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Comparison of two multiplex PCR assays for the detection of *Listeria* spp. and *Listeria monocytogenes* in biological samples

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Abstract

Introduction: The aim of the study was to optimise and compare two multiplex PCR assays for the detection of *Listeria* spp. and *Listeria monocytogenes* in biological samples including the liver, brain, and blood. **Material and Methods:** Three strains of *L. monocytogenes* and single strains of each of the species: *L. ivanovii*, *L. innocua*, *L. grayi*, *L. welshimeri*, and *L. seeligeri* were used. Additionally, five other species of bacterium were used to evaluate the specificity of the tests. **Results:** Specific amplification products were obtained for both multiplex PCR assays, which confirmed the tested strains as *Listeria* spp. and *L. monocytogenes*, respectively. Isolates of other species did not yield PCR products. **Conclusion:** Both multiplex PCR assays proved to be significantly sensitive and highly-specific methods for the detection of *Listeria* strains.

Keywords: *Listeria*, listeriosis, biological samples, PCR.

Introduction

Listeria monocytogenes is widely distributed in the natural environment. This organism can cause infections both in humans and animals. In humans, the elderly, the chronically ill, people with an impaired immune system, pregnant women, and newborns are most vulnerable to infection with *L. monocytogenes*. In pregnant women it induces flu-like symptoms and is associated with spontaneous abortions. In newborns listeriosis takes the form of granulomatosis infantiseptica, the mortality rate of which can reach 100%. Clinically, the disease occurs most often in the form of encephalitis and meningitis. Other forms of listeriosis in humans include skin lesions, conjunctivitis, lymphadenitis, endocarditis, osteomyelitis, pneumonia, and gastroenteritis (5, 14, 24, 25).

In animals, the symptoms of the disease depend on the age and physiological condition of the affected specimen. Listeriosis can affect the central nervous system, the reproductive system, internal organs, or can take the form of septicaemia. Listeriosis in the reproductive system leads to spontaneous abortion. The majority of clinical cases of the disease are noted in sheep, goats, and cattle, whereas in horses, pigs, cats, dogs, and fowls they occur very rarely. Among fur animals, chinchillas are particularly sensitive to this disease (12, 18, 20).

Bacteriological examination is the “gold standard" in the isolation and identification of *L. monocytogenes* from biological material. The laboratory identification of the microorganism is based on the culture on selective enrichment broths and selective agars, and then on the evaluation of colony morphology, and physiological and biochemical properties of the isolate. The examination is time-consuming and requires specialised tests (9).

Genotyping methods for the identification of bacteria are free of constraints that exist in traditional phenotype techniques. The tests based on the analysis of nucleic acids allow a direct detection of microorganisms in bacterial cultures or clinical samples. These tests increase the sensitivity of the method and reduce the time of identification. PCR with primers designed to identify microorganisms at the strain, species, or genus level is considered particularly useful. The method is used for rapid and sensitive detection of *L. monocytogenes* (19, 26, 27).

The aim of this study was to optimise and compare two selected multiplex PCR assays enabling

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the simultaneous detection of *Listeria* genus and species *of L. monocytogenes* in experimentally contaminated biological samples (bovine liver, brain, and blood).

Material and Methods

Bacterial strains. The study involved three reference strains of *Listeria monocytogenes*: *L. monocytogenes* ATCC 7644, *L. monocytogenes* ATCC 13932, and *L. monocytogenes* ATCC 19112. To assess the specificity of the tests, strains of other species of the *Listeria* genus, including single strains of *L. ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *L. grayi* ATCC 19120, *L. welshimeri* ATCC 35897, and *L. seeligeri* originating from the collection of the Department of Microbiology, National Veterinary Research Institute in Pulawy, Poland, were used. In addition, the experiments involved strains of other species, including *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, and *Klebsiella pneumoniae* ATCC 13883.

Isolation of DNA. Each strain was streaked onto TSYEA medium. Cultures were incubated for 24 h at 37ºC and then a bacterial suspension with a density of 1 McFarland Standard was prepared. Samples of 1 mL were centrifuged for 10 min at 13,000 rpm. The supernatant was removed and the pellet was used to contaminate the liver, brain, and blood. To isolate DNA a DNeasy Blood and Tissue Kit (Qiagen, USA) was used.

The sensitivity of the PCRs was determined by a 10-fold dilution technique. The initial concentration of *L. monocytogenes* ATCC 13932 was about 1.5×10^6 CFU/reaction.

Primers. Sequences of primers for amplification were based on the literature data, for the first multiplex PCR assay in accordance with Chen and Knabel (2), and for the second one according to Doumith *et al.* (4) and Gouws and Liedemann (11). The primers were synthesised in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAN (Poland). The characteristics of the PCR primers are shown in Tables 1 and 2.

PCR assays. In both multiplex PCR assays two different reaction mixtures were used. The first contained 200 μM of each deoxynucleotide triphosphate (dNTP) (Thermo Scientific, USA), 1 × PCR buffer containing 2 mM of $MgCl_2$, and 1 U of DNA polymerase (Biotools, Spain). The second was the QIAGEN Multiplex PCR Kit reaction mixture containing 3 mM of $MgCl_2$ (Qiagen, USA). PCR assays were performed in a 25 μL reaction mixture volume containing 5 μL of the DNA and a defined amount of each primer. Primer concentrations (0.01 μM, 0.03 μM, 0.05 μM, 0.10 μM, 0.20 μM, 0.50 μM, 1.00 μM, and 1.50 μM) were evaluated to optimise the reactions.

PCR assays were performed in a TProfessional Basic Thermocycler (Biometra, Germany) using the following parameters: initial denaturation at 95ºC for 15 min, followed by 15 cycles of 94ºC for 90 s, 3 cycles from 55ºC to 51ºC per temperature for 90 s, 72ºC for 90 s, 15 cycles of 94ºC for 90 s, 50ºC for 90 s, 72ºC for 90 s, and a final elongation step at 72ºC for 8 min.

Electrophoretic separation of the amplification products was performed on a 2% agarose gel in TBE buffer at a constant voltage of 95 V, in the Wide Mini-GT Sub Cell apparatus (Bio-Rad, USA). The 100 bp Plus DNA Ladder (Thermo Scientific, USA) was used as a molecular weight marker. After electrophoresis, the gels were stained with ethidium bromide and photographed using the Vilber Lourmat (Germany) recording system.

Table 1. The characteristics of the primers for multiplex PCR according to Chen and Knabel (2)

Gene target	Primer sequence (5' – 3')	Product size	Concentration	
			Reaction mixture containing 2 mM of $MgCl_2$	Qiagen reaction mixture containing 3 mM of $MgCl_2$
iap	(F) ATGAATATGAAAAAAGCAAC	1450–1600 bp	0.10 μM	0.20 μM
	(R) TTATACGCGACCGAAGCCAAC			
lmo2234	(F) TGTCCAGTTCCATTTTTAACT	420 bp	0.03 μM	0.05 μM
	(R) TTGTTGTTCTGCTGTACGA			

Table 2. The characteristics of the primers for multiplex PCR according to Doumith *et al*. (4) and Gouws and Liedemann (11)

Gene target	Primer sequence (5' – 3')	Product size	Concentration	
			Reaction mixture containing 2 mM of $MgCl_2$	Qiagen reaction mixture containing 3 mM of $MgCl_2$
prs	(F) GCTGAAGAGATTGCGAAAGAAG	370 bp	0.20 μM	0.20 μM
	(R) CAAAGAAACCTTGGATTTGCGG			
hly	(A)CATTAGTGGAAAGATGGAATG	730 bp	0.50 μM	0.50 μM
	(B)GTATCCTCCAGAGTGATCGA			

Results

In the first stage, a multiplex PCR was performed using the *iap* and lmo2234 genes (Table 1) with the template DNA obtained from biological samples contaminated experimentally with *L. monocytogenes*. The *iap* gene allowed the confirmation of the strains as *Listeria* spp., and the lmo2234 gene served for confirmation as *L. monocytogenes* species. In the case of these genes, fragments of 1450–1600 bp and 420 bp were obtained. The first stage focused on the influence of different primer concentrations with the use of two reaction mixtures. The optimal concentrations of primers for the reaction mixture containing 2 mM of $MgCl_2$ were 0.10 μM for the *iap* gene and 0.03 μM for the lmo2234 gene. Using the reaction mixture containing 3 mM of $MgCl_2$ the optimal concentrations were 0.20 μM and 0.05 μM, respectively. The amplified fragments were clearly seen in assays on the liver, brain, and blood samples contaminated with the tested strains of *L. monocytogenes*, with both the reaction mixture containing 2 mM of $MgCl_2$ and the reaction mixture containing 3 mM of $MgCl_2$.

Then the sensitivity of this PCR was evaluated. The examination of the DNA from contaminated liver, brain, and blood samples revealed the same sensitivity of 1.5×10^4 CFU/reaction for both reaction mixtures (Fig. 1).

In the next step, the amplification was performed using the *prs* and *hly* genes (Table 2). Confirmation of the strains as *Listeria* spp. was possible by detecting the *prs* gene, whereas detecting the *hly* gene allowed the identification of *L. monocytogenes* species. In the case of these genes, products of 370 bp and 730 bp were obtained, respectively. The optimum primer concentration was 0.2 μM for the *prs* gene and 0.5 μM for the *hly* gene in both the reaction mixture with 2 mM and the mixture with 3 mM of $MgCl_2$. Both reaction mixtures allowed to detect the presence of the amplified fragments found in the liver, brain, and blood samples contaminated with the tested *L. monocytogenes* strains.

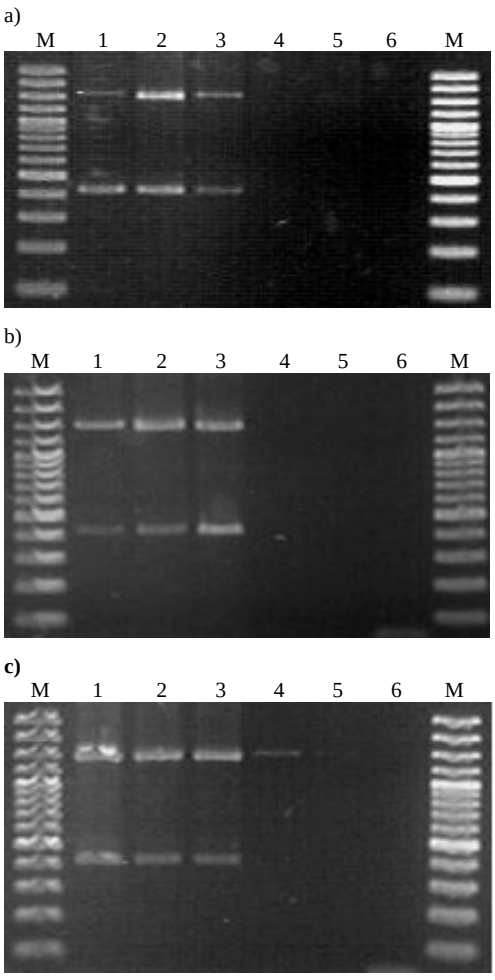

Fig. 1. Determination of the sensitivity of a multiplex PCR according to Chen and Knabel (2) using the reaction mixture containing 2 mM of $MgCl_2$ for the detection of *L. monocytogenes* ATCC 13932 strain in the liver (a), brain (b), and blood (c): 1.5×10^6 CFU/reaction (1), 1.5×10^5 CFU/reaction (2), 1.5×10^4 CFU/reaction (3), 1.5×10^3 CFU/reaction (4), 1.5×10^2 CFU/reaction (5), negative control (6), and molecular weight marker (M)

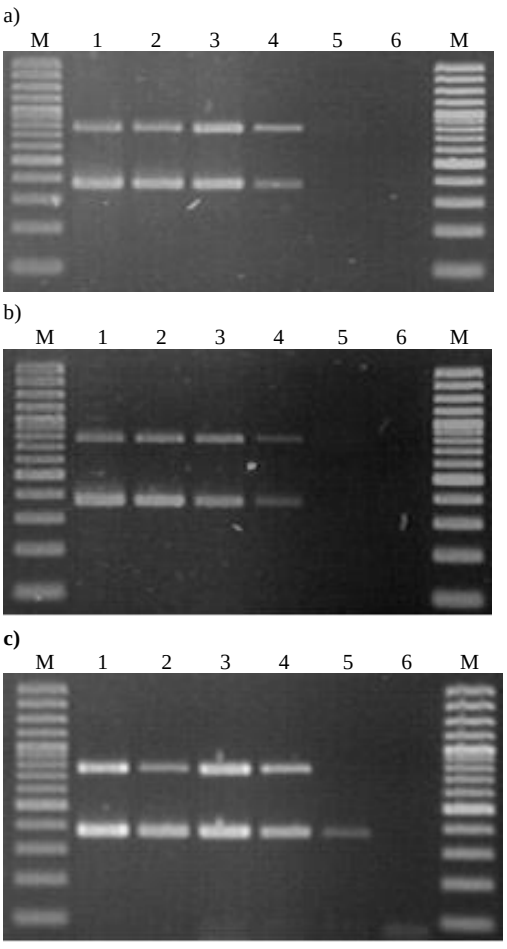

Fig. 2. Determination of the sensitivity of multiplex PCR according Doumith *et al*. (4) and Gouws and Liedemann (11) using the reaction mixture containing 3 mM of $MgCl_2$ for the detection of *L. monocytogenes* ATCC 13932 strain in the liver (a), brain (b), and blood (c): 1.5×10^6 CFU/reaction (1), 1.5×10^5 CFU/reaction (2), 1.5×10^4 CFU/reaction (3), 1.5×10^3 CFU/reaction (4), 1.5×10^2 CFU/reaction (5), negative control (6), and molecular weight marker (M)

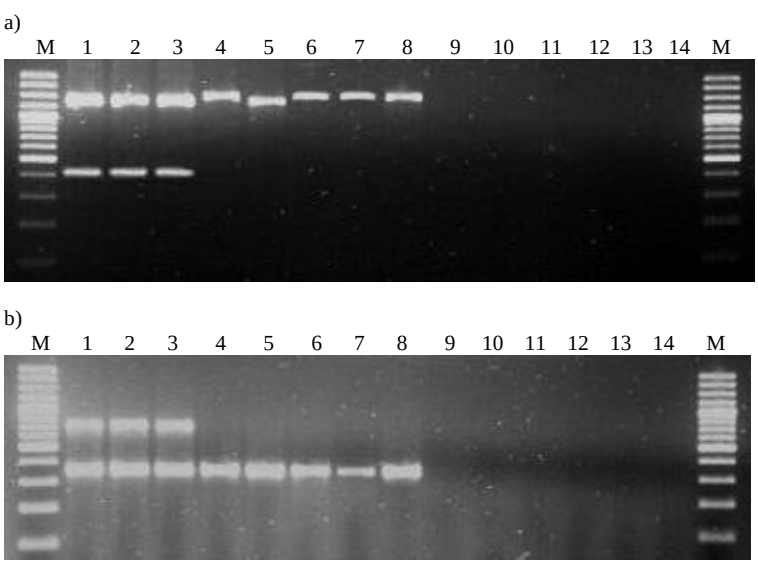

Fig. 3. Determination of the specificity of multiplex PCRs according to Chen and Knabel (2) in contaminated liver samples (a) and Doumith *et al*. (4) and Gouws and Liedemann (11) in contaminated brain samples (b): *L. monocytogenes* ATCC 7644 (1), *L. monocytogenes* ATCC 13932 (2), *L. monocytogenes* ATCC 19112 (3), *L. ivanovii* ATCC 19119 (4), *L. innocua* ATCC 33090 (5), *L. grayi* ATCC 19120 (6), *L. welshimeri* ATCC 35897 (7) and *L. seeligeri* (8), *Bacillus cereus* ATCC 11778 (9), *Staphylococcus aureus* ATCC 6538 (10), *Escherichia coli* ATCC 25922 (11), *Salmonella* Typhimurium ATCC 14028 (12), *Klebsiella pneumoniae* ATCC 13883 (13), negative control (14), and molecular weight marker (M)

As the next stage, the sensitivity of this multiplex PCR was evaluated in subsequent studies. The same sensitivity of 1.5×10^3 CFU/reaction was found for each type of biological sample (liver, brain, and blood) with the use of either reaction mixture (Fig. 2).

To demonstrate the specificity of both multiplex PCR assays, the genetic material obtained from biological samples contaminated with three strains of *L. monocytogenes*, one strain of each of *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*, and the other microorganisms *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhimurium, and *Klebsiella pneumoniae* were examined (Fig. 3). The primer sets used were highly specific. An electrophoretic analysis revealed that in both multiplex PCR assays for all strains of *L. monocytogenes* two products were obtained. The strains of *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri* showed the presence of a fragment specific for the genus *Listeria*. The isolates of other microbial species failed to yield PCR products.

Discussion

PCR is an effective molecular method, which allows quick detection, amplification, and identification of small quantities of nucleic acids (26, 27). PCR assays provide a rapid and sensitive alternative to conventional identification based on examination of morphological, physiological, and biochemical features of *L. monocytogenes* (9). Virulence factors including internalins (Inl), listeriolysin O (LLO), protein p60 (Cwh A), protein ActA, phospholipase A (PlcA), and phospholipase B (PlcB) are involved in the process of pathogenesis of listeriosis (1, 13). These factors play a key role in the invasion and intracellular proliferation in animal tissues. The use of the PCR method enables identification a number of genes specific for *L. monocytogenes* species, such as *hly*, *iap*, *prs*, *prf*A, *fla*A, and *act*A (3, 8, 10, 13, 15, 17, 21).

The PCR efficiency depends on the proper optimisation of reaction conditions. In our study, the influence of the reaction mixture and the concentration of primers for multiplex PCR assays were examined. In order to achieve the desired results a reaction mixture containing 2 mM of $MgCl_2$ and a Qiagen reaction mixture containing 3 mM of $MgCl_2$ were used. In the case of both multiplex PCR assays comparable results were obtained using the two reaction mixtures. Satisfactory results were also achieved by Douminth *et al.* (4), who used a magnesium ion at a concentration of 2 mM in the reaction mixture in the case of the *prs* gene, and Chen and Knabel (2) who established the final concentration of magnesium ions at a level of 3 mM in the reaction mixture for the *iap* and lmo2234 genes.

Our findings indicated that in the multiplex PCR performed by Chen and Knabel (2) the optimal concentration of primers was different for the two reaction mixtures tested whereas the concentration was at the same level for both reaction mixtures in the case of the multiplex PCR according to Doumith *et al.* (4) and Gouws and Liedemann (11). Several researchers used different concentrations of primers depending on the reaction mixtures applied and conditions of PCR (1, 2, 4, 6, 22, 23).

In our examinations the sensitivity of multiplex PCR according to Chen and Knabel (2) for the two reaction mixtures was at the level of 10^4 CFU per reaction irrespective of the type of biological sample (liver, brain, and blood). In the case of the test under Doumith *et al*. (4) and Gouws and Liedemann's protocols (11) the sensitivity was at a slightly higher level (10^3 CFU per reaction). Similarly Dümen *et al*. (6) obtained a detection limit of 10^3 CFU/0.5 mL for *L. monocytogenes* in milk. In another report, Wernas *et al.* (28) also achieved a detection limit of 10^3 CFU/0.5 g in some cheeses, whereas the presence of 10^8 CFU/0.5 g was not detectable in others. In turn, these researchers detected at a lower limit of 1 to 10 CFU for *L. monocytogenes* in pure cultures. Fitter *et al*. (7) also obtained a detection limit of 50 to 500 CFU in pure cultures.

In our study, in the case of the lmo2234 and *hly* genes, specific amplification products allowing the confirmation of the species as *L. monocytogenes* were obtained, and regarding the *iap* and *prs* genes, the presence of fragments specific for the genus *Listeria* was found. No specific products were found when isolates of other species of bacteria were examined. Paziak-Domańska *et al.* (22) and Chen and Knabel (2) also reported 100% specificity of PCR during examinations of *L monocytogenes* and other strains of the genus *Listeria.*

The two multiplex PCR assays demonstrated good sensitivity and high specificity. In the next stage of studies real-time PCR for the detection of *Listeria* spp. and *L. monocytogenes* in biological samples will be applied (16). This method may also improve detection sensitivity and specificity.

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