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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) METHOD FOR FAST DETECTION OF *CAMPYLOBACTER* SPP IN MEAT FOOD PRODUCTS AND ENVIRONMENTAL OBJECTS OF A PROCESSING PLANT'S

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Abstract

There is constant necessity of developing the accurate and fast methods for detection of foodborne pathogens. Microorganisms of Campylobacter genus are one of the main causes of foodborne diseases worldwide. Fast identification of Campylobacter at all stages of the food life cycle is an efficient strategy to control foodborne campylobacteriosis. This article the authors evaluated a commercial loop-mediated isothermal amplification (LAMP) system with bioluminescence, called as the 3M™ Molecular Detection Analysis (MDA), which was used to find Campylobacter in food products with the help of a certain standard method, which is referred to as the reference method. The results of this study showed that the commercial LAMP-based method is as efficient as the reference method, and features high specificity and minimum determinability (sensitivity). The LAMP-based method has been shown to be a fast and reliable method for detection of Campylobacter spp. scarce presence (10 CFU/25 g) in meat, meat products, as well as carcass swabs and production facilities' environment. The LAMP analysis required about 24–27 hours to achieve a result. However the LAMP-based method will facilitate the detection of Campylobacter, as it provides much easier and faster detection of Campylobacter spp., including Campylobacter jejuni/Campylobacter coli, than standard microbiological methods. The LAMP-based method is an efficient tool to prevent the spreading of Campylobacter spp. contamination in food products.

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Introduction

Campylobacteriosis is one of the main causes of bacterial diarrhea all over the world [1,2,3]. Two species of bacteria *Campylobacter jejuni* and *Campylobacter coli* are considered the main causes of campylobacteriosis in human [4]. Poultry products remain the main sources of pathogens which cause this disease. Organisms of the genus *Campylobacter* spp. are not usually pathogenic in adult poultry and are considered to be commensal microorganisms of the poultry's gastrointestinal microbiota. Therefore, campylobacteriosis spreads quickly and asymptomatically among the poultry and is extremely difficult to detect and trace [5]. According to the Center for Active Surveillance for Foodborne Diseases (FoodNet), 14 cases of campylobacteriosis are diagnosed per 100,000 population in the United States (USA) (approximately 1.3 million people) and 71 cases per 100,000 population in the European Union (EU) (approximately 200,000 people) every year [1,6]. Campylobacteriosis is rarely a fatal disease and rare reports of mortality are usually recorded for the old age groups (elderly consumers) and/or immunocompromised patients (WHO).

The main target of activity to reduce the number of infections is the timely identification of *Campylobacter* spp. in food products.

In order to identify this pathogen the standard ISO 10272–1:2017 is used [7]. This standard provides for application of modified charcoal cefoperazone deoxycholate agar (m CCDA). However, it has recently been found that *Escherichia coli* and *Pseudomonas* spp, which are widely distributed in poultry, also actively grow together with *Campylobacter* spp. under the same conditions when found in the same sample [8,9]. This is explained by acquired resistance of *Escherichia coli* and *Pseudomonas* spp. to selective factors of nutrient media recommended in the study [7]. This microorganisms' competition can lead to a significant underestimation of the presence of *Campylobacter* spp. in the tested matrix. Thus, there is a need to improve the methodology for detecting *Campylobacter* spp.

in food products. Traditional methods of microbiological cultivation must constantly evolve by expanding the scope of application of selective media and by optimizing the growth conditions to suppress the growth of associated microflora, including new antibiotics [10].

Although methods for isolating *Campylobacter* spp. continue to improve, problems remain that reduce the efficiency of these methodologies. When exposed to stress factors such as refrigeration and freezing, *Campylobacter* spp. can become viable but not culturable (VBNC), thus making it impossible to detect it by many traditional microbiological methods [11,12,13].

Bacteria in the VBNC state can remain dormant for several years and recover in favorable conditions and subsequently cause infections to human [14]. As a result infections and diseases outbreaks caused by VBNC bacteria are underestimated. It has been proven that 13 out of 20 chicken carcasses are likely to contain VBNC *Campylobacter* [15].

Fast PCR-based methods are to eliminate the problems and issues, associated with the certain difficulties of microbiological approaches, and allow fast assessment of degree of *Campylobacter* spp. incidence in various food matrices. Molecular methods for identifying and detecting foodborne pathogens are more sensitive. One of the most valuable methods, used in the food safety industry and science in general, is the polymerase chain reaction (PCR) [10].

The advanced and potential integrating systems such as LAMP-based microdevices, microchips, and other CD-LAMP-based systems are being developed today. These systems provide high specificity, high speed, multiple detection of foodborne pathogens and maximal minimum determinability (sensitivity). In addition, these LAMP-based systems provide the detection of opportunistic and pathogenic microorganisms [16].

The aim of this study is to evaluate the performance of a loop-mediated isothermal amplification (LAMP) based method with bioluminescence, called the 3M™ Molecular Detection System (MDS), used for fast detection of *Campylobacter* spp. in meat, including poultry meat samples and samples of the environmental objects and facilities.

Objects and methods

To determine the relative level of bacteria detection, the artificially contaminated samples of minced pork and minced chicken were used. Samples (minced pork and minced chicken) were purchased from a local supermarket in the central region of the Russian Federation in February-March, 2021.

To establish inclusion and exclusion of the pure cultures of 20 strains were used, including *Campylobacter jejuni* subsp. *jejuni* ATCC 8841 and *Campylobacter coli* ATCC 33559, and 18 *Campylobacter* spp. isolates, which were collected from poultry processing plants and previously found and confirmed by tests. On the other hand, exclusion tests were performed with non-target organisms using the 10 strains listed below in the Table 1.

Table 1. Microorganism strains used to define the specificity of the LAMP method

No	Bacterial strains	Source
1	*Bacillus cereus*	ATCC 11778
2	*Citrobacter freundii*	ATCC 43864
3	*Enterococcus faecalis*	ATCC 29212
4	*Escherichia coli*	ATCC 25922
5	*Klebsiella pneumonia*	ATCC 13883, NCTC 9633
6	*Proteus mirabilis*	ATCC 35659
7	*Pseudomonas aeruginosa*	ATCC 27853
8	*Salmonella enterica* subsp. *enterica* serovar *Typhimurium*	ATCC 14028
9	*Listeria innocua* serotype 4a	ATCC 33090, NCTC 11288
10	*Salmonella enterica* subsp. *enterica* serovar *Enteritidis*	11272

Environmental swabs were analyzed to evaluate this method as a tool for monitoring the degree of *Campylobacter* spp. incidence. The swabs were taken at the poultry and meat processing plants in the Central region of Russia.

Definition of inclusion and exclusion

Campylobacter spp. strains were restored in Bolton broth (Merck, Germany), with further reinoculation on blood agar (OOO Sredoff, Russia) at temperature 41.5 °C for 48 hours under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). Microaerophilic conditions were created in the device Anaxomat (Advanced Instruments Inc., USA). Non-target microorganisms were grown on tryptic soy broth (TSB, Merck, Germany) at temperature 37 °C overnight under aerobic conditions, followed by its reinoculation on trypticase soy agar (TSA, Merck, Germany). Each test was performed five repetitions once — i. e. five replications. Then, strains of microorganisms of *Campylobacter* genus were introduced into Bolton broth (Merck, Germany) at a concentration of 100 CFU/225 ml, and non-target strains were inoculated in TSB (Merck, Germany) at a concentration of 1,000 CFU/225 ml. Further studies were carried out according to the method [7] and by the LAMP method, starting from the stage of enrichment.

The relative level of detection of *Campylobacter* spp. the LAMP method used in samples of minced chicken and minced pork was determined using bacterial cells of *Campylobacter jejuni* subsp. *jejuni* ATCC 8841 (from the American Type Culture Collection, Manassas, Virginia, USA). To prove the absence of *Campylobacter* in the food matrix (minced chicken and minced pork) used for further studies, the samples were examined according to ISO 10272–1:2017 [7] and the protocol of the LAMP method. Campylobacter-negative food matrices were used as a negative control sample in the analysis to establish the relative level of bacteria detection.

To prepare the required number of cells, *Campylobacter* spp. genus microorganisms were restored in Bolton

broth (Merck, Germany). Then they were centrifuged in a MiniSpin centrifuge (Eppendorf, Germany) at 3,000 g for 10 min, flushed twice in 0.85% sodium chloride solution (JSC VEKTON, Russia). The obtained cells were diluted by decimal dilutions, and the concentrations of microorganisms of 10^1 and 10^2 CFU/ml were obtained. Before inoculation, the amount of the prepared *Campylobacter coli* suspension was counted by spreading a 100 µl aliquot on mCCD agar (Merck, Germany) in duplicate; and the suspension was incubated at 42 °C for 48 hours under microaerophilic conditions to assess the level of inoculum. After confirming the required number of cells, the minced meat samples were inoculated. There were two inoculation levels for the matrices: the high inoculation level of approximately 100 CFU/25 g and the low inoculation level of approximately 10 CFU/25 g. After inoculation the samples were tested by two methods to reveal the presence of *Campylobacter* spp.

Campylobacter spp. was detected using a commercial LAMP-based kit (3M Molecular Detection Analysis 2 — Campylobacter; 3M). The strain was detected according to the manufacturer's guidelines. 25 g sample was mixed with 225 ml 3M Enrichment broth (3M, USA). Then 20 µl of 3M Enrichment broth was added to the vial with lysis solution. The mixture was heated in a thermoblock (Germany, IKA) at 100 °C for 15 min followed by its immediate cooling at room temperature in a cooling block (3M, USA) for 10 min. After cooling, 20 µl of the lysate was mixed with the lyophilized mixture in the test vial with the reagent included in the kit. The prepared vial with the reagent was put into a molecular detection system (3M, USA) for the detection of *Campylobacter* spp. cells using isothermal amplification and bioluminescence for 75 min. All analyses included negative controls and reagent controls to test the performance of the molecular detection system.

To test for the presence of *Campylobacter* spp. in accordance with ISO 10272–1:2017 [7], 25 g of the sample was homogenized in 225 ml of Bolton broth (Merck, Germany) and incubated under microaerophilic conditions for 4–6 hours at temperature 37 °C, and then 40–48 hours at temperature 41.5 °C. After the end of incubation the enriched material from the broth was streaked onto mCCD agar (Merck, Germany) and Preston selective agar (Oxoid, United Kingdom) with a 3 mm loop, and incubated under microaerophilic conditions at 41.5 °C for 44±4 hours. The colonies typical of *Campylobacter* spp. were inoculated on blood agar and then confirmed with biochemical tests api® Campy (bioMerieux, France).

Results and discussion

Microorganisms of the genus *Campylobacter* spp. are responsible for approximately 17% of human diarrhea worldwide, what makes them one of the leading causes of foodborne gastrointestinal infections. Governments and industry in many countries develop strategies to reduce the level of food contamination with *Campylobacter* spp. [17]. There is an urgent need for fast and simple methods capable to detect *Campylobacter* spp. throughout the entire chain of food production. At this stage of the study, the efficiency of the LAMP method (using a commercial kit) was evaluated in comparison with the method according to ISO 10272–1:2017 [7].

The specificity of the method was assessed in terms of inclusion and exclusion. Twenty bacterial strains (*Campylobacter jejuni* subsp. *jejuni* ATCC 8841 and *Campylobacter coli* ATCC 33559, 18 *Campylobacter* spp. isolates) were tested for inclusion. During the LAMP method testing, the positive results were obtained only with samples containing *Campylobacter* spp., including *Campylobacter jejuni* and *Campylobacter coli*, while no amplification was found in experiments with ten non-target strains of microorganisms (Table 2). In a parallel study according to ISO 10272–1:2017 [7] only target strains of microorganisms were identified and validated.

Table 2. Results of inclusion and exclusion of the LAMP analysis for *Campylobacter* spp.

Strains	NO. of tests			
	LAMP		ISO 10272–1:2017	
	Posit.	Negat.	Posit.	Negat.
Results of inclusivity				
Campylobacter jejuni subsp. *jejuni* ATCC 8841	5/5*	0/5	5/5	0/5
Campylobacter coli ATCC 33559	5/5*	0/5	5/5	0/5
Campylobacter spp.	18/18	0/18	18/18	0/18
Results of exclusivity				
Untargeted strains**	0/10	10/10	0/10	10/10

Notes:
*** number of the test replications;**
**** referred to in the chapter "Objects".**

The data in the Table 2 above show that the LAMP method was highly specific for *Campylobacter* spp., including *Campylobacter coli* and *Campylobacter jejuni* subsp. *jejuni*. The specificity of the method was 100%. The results obtained are also confirmed by a number of specialists' studies, which proved the high specificity of LAMP amplification, since 4 primers should specifically bind to 6 separate sections of the target DNA [18].

To determine the minimum determinability (sensitivity) of the LAMP method, various concentrations of the target *Campylobacter jejuni* ATCC 8841 were tested in the artificially contaminated samples of minced meat (Table 3).

At the levels of inoculation of 10 CFU/25 g and 100 CFU/25 g, the method could detect *Campylobacter* in all samples (sensitivity amounted to 100%), and the result in all samples was confirmed by the reference method. In a similar study, the detection limit for *Campylobacter* spp. by the LAMP method accounted for 1,000 CFU/g after incubation and 1–5 CFU/sample before enrichment. At the same time, the minimum determinability (sensitivity) of the reference method ISO 10272–1:2017 [7] was lower than

that of the LAMP method, which was consistent with the study by Rajagopal et al. [19].

Table 3. The results of the test for the minimum determinability (sensitivity) of the LAMP method for *Campylobacter* spp. in the artificially contaminated samples

Level of inoculation (CFU/g)	Minced meat samples	ISO 10272–1:2017 / LAMP				Minimum determinability (sensitivity) (%)	Specificity (%)
		T_{pos}	T_{neg}	F_{pos}	F_{neg}		
10^0 (not inoculated)	Poultry	0/5	5/5	0/5	0/5	100	100
	Pork	0/5	5/5	0/5	0/5	100	100
10^1	Poultry	5/5	0/5	0/5	0/5	100	100
	Pork	5/5	0/5	0/5	0/5	100	100
10^2	Poultry	5/5	0/5	0/5	0/5	100	100
	Pork	5/5	0/5	0/5	0/5	100	100

A significant number of studies have reported that the minimum determinability (sensitivity) of PCR methods is often higher than the one of classical microbiological methods [20, 21]. When examining a non-inoculated sample (the negative control sample), both methods did not detect *Campylobacter* spp. No false negative results were obtained.

To confirm the sensitivity and performance of the LAMP method, the researchers monitored the rate of *Campylobacter* spp. detection in the objects of the production plant's environment (poultry processing plant and meat processing plant). In total 308 samples were collected and tested (Table 4).

Table 4. Minimum determinability (sensitivity) results of the LAMP method compared to the method ISO 10272–1:2017, used for detection of *Campylobacter* spp. in the samples of native contamination

Samples (swabs)	LAMP / ISO 10272–1:2017				Minimum determinability (sensitivity) (%)	Specificity (%)
	T_{pos}	T_{neg}	F_{pos}	F_{neg}		
Meat processing plant	14/152	138/152	14/152	138/152	100	100
Poultry processing plant	60/156	96/156	57/156	99/156	100	94

Note:
T_{pos}, T_{neg} are true-positive and true-negative samples confirmed by both ISO 10272–1:2017 [7] and LAMP methods; F_{pos} and F_{neg} are false-positive and false-negative samples confirmed only by the LAMP method or the ISO 10272–1:2017 method [7], respectively;
*** after the slash, the number of samples tested at the corresponding level of contamination is shown.**

During the analysis by the LAMP method, 14 positive samples were found of 152 samples of the environment in the meat processing plant; and 60 positive samples were found of 156 samples of the environment in the poultry processing plant. All positive samples, obtained by LAMP method, were confirmed according to the method ISO 10272–1:2017 [7] starting from their reinoculation from enrichment media to solified media. 3 samples (swabs) were taken from a poultry processing plant, that proved to be positive for *Campylobacter* spp. when using the LAMP method, however they gave a negative result when analyzed by traditional methods. The discrepancy in results may have been caused by the low number of *Campylobacter* spp. in the enrichment media, which was insufficient for their detection by the conventional culturing methods. Another reason could be the increased antimicrobial resistance in representatives of *Enterobacteriaceae* in recent years. The problem of *Campylobacter* detection in poultry meat samples is confirmed by a wide number of publications [22, 23, 24, 25]. It was shown that β-lactamase-resistant *Escherichia coli,* that is widespread in poultry, significantly outgrow *Campylobacter* spp. when both organisms are present in the same sample [8]. In addition, not only *E. coli*, but also other bacteria such as *Pseudomonas* spp. were found in large numbers in poultry samples and poultry processing plant's environment, and it may impede the detection of *Campylobacter* spp. [9].

The group of researchers Sabike et al. [26] evaluated the Loop-mediated Isothermal Amplification (LAMP) method, applied for direct screening of naturally contaminated chickens' cloacal swabs for *Campylobacter jejuni/Campylobacter coli* to compare this analysis with conventional quantitative microbiological methods. In the comparative study LAMP analysis of 165 broilers showed a sensitivity of 82.8% (48/58 in conventional culturing), 100% (107/107) specificity, 100% (48/48) positive predictive value, and 91.5%. (107/117) negative predictive value. LAMP analysis took less than 90 minutes from the moment of faecal samples obtaining till getting the final results from the laboratory. This suggests that LAMP analysis would facilitate the identification of *Campylobacter jejuni/Campylobacter coli.*-positive broiler flocks at the farm or at slaughterhouses prior to slaughter, making it an efficient way to prevent the spread of *Campylobacter* infection [26].

In the course of this study, it was noted that the LAMP method showed high specificity with a significantly shorter time of analysis: 24–27 hours in comparison with 5–7 days of analysis by the conventional control method of culturing.

It is reported that the sensitivity of PCR methods is often higher than that of classical microbiological methods [20,21]. In another study, an optimized LAMP analysis was tested on the cells of *Campylobacter jejuni* and *Campylobacter coli* inoculated into the samples of chicken faeces. High sensitivity has also been demonstrated in the LAMP analysis, where LOD of 1 CFU/reaction (corresponding to 50 CFU/mL) was observed for both *Campylobacter jejuni* and *Campylobacter coli* within 30 minutes of amplification [4]. This suggests that LAMP analysis would facilitate the identification of *C* at the farm or at pre-slaughter stage in

the slaughterhouses, thus making it an efficient means of preventing the spread of *Campylobacter* spp.

Detection of foodborne pathogens using traditional culturing methods is a reliable approach, but to obtain the results it is necessary to use the specialized laboratories and wait for several days, so there is no real-time information on pathogens presence or absence [27]. Molecular analysis based on LAMP is used to detect a wide range of pathogens [28,29].

Fresh chicken meat suspected of being contaminated with *Campylobacter jejuni* / *Campylobacter coli* was subjected to analysis. For these samples, analysis takes approximately 24–48 hours from the start of the cumulative phase till the pathogen was finally detected. The sensitivity of the LAMP analysis has been found to be ten times higher than the sensitivity of the equivalent PCR analysis. The increase of LAMP sensitivity can be proven both by turbidimetric analysis and by visual assessment with the naked eye. The LAMP analysis is faster and easier to perform than conventional PCR analyses, and is also more specific and requires only a simple heating block or constant temperature water bath [30]. The development of molecular methods, including the LAMP-based methods, is currently receiving a lot of attention. In the research of Kreitlow et al., the developed *LAMP analysis system based on the rplD and cdtC-gyrA genes* provided a fast, sensitive, and highly specific method for detecting and differentiating *Campylobacter jejuni* and *Campylobacter coli* in meat products [31].

Conclusion

This study represents data of validation the performance of a new fast method for detection of *Campylobacter* in poultry carcasses compared to conventional methods of culturing based on the standard ISO 10272–1:2017.

Thus, the present study describes a simple and fast LAMP analysis used for the detection of *Campylobacter jejuni* and *Campylobacter coli* in the analyzed samples. Validation of the LAMP method in comparison with the standard method of mCCDA coating showed that LAMP has a certain limit of detection (LOD) of 10 CFU/25 g. This method features good specificity as all strains of *Campylobacter jejuni* and *Campylobacter coli* were detected without failure, and no false positive results were obtained in analyses of the other tested bacterial control strains. These parameters were determined by analyzing 152 environmental samples obtained from the pork and poultry processing plant, as well as naturally contaminated pork and poultry carcasses. Only 3 samples that were found *Campylobacter* positive by conventional LAMP methods, turned out to be culture negative, ie. false negative. To validate the alternative method, all positive results were validated by the standard method ISO 10272–1:2017.

LAMP method and the reference method showed very good compliance between them. It was evident that the LAMP method was very specific with a much shorter time of analysis: 24–27 hours compared to 5–7 days of the reference method of culturing. The performance of the LAMP method was equivalent to the reference method.

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