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PERFORMANCE EVALUATION OF A MOLECULAR METHOD TO SIMULTANEOUSLY DETECT *Salmonella* Enteritidis/*Salmonella* Typhimurium IN A CHICKEN MEAT MATRIX INSTEAD OF CONVENTIONAL SEROLOGY

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ABSTRACT

Salmonella Enteritidis (SE) and *Salmonella* Typhimurium (ST) are the major agents involved in food outbreaks worldwide. Developing quick tests to better detect this pathogen in food is crucial to ensure safety and meet the growing demand for animal products. Therefore, this study aimed to evaluate the performance of a molecular method to simultaneously detect SE/ST in a chicken meat matrix instead of conventional serology. For this, each pathogen-free sample was contaminated, being single or combined (SE, ST, *Salmonella* spp.). Protocols for NewGene FastX extraction, simultaneous qPCR SETAmp detection, and *Salmonella* SERef and STRef positive controls were followed, and out of eight samples analyzed,five presented 100% agreement with the expected result.The samples were also tested with *Escherichia coli*, *Klebsiella aerogenes*, and *Citrobacter freundii*, which did not amplify, thereby confirming the kit's specificity. There are several kits for simultaneous detection marketed by international companies, although this study is one of the first to validate a Brazilian produced and marketed kit. This is an alternative diagnostic procedure that may replace the serology stage of the conventional *Salmonella* spp. method.

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INTRODUCTION

Salmonella Typhimurium (ST) and *Salmonella* Enteritidis (SE) serovars are recognized as the main etiological agents of salmonellosis in foodborne disease outbreaks reported in humans worldwide (De Melo *et al*., 2017; Rubio *et al*., 2019). It is estimated that, worldwide, 93.8 million cases of non-typhoid gastroenteritis and 155,000 cases of mortality occur annually (Heymans *et al*., 2018). Most infected people present diarrhea, fever, and stomach cramps (CDC, 2019). This bacterium is widely disseminated in poultry farms in Brazil, especially in eggs, chickens, and the environment (Corrêa *et al*., 2018). Birds are generally infected by consuming contaminated feed, cross-contamination in breeding sites, or during slaughter and processing (Paião *et al*., 2013). In the case of poultry meat exports, the current laws of the country to which the food is destinedmust be followed. In Brazil, the legislation establishes a list of microbiological standards for food to be ready for consumers and requiresthat five randomly selected samples of raw poultry meat or offal must be absent of SE and ST(ANVISA, 2019). Similarly, the European Union legislation mandates that for fresh poultry meat, the analysis of five random samples should present absence of SE and ST colonies following the ISO 6579-1:2017 analysis method for detection and the Kauffmann-White-Le Minor system for serotyping (ISO 6579-3:2014; European Union, 2005). As this bacterial genus is synonymous with severe intoxications, both legislations recommend the absence of this microorganism in the aforementioned matrices. The standard *Salmonella* detection for quality control of animal products is the conventional bacteriological method that consists in the stages of pre-enrichment, selective enrichment, isolation and selection, biochemical identification, and seroagglutination test. According to Grimont & Weill (2007), the serotyping technique is

based on different antigenic formulas involving capsular, somatic, and flagellar antigens responsible for classifying *Salmonella* into serotypes. Specific antibodies are used for the antigenic structures present on the bacterium's cell surface to evaluate which antigens are present. What is more, the time required to process the samples and the final diagnosis is long and can last from five to seven days,in addition to other limitations such as low accuracy, specificity, and sensitivity, and the interpretation being quite subjective depending on the reaction with the antiserum.Hence, developing more modern alternative methods for laboratories of the food industryis pivotal to ensure the products are availableat low costs, free of pathogens, and as soon as possible (Gouvêa *et al*., 2012; Corrêa *et al*., 2018; Scopes *et al*., 2018; Wang *et al*., 2018). Polymerase chain reaction (PCR), including reverse transcription PCR (RT-PCR), quantitative real-time PCR (qPCR), and multiplex PCR, have been widely applied as quick and specific forms of detecting *Salmonella* in perishable foods such as milk, meat, eggs, and vegetables (Li *et al*., 2017), taking around 24 h to obtain the final diagnosis (Flores *et al*., 2001). According to Heymans *et al*. (2018), the detection rate of these molecular methods is greater than or equal to conventional detection methods based on phenotypic characteristics. These characteristics are expressed at the moment and may vary depending on cultivation conditions. Molecular methods minimize the disadvantages of phenotypic methods concerning the reproducibility and typicality of the technique. One molecular technique that can be used to differentiate the two *Salmonella* serotypes is real-time PCR (qPCR) (Paião *et al*., 2013; Afshari *et al*., 2018; Saeki *et al*., 2013). The qPCR method provides several advantages, including ease of quantification, greater sensitivity, reproduction and accuracy, shorter processing times, greater process control, and lower risk of contamination (Melo *et al*., 2018). Therefore, this study aimed to evaluate the performance of a molecular method to simultaneously detect *Salmonella* Enteritidis and *Salmonella* Typhimurium in a chicken meat matrix instead of conventional serology.

METHODOLOGY

Sampling method: Eight chicken carcasses were randomly purchased from a supermarket chain in Lajeado, Rio Grande do Sul State (southern Brazil), between April and May of 2021. The carcasses were first analyzed according to the Qualitative Determination Method of the MDS2 3M^{TM}presence/absence technique (AOAC, 2019), which is based on the combination of isothermal DNA amplification with bioluminescence detection. *Salmonella* spp. was detected in one of the samples tested, and the other sevendid not show signs of the pathogen.

Strain activation and quantification: *Salmonella* strains (*S*. Typhimurium ATCC 14028, *S*. Enteritidis ATCC 13076, and *Salmonella* spp.) isolated from a positive chicken meat sample were stored in slant nutrient agar (OXOID®) and kept in a refrigerator. For activation, a loop was removed from each tube, transferred to a brain heart infusion broth (OXOID®), and incubated in a bacteriological incubator for 24 h at 36 ºC. The strains were quantified through several decimal dilutions, and the lowest dilutions were plated to establish the number of colony-forming units (CFU) of each strain in the stationary phase. Then, the mean and standard deviation were calculated, being 3.9 ± 1.5 CFU/25 g for SE, 6.1 ± 1.5 CFU/25 g for TS, and 17.3 ± 2.5 CFU/25 g for *Salmonella* spp.

Contamination of chicken carcasses: Chicken carcasses free of *Salmonella* spp. were artificially contaminated (Table 1) in duplicates with *S*. Typhimurium (ATCC 14028), *S*. Enteritidis (ATCC 13076), and *Salmonella* spp. isolated from the carcass. Approximately 3 to 6 *Salmonella* CFU were used per sample (ISO 16140-3). Then, 25 g of the sample was weighed in a Stomacher® bag (Tecnal Mark, M2202 Scale), followed by adding 225 ml of the buffered peptone water (BPW) enrichment broth (OXOID®) and contamination of each sample using the relevant combinations (Table 1).

Table 1. Contamination applied to chicken carcasses

Sample	Microorganisms used
1	*S*. Typhimurium
2	*S*. Enteritidis
3	*Salmonella* spp.
4	*S*. Typhimurium + *S*. Enteritidis
5	*S*. Typhimurium + *Salmonella* spp.
6	*S*. Enteritidis + *Salmonella* spp.
7	*S*. Typhimurium + *S*. Enteritidis + *Salmonella* spp.

Source: The authors (2021).

The aliquotsfrom the 10^{-8} dilutions of the stationary phase were used (0.4 ml of *Salmonella* Typhimurium, 0.4 ml of *Salmonella* Enteritidis, and 0.5 ml of *Salmonella* spp.).A blank was made with all samples (25 g of chicken meat and 225 ml of BPW). The chicken carcass that presented *Salmonella* spp. underwent the same weighing process and BPW addition, although no strain was added as it had already been naturally contaminated. A triplicate of this sample was carried out. Each weighed sample, alongside the BPW and microorganisms, was placed in a stomacher (Interscience, BagMixer 400) for 60 s. The samples were then incubated at 37 ± 1 ºC for 18 ± 2 h according to the International Organization for Standardization (ISO 6579-1:2017). After 18 h of incubation, the bags with chicken samples were homogenized, and an aliquot (1 mL) was transferred in sterile DNA/RNA-free Eppendorf tubes. The pour plate method was used(in duplicate) to prove the strain quantification with the plate count agarculture medium (OXOID®). The added quantities were 0.4 ml of *Salmonella* Typhimurium, 0.4 mL of *Salmonella* Enteritidis, and 0.5 ml of *Salmonella* spp. (in separate plates) from the 10^{-8} dilutions of the stationary phase. After, the plates were incubated at 35 °C for 48 h.

DNA extraction and quantification: The NewGene FastX kit was used for the extraction process. Approximately 100 µl of the sample was added to a sterile DNA/RNA-free Eppendorf tube and centrifuged (Thermo Scientific Pico 21) at 10,000 rpm for 3 min.The supernatant was carefully discarded using a pipette to preserve the pellet at the bottom of the tube. Subsequently, 100 µl of NewGene FastX was added to the tube and homogenized (Solution Agitator AP 59; Phoenix Luferco) until the pellet was dissolved. The Eppendorf tubes were placed in a dry bath (Thermo Scientific Digital) at 95 °C for 10 min, followed by centrifugation for 3 min at 10,000 rpm and 2 µl added to the qPCR reaction. The DNA purity was measured in the UV-VIS spectrophotometer (Thermo Scientific, NanoDrop One), and approximately 50 to 190 ng/µl of the DNA sample was added to the SETAmp kit's qPCR reaction mixture.

qPCR experiment: The NewGene SETAmp kit is specific for SE/ST detection and differentiation. The mastermix component was fractionated according to the need for reactions (27.8 µl for 1 sample). After the enzyme (0.2 µl) was added to the compound, it was centrifuged for 30 s at 10,000 rpm, and 28 µl was then added to the well (PCR plate) along with 2 µl of the DNA extraction sample. The positive controls NewGene SERef (*Salmonella* Enteritidis) and NewGene STRef (*Salmonella* Typhimurium) 2 µl were added to their own PCR reaction tubes with 28 µl of the mastermix. The plate (containing the respective mixtures) was brought to the qPCR, which used reporter FAM and quencher IOWA BLACK FQ (none) for the *S*. Enteritidis, and reporter HEX (VIC) and quencher IOWA BLACK FQ (none) for the *S*. Typhimurium. Cycling occurred with initial denaturation of 95 ºC for 3 min, followed by the PCR step with denaturation at 95 ºC for 15 s and annealing at 60 ºC for 60 s (repeated 40 times). The FAM and VIC fluorescence signals were captured in the annealing step.

RESULTS AND DISCUSSION

PCR methods are highly effective for detecting *Salmonella* after food pre-enrichment (Wang *et al*., 2018). Seven samples were artificially contaminated in this study and used for the NewGene SETAmp kit verification test (Table 1).

Table 2. Average Ct value obtained by the QuantStudio 5 software during sample amplification/detection (reads I for repetition 1, II for repetition 2, and B for blank). Naturally contaminated samples weretested in triplicate and identified as C-I, C-II and C-III

Samples identification	Expcted result	Obtained result (Ct value)		Detection Agreement (%)
		S.Enteritidis	*S*. Typhimurium	
1 - I	ST	n.d.	35.2	100
1 - II	ST	n.d.	34.9	100
1 - B	n.d.	n.d.	n.d.	100
2 - I	SE	24.5	n.d.	100
2 - II	SE	23.3	n.d.	100
2 - B	n.d.	n.d.	n.d.	100
3 - I	S.spp.	n.d.	n.d.	100
3 - II	S.spp.	n.d.	n.d.	100
3 - B	n.d.	n.d.	n.d.	100
4 - I	SE and ST	n.d.	n.d.	0
4 - II	SE and ST	n.d.	39.2*	0
4 - B	n.d.	n.d.	n.d.	100
5 - I	ST and S.spp.	n.d.	n.d.	0
5 - II	ST and S.spp.	n.d.	n.d.	0
5 - B	n.d.	n.d.	n.d.	100
6 - I	SE and S.spp.	34.1	n.d.	100
6 - II	SE and S.spp.	32.2	n.d.	100
6 - B	n.d.	n.d.	n.d.	100
7 - I	SE, ST and S.spp.	34.2	36.3	100
7 - II	SE, ST and S.spp.	n.d.	37.4	50
7 - B	n.d.	n.d.	n.d.	100
C - I	S.spp.	n.d.	n.d.	100
C - II	S.spp.	n.d.	n.d.	100
C - III	S.spp.	n.d.	n.d.	100
SERef	SE	29.8	n.d.	100
STRef	ST	n.d.	34.9	100

Source: The authors (2021).
SERef: *Salmonella* Enteritidis Referenece; STRef: *Salmonella* Typhimurium Referenece; S.spp.: *Salmonella* spp.; *invalid Ct value (sensitivity limit);n.d.: not detected.

All samples, except the blank (B), were performed in duplicates (I and II), and the naturally contaminated sample was tested in triplicate. The detection cycle threshold (Ct) mean values using samples are listed in Table 2. The average Ct value was obtained using the QuantStudio 5 software (Thermofisher) during sample amplification/detection (reads I for repetition 1, II for repetition 2, and B for blank. This was also performed for the ST for *S*. Typhimurium, SE for *S*. Enteritidis, and *S*. spp for *Salmonella* spp. The naturally contaminated sample was analyzed in triplicate and identified as C-I/C-II/C-III). Amplification curves with Ct above 37 were considered negative as informed by the manufacturer of the kit. Out of the eight analyzed samples, five (1-I/1-II/1-B; 2-I/2-II/2-B; 3-I/3-II/3-B; 6-I/6-II/6-B; C-I/C-II/C-III) presented 100% agreement with the expected result. Two samples (4-I/4-II/4-B; 5-I/5-II/5-B) did not amplify as predicted, and one sample (7-I/7-II/7-B) presented 66.6% of agreement. This may have occurred due to divergent results in the same sample, including problems in sample pipetting due to human errors, inadequately calibrated pipettes, or inadequate tips (Salgado *et al*., 2013). Moreover, we also noticed that the kit used in this experiment presented a better result with samples contaminated with only one *Salmonella* serotype and not in conjunction with others, which does not prevent the use of the kit as it is uncommon to find samples naturally contaminated with both SE and ST serotypes. An extra test was carried out to confirm the specificity of the NewGene SETAmp kit for the SE and ST serotypes using three DNA samples and one negative control sample (no template control). Enterobacteria were used to identify any interference from other strains of the same family. Three different DNA samples from *Escherichia coli* (ATCC 25922), *Klebsiella aerogenes* (ATCC 13048), and *Citrobacter freundii* (ATCC 8090) were tested for exclusivity. None of the samples amplified, thereby confirming their specificity for SE/ST. According to Kawata *et al*. (2010), the optimal value for the amount of DNA in the sample is >50 ng/µl, while lower values may cause amplification failures in PCR reactions. All analyzed samples presented concentrations of ≥55 ng/µl, indicating a good recovery. What is more, high-quality DNA is necessary for PCR reactions, in addition to knowing the efficiency of each process in the DNA analysis (e.g., extraction, purification, and quantification). We observed that the quality of the DNA extraction genetic material presented an average of 2.03 (A260/280), indicating adequate purity of DNA extractions; nevertheless, it is pivotal to include a DNA purification stage for better qPCR results. Regarding the SETAmp kit used for amplification and specific detection of the two serotypes (SE/ST), the components of the PCR reaction mixture must be adequate. In this sense, the enzyme must be providedat an adequate concentration for the PCR amplification to occur. Arezi *et al*. (2003) and Purzyck *et al*. (2006) reported that different enzymes may present significantly higher efficiencies than others. Moreover, Zanetti *et al*. (2015) founda high agreement between specific PCR detection and serological analyses and demonstrated the effectiveness of PCR tests in the specific detection of *Salmonella* isolates from serotypes associated with typhoid and pullorum disease outbreaks in birds. The authors pointed out that PCR tests are an efficient alternative to replace the current biochemical and serological methods. Furthermore, Gaspar *et al*. (2019) reported that the qPCR method had superior detection capacity than conventional microbiology, which was less sensitive. The serotyping process is usually performed by testing a colony presumed to be *Salmonella*, and in this study, the kit's performance was verified using the enrichment broth (single or combined strain), as the extraction kit suggests these two possibilities (broth and agar). It is hypothesized that if the detection was conducted directly from the agar, the percentage of the agreement would have likely been different. The commercial kits evaluated herein showed auspicious results in the poultry meat matrix artificially contaminated with SE and ST. Despite the promising findings presented herein, there is still little dataon a simultaneous SE/ST detection kit for rapid and accurate diagnosis in the differentiation of serotypes of public health interest. There are several kits to simultaneously detect *Salmonella* spp., SE, and ST marketed by international companies, although this study is one of the first to validate a kit produced and marketed in Brazil.

CONCLUSION

The kit for detecting and differentiating*Salmonella* DNA, serotypes Enteritidis and Typhimurium, by qPCR from samples previously processed with NewGene FastX and NewGene Preamp was easily employed and proved to be highly practical. The protocols used were effective in amplifying the fragments. Both the extraction process and

the pipetting of the reaction mixture components for the PCR required few transfers, which may lead to higher productivity in cases of high laboratory demand. Given the above,this study demonstrated an alternative diagnostic procedure to detect/identify pathogens andproved to bea solid candidate to replace the serology step of the conventional method.

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