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REAL-TIME PCR OPTIMIZATION

FOR *LISTERIA MONOCYTOGENES* GENOME DETECTION

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SUMMARY

Listeria monocytogenes is one of the major food contaminants causing the illness, called listeriosis. Listeriosis incidence is much less than the number of salmonellosis and campylobacteriosis cases, but the clinical disease is significantly more severe and has a higher mortality. That's why the development of species-specific PCR techniques to detect *L. monocytogenes* genome is a topical task. *L. monocytogenes* bacteria genome detection technique using real-time polymerase chain reaction (qRT-PCR) was improved. The amplification target was a highly specific and suitable for qualification of all strains *iap* gen, coding *L. monocytogenes* p60 surface protein. Optimum magnesium concentration (6 mM) and primer annealing temperature (57 °C) were selected. The sensitivity and specificity of the technique were identified. Detection threshold was 120 target molecules. The results obtained demonstrate that optimized qRT-PCR version, based on *iap* gen amplification, enables to detect *L. monocytogenes* in animal product and food samples. Optimized qRT-PCR-based screening tests ensure rapid and reliable results.

Key words: qRT-PCR, *Listeria monocytogenes*, food products of animal origin.

INTRODUCTION

Listeria monocytogenes is the facultative intracellular pathogen, one of major food product contaminants causing rare but lethal disease in immunocompromised individuals called listeriosis. Listeriosis is not widespread infection. Number of detected listeriosis cases is considerably less than those of salmonellosis and campylobacteriosis but they are more clinically severe and the lethality is higher [1]. *L. monocytogenes* commonly co-exists with other species of this genus such as nonpathogenic *Listeria innocua* that can be used as an indicator of possible *L. monocytogenes* presence in food products [15]. Safety rules of the majority of countries do not allow presence of *L. monocytogenes* in food products [6, 18].

Identification of *L. monocytogenes* in accordance with current regulations is performed with conventional microbiological (microscopy, inoculation on nutrient media) and serological (agglutination tests, complement fixation test) methods [4, 8].

However, time required for testing with conventional methods varies from 3 to 4 days in case of negative results and it takes up to 7 days to confirm the positive result [24]. Detection of *L. monocytogenes* with microbiological method in animal products is hampered due to high competitive bacteria concentration and low *L. monocytogenes* levels and presence of listeria-inhibiting food components [23]. Real-time PCR is a rapid and feasible alternative to microbiological method. Therefore, development of species-specific PCR techniques for *L. monocytogenes* detection is a highly important task.

Molecular methods such as PCR allow rapid detection and identification of bacterial pathogens [27]. Several traditional PCR techniques for *L. monocytogenes* detection were reported [13, 15, 19, 21]. Moreover, rt-PCR variants for *L. monocytogenes* detection were described [2, 12, 16, 26, 28].

Various genes, 16S and 23S rRNA, *prs*, *gyrB*, *rpoB*, *hly*, *inlA* and *inlB*, *plcA*, *iap*, etc., are currently used as targets for PCR [3, 7, 10, 11, 14, 15, 17, 20, 22, 30].

The study was aimed at optimization of qualitative rt-PCR for detection of *L. monocytogenes* genome in tested products of animal origin and food products.

MATERIALS AND METHODS

Bacterium strains. The following reference strains obtained from the American Type Culture Collection (ATCC) were used: *Listeria monocytogenes* ATCC 19115, *Listeria*

Table 1
Structure of primers and probe [according to D. Rodriguez-Lazaro, 2004]

Name	Sequence
iapQF	[5′-AATCTGTTAGCGCAACTTGGTTAA-3′]
iapQR	[5′-CACCTTTGATGGACGTAATAATACTGTT-3′]
iapQProbe (FAM-RTQ1)	[5′-CAACACCAGCGCCACTACGGACG-3′]

innocua ATCC 33090, *Listeria ivanovii* ATCC 19119, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 19433, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538-P, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 11060, P*roteus mirabilis* ATCC 29906, *Yersinia kristensenii* ATCC 35669.

Nutrient media and conditions for bacteria cultivation. All reference strains were cultivated on Columbia agar (manufactured by HiMedia Co., India) at temperature of 37 °C for 24 hours.

Nutrient broth for *Listeria* (NBL) (Obolensk) was used as a medium for the first *Listeria* enrichment.

Double-strength NBL (Obolensk) was used as a medium for the second enrichment.

Samples of food products. One hundred samples of food products submitted to the FGBI "ARRIAH" in 2017 for testing were used. Samples represented the following groups of animal products:

– meat (beef – 8 samples, pork – 10 samples, poultry meat – 10 samples);

– semi-processed products (meat preparations – 18 samples, chicken meat preparations – 7 samples);

– fish and fish products – 12 samples;

– pasteurized milk – 6 samples;

– butter – 29 samples.

Preparation of samples for testing. A weighed portion of sample (25 g or 25 cm^3) prepared for testing was added to 225 cm^3 of the medium for the first *Listeria* enrichment and incubated at temperature of (30 ± 1) °C for (24 ± 3) hours. After the first enrichment, 0.1 cm^3 of the sample, regardless of any changes in the medium was added to a tube containing 10 cm^3 of the medium for the second enrichment and incubated at a temperature of (37 ± 1) °C for (48 ± 2) hours. After incubation 250 µl of suspension were transferred to 1.5 ml polypropylene Eppendorf tubes that were used for rtPCR tests.

DNA extraction. DNA was extracted from pure culture samples using SORB-GMO-A kit (Sintol, Moscow) in accordance with its manufacturer's instructions.

Oligonucleotides. Primers and probe for *L. monocytogenes* identification previously described by D. Rodriguez-Lazaro *et al.* [25], (Gen Bank AY174670 – AY174682) were synthesized by Sintol Company. Primary structures of oligonucleotides are given in Table 1.

Conditions for rtPCR. rtPCR reagent kit (Sintol, Moscow) was used for PCR tests. The following components as per one sample (20 µl) were used to prepare reaction mixture: 10× PCR buffer – 2.5 µl; dNTP 2.5 mM – 2.5 µl; $MgCl_2$ 25 mM – 2.5 µl; mixture of primers and probe (10 pmol/µl of each) – 0.75 µl; SynTaq DNA-polymerase 5 U/µl – 0.5 µl; ddH_2O – 11.25 µl.

CFX-96 thermostat (BIO-RAD, USA) was used for amplification under the following conditions: 20 s at 95 °C (1 run) and 20 s at 95 °C (45 runs), 20 s at 57 °C (45 runs) and 20 s at 72 °C (45 runs).

Determination of rtPCR sensitivity. DNA extracted from reference *L. monocytogenes* strain suspension (ATCC 19115, optical density – 1 McFarland unit that corresponds to 3×10^8 CFU/cm^3) was used for the primer sensitivity assessment. Concentration of extracted DNA was determined with Implen NanoPhotometer P-Class P-360 spectrophotometer (IMPLEN, Germany). Dilutions (100 µl) of extracted DNA were made with TE buffer.

Statistical processing of data. Results of rtPCR were analyzed with v3.1 CFX Manager Software.

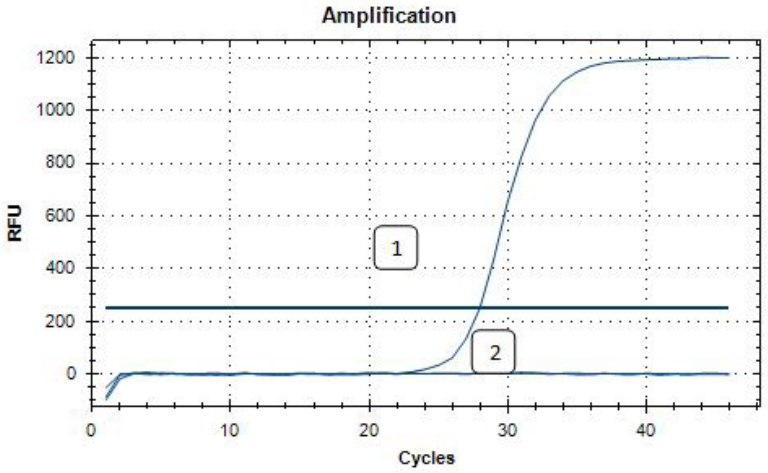

Fig. 1 Amplification of Listeria monocytogenes DNA
1 – positive results; 2 – negative result.

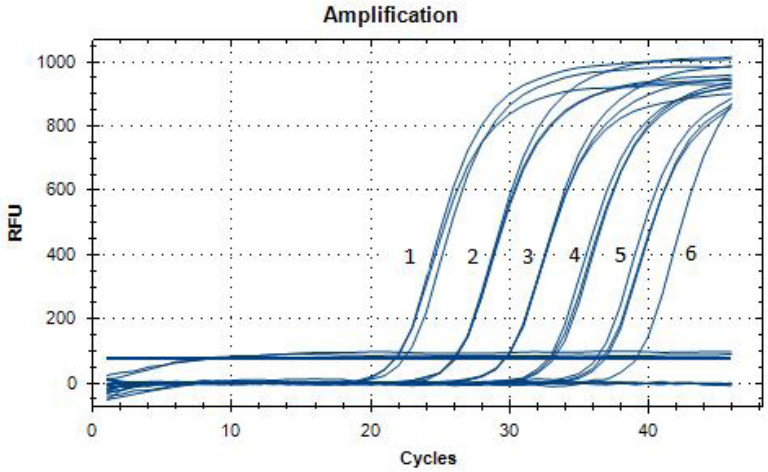

Fig. 2 Determination of analytical sensitivity of rtPCR for L. monocytogenes genome identification (n = 3)
Bacterial cell concentration: 1 – 12 × 105; 2 – 12 × 104; 3 – 12 × 103; 4 – 12 × 102; 5 – 120; 6 – 12.

RESULTS AND DISCUSSION

Selection of primers. The *iap* gene (GenBank, No. X52268) coding for p60 surface protein also known as cell wall hydrolase was selected based on literature data on rtPCR tests [29]. The reason for this selection is the said protein has been shown to have two unique regions of sequences in *L. monocytogenes* that are absent in other *Listeria* spe-

Table 2
rtPCR detection limit for Listeria monocytogenes strain ATCC 19115
($n = 3$)

Sample	Mean value Cq	Standard deviation Cq	End RFU	Result
Neg Ctrl	0.00	0.000	-1.45	(–) Negative
12×10^5	21.61	0.260	970	(+) Positive
12×10^4	26.03	0.072	1,072	(+) Positive
12×10^3	29.67	0.040	1,045	(+) Positive
12×10^2	33.00	0.170	1,004	(+) Positive
120	36.59	0.346	940	(+) Positive
12	39.04	0.000	0.196	(–) Negative
1.2	0.00	0.000	-1.55	(–) Negative

cies that makes it highly specific and suitable for accurate qualitative assessment of all *L. monocytogenes* strains [1].

Comparing *iap* genes in different *Listeria* species A. Burbert *et al.* [15] defined common and variable regions within the said genes specific for each *Listeria* species. PCR primers were designed for *Listeria monocytogenes* (Table 1) based on these *iap* gene sequences [25].

Optimization of rtPCR for specific Listeria monocytogenes detection. Optimization of rtPCR includes the following steps: determination of correct temperature for primer annealing and determination of magnesium ion concentration that produces the highest fluorescent signal response at high specificity. Optimal annealing temperature depends on the primer structure and generally varies from 55 to 72 °C [9].

The following formula is used for estimation of primer annealing temperature

$$Tm = 2 \times (A + T) + 4 \times (G + C),$$

where Tm – annealing temperature, °C;
A, T, C, G – nucleotide bases [9].

Experimentally determined annealing temperature was to be 66 °C for upstream primer and 76 °C for downstream primer. For a pair of primers having different annealing temperatures the least temperature was selected for PCR procedure development. To determine optimal temperature rtPCR was run using ±10 °C gradient starting from the estimated primer annealing temperature and at magnesium ion concentration of 3–6 mM that was increased in a 0.5 mM increment. The PCR results were identical when magnesium concentration of 4–6 mM was used so magnesium concentration of 6 mM was selected. The test results were identical for all temperatures within the range of 56 to 75 °C but decrease in the reaction performance was observed at 76 °C. Annealing temperature of 57 °C was selected based on the experiment results.

We optimized concentrations of primers and probe for specific analysis using the same cycles as in previously developed rtPCR test [5]. Optimal conditions providing the least threshold cycle value (Ct) at the least primer and probe concentrations (Fig. 1.) are given in Materials and methods.

Determination of optimized rtPCR sensitivity. Sensitivity is an important characteristic of the developed PCR procedure. Extracted DNA at concentration of 4 ng/µl was used for the PCR sensitivity assessment.

Providing the medium genome size in various tested microorganisms 1 ng of genomic DNA corresponds to at least 3×10^5 cells [28]. Thus, DNA concentration of 4 ng/µl corresponds to about 12×10^5 bacterial cells.

rtPCR sensitivity was determined with PCR runs using 4-fold dilutions of extracted DNA starting with maximum concentration of 4 ng/µl (that corresponds to 12×10^5 bacterial cells in the reaction) up to 4×10^{-6} ng/µl (that corresponds to 1.2 bacterial cells in the reaction). Amplification reaction was performed in triplicate (Fig. 2).

Obtained results show that the optimized PCR detects *L. monocytogenes* genome at concentration of about 120 bacterial cells in a sample that is consistent to other researchers' findings [12, 25]. Only one out of three rtPCRs was capable of detecting *L. monocytogenes* DNA content of about 12 bacterial cells (Table 2).

Determination of specificity. Primers were tested for their specificity using target (*L. monocytogenes*) and 13 non-target bacteria strains (Table 3). Only target *L. monocytogenes* strain was identified with the reaction. No false positive results were registered that confirmed the species-specificity of proposed rtPCR.

L. monocytogenes genome detection in products of animal origin. One hundred samples of animal products were tested with the optimized rtPCR (Table 4) and conventional microbiological method. *L. monocytogenes* genome was detected in 16% of samples. When the same samples were tested with the microbiological method only 8% of samples were found positive. Differences in test results can be accounted for the PCR ability to detect genetic materials of not only live microorganisms but also dead ones. Microbiological method is capable of detecting only live microorganisms.

Optimized PCR-based screening tests are shown to provide rapid and reliable results. The main advantage of the optimized rtPCR for screening tests is rapid detection of samples that are negative for target pathogen i. e. *L. monocytogenes* genome. However, this method does not allow differentiation between viable and non-viable bacterial cells. Therefore, the rtPCR positive results must be obligatory confirmed by microbiological method.

Table 3
Determination of primer specificity for *Listeria* monocytogenes genome detection

No.	Bacteria species	Strain	Gram staining	*iap*-specific primers
1	*Listeria monocytogenes*	ATCC 19115	+	+
2	*Listeria innocua*	ATCC 33090	+	–
3	*Listeria ivanovii*	ATCC 19119	+	–
4	*Bacillus subtilis*	ATCC 6633	+	–
5	*Bacillus cereus*	ATCC 11778	+	–
6	*Enterococcus faecalis*	ATCC 19433	+	–
7	*Escherichia coli*	ATCC 25922	–	–
8	*Lactobacillus casei*	ATCC 393	+	–
9	*Proteus mirabilis*	ATCC 29906	–	–
10	*Pseudomonas aeruginosa*	ATCC 9027	–	–
11	*Salmonella typhimurium*	ATCC 14028	–	–
12	*Shigella flexneri*	ATCC 12022	–	–
13	*Staphylococcus aureus*	ATCC 6538 P	+	–
14	*Yersinia enterocolitica*	ATCC 9610	–	–

CONCLUSION

rtPCR for *L. monocytogenes* genome detection was optimized. rtPCR analytical sensitivity and specificity were determined; the established *L. monocytogenes* genome detection limit was 120 target molecules in tested samples.

It was demonstrated that the optimized rtPCR could be used for *L. monocytogenes* genome detection in animal product samples during screening testing.

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Table 4
Detection of *L. monocytogenes* in food product samples with rtPCR and microbiological method

No.	Name of sample	Number of samples	Number of positive samples	
			PCR number	Microbiological method number
1	Beef	8	4 (50%)	1 (12%)
2	Pork	10	2 (20%)	1 (10%)
3	Meat preparations	18	4 (22%)	3 (17%)
4	Poultry meat	10	3 (30%)	2 (20%)
5	Chicken meat preparations	7	3 (43%)	1 (14%)
6	Fish and fish products	12	0	0
7	Pasteurized milk	6	0	0
8	Butter	29	0	0
	Total	100	16 (16%)	8 (8%)

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