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Validation of a Commercial Loop-Mediated Isothermal Amplification (LAMP)-Based Kit for the Detection of *Salmonella* spp. According to ISO 16140:2016

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Featured Application: The validated LAMP kit provides an accurate method for the rapid detection of *Salmonella* spp., offering significant advantages over the traditional method, as it is characterised by a high sensitivity, easiness of use for laboratory testing, and a large reduction in the analysis time, making it a valuable asset to the food industry.

Abstract: The traditional cultural method (PCR and Real-Time PCR) for *Salmonella* spp. detection and identification is laborious and time-consuming. A qualitative LAMP method detecting *Salmonella* spp. was validated in compliance with ISO 16140:2016. The results show a relative accuracy, sensitivity, and specificity of 100% in comparison with the reference method ISO 6579-1:2017; the LOD50 was set as 0.4 CFU/g. Additionally, a field study was carried out comparing the LAMP kit, a commercially available Real-Time PCR kit (FoodProof *Salmonella*, Biotecon Diagnostics), and the reference cultural method. The *Salmonella* spp. LAMP kit was suitable for reliable detection of *Salmonella* spp., simplifying and reducing the extent and the steps of the analytical process. A total of 105 samples of raw poultry meat were screened for the presence of *Salmonella* spp. according to three methods: the LAMP kit *Salmonella* spp. (Enbiotech), the Real-Time PCR kit FoodProof *Salmonella* (Biotecon), and the reference cultural method. Using these three methods, only one sample out of the 105 (0.95%) tested was positive for *Salmonella* spp. This sample was further investigated using the reference method described in ISO 6579-3:2014, in order to characterise the *Salmonella* strain. Following this further biochemical identification and serological typing, the isolate was characterised as *Salmonella* Infantis.

Keywords: LAMP; *Salmonella*; ISO 16140:2016; food-borne; validation; specificity; sensitivity; accuracy; kit

1. Introduction

Salmonella is a highly relevant food-borne pathogen of large economic significance for both animals and humans. *Salmonella* outbreaks caused 94,530 human cases in the EU only in 2016, with the highest burden relating to the number of hospitalizations (1766) and deaths (10) [1]. *S.* enteritidis, in particular, accounted for 59% of all *Salmonella* infection cases originating in the EU. The principal reservoirs are the intestines of a wide variety of animals, resulting in the contamination of different foodstuffs, both of animal and plant origin [2,3]. Indeed, *Salmonella* is associated mainly with raw food, subject to faecal contamination, including poultry, raw meat, seafood, egg, and dairy products [4]. In addition, it was also found to be the most common bacterial pathogen responsible for produce outbreaks [5].

Therefore, in EU countries, surveillance of *Salmonella* infections in humans is compulsory, and also for food-producing animals and food thereof. In order to guarantee food security, the availability of reliable methods to identify this pathogenic bacterium is becoming increasingly relevant to the food industry, as well as for official controls.

The traditional cultural method [6] requires more than five days to determine a positive result, besides being laborious and time-consuming. For this reason, to use the traditional cultural method is not very suitable for high-throughput screening of large numbers of food samples for the presence of *Salmonella* cells [7,8]. Less laborious and faster alternative methods for pathogen detection in foods have been developed [8–12]. Among them, the method that combines loop-mediated isothermal amplification (LAMP) with bioluminescence detection stands out as a reliable, faster, and simpler approach than conventional culture methods. LAMP was developed by Notomi et al. in 2000 [9]. It is a method that amplifies DNA with high specificity, efficiency, and rapidity, utilizing a DNA polymerase enzyme with high strand displacement activity and two pairs of primers recognizing six independent sequences of a target gene under isothermal conditions [12]. Subsequently this method has been implemented by Nagamine et al. in 2002, incorporating forward loop primers that accelerate the LAMP reaction and reducing costs as a consequence [13]. Due to its high sensitivity and low cost, LAMP has been applied for pathogen detection screening of large numbers of food samples, and has successfully been used to detect many pathogens, including *Salmonella* spp. [14]. In recent years, some kits based on LAMP have been commercialised and their performance has been positively evaluated [8,12]. Hence alternative methods are catching on, as molecular methods are more rapid and have an interesting potential to be used for screening, revealing a preliminary result, even if ISO 6579-1:2017 [6] still remains necessary to isolate the microorganisms for further characterization. ISO 16140-2:2016 [15] defines the procedures for validation of alternative microbiological methods against the cultural method, measuring the concordance of the results for both methods.

A *Salmonella* LAMP (loop-mediated isothermal amplification) assay was validated in this study as a novel specific and cost-effective nucleic acid amplification method for bacterial detection and identification. This innovative method is characterised by six primers that specifically recognise eight different regions on the target gene [9].

In comparison to PCR and Real-Time PCR, LAMP has many advantages: reaction simplicity, as it can be performed by semi-skilled staff, even in a heating block without any thermal cycler; and detection sensitivity, displaying a 10–100-fold higher sensitivity than PCR [16]. In addition, LAMP shows a higher amplification efficiency and the enzyme commonly involved, Bst DNA polymerase, has shown not to be inhibited by the presence of anticoagulants, NaCl, hemin, and other PCR-interfering substances [17]. Due to its simplicity, the LAMP technique has initially been applied to diagnosis, but recently it has also been extended to genetically modified organisms and identification of meat and fish species in food products [18]. For these reasons, it represents an ideal candidate for point-of-care diagnosis and when rapid results are needed, such as in food industry, where *Salmonella* spp. positive samples need to be immediately blocked for public health and safety.

In this study, we evaluated the LAMP method through validating the kit by comparing it to different methods that are currently the most commonly used.

2. Materials and Methods

All the processed samples used for the method optimisation and validation came from a large-scale distribution, in order to reduce any bias from local food specialities and extend the range of the validation. Samples were chosen as positive samples for the validation of the method and for matrix effects evaluation. All the food samples came from Italian supermarkets.

2.1. DNA Extraction Genomic

Several samples were tested, such as heat-processed milk and dairy products, raw poultry and ready-to-cook poultry products, eggs and egg products (derivate), ready-to-eat and ready-to-reheat fishery products, as well as fresh produce and fruits.

The kit *Salmonella* spp. (Enbiotech) provides a rapid preliminary DNA extraction from food matrices. In the pre enrichment phase, 25 g of a sample was taken and homogenated with 225 mL of Buffered Peptone Water (BPW). After a 22 ± 2 h enrichment in Buffered Peptone Water (BPW) at 37 °C.

The DNA extraction was performed using a ready-to-use buffer contained in the Salmonella Screen Glow kit (Enbiotech). Then, 250 ± 50 mg of a sample was directly placed into 15 mL tubes containing 4 mL of the ready-to-use extraction buffer (Enbiotech) and then incubated for 40 ± 5 min at room temperature.

Genetic amplification using LAMP technology and real-time detection of the results using the dedicated device ICGENE mini (Enbiotech). The kit is ready-to-use and includes:

- DNA extraction buffer, through chemical lysis;
- Tubes strip containing lyophilised primers;
- Amplification master mix;
- Mineral oil;
- Positive control;
- Negative control;
- Sterile water.

2.2. LAMP Assays

The analytical and diagnostic assays to recognise *Salmonella* spp. DNA was performed using the Salmonella Screen Glow commercial kit (Enbiotech) with an ICGENE mini portable instrument (Enbiotech), consisting of a real-time fluorimeter, monitored and regulated by the the ICGENE application (Enbiotech), and downloadable on various smart devices. The Salmonella Screen Glow commercial kit includes ready-to-use reaction tubes (containing primers, fluorescent dye, etc.) to achieve a rapid amplification of the DNA template. The protocol to obtain the specific amplification of the target *Salmonella* spp. DNA was carried out in a mixture with a final volume of 55 µL, including 22 µL of the Salmonella Screen Glow LAMP mix (Enbiotech), 30 µL of mineral oil, and 3 µL of the extracted DNA samples. The mineral oil was added to the top of the reaction mixture to prevent evaporation. The amplification was optimised and performed at 65 °C for 35 min. Real-time monitoring of the fluorescence associated with the amplification was possible using the fluorimeter of the ICGENE portable instrument and the ICGENE application interface.

2.3. Validation Plan

The study was carried out at the Food Microbiology Laboratory of the Istituto Zooprofilattico Sperimentale of Sicily, Palermo (Italy), according to the validation process explained by ISO 16140-2:2016 [15]. According to ISO 16140, we demonstrated that the results obtained with the alternative method (LAMP kit) were comparable (at least equivalent) to the results obtained with the reference method. Following the validation protocol described by ISO 16140-2:2016 [15], we did a comparative study of the alternative method with the

corresponding reference method, conducted by the Food Microbiology Laboratory of the Istituto Zooprofilattico Sperimentale of Sicily, Palermo (Italy). Validation determined the following parameters: relative limit of detection (RLOD), inclusivity, exclusivity and a method comparison study including relative accuracy (AC), relative specificity (SP), and sensitivity (SE). Examining the samples both with the alternative (LAMP kit) method and the reference method, the parameters of AC, SP, and SE were calculated as follows:

$$AC = (PA + NA)/N \times 100\% \quad (1)$$

$$SP = (NA)/N_{-} \times 100\% \quad (2)$$

$$SE = (PA)/N_{+} \times 100\% \quad (3)$$

where PA = positive agreement; NA = negative agreement; N = total number of samples; N_{-} = number of negative samples; and N_{+} = number of positive samples.

The RLOD was calculated as follows:

$$RLOD = LOD50 \text{ alternative method}/LOD50 \text{ reference method} \quad (4)$$

where LOD50 = the limit of detection (LOD) of 50% = the smallest amount of analyte that can be detected but not quantified with a 50% probability. Therefore, LOD50 is the level of detection for which 50% of tests give a positive result [19].

RLOD, SP, and SE were performed against the reference method ISO 6579-1:2017 [6] "Microbiology of the food chain-Horizontal method for the detection, enumeration and serotyping of *Salmonella*-Part 1: Detection of *Salmonella* spp."

2.4. Bacterial Strains

Bacterial strains were maintained on cryogenic beads at –20 °C; before use, the beads were placed in Columbia Blood Agar (CBA-Microbiol) plates and incubated at 37 °C for 18–24 h. Subsequently, the bacterial strains were placed in Tryptone Soya Agar (TSA-Microbiol) tubes, incubated at 37 °C for 18–24 h and maintained at 4 °C for two weeks. For RLOD and the comparative studies, a strain of *Salmonella* enteritidis ATCC 13,076 was used. For the inclusivity study, field strains cultures coming from food samples were used, which were identified and confirmed by ISO/TR 6579-3:2014 [20].

Inclusivity of the LAMP method was evaluated by testing 25 pure cultures of the target microorganisms, while exclusivity was determined by testing 30 pure cultures of species other than *Salmonella* spp. (Table 1).

A comparative study was performed comparing the LAMP kit and the reference method. In particular, this study allowed evaluating the relative accuracy, relative specificity, and sensitivity. Food samples were chosen based on the categories given in Table A1 of ISO 16140-2:2016 [15].

In particular, five categories were chosen among the most relevant:

- Heat-processed milk and dairy products;
- Raw poultry and ready-to-cook poultry products;
- Eggs and egg products (derivate);
- Ready-to-eat, ready-to-reheat fishery products;
- Fresh produce and fruits.

For each category, 60 samples were tested, made up of 3 specific typologies with 20 samples representative of each typology (3 typologies × 20 samples for each = 60 samples per category). Of tested samples per typology, 50% (i.e., 10) were negative and 50% were spiked and hence positive.

RLOD tests were run on the same five food matrices of the comparative study: heat-processed milk and dairy products; raw poultry and ready-to-cook poultry products; eggs and egg products (derivates); ready-to-eat, ready-to-reheat fishery products; and fresh produce and fruits.

Table 1. *Salmonella* spp. tested for inclusivity and species used for exclusivity testing.

Microorganism				
	Tested for Inclusivity		**Species Used for Exclusivity Testing**	**Code**
1	*S. Livingstone*	1	*Aeromonas hydrophila*	ATCC 35650
2	*S. Heron*	2	*Arcobacter butzleri*	NCTC 12481
3	*S. Corn*	3	*Bacillus cereus*	ATCC 11778
4	*S. Madelia*	4	*Bacillus cereus*	B25052
5	*S. Typhimurium (monophasic)*	5	*Bacillus subtilis*	BCS51
6	*S. Thompson*	6	*Campylobacter coli*	ATCC 33559
7	*S. Virchow*	7	*Campylobacter jejuni*	ATCC 33291
8	*S. London*	8	*Citrobacter freudii*	ATCC 8990
9	*S. Typhimurium*	9	*Clostridium bifermentans*	CBIF107
10	*S. Kissi*	10	*Clostridium perfringens*	ATCC 13124
11	*S. Blocklei*	11	*Escherichia coli*	ATCC 25922
12	*S. Toulon*	12	*Escherichia coli O157*	ATCC 35150
13	*S. Halle*	13	*Enterobacter cloacae*	Not available
14	*S. Abony*	14	*Enterococcus faecium*	EFC49
15	*S. Messina*	15	*Enterobacter sakazakii*	ATCC 29544
16	*S. Montevideo*	16	*Listeria innocua*	ATCC 33090
17	*S. Potsdam*	17	*Listeria ivanovii*	ATCC 19119
18	*S. Muenster*	18	*Listeria monocytogenes*	ATCC 7684
19	*S. Larochelle*	19	*Listeria seeligeri*	Not available
20	*S. Newport*	20	*Micrococcus luteus*	ATCC 9341
21	*S. Hadar*	21	*Psudomonas aeruginosa*	ATCC 10145
22	*S. Poona*	22	*Rhodococcus equi*	ATCC 6939
23	*S. Muenchen*	23	*Staphylococcus aureus*	ATCC 25923
24	*S. Derby*	24	*Staphylococcus aureus*	ATCC 38862
25	*S. Kottbus*	25	*Shigella sonnei*	ATCC 9290
		26	*Streptococccus agalactiae*	STRA41
		27	*Vibrio cholerae*	ATCC 1473A
		28	*Vibrio parahaemolyticus*	ATCC 17802
		29	*Vibrio vulnificus*	ATCC 27562
		30	*Yersinia enterocolitica*	ATCC 23715

From TSA, *Salmonella* enteritidis was inoculated in Xylose Lysine Deoxycholate agar (XLD-Microbiol) plates to obtain isolated colonies. XLD agar plates were incubated at 37 °C for 24 h.

In order to calculate the RLOD value, each of the five categories was spiked with the target microorganisms at three levels of contamination. In particular, they were 5 replicates of negative samples (0 CFU), 20 replicates of the lowest detection level (0.4 CFU/g), and 5 replicates of a higher contamination level (4 CFU/g), for a total of 30 contaminated samples for each food category. In total, 25 g of each the different food samples were inoculated with the corresponding level of contamination and the samples were then stabilized at room temperature or 4 °C, depending on food typology and its storage temperature. Subsequently, the reference and the alternative methods were performed.

3. Results

The method was optimised for the DNA extraction phase by testing in triplicate the initial weight of the samples at 250 mg. The extract was tested with three levels of contamination: 0, 0.4, and 4 CFU/g.

3.1. Validation

As for inclusivity and exclusivity, the results showed all samples were correctly recognised; as a matter of fact, all target microorganisms were identified, while the relevant range of other species tested did not interfere.

The relative accuracy, specificity, and sensitivity of each food category are reported in Table 2.

Table 2. Relative accuracy, relative sensitivity, and relative specificity of the alternative method (*Salmonella* spp.).

Category	PA	NA	PD	ND	N	AC	SE	SP
Heat-processed milk and dairy products	30	30	0	0	60	100%	100%	100%
Raw poultry and ready-to-cook poultry products	30	30	0	0	60	100%	100%	100%
Eggs and egg products (derivates)	30	30	0	0	60	100%	100%	100%
Fresh produce and fruits	30	30	0	0	60	100%	100%	100%
Ready-to-eat, ready-to-reheat fishery products	30	30	0	0	60	100%	100%	100%
Heat-processed milk and dairy products	30	30	0	0	60	100%	100%	100%
Raw poultry and ready-to-cook poultry products	30	30	0	0	60	100%	100%	100%
Eggs and egg products (derivates)	30	30	0	0	60	100%	100%	100%
Fresh produce and fruits	30	30	0	0	60	100%	100%	100%
Ready-to-eat, ready-to-reheat fishery products	30	30	0	0	60	100%	100%	100%
Heat-processed milk and dairy products	30	30	0	0	60	100%	100%	100%

The results showed 100% for all three parameters for all the food categories; hence, neither false-positive nor false-negative samples were detected, with this 100% performance consistent with the reference method.

The RLOD value obtained was 1 for all the categories; therefore, the same LOD was reached both for the LAMP kit and for the reference method, while the detection limit was set at 0.4 CFU/g for all food categories (Table 3). At this concentration, false-negative results were found with the LAMP method with the commercialised kit: a sample of the heat-processed milk and dairy products and three samples of raw poultry and ready-to-cook poultry products (Table 3). For the detection limit of 0.4 CFU/g, the alternative (LAMP kit) method proved to be less sensitive than the traditional ones.

Table 3. Data for RLOD calculation (N. tot = total number of samples; N. pos ref = number of positives with the reference method; N. pos kit = number of positives with the *Salmonella* spp.).

Category	Contamination Level	CFU/g	N. Tot	N. Pos Ref	N. Pos Kit
Heat-processed milk and dairy products	1	0	5	0	0
	2	0.4	20	20	19
	3	4	5	5	5
Raw poultry and ready-to-cook poultry products	1	0	5	0	0
	2	0.4	20	20	17
	3	4	5	5	5
Eggs and egg products (derivates)	1	0	5	0	0
	2	0.4	20	20	20
	3	4	5	5	5
Fresh produce and fruits	1	0	5	0	0
	2	0.4	20	20	20
	3	4	5	5	5
Ready-to-eat, ready-to-reheat fishery products	1	0	5	0	0
	2	0.4	20	20	20
	3	4	5	5	5

3.2. Field Study

A total of 105 samples of raw poultry meat were screened for the presence of *Salmonella* spp. according to three methods: the LAMP kit *Salmonella* spp. (Enbiotech), the Real-Time PCR kit FoodProof *Salmonella* (Biotecon), and the reference cultural method. Using these three methods, only one sample out of the 105 (0.95%) tested was positive for *Salmonella* spp. This sample was further investigated using the reference method described in ISO.

4. Discussion

In recent years, different diagnostic approaches have been developed for detecting various food pathogens, including innovative molecular methods.

Salmonella infections have been declining constantly since the implementation of EU control measures in poultry in 2007, although the data for 2016 showed a relevant increase of 11.5% in the number of cases compared to the previous year [1], underlining the need for continued risk management plans both at the state and at the food industry level.

In this context, the use of alternative methods, such as the "*Salmonella* spp." kit that can rapidly identify pathogenic bacteria, is of great relevance, provided they are validated against the standardized reference method as stated by ISO 16140-2:2016 [15].

The results obtained by the validated method in the comparative studies were equivalent to the microbiological reference method, hence providing a valid alternative to the cultural method. The relative sensitivity was found to be 100% for all the food typologies examined, confirming the absence of inhibition by different kinds of substrates. Moreover, the kit is characterised by a 100% specificity, as it does not amplify the other species tested, and it is inclusive of at least 25 serovars of *Salmonella* evaluated. At the detection limit of 0.4 CFU/g, the LAMP kit showed false-negative results for 4 out of 100 samples; but, even though there is a lower sensitivity than the traditional methods, it still has the advantage in terms of speed of execution and ease of use.

During this study, we also compared the LAMP kit with another commercially available diagnostic method: Real-Time PCR in raw poultry samples. The results indicate that all the methods were in good agreement, even if a limitation of this study is the scarcity of positive results (i.e., 1 sample) that could hinder a more deepened evaluation. No problems of PCR inhibition were found using the internal amplification control provided in the kit; hence, in negative samples, the absence of pathogenic microorganisms was effectively determined.

Another feature of this study is that out of the 105 poultry samples screened for the presence of *Salmonella* spp., only 1 positive sample occurred, with a prevalence of 0.95%. Samples were bought in different retail markets in order to have a more realistic representation of Sicilian poultry contamination. In fact, poultry flocks, particularly chicken, are frequently colonized with *Salmonella* without any detectable symptoms by horizontal and vertical transmission at the primary production level [21].

In European countries, the percentage of *Salmonella*-positive samples from fresh broiler meat is quite higher (4.85%) [22], even if, besides retail, also samples from slaughterhouse and processing plants are included.

Although our prevalence is rather low, this should be taken as an additional motivation for the continuous control of this pathogens, as an effective implementation of control measures could still decrease the prevalence, producing safer food. Constant monitoring is mandatory to avoid new difficulties, such as the increasing antibiotic resistance in *Salmonella* spp. that has become a severe issue for public health at a global level [23].

5. Conclusions

The data in this study support the suitability of the *Salmonella* spp. kit for commercial use on different food samples, including egg products and poultry meat, which are the foods most associated with salmonellosis.

Therefore, the validated LAMP kit provides an accurate method for the rapid detection of *Salmonella* spp., offering significant advantages over the traditional method, as it is characterised by a high sensitivity (up to 0.4 CFU/g), easiness of use for laboratory testing, and a large reduction in the analysis time (about 26 h to obtain definitive results), making it a valuable asset to the food industry. Despite the LAMP kit being less sensitive than the traditional methods, the great rapidity and ease of use suggest that the LAMP assay can be a valid alternative for routine examination in the food sector and for screening of large numbers of food samples.

In our study we isolated the *S.* Infantis strain from the positive sample. In the EU, an increased occurrence of various serotypes implicated in human infections, including *S.* Infantis, has been reported, related to poultry meat [24]. The increase in *S.* Infantis has been associated with the propagation of various clones of broiler origin in different European countries, including the dominant Hungarian clone [25].

Continuous monitoring to detect *Salmonella* along the food chain is of critical importance for public health, above all in the poultry meat industry, as poultry meat is one of the most consumed meats globally and thus one of the most traded meat products.

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