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FOOD MICROBIOLOGY - RESEARCH PAPER

Salmonella detection with LAMP and qPCR and identification of serovars of interest by multiplex qPCR in poultry carcasses

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Abstract

Salmonella is present in the poultry production chain and is a major challenge in terms of food safety and animal health. The early *Salmonella* detection is one of the main tools to control and prevent the transmission of this pathogen. Microbiological isolation and serotyping to identify and differentiate *Salmonella* serovars are laborious processes, time-consuming, and expensive. Therefore, molecular diagnostic methods can be rapid and efficient alternatives to the detection of this pathogen. Thus, the aim herein was to standardize and evaluate the use of loop-mediated isothermal amplification (LAMP) in comparison with real-time PCR (qPCR) for detection of *Salmonella* associated with a multiplex qPCR for simultaneous identification and differentiation of *S*. Enteritidis, *S*. Typhimurium, *S*. Pullorum, and *S*. Gallinarum. The LAMP, qPCR, and multiplex qPCR assays were comparable in specificity. The three techniques were evaluated for specificity for 16 different serovars of *Salmonella* and for 37 strains of the serovars of interest. The limit of detection and the efficiency of the LAMP, qPCR, and multiplex qPCR reactions were determined. The techniques were applied to 33 samples of chicken carcasses and compared to the results of conventional microbiology for validation. As results, LAMP was specific in the detection of different *Salmonella* serovars but presented lower limit of detection ranging from 10^1 to 10^4 CFU/reaction. In comparison, qPCR could detect less cells (10^0 to 10^2 CFU/reaction), reaching equal specificity and better repeatability in the assays. The qPCR multiplexing for identification of the different serovars also showed good specificity, with the detection threshold between entre 10^1 and 10^2 CFU/reaction. The results obtained in the analyses on poultry carcasses suggested a correspondence between the results obtained in molecular methods and in conventional microbiology. Thus, the proposed assays are promising for the diagnosis of *Salmonella* in poultry carcasses, already proved to be faster and more efficient than conventional diagnostics techniques, being of great interest for poultry production, animal, and public health.

Keywords Pathogen · Molecular biology · One health · Food microbiology

Introduction

Brazil stands out in global poultry production, being the third largest producer and largest exporter; however, *Salmonella* stands as a major challenge, leading to economic losses, trade embargoes, and productivity decrease [1, 2]. Currently, faster and accurate diagnostic procedures are needed, mainly to ensure food safety [3, 4]. For the diagnosis of *Salmonella*, the conventional method of isolation has been used in a standardized way; however, it is time-consuming, taking between 5 and 7 days, and several steps to complete the analysis [4, 5]. Thus, molecular methods have been commonly used for the detection of *Salmonella*, with promising results [6–9].

I declare that this article was prepared exclusively by the authors and that it has not been submitted to other publications.

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One of the most innovative molecular techniques is the loop-mediated isothermal amplification (LAMP), which is used in the diagnosis of several pathogens, including *Salmonella* [7, 10–13]. This technique is a DNA amplification method under isothermal conditions that consists of using a set of specific primers (two or three pairs of primers) for the DNA target, taking around 40 min per reaction, at a temperature of 60–65 °C. Furthermore, it does not require sophisticated equipment and is described as a fast alternative with good specificity and sensitivity in detecting microorganisms [14–16]. In a study that evaluated LAMP using two detection methods (turbidimetry and fluorescence) on 247 strains of *Salmonella*, the results demonstrated that LAMP assays showed 100% specificity, and the detection limits ranged from 1.3 to 28 cells. In this same study, the LAMP technique exhibited comparable performance to the qPCR method, with the additional advantage of providing a shorter analysis time [7]. In another study, also using the LAMP technique with fluorescence detection for *Salmonella*, the isothermal approach yielded highly specific results, with a detection limit of 20 CFU/reaction. This demonstrated greater sensitivity compared to the conventional PCR method [10].

Another important technique is the multiplexing of PCR and qPCR. The great advantage associated with multiplexing is the use of pairs of primers that allow the simultaneous detection and identification of different specific DNA sequences in the same sample. Multiplex qPCR has also been used to detect *Salmonella* and differentiate serovars [17–20].

The mentioned techniques can constitute a promising tool for the diagnosis of *Salmonella* at national level. However, there is a scarcity of studies addressing the LAMP technique in the diagnosis of *Salmonella* in chicken carcasses in Brazil. Therefore, studies like this are important, as they explore new methodologies such as LAMP, along with well-established molecular biology techniques like qPCR. Thus, this study aims to evaluate the use of LAMP in comparison with qPCR for detection of *Salmonella* associated with multiplex qPCR for simultaneous identification and differentiation of key serovars whose monitoring is recommended by Brazilian sanitary law: Enteritidis and Typhimurium (serovars of public health importance) and Gallinarum and Pullorum (serovars of interest to animal health). In summary, this study seeks to establish standardized tests in the diagnosis of this pathogen, with rapid detection as a screening and the simultaneous identification of serovars by multiplex qPCR.

Materials and methods

Bacterial strains

A total of 53 *Salmonella* strains (12 *S*. Enteritidis strains, 11 *S*. Typhimurium strains, 9 *S*. Gallinarum strains, 5 *S*. Pullorum strains, and 16 different isolated serovars) and 7 non-*Salmonella* strains (three strains *Escherichia coli*, two strains *Pseudomonas* spp. and two strains *Listeria* spp.) were used in this study. In addition to the four serovars of interest (Enteritidis, Typhimurium, Gallinarum, and Pullorum), the different serovars included in the study were Anatum, Adelaide, Bovismorbificans, Bredeney, Coeln, Derby, Give, Havana, Heidelberg, Minnesota, S.I.4,5,12: i:-, Panama, Senftenberg, Mbandaka, Miami, and Muenchen. The strains were provided by the School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP) and University of Paraná State (UFPR). The different serovars used in the study were sent for serotyping to the Enteropathogens Laboratory of the Instituto Adolfo Lutz, for confirmation and determination of the serotype with agglutination of specific *Salmonella* antisera produced by the institution according to standardized methodology.

DNA extraction

The isolates were stored in Nutrient Agar and recovered in Luria Bertani medium (LB) with incubation at 37 °C for 24 h under agitation. After 24 h of incubation and the turbidity of the medium was verified, the genomic DNA was extracted with a magnetic beads based in house protocol [21]. Extracted DNAs were stored in −20 °C freezers until processing.

Selection of primers for LAMP, qPCR, and qPCR multiplex

The primers used in the molecular techniques (LAMP, qPCR, and multiplex qPCR) were described in previous studies, showing satisfactory results in the rapid, sensitive, and specific detection of *Salmonella* and the serovars of interest (Enteritidis, Typhimurium, Gallinarum, and Pullorum). For the detection of *Salmonella*, the primers used in the LAMP and qPCR are described in Table 1.

For the multiplex qPCR that differentiates the four serovars, as described in the studies that used this set of primers, the *S*. Enteritidis samples amplified only for the SE target; *S*. Typhimurium samples amplified only for the ST target; for the samples from *S*. Pullorum and *S*. Gallinarum, amplification for the SGP target occurs in both; for the differentiation of these two serovars *S*. Gallinarum also amplifies for the SG target. The primers used in the multiplex qPCR are described in Table 2.

LAMP protocol

For the LAMP technique, WarmStart® LAMP 2X Master Mix (DNA & RNA) was used. A fluorescent agent was

Table 1 Particulars of the primers used for LAMP and qPCR detection

Primers	Targeted gene	Primer sequence (5′ to 3′)	References
LAMP			
FIP	*invA*	GACGACTGGTACTGATCGATAGTT TTTCAACGTTTCCTGCGG	Hara-Kudo et al., 2005 [22]; Xin et al., 2021 [19]
BIP		CCGGTGAAATTATCGCCACACAAA ACCCACCGCCAGG	
F3		GGCGATATTGGTGTTTATGGGG	
B3		AACGATAAACTGGACCACGG	
LOOP-F		GACGAAAGAGCGTGGTAATTAAC	
LOOP-B		GGGCAATTCGTTATTGGCGATAG	
qPCR			
INVA5F	*invA*	GATTTGAAGGCCGGTATTATTG	Barbau-Piednoir et al., 2013 [23]
INVA5R		ATAAACTTCATCGCACCGTCA	

added to reveal the results. For the reactions, 12.5 µL of WarmStart® LAMP 2X Master Mix (DNA & RNA), 1.6 µM of FIP, and BIP primers were used; 0.2 µM of primers F3 and B3; and 0.4 µM of the loop-F and loop-B primers, 2 µL of the extracted sample, and nuclease free water until the volume was completed, totaling 25 µL per reaction. Incubation conditions were 30 min at 65 °C followed by a further 5 min at 85 °C. After incubation, 1 µL of SYBR® Green I (Sigma–Aldrich, USA) diluted at 1:100 was added to observe the results.

qPCR protocol

To perform the qPCR, 5 µL of extracted DNA, 10 µL of GoTaq® qPCR Master Mix (Promega, Madison, USA), 0.6 µL of each primer (10 µM), and nuclease free water were used to complete the volume, totaling 20 µL of reaction. The qPCR reaction was cycled at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min; ending with the melting curve, the analysis were performed in the AriaMX real time PCR system (Agilent, Santa Clara, CA, USA).

Table 2 Particulars of the primers used for multiplex qPCR in the differentiation of serovars

Primers	Targeted gene	Primer sequence (5′ to 3′)	Target	Serovar detection	References
SE-F	*lygD*	TCTGGGACGCCAAAAAGC	SE	Serovar Enteritidis	Agron et al., 2001 [24] ; Xin et al., 2021 [19]
SE-R		TGACGGTAGATTGTGTCT CAAAGC			
PROBE SE		Cy5-TCAAACTTACTCAGG AGATCGCCGCTG-BHQ2			
ST-F	*STM4495*	GTTCAGCTCCGGTAAAGA GAA	ST	Serovar Typhimurium	Akiba et al., 2011 [25]; Xin et al., 2021 [19]
ST-R		AGCAGCGGCACTACATAT TC			
PROBE ST		Cy3-CGTTTGAGTGCCTGG TCTATCTGA-BHQ2			
SGP-F	*glgC*	GGATGTCCACGCTCATTT CTC	SGP	Serovars Pullorum and Gallinarum	Adapted from Kang et al., 2011 [26]; Xin et al., 2021 [19]
SGP-R		TGAAAGCTGGCGTTACGG TTA			
PROBE SGP		FAM-CGTCAGGCCCACCGC CGACAG-BHQ1			
SG-F	Deletion in the *glgC* gene	CAGGCGATCATATCTACA AGCAGG	SG	Serovar Gallinarum	
SG-R		TCTTGTCGCTTTCATCGA CCGC			
PROBE SG		ROX-ACTCGCGTATGTTTT GAAAAGGGC-BHQ1			

Multiplex qPCR protocol

For multiplex qPCR, 5 µL of extracted DNA and 10 µL of GoTaq® Probe qPCR Master Mix Protocol (Promega, Madison, USA) were used. The concentrations of primers and probes were 0.05 µM SGP primers and probe, 0.1 µM SG, SE, and ST primers, and 0.05 µM for SG, SE, and ST probes. A volume of nuclease free water was added to 20 µL of reaction. The multiplex qPCR reaction was cycled at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 57 °C for 40 s; the analyzes were performed in the AriaMX real-time PCR system (Agilent, Santa Clara, CA, USA).

Specificity of the LAMP, qPCR, and qPCR multiplex assay

The entire collection of *Salmonella* strains (53 strains), including non-*Salmonella* strains, were used to assess the specificity of the assays.

Limit of detection (LOD) and efficiency of the LAMP, qPCR, and qPCR multiplex assay

The LOD of the multiplex LAMP, qPCR, and qPCR assays was assessed by preparing inoculum of each of the serovars of interest (*S*. Enteritidis, *S*. Typhimurium, *S*. Gallinarum, and *S*. Pullorum). For inoculum formation, 4 *Salmonella* strains were resuspended in LB and incubated at 37 °C for 18–24 h, and the CFU/mL were estimated by XLD agar plating. After the colony count was performed in each of the dilutions, inocula of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/mL were selected for extraction in triplicate of each of the four serovars of interest. Subsequently, the amount of CFU per reaction of each inoculum was estimated. Furthermore, to evaluate the performance of the reaction, the efficiency of the tests was calculated using the values of the slope of the curve (slope), applying the formula: $E = 10^{(-1/\text{slope})} - 1$. Correlation coefficient (R^2) was also evaluated looking for values close to 0.99.

Detection in naturally contaminated samples

The LAMP, qPCR, and multiplex qPCR assays were applied to evaluate the presence of *Salmonella* in 33 samples of chicken carcasses that were sent for *Salmonella* research by conventional methodology or by the Laboratory of Food Sanitary Inspection SOAP/FMVZ/Botucatu. Samples for the abovementioned molecular methods were obtained after the pre-enrichment step in Buffered Peptone Water (APT) with a 24-h incubation period. Meanwhile, each sample was also subjected to standard bacterial isolation methods with the protocol recommended by the Bacteriological Analytical Manual/BAM-USDA [27].

All analyses were conducted in strict accordance with animal welfare guidelines, with approval from the Ethics Committee on the Use of Animals - CEUA of the Faculty of Veterinary Medicine and Animal Science at UNESP, Brazil (Protocol number 0111/2022).

Results

Evaluation of sensitivity of LAMP, qPCR, and qPCR multiplex protocol

In the evaluation of the specificity of the techniques, all *Salmonella* specimens were detectable in the LAMP and qPCR techniques for the *invA* gene, the non-*Salmonella* strains were not detectable. For multiplex qPCR specificity, only *S*. Enteritidis, *S*. Typhimurium, *S*. Gallinarum, and *S*. Pullorum produced the corresponding amplified signals, non-target bacteria, including other *Salmonella* serovars and non-*Salmonella* strains, were negative in multiplex qPCR. The sample of *S*. 4,[5],12:i:- showed amplification for the ST target (Cq = 21.47); this serovar is antigenically similar to serovar Typhimurium which justifies its amplification. No false positives were found, indicating that the assays are specific.

Evaluation of the limit of detection and efficiency of the LAMP, qPCR, and qPCR multiplex assay

To determine the LOD of the molecular techniques, the inoculum prepared containing DNA extracted from each of the serovars at concentrations 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/reactions were tested in triplicates. The limit of detection for LAMP was from 10^1 to 10^4 CFU/reactions, while the qPCR for the *invA* gene was from 10^0 a 10^2 CFU per reaction, and the multiplex qPCR was from 10^1 a 10^2 CFU per reaction; the detailed results are shown in the Fig. 1 and Table 3.

The standard curve of the qPCR and qPCR multiplex assays was constructed using the mean Ct values for the inoculums of each of the serovars. For the qPCR for the *invA* gene, the slopes of the standard curves for *S*. Enteritidis, *S*. Typhimurium, *S*. Gallinarum, and *S*. Pullorum were −3.208, −3.583, −3.366, and −3.446, respectively. Correlation coefficients (R^2) were above 0.98, and amplification efficiencies ranged from 90 to 105%, indicating high linearity in the assays (Fig. 2).

For multiplex qPCR, the slopes for *S*. Enteritidis (SE target), *S*. Typhimurium (ST target), *S*. Gallinarum (SGP and SG target), and *S*. Pullorum (SGP target) were −3.455, −3.794, −3.657, − 3.292, and −3.758, respectively. Correlation coefficients (R^2) were above 0.98, and amplification efficiencies ranged from 83 to 102% (Fig. 3).

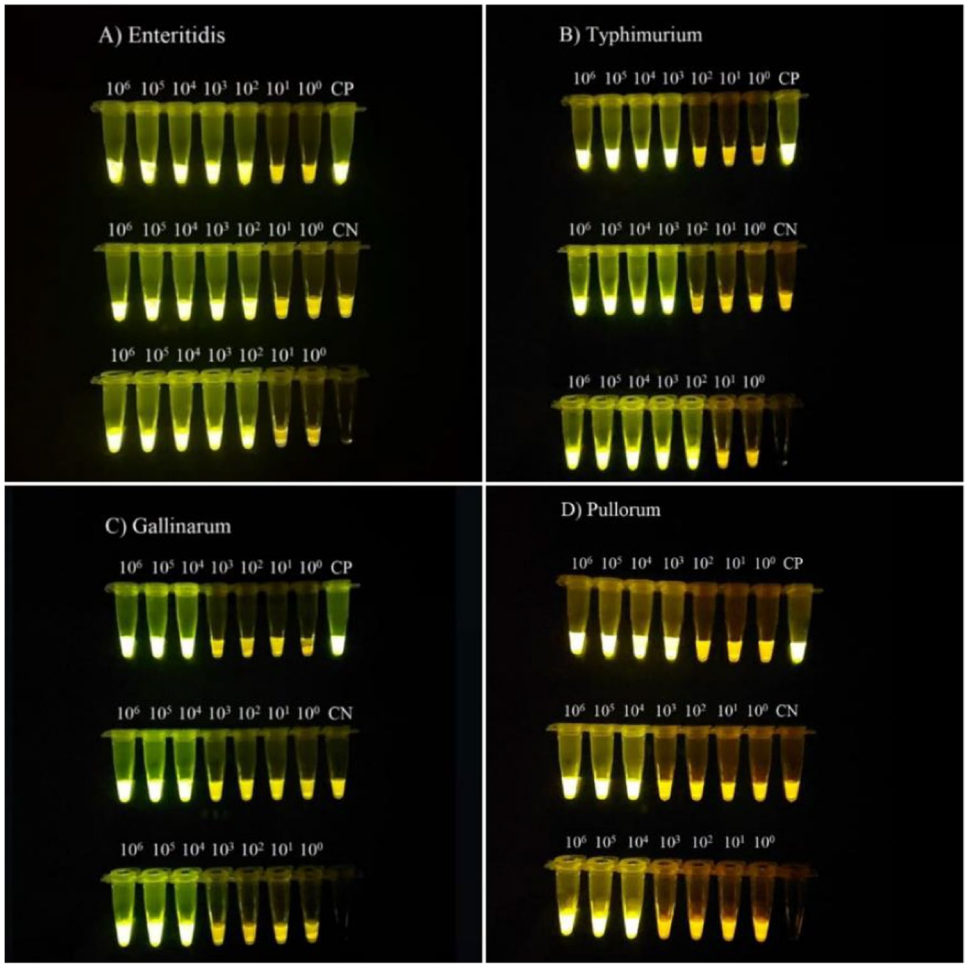

Fig. 1 Limit of detection of the LAMP technique using inoculum of the serovars of interest. **A** Triplicate of *S*. Enteritidis inoculum. **B** Triplicate of *S*. Typhimurium inoculum. **C** Triplicate of *S*. Gallinarum inoculum. **D** Triplicate of *S*. Pullorum inoculum

Table 3 LOD values found in each of the molecular diagnostic methods

LOD molecular diagnosis (CFU/reaction)						
Bacterial suspensions	LAMP	qPCR for the *invA* gene	qPCR multiplex			
			Target SE	Target ST	Target SGP	Target SG
Inoculum *S*. Enteritidis	10^2	10^1	10^1			
Inoculum *S*. Typhimurium	10^3	10^2		10^2		
Inoculum *S*. Gallinarum	10^4	10^0			10^1	10^1
Inoculum *S*. Pullorum	10^4	10^1			10^1	

Evaluation of LAMP, qPCR, and qPCR multiplex for Salmonella detection in naturally contaminated samples

To evaluate the discernibility and applicability of the proposed assays, naturally contaminated samples were analyzed. A total of 33 suspect samples were collected and evaluated in the LAMP, qPCR, and qPCR multiplex assays. Of those, 7/33 (21%) presented amplification for the *invA* gene, and four were detectable in LAMP and conventional microbiology; two samples were detectable only in qPCR with no *Salmonella* presence confirmed by conventional microbiology, and one sample was detectable in LAMP and qPCR, and its presence was not confirmed by conventional microbiology (Table 4). None of the samples showed amplification in the multiplex qPCR.

Discussion

LAMP is described as a screening method in the *Salmonella* diagnostic routine with promising results [7, 28, 29]. In most studies, LAMP demonstrates similar specificity and sensitivity when compared to PCR and its variations [7, 22]. In the

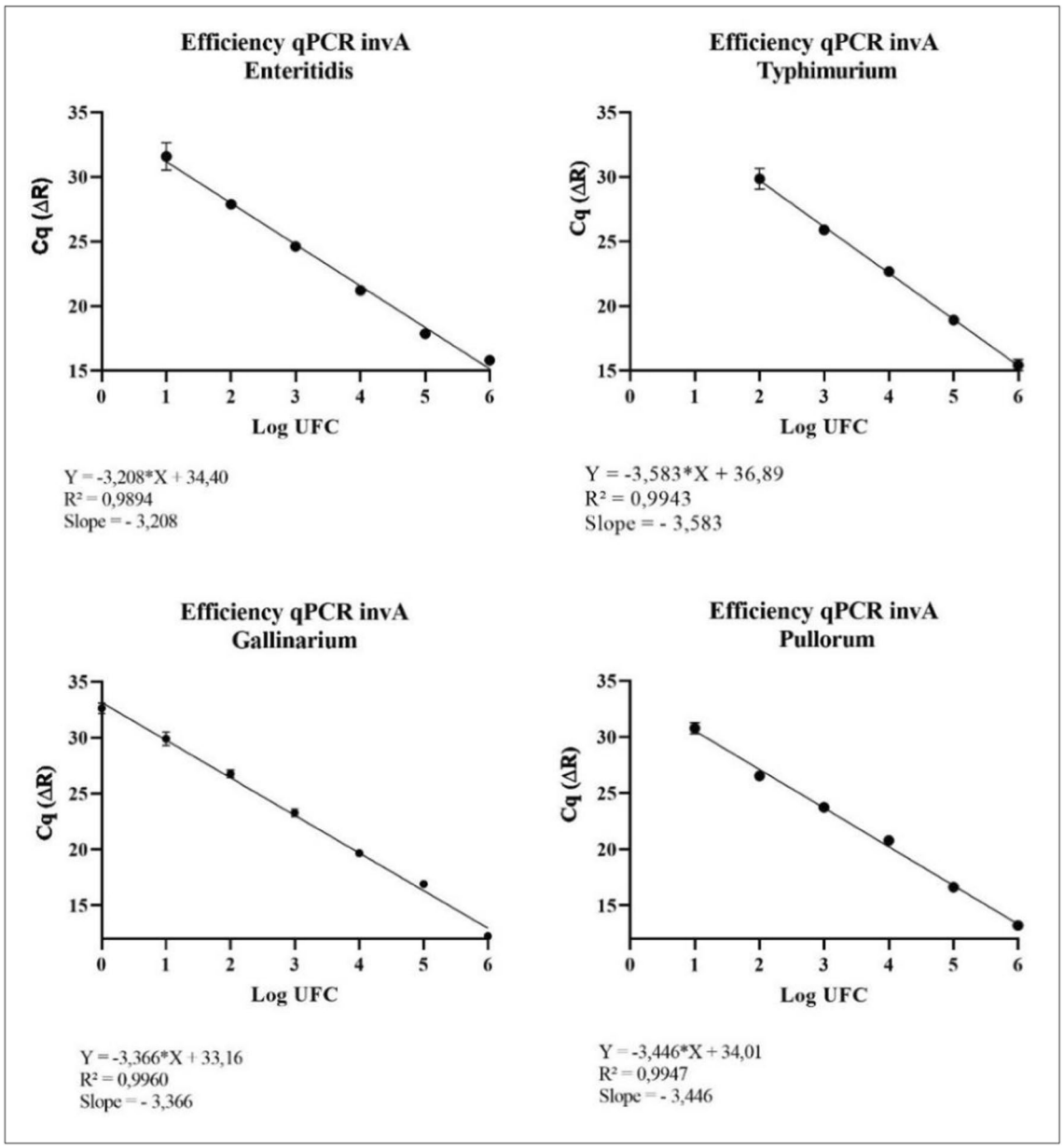

Fig. 2 Standard curves of the real-time PCR for the *invA* gene for each of the serovars

present study, the specificity between the assays (LAMP, qPCR for the *invA* gene and qPCR multiplex) for the tested strains was the same, but qPCR detected less cells, being more sensitive, when compared to the detection limit of LAMP. LAMP detection limits described in other studies are between 1.3 and 28 CFU/reaction in pure culture [7, 22], 5 CFU/reaction in artificially contaminated samples [29], and a lower limit of detection also been reported around 10^4 CFU/reaction [30, 31]. Although the technique is quick and easy to perform and does not demand sophisticated equipment, LAMP has some limitations, such as the high risk of contamination of the assays, which can reveal false-positive results in negative controls. Furthermore, it also demands a precise design of the primers and a rigorous optimization of the assay in addition to a difficulty in multiplexing and in the quantification of the target DNA after the reaction [32–35].

In the detection of *Salmonella* by qPCR, the *invA* gene has been effectively used and is recognized as a standard gene in the detection of this pathogen, having been successfully applied in several studies [23, 36–40]. In this sense, the present study obtained good specificity in the tests, which agrees with other studies in the high specificity of identification of the isolates with the use of primers designed for the *invA* gene [23, 39, 41, 42]. For LOD, studies describe very

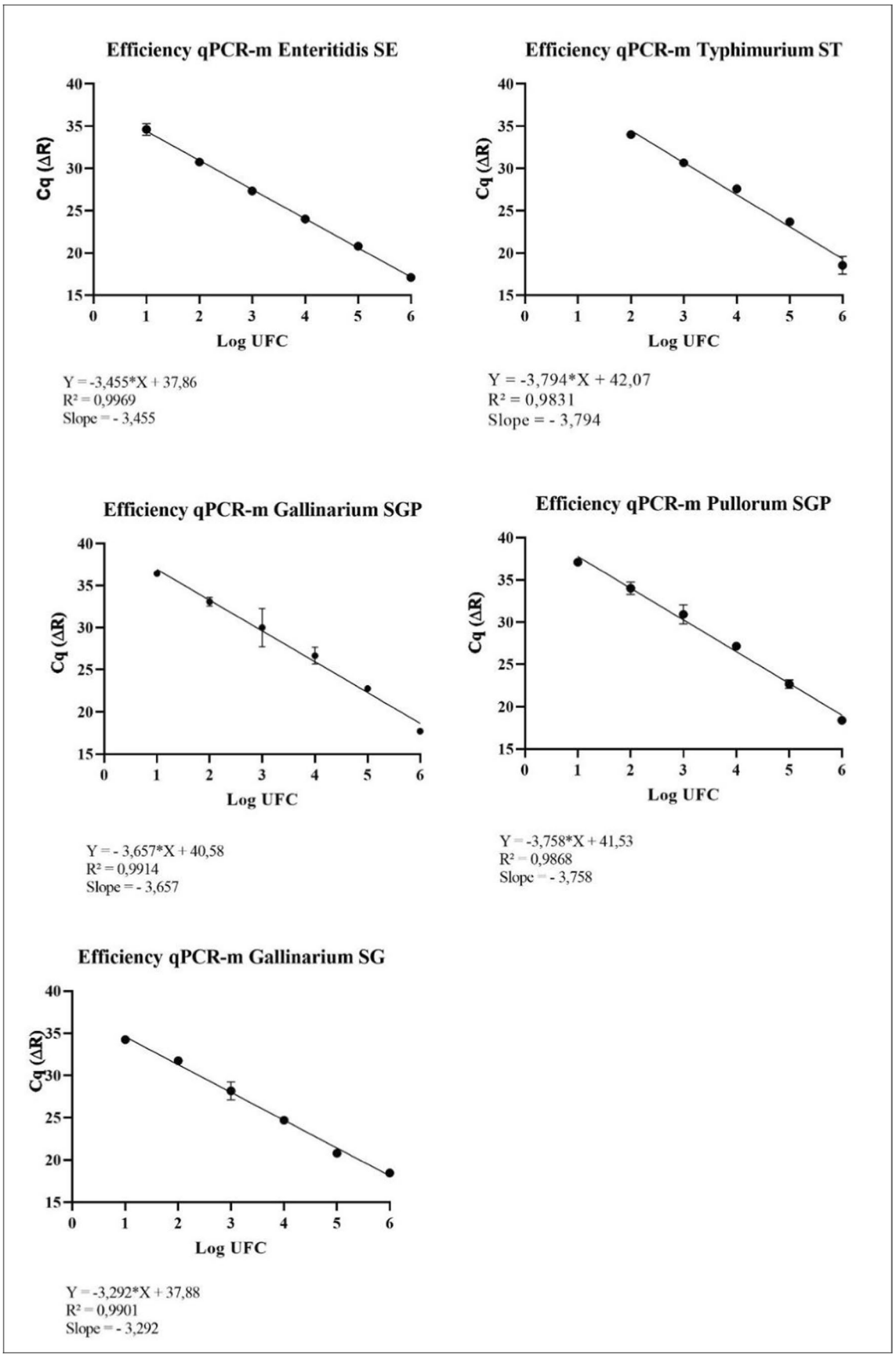

Fig. 3 Standard curves of the multiplex real-time PCR for each of the serovars

Table 4 Results of the evaluation of molecular biology and traditional microbiology techniques for clinical samples of chicken carcasses

No. of analyzed samples	Conventional microbiology	LAMP	qPCR for the *invA* gene
4	Detectable	Detectable	Detectable
1	Not detectable	Detectable	Detectable
2	Not detectable	Not detectable	Detectable
26	Not detectable	Not detectable	Not detectable

sensitive detection values, being detectable up to 8.5×10^0 CFU/mL in artificially contaminated and enriched chicken meat [43]; also reporting 3–5 detectable copies for qPCR searching for the *invA* gene [23]. Another study detected 1 to 2 copies of *Salmonella* DNA per reaction [44]. In a sample of chicken meat, another work obtained detection of up to 5 CFU in 25 g of sample [45]. A sensitivity of 5.2×10^3 CFU/mL for pure culture without enrichment has also been described [46]. In a study of the analytical precision analysis of primers that target the *invA* gene, a limit of 10^4 CFU/ml (50 CFU/reaction) was described [47].

The multiplexing qPCR assay has been described as an interesting and useful tool for identifying and differentiating *Salmonella* serovars [19, 48–50]. In the study, the multiplex qPCR directed to the four target serovars of interest showed satisfactory specificity, having amplified 100% of the serovars of interest tested and no false positives; the specificity of the primers and probes continued to show good results, as found in other works [19, 24–26]. LOD for multiplex qPCR targeting, the same four serovars of interest, one study obtained thresholds of 500 CFU/g in artificially contaminated samples without the enrichment step [19]. For the *S.* Enteritidis and *S.* Typhimurium duplex assay, the detection threshold was 10^1 CFU/mL [49], as well as the threshold of 40 CFU/reaction has also been reported [51]. In a study that standardized a multiplex qPCR for five *Salmonella* serogroups (B, C1, C2, D, and E) and for serovars *S.* Enteritidis and *S.* Typhimurium, limits of approximately 10^3 to 10^4 CFU/mL were found in pure culture and 10^4 to 10^5 CFU/g in a chicken meat sample with the enrichment step [50]. Also, 100 CFU/reaction per reaction for bacterial culture for the same serovars has been described [18]. In the analysis of the duplex *S.* Gallinarum and S. Pullorum, detection was obtained up to 10^1 CFU/mL [52]; another threshold found was also 100 CFU/reaction for the same serovars [53].

In the analyses of naturally contaminated samples, there was agreement between the results found from molecular assays and conventional microbiology, which has been reported in the literature [4, 31, 54–56]. The differences between the results found in the study may be associated with the lack of distinction between viable and dead cells in qPCR, implying the amplification of any target genetic material present in the sample, regardless of the cell condition [56–58]. In the study, the serovars recommended in the qpCR multiplex were not detectable in the naturally contaminated samples; the reduced number of samples may have contributed to this result, but it is also worth mentioning the change in *Salmonella* serotypes related to poultry and poultry production today. Other studies describe serovars such as Minnesota, Mbandaka, Senftenberg, Agona, Schwarzengrund, Infantis, and Panama as prevalent serovars in the Brazilian poultry industry [59, 60]. Recent studies describe serovars Heidelberg and Minnesota as more prevalent in the current poultry setting [61–63].

In conclusion, *Salmonella* is a global zoonotic problem in poultry farming. It is crucial to develop rapid and cost-effective alternatives for its diagnosis. This study standardizes and evaluates molecular techniques (LAMP, qPCR, and multiplex qPCR). Although LAMP is a promising technology, with several operational advantages, regarding the necessary equipment and time, qPCR has shown to be more sensitive (limit of detection ranging from 10^0 to 10^2 CFU/reaction) for *Salmonella* detection and exhibits fewer limitations, making it a more robust technique in the context of this study. Multiplex qPCR is also promising for identifying important serovars for the health of Brazilian poultry. These techniques enable fast and practical diagnoses, making them attractive for routine laboratory analyses.

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Declarations

Conflict of interest The authors declare no competing interests.

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