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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR RAPID DETECTION OF *L. MONOCYTOGENES* IN MEAT

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ABSTRACT

There is a continued need to develop improved rapid methods for detection of foodborne pathogens. Rapid and sensitive methods for enumeration of *Listeria monocytogenes* are important for microbiological food safety testing purpose. The aim of this project was to evaluate a commercial loop-mediated isothermal amplification (LAMP) based system with bioluminescence, named as 3M™ Molecular Detection Assay (MDA), was validated for the detection of *L. monocytogenes* in food products with a standard GOST 32031-2012 method as reference. The results of this study revealed that a commercial LAMP-based method performed equally effective compared with method, showing from 94% to 100% specificity and sensitivity, respectively. The LAMP-based method was shown to be rapid and reliable detection technique for *L. monocytogenes* present at low numbers (10 CFU.g^{-1}) on raw meat and meat products and can be applicable in meat industry. Thus, compared with the microbiological method based GOST 32031-2012, the LAMP assay is a relatively rapid and highly sensitive method for detecting *L. monocytogenes* and will facilitate the surveillance for contamination of *L. monocytogenes* in food. The 3M MDS result and culture-based detection (GOST 32031-2012) did not differ significantly (p >0.05) regarding the number of positive samples.

Keywords: meat; *L. monocytogenes* detection; LAMP-method

INTRODUCTION

Listeria monocytogenes is a pathogen that causes the severe foodborne disease such as listeriosis **(Swaminathan and Gerner-Smidt, 2007; Warriner and Namvar, 2009)**. There is approximately 1600 illnesses and 260 deaths each year are due to listeriosis in USA **(Centers for Disease Control and Prevention, 2014)**. European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) reported that in 2008 – 2016 in Europe an increasing trend of human listeriosis cases was observed with 2536 cases of which 97.7% were hospitalized and 16.2% were with case fatality **(EFSA and ECDC, 2017)**. A major concern for processors of risk food products is a survive, multiply; persist under harsh conditions in food processing environments **(Gandhi and Chikindas, 2007; Carpentier and Cerf, 2011)**. *L. monocytogenes* can occur in raw or processed foods that are contaminated during processing. **(Koreňová and Oravcová, 2011; Bogdanovičová et al., 2015)**. It is estimated that more than 99% of human listeriosis results from consumption of contaminated food, particularly ready-to-eat (RTE) foods, such as dairy products, smoked fish **(Allerberger and Wagner, 2010; Koch et al., 2010)**.

Indrawattana et al. (2011) reported that 15.4% from 104 of the raw meat samples collected from supermarkets and open markets in the Bangkok metropolitan area were contaminated with *L. monocytogenes*. In Morocco, for instance, *L. monocytogenes* was present in 2.3% of 426 poultry and red meat samples collected in 2008 **(Ennaji et al., 2008)**.

The official methods for the detection of this pathogen in foods, are based on culture techniques **(Law et al., 2015)** are reliable but present disadvantages, such as time-consuming and lengthy. This is a major drawback of particular importance for food products with short shelf-life, for performing outbreak analysis, and for self-monitoring in production plants **(Garrido-Maestu et al., 2017; Shan et al., 2012)**. The appearance of molecular methods, such as the Polymerase Chain Reaction (PCR), and real-time PCR (qPCR), has allowed to overcome these limitations. More recently, isothermal DNA amplification approaches are gaining interest, being among the most popular loop-mediated isothermal amplification (LAMP). It presents several advantages over PCR/qPCR, such as being performed at constant temperature or having higher specificity due to the present of a several number of primers **(Abdulmawjood et al., 2016; Wang et al., 2015)**. LAMP

can be monitored in real-time by measuring the increase in fluorescence of DNA binding dyes **(Seyrig et al., 2015)**.

The objective of this study was to evaluate the performance of a commercial loop-mediated isothermal DNA amplification (LAMP) based method with bioluminescence named as 3M™ Molecular Detection System (MDS) for the detection of *L. monocytogenes* in raw meat and ready-to-cook (RTC) meat products using the 3M™ Molecular Detection Assay (MDA). The study was conducted for the detection of low inoculum levels of *L. monocytogenes* in comparison to the GOST 32031 method to validate LAMP-based method.

Scientific hypothesis

LAMP-based system provides rapid and reliable results for the detection *L. monocytogenes* in raw meat and meat products and can be applicable in meat industry. Sensitivity and specificity shold be more than 90%. Kappa-value should be more than 0.85.

MATERIAL AND METHODOLOGY

Raw meat (beef, pork) and meat products (RTE, RTC) were selected as objects of study. Pork ground and beef ground samples were used for artificial contamination and further detection of method sensitivity threshold. All samples were purchased at a local supermarket in central region of Russian Federation from September 2018 through March 2019.

Cultures preparation

L. monocytogenes ATCC 35152 NCTC 7973 and ATCC 13932 serovars 4b (from American Type Culture Collection (Manassas, VA, USA), were activated in 10 mL of tryptone soya broth (TSB, Oxoid, England) for 24 h at 37 °C. The cultures were centrifuged (Eppendorf, Germany) at 3000 g for 10 min, washed twice with 0.1% (w/v) peptone water (Oxoid, England), and resuspended in 1 mL of 0.1% (w/v) peptone water (Oxoid, England), and then mixed (1:1, v/v) to prepare a 2-strain cocktail (10_1 and 10_2 CFU.mL^{-1}). Before inoculation, the counts of prepared 2-strain cocktail of *L. monocytogenes* diluted in 0.1% (w/v) PW (Oxoid, England) were enumerated by spread plating an aliquot of 100 μL on tryptone soya agar (TSA, Oxoid, England) in duplicate and incubating TSA plates at 37 °C for 48 h to estimate the inoculum levels.

Inoculation procedure

There were two inoculation levels for matrix: a high inoculation level of approximately 100 CFU.g^{-1} and a low inoculation level of approximately 10 CFU.g^{-1}. Also was used uninoculated samples as negatives controls.

L. monocytogenes detection with a commercial lamp-based system

The detection of *L. monocytogenes* cells by the commercial LAMP-based kit (3M Molecular Detection Assay *Listeria monocytogenes*; 3M) was performed according to the manufacturer's manual. Briefly, 25 g of sample were mixed with 225 ml Demi-Fraser broth (3M, USA). Then 20 μL of UVM enrichment was added to a tube with lysis solution. The mixture was warmed in a heat block (Germany, IKA) at 100 °C for 15 min, followed by immediate cooling at room temperature in a chilling block (3M, USA) for 10 min. After mixing by inversion, 20 μL of this lysate was mixed with the pellet in the reagent tube from the assay kit. The reagent tube was placed in a molecular detection system (3M, U.S.A.) for the detection of *L. monocytogenes* cells via isothermal amplification and bioluminescence for 75 min. All analyses included negative and reagent controls to validate the performance of the molecular detection system.

Detection *L. monocytogenes* by GOST 32031

The samples were examined for the presence of *L. monocytogenes* bacteria in accordance with GOST 32031-2012. 25 g of the meat was homogenized in 225 cm^3 of Demi-Fraser broth (Merck, Germany) and incubated at 30 °C for 24 hours. Then 0.1 cm^3 the enriched culture was added to 10 cm^3 of Fraser's broth (Merck, Germany) and cultured at 37 °C for 48 hours. From each broth after the end of incubation with a 3 mm loop, the enriched material was streaked onto a chromogenic agar for *Listeria* (Agar *Listeria* according to Ottaviani and Agost (Merck, Germany)) and selective nutrient agar for *Listeria* PAL (FBUN GNC PMB, Russia) and incubated at 37 °C within 24 – 48 hours. On chromogenic agar for *Listeria*, *L. monocytogenes* grows in the form of blue colonies with an area around; on PAL, brown colonies with black halos. Colonies typical of the genus *Listeria* and *L. monocytogenes* were seeded on tryptone soya agar with yeast extract (TSAYE) and incubated at 37 °C for 18 – 24 hours, which was then confirmed using biochemical tests (Oxoid, England).

Statistic analysis

Sensitivity and specificity of the commercial LAMP-based kit for the detection of *L. monocytogenes* were defined as the number of samples truly positive (T_{pos}) and truly negative (T_{neg}), respectively, compared with the GOST method. The sensitivity, specificity, and accuracy of the commercial LAMP based kit were calculated as follows:

$$\text{Sensitivity} = [T_{pos} / (T_{pos} + F_{pos})]$$

$$\text{Specificity} = [T_{neg} / (T_{neg} + F_{neg})]$$

where T_{Pos} and T_{Neg} are the number of positive and negative samples, respectively, confirmed by both the GOST and commercial LAMP-based kit, and F_{pos} and F_{neg} are the number of positive and negative samples, respectively, confirmed by the commercial LAMP-based kit. Kappa value of concordance, describing the statistical agreement between the two detection methods was calculated, as described. Kappa values were classified as follows: 0.01 indicated no concordance; 0.1 to 0.4 indicated weak concordance; 0.41 to 0.60 indicated clear concordance; 0.61 to 0.80 indicated strong concordance; and 0.81 to 1.00 indicated nearly complete agreement.

A chi-square test (AOAC, Official Methods of Analysis Program Manual) for significant difference was used to determine whether the proportion of positive samples was different between the 3M MDS and the GOST-method.

RESULTS AND DISCUSSION

L. monocytogenes can multiply over a wide range of pH and osmolarity, at low temperatures, and both under aerobic and anaerobic conditions, this is a particular concern and necessitates control along the food chain.

A wide variety of culture and alternative methods have been developed in order to detect or quantify this pathogen in food. In this study, the effectiveness of the commercial LAMP-based kit was evaluated in comparison to the standard culture GOST method for quickly detection of *L. monocytogenes* on different food matrices as artificially contained so and naturally contained.

In this study, a 2-strain cocktail of *Listeria monocytogenes* was used for the detection sensitivity threshold.

L. monocytogenes detection in artificial contamination samples

At the inoculum levels of 10_1 and 10_2 CFU.g^{-1} both methods abled the detection of *L. monocytogenes* in all samples (Table 1), resulting in 100% specificity and sensitivity (kappa value 1). At the inoculum level of 10_0 CFU.g^{-1} (not uninoculated *L. monocytogenes*), both methods were unable to detect *L. monocytogenes*. False negative results were not obtained.

L. monocytogenes detection in samples with native microflora (raw meat and meat products, purchased in local supermarkets)

The results samples show high specificity of the LAMP-method (not less than 90%) (Table 2). The 3M MDS result and culture-based detection (GOST 32031-2012 method) did not differ significantly (p >0.05) regarding the number of positive samples.

In similar studies, the high specificity and sensitivity of the method on artificially infected matrices has also been proven. *L. monocytogenes* was detected in 11 samples of pork by LAMP – method and in 10 samples by GOST 32031. One sample was not confirmed according to the reference method and was identified as false-positive (F_{pos}). The sensitivity of the method in the study of pork samples was 90% (Kappa value 0.93), specificity – 100%.

In another study the LAMP-GNP/DNA probe assay was applied to the detection of 200 raw chicken meat samples and compared to routine standard methods. The data revealed that the specificity, sensitivity, and accuracy were 100, 90.20, and 97.50%, respectively **(Wachiralurpan et al., 2018)**.

Also, in the study of 32 samples of beef ready-to-cook products, 1 false positive result was found by the LAMP method. The sensitivity and specificity of the method were 88% and 100%, respectively, with a Kappa-value of 0.92.

Table 1 Comparison of LAMP-based method and GOST-method for the detection *L. monocytogenes* in artificial contamination samples.

Level Inoculated (CFU.g^{-1})	Matrix	LAMP and GOST		LAMP		Sensitivity (%)	Specificity (%)	Kappa
		T-pos	T-neg	F-pos	F-neg			
10_0	Raw ground beef	0/5	0/5	0/5	0/5	100	100	1
	Raw groud pork	0/5	0/5	0/5	0/5	100	100	1
10_1	Raw ground beef	5/5	5/5	5/5	5/5	100	100	1
	Raw groud pork	5/5	5/5	5/5	5/5	100	100	1
10_2	Raw ground beef	5/5	5/5	5/5	5/5	100	100	1
	Raw groud pork	5/5	5/5	5/5	5/5	100	100	1

Note: T-pos, T-neg are true positive and negative samples, confirmed by both GOST and LAMP-based methods; F-pos and F-neg are false positive and negative samples, confirmed only by LAMP-based technique or GOST method, respectively.

Table 2 Comparison of LAMP-based method and GOST-method for the detection *L. monocytogenes* in native contamination samples.

Food matrix	LAMP and GOST		LAMP		Sensitivity (%)	Specificity (%)	Kappa
	T-pos	T-neg	F-pos	F-neg			
pork	10	25	1	0	90	100	0.93
beef	5	18	0	1	100	94	0.88
RTC pork	3	16	0	0	100	100	1
RTC beef	8	24	1	0	88	100	0.92
RTE	1	16	0	0	100	100	1

Note: T-pos, T-neg are true positive and negative samples, confirmed by both GOST and LAMP-based methods; F-pos and F-neg are false positive and negative samples, confirmed only by LAMP-based technique or GOST method, respectively.

In this case a false positive result could be caused by the DNA amplification on injured or sub lethally cells that cannot be detected by ISO **(Lim et al., 2015)**. Another explanation for false positive results is the using of 4 to 6 primers with a much higher concentration in the LAMP method than in the classical methods based on PCR. In terms of efficiency, the PCR and real-time PCR assays could detect *L. monocytogenes* based on the listeriolysin O gene (*hly*) with a detection limit of 8 – 10 CFU **(Rip and Gouws, 2009)**. However, these assays required sophisticated equipment and post-amplification manipulations that took more time to obtain results **(Gianfranceschi et al., 2014)**.

This could lead to an increase in the possibility of non-specific amplification caused by forming primer dimers **(Wang et al., 2015)**. 23 samples of beef were analysed. 5 positive and 18 negative results were detected and confirmed by both research methods (GOST and LAMP). However, 1 sample was false negative by LAMP-based method compared with GOST 32031-2012. The sensitivity of the method in the study of beef in this case was 100% (Kappa value 0.88), and the specificity – 94%. In other studies, false negative results were also obtained **(Lim et al., 2015)**. These authors showed that 1 naturally contaminated sample of duck wings was presented as false-negative. The validation study also showed 91% sensitivity and 95% specificity, Kappa-value 1.

In the study of 19 ready-to-cook pork samples and 17 meat ready-to-eat products, no significant (p >0.05) differences in the results obtained by the LAMP and GOST 32031-2012 methods were found. The sensitivity and specificity of both methods was 100% with Kappa-value 1. Such a convergence of the two methods can be associated with an enough viable cells of *L. monocytogenes* in the sample to identify them.

Several false-positive and false-negative results were obtained at low levels of inoculum (10_1 CFU/10 cm_2), for the LAMP method have been reported **(Mikš-Krajnik et al., 2015)**. It also reports at a 10_2 CFU/100 cm_2 microbial cell level, both methods were suitable for detection of *L. monocytogenes* and had 100% specificity and sensitivity.

In another study the LAMP method was employed to test 94 retail food samples effectively. Sensitivity in detection of *L. monocytogenes* by the LAMP was higher than that of PCR and none of the conventional method positive samples was missed by the LAMP method **(Shan et al., 2012)**.

Listeriosis outbreaks were seen in many countries including Japan, the United States and countries of Europe **(EFSA, 2011; Miya et al., 2015; Self et al., 2019)**. Human infections caused by *L. monocytogenes* have become a global health concern. The presence of *L. monocytogenes* in processing environment at slaughterhouses, deli meat factories or in retail may be a reason of cross-contamination. *Listeria monocytogenes* can contaminate various foods via food processing environments and contamination of raw materials. Hence, there is a necessity a variety of methods for rapid detection of foodborne pathogens as it is required in many food analyses.

In this study rapid LAMP method of *L. monocytogenes* detection was performed. Despite the having of false-negative and false-positive results, LAMP-based method was effective and easier to perform than some of standardized assays and has the advantage to reduce analysis time (less 2 days comparing with 5 days GOST method). Furthermore, this technique is 10 times more sensitive than the conventional PCR assay as reported **(Wachiralurpan et al., 2017)**. The occurrence of false-positive results can be reduced by preventing cross-contamination, high humidity and temperature when working with reaction mixture tubes **(Bird et. al.,2013, Wang et al., 2015)**.

CONCLUSION

The results of this study revealed that a commercial LAMP-based method performed equally effective compared with method, showing from 94% to 100% specificity and sensitivity, respectively. The LAMP-based method was shown to be rapid and reliable detection technique for *L. monocytogenes* present at low numbers (10 $CFU.g_{-1}$) on raw meat and meat products and can be applicable in meat industry. Loop-mediated isothermal amplification (LAMP) has become a powerful alternative to polymerase chain reaction (PCR) for pathogen detection in food matrices.

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