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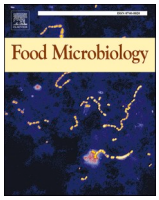

Phage amplification coupled with loop-mediated isothermal amplification (PA-LAMP) for same-day detection of viable *Salmonella* Enteritidis in raw poultry meat

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ABSTRACT

Salmonella Enteritidis is the main serotype responsible for human salmonellosis in the European Union. One of the main sources of *Salmonella* spp. in the food chain are poultry products, such as eggs or chicken meat. In recent years, molecular methods have become an alternative to culture dependent methods for the rapid screening of *Salmonella* spp. In this work, the strain *S.* Enteritidis S1400, and previously isolated and characterized bacteriophage PVP-SE2, were used to develop and evaluate a same-day detection method combining Phage Amplification and Loop-mediated isothermal amplification (PA-LAMP) to specifically detect viable *S.* Enteritidis in chicken breast. This method is based on the detection of the phage DNA rather than bacterial DNA. The virus is added to the sample during pre-enrichment in buffered peptone water, where it replicates in the presence of viable *S.* Enteritidis. The detection of phage DNA allows, on the one hand to detect viable bacteria, since viruses only replicate in them, and on the other hand to increase the sensitivity of the method since for each infected *S.* Enteritidis cell, hundreds of new viruses are produced. Two different PA-LAMP detection strategies were evaluated, a real time fluorescence and a naked-eye detection. The present method could down to 0.2 fg/μL of pure phage DNA and a concentration of viral particles of 2.2 log PFU/mL. After a short Salmonella recovery step of 3 h and a co-culture of 4 h of the samples with phage particles, both real-time fluorescence and naked-eye method showed a LoD_{95} of 6.6 CFU/25 g and a LoD_{50} of 1.5/25 g in spiked chicken breast samples. The entire detection process, including DNA extraction and LAMP analysis, can be completed in around 8 h. In the current proof-of-concept, the novel PA-LAMP obtained comparable results to those of the reference method ISO 6579, to detect *Salmonella* Enteritidis in poultry meat.

1. Introduction

Foodborne diseases are one of the main public health problems worldwide with 600 million cases every year and causing 420 000 deaths of which 125 000 are children under 5 years (World Health Organization, 2015). Also, 110 billion US$ are lost each year as a result of unsafe food in low- and middle-income countries (World Health Organization, 2015). In addition, the emergence of foodborne multidrug-resistant strains results in more complicated infections increasing death rate (Pulingam et al., 2022). This requires an integrated farm-to-fork control of bacterial hazards. *Salmonella* spp. is one of the major foodborne pathogens worldwide. In the case of Europe, it is the second most frequently reported foodborne pathogen. In 2021, 60 050 cases were confirmed (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). This genus is composed by more than 2600 different serotypes being *S.* Typhimurium and *S.* Enteritidis those primarily responsible for human salmonellosis cases. In 2021 *S.* Enteritidis accounted for 54.6% of the human infections reported, while *S.* Typhimurium, and its monophasic variant, were responsible for 20.2% (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). Although *Salmonella* can be present in a wide range of foods, poultry products such as eggs and meat are the main source of this pathogen in the food chain (European Food Safety Authority and European Centre for Disease

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Prevention and Control, 2022). The EU produced 9.8 million tons of chicken meat in 2022, and imported high value poultry products, including breast meat and poultry preparations from Brazil, Thailand and Ukraine (Development, European Commission-Directorate-General for Agriculture and Rural). The production of chicken meat is strategic because it is one of the most economical meats and one of the most consumed worldwide. The European Union established in its Regulation (EC) n° 2160/2003 (European Commission, 2003) control measures to reduce the prevalence of *Salmonella* in poultry production and according to Regulation (EC) n° 646/2007 (European Commission, 2007) the aim is to reduce the prevalence of *S.* Typhimurium and *S.* Enteritidis below 1% in broiler flocks. Regulation (EC) n° 1086/2011 (European Commission, 2011) established the criterion of absence of *S.* Typhimurium and *S.* Enteritidis in 25 g of fresh poultry meat.

The control of *Salmonella* in the food chain has been based on classical culture-based methods for many years. Despite its usefulness, these methods are lengthy and laborious. In the specific case of *Salmonella*, the reference method ISO 6579:2017 (International Organization for Standardization, 2020) described by the International Organization for Standardization (ISO) requires from three to six days only to confirm the Presence/Absence of *Salmonella* in food products and farm environmental samples, including several hands-on-steps. This is a limiting factor for short shelf life products such as chicken meat. It is therefore of crucial importance to develop methods to minimise the time needed for the detection of *Salmonella*.

Molecular methods have emerged in the last years as fast and reliable alternatives to classical microbiology. Most of the methods developed so far, including those commercially available, are based primarily on the use of the Polymerase Chain Reaction (PCR), more specially on quantitative PCR (qPCR) (Chin et al., 2022,Elizaquível et al., 2014) and in more recent years digital PCR (dPCR) for the absolute quantification of DNA molecules (Kim et al., 2023). Although PCR/qPCR may be considered the gold standard among nucleic acid amplification techniques, it requires specific equipment with the capacity to work under different temperatures in the same analysis and with the ability to detect fluorescence. To overcome these complexities, molecular techniques that rely on isothermal amplifications like Recombinase Polymerase Amplification (RPA) (Piepenburg et al., 2006) or Loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), among others have emerge in the last decades. These techniques employ a constant amplification temperature and can be performed in a simple incubator or water bath. In addition, with an appropriate chemistry such as phenol red, malachite green, calcein among others, naked-eye detection can be performed (Garrido-Maestu and Prado, 2022a). Therefore, these techniques can be easily applied in decentralized settings at the point-of-testing (PoT).

A classic limitation of DNA–based techniques is that they are unable to differentiate between viable and non-viable microorganisms. This is due to the stability of DNA, which can be present in the samples long time after bacterial cells have died. To overcome this limitation, different alternatives such as the use of DNA intercalating compounds, including propidium and ethidium monoazide (Qin et al., 2020; Roumani et al., 2021; Roumani et al., 2023) or mRNA detection (Miao et al., 2018; Azinheiro et al., 2022a, 2022b) have been tested. DNA intercalating compounds bind only to free DNA molecules but do not cross the cell membrane, and block their amplification by molecular techniques (Nocker et al., 2006). However, non-viable and inactivated bacteria may not present compromised cell membranes in some situations, and false positives can be obtained. RNA molecules are degraded by intra and extra-cellular RNases, and therefore RNA levels rapidly decline after bacterial death. Thus, only RNA produced by live bacteria are detected. However, it is not demonstrated that all RNA molecules have a short life, moreover, RNA detection requires careful manipulation and the addition of a retrotranscription step (Foddai and Grant, 2020).

Another promising alternative to detect bacterial viable cells in combination with molecular methods is the use of bacteriophages (phages) (Foddai and Grant, 2020), viruses that specifically infect, and replicate, in viable bacterial cells. This approach is based on the detection of phages, which only reproduce if the target bacterium is present, instead of detecting the bacteria themselves (Garrido et al., 2013). Phages, in general terms, are characterized by a narrow infection spectrum, each phage being capable of infecting a bacterial species or even specific strains within a bacterial species (Lin et al., 2017). These viruses have mainly two life cycles, the lytic cycle, where they infect the bacterium, reproduce in it and lyse it to release new viral particles, and the lysogenic cycle, where the phage genome is integrated into the bacterial chromosome and reproduces with it until it enters a new lytic cycle (Lin et al., 2017). The development of detection methods based on the reproduction of the virus, requires the use of strictly lytic phages. The use of these lytic phages has the advantage of detecting only viable cells, a pre-requisite for phage amplification. Also important, is the fact that the infection of each bacterial cell by a single phage gives rise to tens to hundreds of new virus particles (Santos et al., 2018). By detecting the resultant phages instead of the target bacteria means that we are detecting tens to hundreds more particles improving the limit of detection of the method in the same range. *Salmonella* phage vB_SenS_PVP-SE2 (PVP-SE2) previously known as φ38, was selected for the development of the PA-LAMP method. This phage demonstrated the capacity to infect 9 different strains of *S.* Enteritidis of the 13 strains tested (Santos et al., 2010). This broad host range phage has a latent period of 15 min, with a rise period of 15 min, with an average of 240 progeny phages per infected cell (Milho et al., 2018). As a consequence, it is able to reduce the enrichment periods needed for the detection of *Salmonella*. Some works have demonstrated the utility of phage amplification combined with qPCR to detect pathogenic bacteria (Kutin et al., 2009)) and foodborne pathogens as *Salmonella* (Huang et al., 2022; Garrido-Maestu et al., 2019) or *Staphylococcus aureus* (Huang, Zheng, Ding, R. Nugen and Wang, 2023). However, the combination of phage amplification with LAMP has not been reported. While phage specificity is an advantage, it also has some limitations. For example, a single phage may not be able to infect all strains of the same species or serotype. In this case, the use of phage cocktails may be a good strategy to overcome this issue (Li et al., 2022; Kuźmińska-Bajor et al., 2023). This technology can be very useful for the food industry and laboratories dedicated to food microbiology due to its isothermal nature, which facilitates its application in low resource settings, and the possibility for naked-eye results observation enabling the development of assays for implementation at PoT.

In the present work, a novel *S.* Enteritidis detection method was developed that allows a same day (8 h) detection of viable cells. The method was evaluated in spiked chicken breast samples and was based in the combination of phage amplification and detection by LAMP (PA-LAMP). Additionally, two detection strategies were tested, real-time fluorescence and naked-eye colorimetric detection.

2. Material and Methods

2.1. Bacterial strains, bacteriophage and culture media

The *Salmonella enterica* serovar Enteritidis strain S1400 (Sillankorva et al., 2010; Santos et al., 2010; Milho et al., 2018), which belongs to the University of Bristol private collection and was used to develop several detection methods (Azinheiro et al., 2018; Azinheiro et al., 2021; Garrido-Maestu et al., 2019), was selected as reference strain for all the assays carried out in the present study. One isolated colony was picked from Tryptic Soy Agar plates (TSA, Biokar diagnostics S.A, France) and transferred to 4 mL of Nutrient Broth (NB, Biokar diagnostics S.A.) and incubated overnight at 37 °C. The culture was one hundred-fold serially diluted and plated on TSA to determine the concentration of viable bacteria. The data obtained was used as reference for spiking experiments. Buffered Peptone Water (BPW, Biokar diagnostics S.A) was used

for pure phage dilution and spiking experiments. *Salmonella* selective broth Rappaport Vassiliadis (RV, Biokar diagnostics S.A.) and Muller Kauffmann Tetrathionate (MKTT, Biokar diagnostics S.A.) and agar media Xylose Lysine Deoxycholate agar (XLD, Biokar diagnostics S.A) and Chromagar™ *Salmonella* Plus agar (CHROMagar, France) were used when spiked samples were analyzed in parallel following ISO 6579–1:2017/Amd 1:2020 (International Organization for Standardization, 2020).

A fresh phage stock was prepared for subsequent assays. Briefly, 100 μL of an exponential Lysogenic Broth (LB, NZYtech, Portugal) culture of *Salmonella* S1400 was mixed with 10 μL of ten-fold dilutions of a PVP-SE2 phage suspension in SM buffer (100 mM NaCl, 50 mM Tris-HCl, 8 mM $MgSO_4{\cdot}7H_2O$, pH 7.5) and 3 mL of semi-solid TSA (0.7% agar) and poured on a TSA plate, incubated overnight at 37 °C.

A single phage plaque was picked and spread in a double layer agar prepared as detailed above, and the plates were incubated overnight at 37 °C.

Subsequently, 3 mL of SM buffer were added to each plate and incubated at 4 °C, and 90 rpm, overnight to resuspend the phages. SM buffer with the phages was collected and placed in a 15 mL tube, and chloroform to a final concentration of 10% was added. Tubes were centrifuged at 9000×*g* and 4 °C for 10 min, and aqueous phase was collected and filtered using a 0.22 μM syringe filter (PES filter, GE Healthcare). Filtrate was used as fresh phage stock. Ten-fold dilutions in SM buffer were prepared and 10 μL dispensed in double layer agar to determine stock concentration.

2.2. Primer design

The phage annotated genome sequence, downloaded from NCBI with accession number MF431252.1, was used for LAMP primer design. Endolysin and capsid phage genes were selected for the present study. Primers were designed with Primer Explorer V5 (https://primerexplorer.jp/e/index.html) and are listed in Table 1.

2.3. Nucleic acid extraction

A mere thermal lysis based nucleic acid extraction protocol was used to simplify the workflow and reduce the turnaround time and reagent consumption. Briefly, 1 mL of enriched sample was placed in a 1.5 mL tube, heated at 99 °C for 5 min under agitation (1400 rpm) in a Thermomixer comfort (Eppendorf AG, Germany), after which the samples were centrifuged at 16 000×*g* for 1 min at 4 °C, and the supernatant containing the DNA was transferred to a clean tube. For short term storage, the samples were refrigerated at 4 °C, while for long term they were frozen at −80 °C.

Table 1
Primers designed for Loop-Mediated Isothermal Amplification assays.

Phage gene	Primer name	Sequence (5′→ 3′)
Endolysin	FIP-Ph_end-Salm	GTC TAC CGC GGC TAC AGC CTT A-GAG GGC CAG AAG ATT AC CGA
	BIP-Ph_end-Salm	GCG CAT CCG TCG CTC AAT CA-CCA GCG TTA TAC ACC AGG TC
	F3-Ph_end-Salm	AAT TGG CTA CGG CCA CTA C
	B3-Ph_end-Salm	GAA GCC GCA ATC ACA CCG
	LF-Ph_end-Salm	CCC TGT TCA GTA GCA AAA GAC CCT G
	LB-Ph_end-Salm	GTC ACA GTT CGA TGC GAT GTG C
Capsid	FIP-Ph_cap-Salm	CGG CGC TGG TGA AAC TGT ATC C-GTG GTG GTG TTG AAA CCC T
	BIP-Ph_cap-Salm	CTA GCT GGC AGG ACC TGG CT-GGC ACA TGC TTA CGG TCT AC
	F3-Ph_cap-Salm	AGC GTG AAG CAT CTC GTG
	B3-Ph_cap-Salm	CCA GTT ACC AGG AAC GCA AT
	LF-Ph_cap-Salm	GGT GCA GCA ACC AGG TCT T
	LB-Ph_cap-Salm	CCA CCA ACT GGA ACC GTG TA

Sequence MF431252.1 obtained from NCBI was selected as a reference for primer design.

2.4. LAMP assay optimization

The optimal amplification temperature was evaluated in a temperature range between 62 °C–67 °C. Due to the presence of non-specific amplifications when using the capsid primers, a mix of primers with the absence of one of the Loop primers was evaluated. The dynamic range of LAMP assay was determined by performing 10-fold dilutions of pure phage DNA and 10-fold dilutions of phage particles in BPW. The specificity of the assay was confirmed by analyzing 17 bacterial strains and 7 phages listed in Table 2. To determine whether mRNA amplification of the endolysin and capsid genes could increase the analytical sensitivity of the reaction and reduce the detection time, LAMP reactions were supplemented with 0.25 units of Reverse Transcriptase (RT) (Reverse Transcriptase for isothermal amplification OptiGene, UK).

Fluorescent LAMP assays were performed in a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, CA, USA). After optimization, 20 μL reactions were composed by 12 μL of Fast Master Mix (ISO-004, OptiGene, UK), 800 nM FIP/BIP primers, 400 nM LB/LF primers and 200 nM F3/B3 primers (endolysin (E), capsid (C), and endolysin + capsid (E + C) primer combination were tested), 50 nM of ROX (Invitrogen™, ThermoFisher Scientific), 2 μL of template DNA, and the remaining volume was completed with nuclease-free water. Experiments were run at 66 °C for 30 min with fluorescence detection each 30 s. After the amplification, melt-curve analysis was performed. To this end, samples were heated at 95 °C for 1 s, 85 °C for 20 s and heated up to 95 °C with temperature increments of 0.05 °C/s and fluorescence acquisition after each temperature increment.

Colorimetric LAMP assays were performed in 0.2 mL PCR tubes in a Veriti Thermal Cycler (Applied Biosystems, ThermoFisher Scientific, CA, USA) at 66 °C for 30 min. Two different Master Mixes were tested. In the first place, WarmStart® Colorimetric LAMP 2x Master Mix with UDG (New England Biolabs, MA, USA) was evaluated. The 25 μL reactions were composed by 12.5 μL of Master Mix, 1600 nM FIP/BIP primers, 400 nM LB/LF primers and 200 nM F3/B3 primers, 2 μL of DNA and remaining volume was completed with nuclease-free water. The second type of reaction was a modification of the Fluorescent LAMP based on Sukphattanaudomchoke et al. (2020). The 20 μL reactions were loaded in 0.2 ml 8-tube PCR strips. The top of the tubes was cover with Parafilm® leaving an opening of 1 mm on the hinge side of the tube. Then, 1 μL of a 1,000X SYBR Green I solution (Invitrogen™, ThermoFisher Scientific, Waltham, Massachusetts, USA) was placed in the lid and the tubes were carefully closed. After the reactions, the tubes were shacked to allow SYBR Green placed in the lid to mix with the reaction. In the presence of double-stranded amplicons, SYBR Green binds to it and the reaction turns greenish at naked-eye and emits fluorescence that can be seen under UV light. When no amplicons are produced in the reaction, the solution remains orange revealing a negative sample.

2.5. Evaluation of the LAMP methodology

Phage infectivity was evaluated, first in BPW contaminated with 10^4 CFU/mL of *S.* Enteritidis S1400 and 10^3 PFU/mL. Samples of 1 mL were taken every hour up to 6 h, and the DNA was extracted as described in Material and Methods 2.3. DNA samples were analyzed by real-time fluorescence LAMP. In this case, LAMP samples were analyzed with and without the supplementation of the reaction with RT.

Afterwards, the methodology was evaluated in spiked chicken breast

Table 2
List of bacteria and phages selected for the inclusivity/exclusivity assay and interpretation of the result according the melting temperature.

		Fluorescence LAMP amplification		Melting Temperature (°C)		Result Interpretation	
Microorganism	Code	Endolysin	Capsid	Endolysin	Capsid	Endolysin	Capsid
Salmonella phage	vB SenS_PVP SE 2	+	+	91.10 ± 0.31[a]	91.05 ± 0.29[a]	+	+
E. coli phage	S40	+	+	90.33	90.20	–	–
E. coli phage	S3	–	–	–	–	–	–
Salmonella Enteritidis	S1400	–	–	–	–	–	–
K. pneumoniae phage	S39	–	–	–	–	–	–
E. faecalis phage	S7	–	+	–	89.41	–	–
A. baumannii phage	S2	+	–	90.26	–	–	–
P. aeruginosa phage	B	+	+	89.02	89.92	–	–
Salmonella Enteritidis	AMC 82	–	–	–	–	–	–
Salmonella Typhimurium	CECT 4594	–	–	–	–	–	–
Salmonella Typhimurium	ACM 96	–	–	–	–	–	–
Salmonella Typhimurium	AMC 238	–	–	–	–	–	–
Salmonella Montevideo	ACM 28	–	–	–	–	–	–
Salmonella Wentworth	ACM 84	–	–	–	–	–	–
Salmonella Rissen	ACM 90	–	–	–	–	–	–
Salmonella Wentworth	AMC 200	–	–	–	–	–	–
Salmonella spp.	AMC 255	–	–	–	–	–	–
Salmonella spp.	ACM 260	–	–	–	–	–	–
Escherichia coli	AMC 73	–	–	–	–	–	–
Escherichia coli	AMC 75	–	–	–	–	–	–
Escherichia coli	AMC 81	–	–	–	–	–	–
Escherichia coli	AMC 171	–	–	–	–	–	–
Escherichia coli	AMC 178	–	–	–	–	–	–
Escherichia coli	AMC 190	–	–	–	–	–	–

[a] Melting temperature calculated for the endolysin and capsid amplicons. For the result interpretation, if the melting temperature of the sample was between the range of melting temperature for endolysin or capsid primer, the sample was considered positive. If melting temperature was out of range, or there was no melting curve because of absence of amplification, the sample was considered negative.

samples. Briefly, 25 g of chicken breast were inoculated with different concentrations of *S.* Enteritidis S1400 and homogenized with 225 mL of BPW pre-warmed at 37 °C for 30 s in a Stomacher 400 Circulator (Seward Limited, West Sussex, UK). Different pre-enrichment times (1–3 h) at 37 °C were evaluated following a similar procedure described by Garrido-Maestu et al. (Garrido-Maestu et al., 2019). This was done to determine if *Salmonella* recovery time could be reduced. After this step, 10^3 PFU/mL of phage PVP-SE2 were added to each sample, and homogenized again for 30 s. The samples were incubated again at 37 °C and 1 mL samples were collected every hour from 0 h to 6 h and processed as described in sections 2.3 and 2.4.

Once the pre-enrichment and phage co-culture time were optimized the Limit of Detection (LoD) with 95% of confidence (LoD_{95}) was determined as described by Wilrich and Wilrich (2009). For that purpose, chicken samples were spiked with different concentrations of *S.* Enteritidis S1400 (Table 3). All the samples were analyzed by the real-time fluorescence and colorimetric LAMP versions. To compare this methodology against a reference method, the samples were analyzed following the ISO 6579:2017–1 for the detection of *Salmonella* spp. in food and feed. Briefly, BPW was incubated for 18 h at 37 °C. After the pre-enrichment, 100 μL were transferred to RVS (Biokar diagnostics S. A., France) and 1 mL to MKTTn (Biokar diagnostics S.A., France). These media with the samples were incubated for 24 h at 42 °C and 37 °C respectively. Then, samples were streaked in XLD and Chromagar *Salmonella* and incubated for 24 h at 37 °C. The presence of presumptive *Salmonella* colonies on XLD (red colonies with black centers) and Chromagar™ *Salmonella* (mauve colonies) was evaluated.

According to the results, samples were classified as being in Positive/Negative Agreement (PA/NA), if the results matched, and Positive/Negative Deviations (PD/ND) if the results did not match to those of the reference method (ISO 6579:2017–1). The parameters SE (relative sensitivity, percentage of positive samples giving a correct positive signal), SP (relative specificity, percentage of negative samples giving a correct negative signal), AC (relative accuracy, degree of correspondence between the response obtained by the expected results and the method on identical samples), PPV and NPV (Positive, and Negative, predictive values) and the Cohen's Kappa (κ) (degree of concordance

Table 3
Sample inoculation pattern and fluorescence and colorimetric LAMP results as well as result of the reference method ISO 6579:2017–1.

Stress	N	Inoculation level (CFU/25 g)	Fluorescence LAMP	Colorimetric LAMP	ISO 6579:2017–1
No	1	7.8×10^3	+	+	+
No	1	7.5×10^2	+	+	+
No	1	9.9×10^1	+	+	+
No	1	6.6×10^1	+	+	+
No	1	5.5×10^1	+	+	+
No	2	5.1×10^1	+	+	+
No	1	4.4×10^1	+	+	+
No	1	3.7×10^1	+	+	+
No	1	3.3×10^1	+	+	+
No	1	2.9×10^1	+	+	+
No	1	2.7×10^1	+	+	+
No	1	2.5×10^1	+	+	+
No	1	2.0×10^1	+	+	+
No	1	1.9×10^1	+	+	+
No	1	1.5×10^1	+	+	+
No	1	1.4×10^1	+	+	+
No	1	9.0×10^0	+	+	+
No	1	7.0×10^0	+	+	+
No	1	4.5×10^0	+	+	+
No	1	2.3×10^0	+	+	+
No	1	1.1×10^0	+	+	+
No	4	1.0×10^0	–	–	+
Cold	1	5.6×10^1	+	+	+
Cold	2	4.9×10^1	+	+	+
Cold	1	4.4×10^1	+	+	+
Cold	1	3.7×10^1	+	+	+
Cold	1	2.9×10^1	+	+	+
Cold	1	1.9×10^1	+	+	+
Cold	1	1.5×10^1	+	+	+
No	4	Not inoculated	–	–	–

N: number of inoculated samples at the indicated concentration. The column stress indicates if inoculated samples were exposed to some type of stress after being contaminated with S. Enteritidis and before being tested.

between the alternative and the reference method) were calculated attending to formulae and definitions previously described (Tomás et al., 2009; Anderson et al., 2011; Garrido et al., 2013).

2.6. Statistical analysis

The software Graphpad Prism 9 (Boston, MA, USA) was used for statistical analysis and for the preparation of graphs. One-way Anova analysis with post-hoc Tukey test was used to determine the existence of differences between groups ($p < 0.05$).

3. Results

3.1. LAMP assay optimization

LAMP primers were evaluated with pure phage DNA. When using the C primers we observed unspecific amplification in negative controls, thus the assay was optimized by eliminating one of Loop primers. Best results, faster amplification without lack of unspecific amplification, were obtained when only one loop primer, LB, was used. Consequently and to avoid those unspecific amplifications, LF was removed from the assay. The range of temperature between 62 °C–67 °C was evaluated with the E and C primers. The time to threshold (*Tt*) decreased as temperature increased stabilizing at 65 °C and above. One-way Anova analysis showed that there were no significant difference ($p > 0.05$) for E and C primers between 65 and 67 °C.

The dynamic range of the LAMP assay with E, C and E + C primers was determined by using ten-fold dilutions of pure phage DNA and phage particles in BPW (Fig. 1). There was a consistent detection between 2 ng/μL and 0.2 fg/μL of pure phage DNA and between 9.2 log PFU/mL and 2.2 log PFU/mL of pure phage particles. Detections times

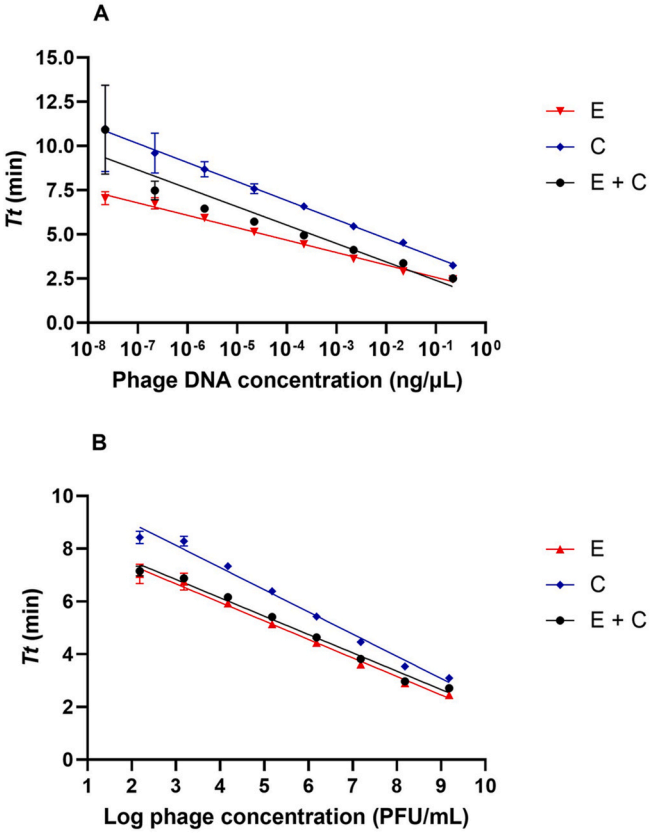

Fig. 1. Determination of the dynamic range of LAMP assay with A) pure phage DNA and B) phage serially diluted in BWP with endolysin (E), capsid (C) and endolysin and capsid (E + C) primers.

were significantly ($p < 0.05$) higher with C primers with pure DNA in comparison with E primers in all concentrations of pure DNA tested except in the last dilution tested. This is probably due to the use of only one loop primer. However, E primers showed better performance with pure phage DNA in the last dilution. With 2 fg/μL there was a difference lower than 1 min (range 6.86–7.47 min) between replicates with E primers while with C (range 9.01–13.72 min) and E + C (8.77–13.70) primers, there was a difference of approximately 4–5 min between replicates. It was expected that the combined use of E and C primers in the same reaction would reduce the detection time compared to the detection of a single gene. However, this was not the case, and even between 22 and 0.022 pg/mL of pure DNA the detection time was significantly shorter ($p < 0.05$). Thus, in the case of phage particles diluted in BPW, the detection times with E primers where significantly lower ($p < 0.05$) in comparison with C primers.

The inclusivity/exclusivity of LAMP assay was determined by using a panel of *Salmonella* and *E. coli* from different serotypes and different double-stranded DNA phages (Table 2). No amplification was observed for any of the bacterial strains evaluated in the study. However, unspecific amplifications were found for endolysin and capsid genes in an *Escherichia coli* and *Pseudomonas aeruginosa* phage, for endolysin gene in an *Acinetobacter baumannii* phage and for capsid gene in *E. faecalis* phage (Table 2). The melting temperature with *Salmonella* phage PVP-SE2 for E primer was 91.10 ± 0.31 and for C primer was 91.05 ± 0.29. Therefore, for a sample to be considered positive the melting curve should be in that range of melting temperature. As observed in Table 2, the melting temperature for the other phages was below those temperature in both E and C primers and therefore those unspecific amplifications can be differentiated of an amplification of the desired target.

In an attempt to reduce the detection times, LAMP reaction was supplemented with reverse transcriptase (RT). By adding this enzyme there is an amplification of the RNA in addition to DNA and may increase the sensitivity of the reaction. *S.* Enteritidis S1400 was incubated with *Salmonella* phage PVP-SE2 for 6 h, and every hour from time 0, a sample was taken and analyzed by LAMP to determine the effects of RT supplementation. The results are showed in Fig. 2. This experiment also served to determine the effect of host-phage co-culture time in the LAMP detection times. This co-culture time is the one required for the phage to replicate in the *Salmonella* cells and achieve a concentration that allows its detection. Two-way ANOVA showed that the addition of RT had no significant effects ($p > 0.05$) in time detection with E, C and combination of both targets in any of the time points included in the assay. However, the co-culture time of phage and host did significantly ($p < 0.05$) influence the detection time as expected. Once determined that the addition of RT did not reduce the detection time, the effect of co-culture time was determined only in samples without RT. Table 4 summarizes the detection times for the 6 h of incubation, and with the three primer combinations, as well as the significant differences observed with one-way ANOVA analysis. Interestingly, detection times where significantly higher at 1 h incubation compared to 0 h, and there were no significant differences between 0 h and 2 h. From this hour onwards, detection times start to decrease. Shortest detection times were observed at 5 h, but there were no significant differences between detection times at 4 h, 5 h and 6 h. Between 3 h and 4 h there were no significant differences with E + C primers, although detection times were considerably shorter at 4 h. For the different primer combinations, the detection times at each sample point were also compared (Table 4). In all hours tested, detection times were significantly higher for C primers than for E and E + C primers. No differences were observed between the later two. Due to the results observed in this part of the work, the endolysin primers were selected for the analysis of inoculated samples as the detection times were shorter than for the C primers. Furthermore, as there was no difference between the use of the endolysin primers alone and the combined use of E and C primers, there was no need to use this combination since it would increase the costs associated to this method. Also, after analyzing the results of the co-culture times, it was decided to evaluate

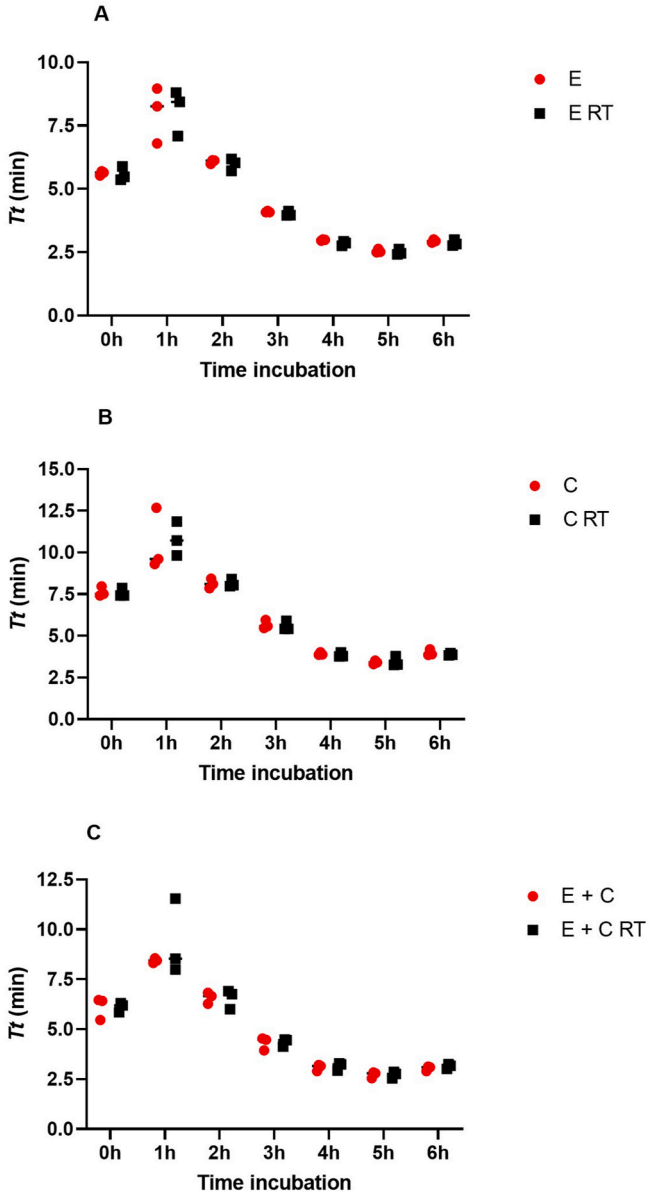

Fig. 2. Fluorescence LAMP Time to threshold (*Tt*) of phage/bacteria co-culture incubation time in normal reaction and supplemented with reverse transcriptase for A) endolysin, B) capsid and C) endolysin + capsid primers.

the range between 3 h and 6 h h in spiked chicken samples.

3.2. Evaluation of the LAMP methodology

3.2.1. Optimal pre-enrichment and co-culture time determination

In addition to determining the co-culture time of the phage and host, it was also necessary to determine the enrichment time of the chicken sample in BPW. Three different pre-enrichment times at 37 °C were evaluated, 1 h, 2 h and 3 h. The objective was to determine how much the recovery of the *Salmonella* could be reduced. After the pre-enrichment, the phages were added to the samples, incubated again and aliquots were taken every hour between 3 h and 6 h of co-culture. Two chicken breast samples were spiked with 1×10^3 CFU/25 g and 1×10^4 CFU/25 g respectively and incubated 1 h at 37 °C before phage addition. In this case, LAMP amplification was only observed after 6 h of phage-sample co-culture at 37 °C. In a second experiment, three chicken breast samples were contaminated with 4.5×10^2, 4.5×10^3 and 4.5×10^4 CFU in 25 g respectively and incubated 2 h at 37 °C before adding the phage. In this case amplification was detected in all samples after 5 h of co-culture. Finally, three chicken breast samples were contaminated with 7.8×10^3, 7.5×10^2 and 2.5×10^2 CFU/25 g and incubated 3 h at 37 °C before phage addition. After 3 h of phage-sample co-culture there was amplification only in the two samples with the highest contamination, but after 4 h there was amplification in all the samples. With 1 h, 2 h and 3 h of pre-enrichment, a total of 7 h of sample incubation (including co-culture time) was required to detect phage amplification by LAMP. Considering these results, the 3 h incubation was selected, as it would allow for higher growth of *Salmonella,* if present in the sample, and better recovery of stressed cells.

3.2.2. Determination of LoD

A total of 34 breast chicken samples were contaminated with decreasing concentration of *S.* Enteritidis S1400, see Table 3, and the number of positive and negative samples for each spiking level were inputted in the model to calculate the LoD. Seven of these 34 samples were subjected to thermal stress. In brief, these samples were contaminated with different concentrations of *Salmonella* and were stored at 4 °C for 24 h before starting the analysis. In addition, 4 non-spiked samples were also analyzed. After adding BPW, samples were homogenized and pre-enriched for 3 h at 37 °C. After this time, phage was added at a final concentration of 10^3 PFU/mL and samples were incubated again at 37 °C. After 4 h of co-culture, 1 mL of sample was collected, DNA was isolated and analyzed by LAMP:

At this point, the limit of detection was determined. Chicken breast samples were contaminated with different concentrations of *S.* Enteritidis S1400 as indicated in Table 3, including samples with cold stressed *Samonella* cells, and incubated 3 h at 37 °C. Then, phages were added and samples were incubated another 4 h at 37 °C. Samples were analyzed in parallel following the reference method ISO 6579–1:2017/a1:2020. It was possible to detect down to 1.1 CFU/25 g, however it was not possible to detect phage amplification in four samples contaminated with 1 CFU/25 g, but those samples were positive with the reference method. The LoD_{50} of the method was determined to be 1.5 CFU/25 g and the LoD_{95} was 6.6 CFU/25 g. The turnaround time of the method, considering pre-enrichment, phage-host co-culture, DNA extraction and LAMP detection was of ~8 h.

3.2.3. Evaluation of the PA-LAMP methodology

The calculation of the performance parameters was based on the data of the samples spiked above the LoD_{50}. A total of 38 samples were

Table 4
Fluorescence LAMP Time to threshold (*Tt*) measured in min, of phage/bacteria co-culture incubation time.

	Co-culture incubation Time						
	0 h	1 h	2 h	3 h	4 h	5 h	6 h
Endolysin	$_{B}5.62 \pm 0.09^{b}$	$_{A}8.01 \pm 1.11^{a}$	$_{A}6.08 \pm 0.08^{b}$	$_{B}4.10 \pm 0.03^{c}$	$_{B}2.98 \pm 0.03^{c,d}$	$_{B}2.54 \pm 0.07^{d}$	$_{B}2.94 \pm 0.06^{d}$
Capsid	$_{A}7.64 \pm 0.29^{b,c}$	$_{A}10.53 \pm 1.87^{a}$	$_{A}8.13 \pm 0.29^{b}$	$_{A}5.67 \pm 0.26^{c,d}$	$_{A}3.92 \pm 0.08^{d,e}$	$_{A}3.41 \pm 0.11^{e}$	$_{A}3.98 \pm 0.19^{d,e}$
Endolysin + capsid	$_{B}6.12 \pm 0.56^{b}$	$_{A}8.44 \pm 0.12^{a}$	$_{A}6.59 \pm 0.29^{b}$	$_{B}4.32 \pm 0.32^{c}$	$_{B}3.09 \pm 0.17^{d}$	$_{B}2.73 \pm 0.15^{d}$	$_{B}3.04 \pm 0.12^{d}$

One way ANOVA analysis with post-hoc Tukey test was performed to determine the influence of co-culture incubation time in Time to threshold (Tt) and another One way Anova analysis to determine the difference between primers. Different lower case letters in the same row indicate significant differences ($p < 0.05$) for the different times of co-incubation in each primer. Different uppercase letters in the same column indicate significant differences ($p < 0.05$) between the different primers.

included in the study, of which 4 were non-inoculated negative samples. Of the remaining 34 samples, 5 were inoculated below the LoD_{50} and excluded for subsequent calculations. Therefore, 29 samples with a range of inoculation between 7.8×10^3 CFU/25 g and 2.3 CFU/25 g were used to calculate the performance parameters SE, SP, AC, PPV and NPV providing values of 100%, and a κ value of 1.00.

3.2.4. Colorimetric LAMP

Regarding the colorimetric LAMP, two different Master mixes were evaluated. WarmStart® Colorimetric LAMP 2x Master Mix amplification detection is based on a color change of the LAMP reaction. Positive reactions change from initial pink to yellow. However, in the present study, after the addition of a DNA sample, there was a slight change of color to yellow before the LAMP reaction. As that change could interfere in the interpretation of the results, this master mix was discarded. As an alternative, the colorimetric method implemented the same Master Mix as of the real-time fluorescence LAMP (without ROX) and the addition of SYBR® Green, which showed a better performance as no initial effect was observed when adding the sample to the reaction mixture, see Fig. 3. There was a complete correspondence in the results between real-time fluorescence and colorimetric LAMP (Table 3).

4. Discussion

In the present study, a successful PA-LAMP method to detect *S.* Enteritidis in chicken samples was developed, taking advantage of two different detection strategies, real-time fluorescence and naked-eye colorimetric observation. Up to 6 primers are commonly used in LAMP reactions. A pair of these primers are called Loop primers and are added to the reaction to accelerate DNA amplification and reduce detection times (Nagamine et al., 2002). In the case of C primers, unspecific amplification was detected during method optimization and it was observed that removal of Loop F completely eliminated the unspecific amplifications. However, this resulted in longer detection times compared to the endolysin primers. For that reason, detection of the endolysin gene was selected for the final method evaluation in spiked chicken samples. For pure phage DNA, the dynamic range covered was between 2 ng/μL and 0.2 fg/μL. This is in line with the results reported by Garrido-Maestu et al. (2019) in the PA-qPCR method developed for *S.* Enteritidis detection, and similar to those observed also in PA-qPCR by Huang et al. targeting the phages of *S. aureus* and *Salmonella* (Huang, Zheng, Ding, R. Nugen and Wang, 2023). The minimum concentration of DNA detected was lower in LAMP methods than in those that target *Salmonella* DNA directly (Azinheiro et al., 2022a, 2022b; Kreitlow et al., 2021,Zhang et al., 2022). For the detection of viral particles, the present method detected from 9.2 to 2.2 log PFU/mL, which is one logarithm less than previously reported by Garrido-Maestu et al. (Garrido-Maestu et al., 2019) with their PA-qPCR assay, and similar to the value observed by Luo et al. (2018) for *A. baumannii*. During the propagation of DNA phages inside bacterial cells, both new DNA strands for new progeny and RNA molecules for the synthesis of viral proteins are formed. For that purpose, highly expressed genes such as endolysin and capsid genes were selected as molecular targets for primer design. Therefore, in an attempt to reduce detection times, LAMP reaction was supplemented with RT to convert mRNA in cDNA, increasing thus the number of the LAMP target DNA copies. However, no differences in the detection time were observed. This could be explained in part by the fact that the DNA polymerase included in the master mix used in this work already presents reverse transcriptase activity (optigene). It is therefore possible that this enzyme is already using mRNA from the sample as a template and the addition of RT to higher levels does not have the expected effect.

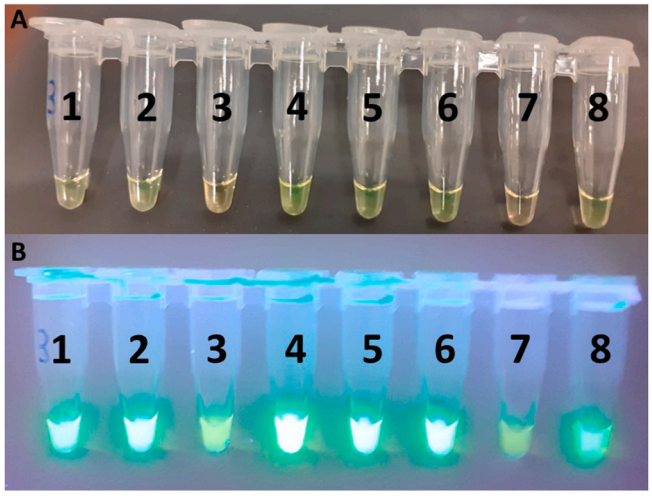

Fig. 3. SYBR Green I PA-LAMP colorimetric method tubes observed a) under white light and b) under UV light. Each tube correspond with a samples spiked with different concentrations of *Salmonella* as follow: 1) 9 CFU/25 g 2) 2.3 CFU/25 g 3) 1 CFU/25 g and cold stressed 4) 49 CFU/25 g 5) 55.5 CFU/25 g 6) 44 CFU/25 g 7) negative control 8) positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In the present method, a simple thermal lysis protocol was successfully implemented for DNA extraction. This simplified extraction method has already proved to be useful in the detection of phage DNA by qPCR (Garrido-Maestu et al., 2019; Huang, Zheng, Ding, R. Nugen and Wang, 2023). The use of this lysis method has several advantages including, a simplified workflow and a shorter analysis time. On top of that, it reduces analysis costs avoiding the use of expensive reagents or extraction kits.

Considering the colorimetric LAMP, the WarmStart® Colorimetric LAMP 2x Master Mix tested for naked-eye detection, bases its color change in the implementation of phenol red to track pH changes in reaction (acid pH turns the reaction yellow while basic keeps it orange/pink (Garrido-Maestu and Prado, 2022b)). A slight color change after the addition of the sample, and before LAMP reaction, was observed. This phenomenon was previously reported by Azinheiro et al. (Azinheiro et al., 2022). The pH of the DNA extract due to, either the sample or compounds of the culture media, may be behind this observation. This is especially important when no purification of the extracted DNA is performed.

Previous studies have evaluated the usage of phage amplification in combination with qPCR for the detection of *Salmonella* (Garrido-Maestu et al., 2019), *A. baumannii* (Luo et al., 2018), or even multiple pathogens at the same time as *Salmonella* and *S. aureus* (Huang, Zheng, Ding, R. Nugen and Wang, 2023), demonstrating its usefulness and its ability to reduce the required analysis time. However, the application of LAMP is novel, bringing the additional advantage of its possible combination with different naked-eye detection strategies. This simplifies the laboratory equipment needed and allows its application for PoT (Garrido--Maestu and Prado, 2022b). Due to the problems observed with the commercial colorimetric master mix, a homemade solution was chosen based on previous studies (Sukphattanaudomchoke et al., 2020; Singh et al., 2017; Nie et al., 2012). Simply adding SYBR Green after the LAMP reaction, a greenish color is observed when a sample is positive and fluorescence is observed under UV light. There were two main reasons to place the SYBR Green in the lid of the tube and only mix after the LAMP reaction has taken place. The first one is that high concentrations, as those needed for visual observation of LAMP amplification, would have inhibited the reaction (Goto et al., 2009), and the second reason relies on the fact that opening the reaction tubes to add the SYBR Green after the amplification could cause cross-contamination (Lai et al., 2021). The total correspondence between fluorescence and SYBR Green-based naked eye detection shows the utility of this method to be used at PoT, and avoids the need of expensive equipment such as for qPCR. Lastly, it is important to note that the phage suspension is added in a

similar way to any classical media supplement, in this way, it may be prepared and quantified in advance, and stored refrigerated until needed, once more simplifying the assay as no complex operations related to the phages will have to be performed at PoT.

The food industry requires rapid detection methods in order to avoid delays in the distribution and marketing of foodstuffs, this is especially important for perishable products. As mentioned above, the ISO 6579 method requires a minimum of three days to confirm a negative sample. The use of short enrichment methods combined with molecular methods can considerably reduce these times. The method developed in the present study has a total incubation time of 7 h (3 h of sample pre-enrichment + 4 h of phage-host co-culture); the DNA extraction can be performed in approximately 10 min; and both, fluorescent and visual LAMP, have a turnaround time of 30 min. Therefore, the whole analysis could be performed in roughly 8 h, which would represent a work shift, allowing a "same-day detection".

The pre-enrichment time of the sample in BPW is of great importance as it allows to recover stressed bacteria, as demonstrated herein with cold stressed *S.* Enteritidis, Table 3, while improving the LoD of the method. The LoD_{95} of the present method was 6.6 CFU/25 g. This is a similar LoD as that observed by Garrido-Maestu et al. (Garrido-Maestu et al., 2019) using PA-qPCR in a 9 h workflow. Huang et al. (Huang et al., 2022) developed a PA-qPCR method using the broad range spectrum *Salmonella* phage T156 with a shorter detection time (3.5 h) but the LoD was 10 CFU/mL. It is also interesting to compare these results with LAMP methods that allow for the direct detection of the pathogen. Garrido-Maestu et al. (Garrido-Maestu et al., 2017) developed a LAMP method to detect *S.* Typhimurium and *S.* Enteritidis serotypes in chicken breast samples obtaining a LoD <10 CFU/25 g after 18 h or enrichment followed by DNA extraction and 60 min of LAMP amplification, and Kreitlow et al. obtained a LoD of 1 CFU/25 g for *Salmonella* spp. in chicken samples but in this case after 20 h incubation of food samples in BPW, proving that although the detection limits reached are similar, these methods do not allow same-day detection (Kreitlow et al., 2021). Yang et al. (Yang et al., 2015) developed a LAMP method with a LoD of 1.1–2.9 CFU/25 g of *Salmonella* in produce after 6–8 h of enrichment in BPW. But as an advantage, the method developed in the present work presents the added value of specifically detecting viable cells, since these are necessary for the multiplication of the phages. Youn et al. (Youn et al., 2017) developed a LAMP method combined with PMA treatment, one of the classic alternatives for detecting viable cells, with a limit of detection of 8×10^1 CFU/reaction (estimated to be 8×10^5 CFU/25 g). This method can be performed in 2 h since no enrichment step was added prior to the LAMP. Although this method stands out for its rapidity, it is important to remember that the enrichment steps are very important to detect *Salmonella* with LoD in accordance with legislation (1 CFU/25 g for *Salmonella* spp.), otherwise false negatives will be obtained.

The results of the current method were compared with those obtained by the reference method ISO 6579. A total of 38 chicken samples were analyzed in the current study, 34 of those were spiked with different concentrations of *Salmonella* and 4 samples were not inoculated. Out of these, only 4 ND were obtained, being these associated with samples inoculated with 1 CFU/25 g, thus below the LoD_{50} (1.5 CFU/25 g) and were not considered in the evaluation of the performance of the method thus, the SE, SP, AC, PPV and NPV obtained a value of 100%. Additionally, the κ value was 1.00 that indicates that the method is in "almost complete concordance" with the reference method (ISO 6579) (Altman, 1990).

The present method used BPW as enrichment media to follow a protocol as close as possible to the reference method for the detection of *Salmonella* spp., ISO 6579, allowing a smooth transfer to this new methodology. In the present study, phage PVP-SE2 showed no limitation in terms of infection and multiplication in BPW. However, it would be interesting to evaluate in future studies different culture media to determine if the phage reproduction rate is higher and therefore reduce incubation times. Likewise, in the future it would be interesting to develop and evaluate a phage cocktail with a wide range of infection, and a latency period as short as possible. In this sense, the use of genetically modified phages could be very useful (Kilcher and Loessner, 2019).

5. Conclusion

In the present work a proof-of-principle PA-LAMP assay for the detection of viable *S.* Enteritidis in chicken breast was successfully developed. In about 8 h, including sample pre-enrichment, DNA extraction and LAMP it was possible to detect 1.1 CFU/25 g of food sample. This makes this method suitable for same-day detection. Furthermore, the development of a visual alternative allows this method to be applied at the PoT with a minimum investment in equipment. This is of particular interest for poultry slaughterhouses in which routine control of *Salmonella* in chicken carcasses is carried out. Taken all together, we have developed a new, fast, simple and non-technical demanding and economic method that can detect *Salmonella* in the same working day with results comparable to the reference method (ISO 6579).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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