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Detection of *Salmonella* in Foods Using a Reference PN-ISO Method and an Alternative Method Based on Loop-mediated Isothermal Amplification Coupled with Bioluminescence

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. *Salmonella*, one of the primary indicators of food safety, is a common cause of food poisoning of an epidemic nature around the world. These microorganisms can colonize the gastrointestinal tract of both people and animals, and next contaminate not only eggs, milk, meat and dairy products, but also vegetables, fruit, grains and even spices.

Objectives. The aim of this study was to analyze the frequency of detection of *Salmonella* spp. in food samples using a reference PN-ISO method and an alternative method based on loop-mediated isothermal amplification (LAMP) coupled with bioluminescence.

Material and Methods. Food samples were obtained in cooperation with the State Sanitary-Epidemiological Station in Wrocław. Dairy products, meat, fish, pastry and confectionery, vegetables, herbs and spices were analyzed. The food samples were examined using a standard culturing method according to PN-ISO 6579:2003 for *Salmonella* spp. and an alternative method based on the isothermal amplification and bioluminescence phenomenon using the 3M MDS device.

Results. In 399 tested food samples in 8 materials, using both the reference and the alternative LAMP-based method, the presence of *salmonella* was confirmed. The results obtained show the 100% sensitivity, specificity and accuracy of the presented alternative, LAMP-based technique compared to the reference PN-ISO method.

Conclusions. The alternative method using isothermal amplification and bioluminescence makes it possible to detect *Salmonella* in foods in a much shorter time than the referential culturing method (**Adv Clin Exp Med 2016, 25, 5, 945–950**).

Key words: *salmonella*, foods, isothermal amplification.

We have recently been observing dynamic changes in microbiology, especially in industrial sectors, including the food industry. Laboratories in Poland and around the world use fast, modern methods for the detection of pathogens in foods and to control the degree of microbiological pollution in the industrial environment. These new methods are particularly important while implementing the HACCP system, which requires systematic monitoring of microbial hazard in foods, making possible the execution of reliable analyses in a short time [1, 2].

Salmonella, as one of the primary indicators of food safety, is a common cause of food poisoning of an epidemic nature around the world. These microorganisms can colonize the gastrointestinal tract of both people and animals, treating it as their reservoir, while the application of natural fertilizers with contaminated animal feces contributes to the spread of salmonella in the whole

environment, contaminating not only eggs, milk, meat and dairy products, but also vegetables, fruit, grains and even spices [3].

Microbiological laboratories use conventional culturing methods as a standard technique for the detection of pathogenic bacteria in food samples, characterized by high sensitivity and specificity [4]. A 3-step assay includes premultiplication, selective multiplication and identification that enables an evaluation of the morphological and biochemical features of the microorganisms tested, and this procedure and the final confirmation of the results requires a suitably equipped laboratory and qualified staff and lasts on average between 7–10 days [5].

The prevalence of bacteria that have lost their ability to grow on solid media, so-called VBNC (viable but nonculturable), but remain metabolically active and retain the characteristics of virulence, and cannot be detected using conventional culturing methods in food samples, cause additional problems [6]. Therefore, methods with the highest sensitivity and specificity, allowing fast detection of pathogenic bacteria, are of particular importance. Immunochemical methods detecting microbial organisms and their toxins involving antibodies are used widely in microbiological diagnosis [7]. In addition to the typical serological tests done in laboratories, automated VIDAS immunoanalyzers (Bio Mérieux), techniques using the bioluminescent chemistry of enzymes or immunomagnetic methods are used [8, 9].

More and more frequently, methods of the molecular identification of pathogenic microorganisms in foods, associated with a specific analysis of their sequence of nucleic acids, are used [10]. In the case of fluorescent *in situ* hybridization (FISH), fluorescently marked DNA probes (short oligonucleotides) are used, which, on the principle of complementarity, bind specifically with ribosomal RNA fragments of the microorganisms being identified [11].

The use of thermostable polymerase and adequate primer sequences allows the detection of microorganisms based on the polymerase chain reaction (PCR), which provides quick and sensitive identification even with a small amount of nucleic acids in a sample of food [12]. Due to the high sensitivity of the polymerase to environmental factors, this method may give false negative results, while the lack of ability to differentiate live microorganisms from dead ones with the PCR method may result in false positive results. A multiplex PCR technique, using more than one set of primers, allows for simultaneous detection of several fragments of the target nucleic acids. Real-time PCR is another modified PCR technique, which allows you to obtain quantitative results by determining the amount of PCR products [13, 14].

The RT-PCR (reverse transcriptase PCR) method allows for the distinction between live and dead microorganisms identified in the sample. The synthesis of single-strand DNA by reverse transcriptase (RT) is possible only on the basis of an mRNA template, which is present only in living cells [15].

The disadvantages of the PCR method include the need to use expensive thermocyclers, its limited specificity and efficiency of amplification, the use of highly purified nucleic acids and electrophoretic separation of reaction products [16].

A much faster, more accurate and cheaper method is loop-mediated isothermal amplification (LAMP), whose reliability has been confirmed in numerous reports and made possible the development of commercially available diagnostic kits [17]. The LAMP method has been included in the list of diagnostic methods officially recommended to perform a routine identification and analysis of pathogens, because it allows for a simple reaction on the heating block and amplification of the selected DNA sequence with a high sensitivity and specificity in isothermal conditions. It makes possible the multiplication of a DNA fragment up to 109 copies an hour, so in less time than the standard PCR procedures.

The LAMP developed for *Salmonella* spp. is based on the specific amplification of the conserved region of DNA. The 3M^{TM} Molecular Detection System links two techniques, isothermal DNA amplification and real-time bioluminescence detection. This combination increases the sensitivity and specificity of the results. Bst DNA polymerase used in the LAMP method possess activity exchange of DNA strands but does not have exonuclease activity, thus the resulting product has a very high specificity for the matrix. Subsequent cycles of extension and strand displacement are carried out using polymerase, and two or three pairs of primers that recognize six or eight different regions of the bacterial genome of the matrix.

The high specificity of the LAMP method in comparison with PCR is associated with a greater number of primers, which can recognize several different sequences on the DNA matrix, while in the PCR method, usually two primers specific to a given DNA sequence are used [18].

Other genetic methods are becoming more and more popular as well: DNA microarrays or different types of biosensor techniques, which are based on the specific relation of DNA probes with target fragments of nucleic acids of the identified microorganism. The detected DNA segments are characteristic of the identified microorganisms or genes coding their virulence factors [19–21].

The aim of this study was to analyze the frequency of detection of bacteria of the genus *Salmonella* spp. in food samples using standard culturing methods according to PN-ISO 6579:2003 *Salmonella* spp. and compare those findings with results obtained by means of an alternative method based on the isothermal amplification and bioluminescence phenomenon using the 3M MDS device – 3M^{TM} Molecular Detection System.

Material and Methods

Food samples were obtained in cooperation with the State Sanitary-Epidemiological Station in Wrocław. Dairy products, meat, fish, pastry and confectionery, vegetables, herbs and spices were analyzed – Table 1.

Two methods were used at the same time: The reference method using standard microbiological mediums and an alternative method based on the application of a molecular diagnostic device produced by 3M, which is intended to work with 3M molecular diagnostic tests, to quickly detect pathogens in foods by using the isothermal amplification and bioluminescence phenomenon – 3M^{TM} Molecular Detection System.

We determined the presence of *salmonella* bacteria with a reference method according to PN ISO 6579:2003 [22]. Premultiplication was done in peptone buffered water (37°C within 18–24 h), and then sieved on the selective medium: Rappaport-Vassiliadis (41.5°C within 18–24 h) and Muller-Kauffmann with tetrathionate and novobiocin (37°C within 18–24 h). After incubation, the bacteria were cultured on the selective-differential medium XLD, containing lysine, xylose and sodium deoxycholate, and a medium defined by Hektoen, with lactose, sucrose, sodium thiosulphate and ammonium ferric citrate. Growth on the selective media made initial identification of these bacteria possible by assessing the morphological features of the colonies grown, which then was confirmed with a biochemical analysis of the analyzed microorganisms.

The alternative method used 3M Molecular Detection Assay for *salmonella* tests, which are used with the Molecular Detection System (MDS) for the quick detection of these bacteria in food samples [23]. Premultiplication was carried out in standard peptone buffered water at 37°C within 18–24 h. The procedure related to the use of 3M MDS tests involved the transfer of the multiplied culture to tubes with a lysing solution and lysis for 15 min at 100°C in the first stage, and then a quick cooling within 10 min at room temperature. In the next stage, the test samples were treated with the lysing solution and transferred to the tubes with a reactive set, in which the isothermal amplification and detection of *salmonella* take place simultaneously within 75 min.

The 3M^{TM} Molecular Detection System device uses the LAMP method in combination with the bioluminescence technique to detect the presence of pathogens in food samples in real-time. The detection process is based on a two-step enzymatic reaction in which pyrophosphate, produced as a by-product of DNA amplification, is converted to adenosine triphosphate (ATP), which is processed by thermostable luciferase in the signal light.

This system provides qualitative results, and indicates only the presence or absence of the pathogen. The 3M^{TM} Molecular Detection System makes it possible to obtain positive results within 75 min when a target sequence is amplified, while the negative ones are presented at the end of the process.

Statistical analyses were performed according to the protocol of the validation of alternative methods [24] using the statistical software package STATISTICA^{TM} v. 10.0. The data obtained was cal-

Table 1. Comparison of alternative LAMP-based and reference PN-ISO methods for the detection of *Salmonella* spp. in food samples

Foods samples (n)	Results confirmed by both reference and alternative methods		Results confirmed only by reference or alternative method		Sensitivity (%)	Specificity (%)	Accuracy (%)
	TP	TN	FP	FN			
I – (107)	4/107	103/107	0/107	0/107	100	100	100
II – (167)	2/167	165/167	0/167	0/167	100	100	100
III – (70)	1/70	69/70	0/70	0/70	100	100	100
IV – (40)	1/40	39/40	0/40	0/40	100	100	100
V – (15)	0/15	15/15	0/15	0/15	NA	100	100

I – meat; II – pastry and confectionery; III – dairy products; IV – vegetables, herbs and spices;
V – fish; TP – true positive; TN – true negative; FP – false positive; FN – false negative; NA – not analyzed.

culated as follows: sensitivity [TP/(TP+FP)] × 100, specificity [TN/(TN-FN)] × 100 and accuracy [(FP+FN)/(TP+TN)] × 100 – Table 1.

Results and Discussion

In the studies on 399 tested food samples, divided into 8 materials, using both the reference and the alternative method, the presence of *salmonella* was confirmed – Fig. 1. The results obtained showed 100% sensitivity, specificity and accuracy of the alternative LAMP-based technique presented, compared to the reference PN-ISO method – Table 1.

Microbiological tests of food samples are routinely performed using standard culturing methods, which, in the case of detecting enteropathogens, require several-day incubation in different media, which with having to confirm the results obtained by using biochemical and serological tests, significantly increases the time of laboratory analysis. Therefore, alternative methods of identifying the most important food-borne pathogenic bacteria, usually by the detection of pathogen-specific DNA sections, are becoming more popular [25].

Salmonella bacilli are obligatory bacterial pathogens, which is why it is very important to detect their presence in food products. Thermal treatment of food and a range of procedures in food production can lead to the destruction of these bacteria or sublethal damage to their cells, so each of the methods identifying these microorganisms in foods is preceded by a phase of premultiplication (non-selective multiplication), which reduces the probability of obtaining a false negative result. Food products also create a specific environment for microorganism development, due to the varied salt content or all the kinds of preservatives that may further inhibit not only the growth

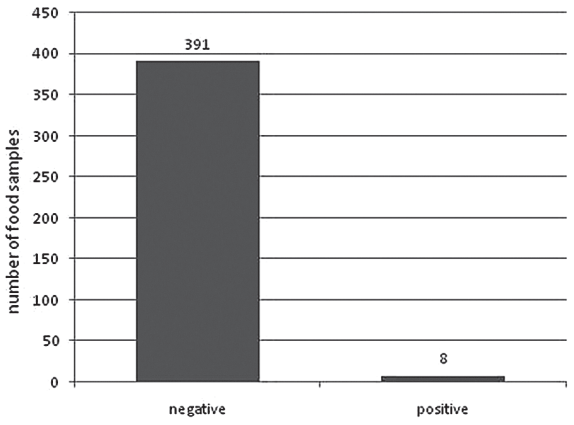

Fig. 1. Detection of *Salmonella* in foods using reference PN-ISO method and alternative method

of bacteria, but also the identification process, in particular one using molecular methods [26].

In tests conducted by Zadernowska et al. [27], the presence of bacilli *Salmonella* spp. in broth cultures was being detected. The results obtained with three methods (FISH, SLMX, ISO) were exactly the same, which confirms the high specificity of the alternative methods. On the other hand, in studies on meat contaminated with a mixture of *Salmonella* spp. and other bacteria of the *Enterobacteriaceae* family, some discrepancies in the results obtained were observed. With both the FISH and enzyme immunoassay methods, bacilli of *Salmonella* spp. in all trials were found, while with FISH, no positive results in trials non-contaminated with *Salmonella* spp. of the *Enterobacteriaceae* family were obtained, and SLMX tests showed false positive results, which, as the authors indicate, may be due to cross reactions with antigens of other bacilli of the *Enterobcteriaceae* family found in the test samples of meat.

Currently, fast techniques for identifying pathogens found in food based on DNA structure analysis are some of the most important technical solutions in molecular microbiology. In experimental studies, Misiewicz et al. [28] contaminated homogenized cheese samples with *Salmonella* in order to detect the microorganisms with the use of alternative molecular methods. In each contaminated food sample, using real-time PCR, bacteria of the genus *Salmonella* were found and an analysis was performed using the classical PCR method to confirm the presence of the bacteria's DNA in the samples. The results confirmed by the classical PCR were consistent with the results obtained in the real-time PCR reaction, however, they were obtained in a much shorter time than in classical PCR as this technique does not require electrophoretic separation.

In other studies, the sensitivity and specificity of *Salmonella typhi* detection with the use of three methods were compared: standard culturing method, conventional PCR and LAMP technology with the use of isothermal polymerase, which yielded 10 times greater sensitivity in the detection of these pathogens, compared to PCR [29].

The results obtained by Chen et al. [30] indicate that the isothermal amplification LAMP method enables quick and sensitive detection of various serotypes of *Salmonella*, which was confirmed when these bacteria-specific genes were used as primers (*Salmonella invE* gene and 3 serotype-specific genes: fliC, lygD and STM4495), which allowed the identification of strains of *S. Choleraesuis*, *S. Enteritidis* and *S. Typhimurium*.

Salmonella spp., as a typical indicator of bacteria in food, are often used as target microorgan-

isms for the evaluation of various new techniques which are developed with the use of DNA biosensors, usually electrochemical or piezoelectric. Currently, there are a number of methods for the detection and identification of microorganisms in food [31]. Taking into account the nature and extent of the risks associated with the spread of infections transmitted by contaminated food pathogens, it is important to use the latest technological advances, particularly in the intensely-developing field of molecular biology in recent years, to develop new tests and equipment to enable fast and effective laboratory diagnosis.

The authors concluded that the alternative method using isothermal amplification and bioluminescence makes it possible to detect bacteria of the genus *Salmonella* in foods in a much shorter time than the referential culturing method.

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