samples of milk and milk products were analysed with 160 positives, corresponding to 2.56%. *Listeria monocytogenes* was the most

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widespread in the samples of raw milk, representing approximately 7.56% and of curds, representing approximately 5.43% (Pástorová et al. 2001). While *Listeria* spp. are ubiquitous in nature, only *L. monocytogenes* is pathogenic to humans (Klein et al. 1997).

Listeria monocytogenes is a non-sporogenic, facultative intracellular gram-positive bacterium that has the ability to survive high and low temperatures, low pH (Sado et al. 1998), and high salinity. It is not acid-resistant and grows well on a tryptose and blood agar (Bubert et al. 1997).

Standard identification of listeriae from samples contaminated with multiple species (e.g. samples of food and faeces or from the environment) relies on selective enrichments and subsequent biochemical analyses and can be difficult, laborious, and time-consuming (Almeida and Almeida 2000). The reliability of PCR detection methods depends, in part, on the purity of the target template and the presence of sufficient numbers of target molecules. With such complex matrices as foods, steps must be taken to limit the effects of any potentially inhibitory compounds present that may limit PCR amplification of the intended target (Bhaduri and Cottrell 1998). Various sample preparation methods have been developed to remove or to reduce the effects of PCR inhibitors in foodstuffs without understanding the mechanisms of inhibition.

The main source of *Listeria monocytogenes* in milk is probably faecal contamination, although the organism is a rare cause of mastitis. *Listeria monocytogenes*, if present in raw milk in numbers of ca 100 per ml, can survive a number of cheese-making processes and can remain viable in the final product for a considerable length of time (Griffiths 1989).

It is known that PCR testing, despite enormous diagnostic potential, has had difficulty moving from research into end-user laboratories. These qualitative assays have the potential to be integrated into testing laboratories for monitoring the microbiological quality of foods.

We used the PCR method for the detection of *Listeria monocytogenes* by Simon et al. (1996). The aim of this study was the detection and differentiation of suspect isolates without proven haemolytic activity of *Listeria monocytogenes.* We describe here protocols that allow the detection of *Listeria monocytogenes* in milk and dairy products in less than 36 h by DNA extraction followed by PCR (Simon et al. 1996). Conventionally, detection of *Listeria monocytogenes* involves selective enrichment subculture on selective agar plates followed by confirmatory identification tests, and this can take several days to complete. It would be useful to have a rapid detection protocol to screen for the presence of *Listeria monocytogenes* in milk and dairy products. In several studies, *prfA*–derived primers have been applied for the identification of *Listeria monocytogenes* (Wernars et al. 1992).

Materials and Methods

Samples

Food samples were collected from 100 various dairy products: 60 samples of various cheeses: semi-soft cheeses: "Parenica"– smoked, "Jasná", "Kriváň", soft cheeses: "cow's nugget", "Olomouc curds", soft cheeses from raw sheep's milk: "sheep's nuggets cheese", "bryndza", 40 raw milk samples from various farms, and 60 suspect *Listeria monocytogenes* strains isolated from milk and cheeses from State Veterinary Institutes in Prešov, Košice and Nitra.

Listeria sp. reference material

The following strains of the *Listeria* species were obtained from Czech Collection of Microorganisms: *Listeria monocytogenes* CCM 5576*, Listeria ivanovii* CCM 5884*, Listeria innocua* CCM 4030*, Listeria seeligeri* CCM 3970.

Primers according Simon et al. (1996) were used.

Cultivation and biochemical typing

For the confirmation and samples incubation were used the *Listeria* enrichment broth – LEB (Oxoid, England), selective media Oxford agar (Oxoid, England), and chromogenic media Rapid *Listeria monocytogenes* (Sanofi, France). The methods were described in norm STN ISO 10560 (1999). Biochemical characteristic of examined samples was determined by fermentation of carbohydrates in accordance with Bergey`s Manual protocol (1994).

Serotyping

For serological typing "Listeria antiserum SEIKEN" (DENKA SEIKEN, Japan) was used by method of glass agglutination by "Listeria antiserum SEIKEN" protocol.

Enrichment and DNA extraction

A total of 25 g or 25 ml of food sample were incubated in 225 ml of *Listeria* enrichment broth (Oxoid, England) at 30 ± 1 °C for 24 and 48 h. For DNA isolation 1 ml of suspension after 24 h and after 48 h was necessary. Homogenate was centrifuged at 1800 × *g* for 5 min and the supernatant was discarded. The pellet was resuspended in 100 µl of 0.5 % TRITON X-100 (Sigma, Germany) and the whole process was repeated. Homogenate was incubated at 95 °C for 5-10 min. 2 µl of Proteinase K [20 mg/ml] (Promega, USA) were added to the homogenate after cooling and it was incubated at 55 °C for 2 h. Proteins were removed with a phenol-chloroform-isoamylalcohol [25:24:1] solution (Sigma, Germany). DNA was precipitated with ice-cold absolute ethanol at -70 °C for 2 h, centrifuged at 3500 × *g* for 10 min. Pellet was dried and DNA was resuspended in 30 µl of sterile distilled water.

PCR amplifications

The first round used primers PRFA1 and PRFA2 (Simon et al. 1996) directed against nucleotides 181-207 and 1462-1482 of the sequence. Each 50 µl of the reaction mixture contained: 5 µl target DNA, 5 µl 10 x PCR buffer (Gibco BRL, USA), 2mM dNTPs (Promega, USA), 50mM $MgCl_2$ (Gibco BRL, USA), 0.5 µmol/l primer (Generi Biotech, Czech Republic) and 1U Taq DNA polymerase (Gibco BRL, USA), sterile distilled water added to the volume 50 µl.

Hot start was at 94 °C for 2 min. The reaction mixtures were subjected to 35 cycles consisting of heat denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and DNA extension at 74 °C for 1 min. Finally, the samples were maintained at 74 °C for 5 min for the final extension of DNA. These incubation conditions were the same for second round-nested PCR, except those LIP1 and LIP2, since these primers required 45 cycles.

The second round employed primers LIP1 and LIP2 (Simon et al. 1996) directed against nucleotides 634-654 and 886-907 of first product amplified by PRFA1 and PRFA2. 2 µl of completed first round reaction mixture were added to each reaction as target DNA. Remaining components were the same as in the first round.

Visualisation of the PCR product

For detection, 10 µl of PCR reaction mixture was electrophoresed on a 2% w/v agarose gel (Gibco BRL, USA), diluted in 1 × TAE buffer (Kaufman et al. 1995), stained with ethidium bromide (Amresco, USA) in concentration 0.1 µg/ml and viewed under the ultraviolet light.

Results and Discussion

We confirmed the specificity of PCR method on reference material from CCM strains of *Listeria innocua, Listeria seeligeri, Listeria ivanovii* that templates DNA. 1060 bp product from first round and 273 bp product from second round of PCR were amplified by using CCM 5576 *Listeria monocytogenes*.

In 12 samples (from total 18 positives) the *Listeria monocytogenes* contamination was confirmed after 24 h of incubation using the first round of PCR. By second round - the nested PCR the *Listeria monocytogenes* contamination was confirmed in all 18 positive samples. The contamination with *Listeria monocytogenes* in all 18 samples was confirmed after 48 h of incubation using only the first round of the PCR protocol. All these results were in agreement with the traditional identification (Table 1, Plate XII, Fig. 1). In this comparison, the

Table 1
Comparison between PCR reaction and traditional method for *Listeria monocytogenes* detection in naturally contaminated raw milk and cheese

Time of enrichment	Nested PCR				Traditional – culture			
	T 24		T 48		T 24		T 48	
Positive / Negative	P	N	P	N	P	N	P	N
Raw milk	5	25	5	25	5	25	5	25
Soft cheese	8	22	8	22	5	25	8	22
Semi - soft cheese	5	25	5	25	3	27	5	25
Total	18	72	18	72	13	77	18	72

T24 – 24-h enrichment culture, T48 – 48-h enrichment culture, P – positive samples, N – negative samples

advantage of PCR method is in its possibility of results ready after only 24 h of incubation. Traditional culture-based methods give the ready results after 48 h of incubation (after 24 h of incubation was by cultivation method as positive only 13 from all 18 positive samples). *Listeria monocytogenes* was detected by PCR in all samples in a course of maximum 2 days. The sensitivity of the detection varied according to the time of sample-incubation, because universally there is a very small number of this pathogen cells in foodmaterial. Sensitivity was adjudged from the results of the second round-nested PCR, because nested round intensify exponentially the sensitivity of PCR detection (Manzano et a. 1996).

Conventional culture-based methods are labour-intensive and time-consuming, in many instances requiring 5 to 10 days to complete (Manzano et al. 1997). This, in food-marketing context (export, import) slows down all distribution processes. The second problem of the standard identification of *Listeriae* is the specific distinguishing of non-haemolysed *L. monocytogenes* strains from *Listeria innocua* (serotype 4 ab), that are most frequently presented in food. There are only 4 possibilities how to solve this problem: pathogenity for mice detection, API system, Rapid *Listeria monocytogenes* medium (Sanofi, France) and PCR. First two methods are very labour-intensive, time and material consuming. Rapid foodstuffs and so classical cultivation techniques still remain the official method (Karpíšková et al. 2000). The detection process of *L. monocytogenes* does not speed up but mainly defines it more exactly.

Using of the term „non-haemolytic strains of *Listeria monocytogenes*" (Allerberger et al. 1997) is incorrect. From this point we used "non-haemolysed strains of *Listeria monocytogenes"* in our paper. Haemolytic activity is one of the initiating mechanisms of this microorganism's pathogenity. From the point of view of genetic information one can say that each *Listeria monocytogenes* genome must contain the gene for haemolysis as well (Wiedmann et al. 1997).

The problem of proving this feature depends on a large amount of *in vitro* environmental factors that partly or completely prevent the expression of gene. This can occur as a reason of weakening or sublethal damage to the microorganism e.g. by processing technique of food production or by chemotherapy of previous host the source of isolated species (Allerberger et al. 1997; Wiedman et al. 1997; Wang 1999). The applied diagnostic material plays the important role, in this case it is blood as a part of cultivation medium (antibody volume in it). All these inputs may cause that *Listeria monocytogenes* appears in vitro as "non-haemolytic".

STN ISO 10560 (1999), used for microbiological examination of *Listeria monocytogenes* in milk and dairy products, does not accentuate haemolytic activity of *Listeria monocytogenes*. This is justifiable and originates from facts listed above. However, it must be said that the species *Listeria innocua* included in the genus *Listeriae* that is really non-haemolytic, shows generally the same biochemical activities as *Listeria monocytogenes*

Table 2
Differentiation of suspicious strains from milk and dairy products using nested PCR and Rapid *Listeria monocytogenes medium* (Sanofi, France)

Isolates from:	Biochemical typing (Bergey's manual)	Serotype	Haemolysis on blood agar	PCR Listeria Monoc.	RAPID L.monocyt. medium
Dairy products 40 ×	+	4 a b	–	–	*Listeria innocua*
Raw milk 18 ×	+	1/2 a	+	+	*Listeria monocytogenes*
Raw milk 2 ×	+	4 a b	–	+	*Listeria monocytogenes*

(Holt et al. 1994). Neither the serotyping is reliable in differentiation of the two species, mainly in serotype 4ab that can occur in both of them. The differentiation is very important because of frequent presence of *Listeria innocua* in foods, and because it is not pathogenic.

In this study we verified the use of PCR method for identification of suspicious strains without haemolytic activity isolated from food (Table 2).

Listeria monocytogenes was found in only two of 42 examined non-haemolysed suspicious strains these were isolated in others laboratories of all Slovakia. Their biochemical profile by Bergey's manual typing was the same as the profile of *Listeria monocytogenes* and the serotype of all 42 strains was determined as 4ab by Listeria antiserum SEIKEN Test. According to STN ISO10560 (1999) guidelines all these strains showing no haemolysis should be considered as *Listeria monocytogenes*-positive!

At the same time we performed control cultivation using the chromogenic medium Rapid *Listeria monocytogenes* (Sanofi, France) to prevent false-negative results of PCR. It is a chromogenic medium – agar for rapid distinguishing between *Listeria monocytogenes* and others species of the genus *Listeria* by determination of fosfolipase and the ability of bacteria to utilise xylose. *Listeria monocytogenes* formed black colonies, *Listeria innocua* formed colourless colonies. The advantage of the PCR method in comparison with Rapid *Listeria monocytogenes* medium is the time of cultivation necessary for the Rapid medium use. Moreover, the PCR method is cheaper than the chromogenic medium with all enrichment and cultivation material, it must be used before Rapid medium cultivation. In all 60 cases (42 without- and 18 with haemolysis) the results were identical with PCR results. The negative strains obtained with PCR method and cultivation on a medium mentioned before were specified as *Listeria innocua*.

With food contaminated by *Listeria monocytogenes*, the PCR is with its sensitivity and reliability comparable with conventional method and, in addition, it solves the problem of interpretation of classical biochemical and serological typing in the only one step without the necessity of using additional examinations.

Furthermore, it is possible to state that in most of the strains isolated from food with a biochemical profile of non-haemolytic *Listeria monocytogenes* on blood agar serotype 4ab are not *Listeria monocytogenes* but a non-pathogenic *Listeria innocua*. This is very important from the point of view of food evaluation following the STN ISO 10560 (1999).

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Stanovenie *Listeria monocytogenes* v mlieku a mliečnych výrobkoch a rozlíšenie podozrivých izolátov bez dôkazu hemolytickej aktivity

Hlavným cieľom našej práce bolo riešenie problémov v oblasti normatívnej diagnostickej metodiky pre dôkaz *Listeria monocytogenes* STN ISO 10560 (1999). Tými sú hlavne dlhá doba vyšetrovania (5-10 dní) a rozlíšenie nehemolyzujúcich kmeňov *Listeria monocytogenes* od nepatogénneho druhu *Listeria innocua* s rovnakým biochemickým a serologickým profilom (serotyp 4ab). K riešeniu druhého zo spomenutých problémov bola použitá PCR ako schválená metóda na vyšetrenie 60 *Listeria monocytogenes*-suspektných izolátov získaných z ostatných laboratórií Slovenska.

Overili sme možnosť detekcie *Listeria monocytogenes* v mlieku a mliečnych výrobkoch metódou nested PCR. Predmetom tohto experimentu bolo vyšetriť 100 rôznych vzoriek tradičnou kultivačnou metódou a porovnať ich s výsledkami PCR. Všetky získané výsledky predstavovali 18 pozitívnych a 72 negatívnych vzoriek. Použitím PCR sme dosiahli výsledky už v priebehu 2 dní, čo je veľmi efektívna možnosť diagnostiky v laboratóriach hygieny potravín.

Na PCR detekciu sme použili dva páry primerov (PRFA 1,2 a LIP 1,2) s afinitou k *prfA* génu dôležitého v regulácii syntézy lysteriolyzínu. Ako výsledok sme dosiahli produkt veľkosti 1060 bp v prvom kroku PCR a produkt veľkosti 273 bp v nested PCR. Citlivosť a spoľahlivosť PCR metódy bola porovnateľná s tradičnými metódami. Zároveň metóda riešila problém interpretácie výsledkov z biochemických a serologických testov. Z toho vyplýva, že izolovaný, s biochemickým profilom pre druh *Listeria monocytogenes,* kmeň serotypu 4ab a pomnožený na krvnom agare, nepotvrdil PCR metódou druh *Listeria monocytogenes* ale nepatogénny druh *Listeria innocua*. Ide o dôležitý krok z pohľadu vyhodnotenia výsledkov podľa STN ISO 10560 normy (1999).

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Plate XII
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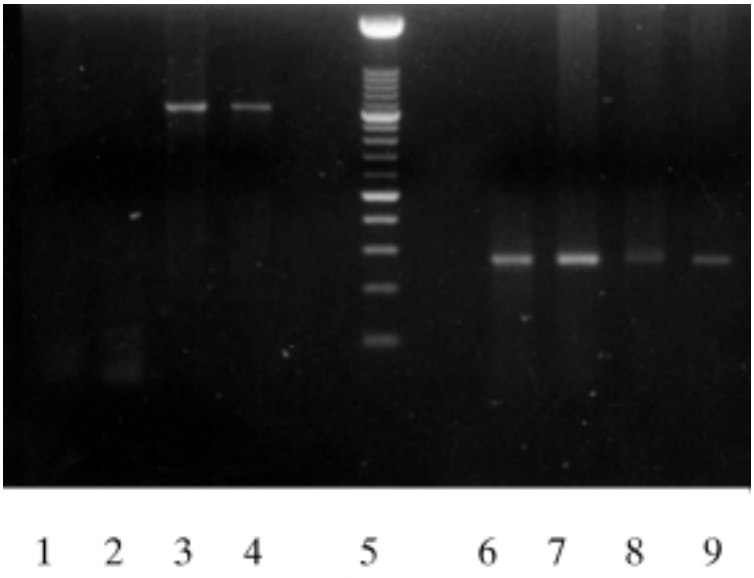

Fig.1. PCR and nested PCR
Lanes 1-4 – PCR products from first step of PCR reaction. Lanes 1 and 2 - samples after 24 h enrichment; Lanes 3 and 4 - samples after 48 h enrichment; Lane 5 – 100-bp molecular mass marker (Amersham Pharmacia Biotech); Lanes 6-9 - PCR products from nested PCR with same samples then in lanes 1-4