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Rapid DNA Detection of *Salmonella enterica* Typhimurium and Heidelberg from Poultry Samples

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Abstract: The *Salmonella enterica* serovars Typhimurium (*S.* Typhimurium), Heidelberg (*S.* Heidelberg), and their monophasic variants (*S.* 1,4,[5],12:i:-, S. 1,4,[5],12:r:- and S. 1,4,[5],12:-:1,2) are highly disseminated in poultry farming and can contaminate chicken meat, eggs, and other foods of avian origin. A time-consuming bacteriological and serological analysis is usually required to identify serovars by traditional methods. Incomplete and inconclusive serological results are frequent in routine analysis, mainly due to the occurrence of bacterial isolates presenting similar antigenic profiles. Molecular biology assays have been developed to improve the detection of specific *Salmonella* serovars and strains. This study aimed to develop a multiplex real-time PCR (SHTAmp) for the rapid DNA detection of *S.* Typhimurium, *S.* Heidelberg, and their monophasic variants from poultry samples. The methodology was used in the analysis of 147 field isolates from Brazilian poultry flocks previously evaluated with serological analysis. The results demonstrated that it was able to specifically and rapidly detect 21 *S.* Typhimurium and 57 *S.* Heidelberg isolates with complete antigenic formulae. Furthermore, SHTAmp was able to differentiate nine *S.* Typhimurium and 44 *S.* Heidelberg isolates with incomplete serological formulae (monophasic and aphasic variants). The complete methodology was also successfully used to detect these bacteria directly from 34 poultry samples after pre-enrichment in buffered peptone water (BPW). In conclusion, SHTAmp is a fast and accurate method to detect the two frequent and concerning serovars *S.* Typhimurium and *S.* Heidelberg directly from poultry samples.

Keywords: molecular diagnosis; multiplex real-time PCR; *Salmonella* Typhimurium; *Salmonella* Heidelberg

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1. Introduction

Salmonella is one of the most important enteric bacterial pathogens in human and animal health. This bacterial genus can infect humans through the consumption of foods of poultry origin. *Salmonella* is present in these foods due to its wide spread in intensive commercial poultry farms. It infects the intestinal microbiota of birds in flocks without the presentation of clinical signs, and can contaminate chicken meat and eggs. Human contamination by this bacterium can result in enteric diseases and even systemic infection and septicemia [1].

Taxonomically, *Salmonella* belongs to the family Enterobacteriaceae and has two species: *S. enterica* and *S. bongori*. *S. enterica* is further subdivided into the six subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. *Salmonella* spp. is also classified into approximately 2600 serovars, some of them highly adapted to chickens and with high prevalence in poultry flocks around the world [2]. Two of these serovars, Typhimurium (*S.* Typhimurium) and Heidelberg (*S.* Heidelberg), are highly frequent in broiler and

layer chicken farming and have frequently been associated with outbreaks of foodborne infections around the world [3–6]. *S.* Typhimurium has been a concerning serovar for a long time, with a wide spread in poultry and other livestock production systems, such as pig and cattle farming. Moreover, the *S.* Typhimurium monophasic variant (antigenic formula 1,4,[5],12:i:-) emerged in poultry and pig farming recently, and is often associated with salmonellosis and human outbreaks [7]. *S.* Heidelberg has also been considered a cause of concern for human diseases because it is frequently detected in foods of animal origin and can result in foodborne outbreaks [8,9]. This serovar has also assumed greater importance in recent years due to its dissemination on poultry production farms [6,9]. Bacterial isolates from these two *Salmonella* serovars (and their monophasic variants) are even more concerning because they generally exhibit resistance to several antimicrobials [6,8,9].

Laboratory procedures for detecting the presence of *Salmonella* and the additional identification of serovars have been routinely used to analyze samples collected from poultry farms, slaughterhouses, egg processing industries, and foods sold on the public market. These procedures aim to mitigate the risk of human contamination [10–12]. Some poultry-producing countries have adopted specific control programs to reduce the spread of *Salmonella* and prevent foodborne contamination. In Brazil, the National Program for Poultry Health (*Programa Nacional para Sanidade Avícola*—PNSA, *Ministério da Agricultura e Pecuária*—MAPA) regulates the control of the main pathogens in poultry farming. The PNSA determines that the diagnosis of *Salmonella* in poultry samples must be carried out through traditional microbiological bacterial isolation, serological identification, and/or detection by molecular biology. Furthermore, it establishes that the identification of the serovars Gallinarum, Enteritidis, and Typhimurium is necessary after the detection of any *Salmonella* strain in poultry samples [13–15].

The traditional bacteriological method to detect *Salmonella* includes culture isolation and the biochemical/antigenic characterization of the isolates. Additional serological tests are necessary to identify the *Salmonella* serovars. Currently, the multi-step and complex White–Kauffman–Le Minor (WKL) scheme is the gold standard serological method [16]. In this laboratorial method, *Salmonella* isolates are usually serotyped with slide agglutination tests using several antisera against somatic (O) and flagellar (H) antigens to determine the isolate's antigenic formula and the specific serovar. Also, it is necessary to carry out the phase change bacteriological technique to define the two flagellar proteins expressed in motile serovars [2]. The occurrence of monophasic and aphasic variants of some serovars brings additional complexity into the identification, since some isolates cannot present some H antigens and the antigenic formula results are incomplete [17]. Specifically, *S.* Typhimurium and *S.* Heidelberg have similar antigenic formulae with one unique difference (phase 1 flagellar protein "i" in Typhimurium and "r" in Heidelberg), making their antigenic differentiation in the WKL scheme, mainly for monophasic isolates, more difficult [16].

Polymerase chain reaction (PCR) assays have been described and used to detect *Salmonella* and some of the main concerning serovars and strains [17–22]. This method has high specificity and sensitivity, providing more accuracy in the laboratory analysis. In addition, the complete *Salmonella* laboratory procedure (with serovar identification) can be performed in one day, increasing the overall productivity of the poultry industry and supplying safe food products for human consumption. A highly qualified method of detection of *Salmonella* in poultry farms and industries is also necessary to avoid the spread of bacterial pathogens [11,23].

The present study presents the development and routine use of a specific multiplex real-time PCR to detect two concerning *Salmonella* serovars (Typhimurium and Heidelberg) and their monophasic flagellar variants. The developed method was used to rapidly identify these serovars from *Salmonella* isolates directly from pre-enriched environmental samples collected in poultry farms.

2. Materials and Methods

2.1. Bacterial Isolates

The overall sampling included 147 isolates of *Salmonella enterica* subspecies *enterica* recovered as part of this study and obtained from Brazilian poultry flocks (broilers, broiler breeders, and quails) analyzed by PNSA standard methods [24] from January 2019 to December 2020. They were sourced from the flocks' environments after sampling performed using drag and boot swabs (n = 102), feces (n = 29), feed (n = 5), and litter (n = 11) in farms from different poultry-producing regions in Brazil. All *Salmonella* strains were obtained using classical bacteriological methods for isolation and characterization. Briefly, 25 g of each environmental sample was pre-enriched in 225 mL of 1% buffered peptone water–BPW (Laborclin, Pinhais, Brazil) in a sterile plastic bag and incubated at 37 °C for 24 h. After this period, the next step was selective enrichment into Rappaport–Vassiliadis broth media (Difco, Detroit, MI, USA) in a ratio of 1:100 and Selenite Cystine (Difco, Detroit, MI, USA) in a ratio of 1:10. From each of these two selective enrichment cultures, aliquots were transferred aseptically on plates containing Brilliant Green, MacConkey, and *Salmonella*–Shigella agars (Difco, Detroit, MI, USA) at 37 °C and incubated for 24 h. Presumptive identification was carried out through cultures obtained from selective plating. After this, two and three colonies suggestive of *Salmonella* were chosen and incubated in tubes containing Triple Sugar Iron and Lysine Iron slant agars (Difco, Detroit, MI, USA). Subsequently, the isolates were classified according to their biochemical characteristics using indole and citrate, followed by incubation at a temperature of 37 °C for a period of 24 h [24]. Colonies biochemically classified as *Salmonella* were additionally evaluated by serology with polyvalent anti-*Salmonella* somatic (O) and flagellar (H) antisera (Bio-Rad, Marnes-la-Coquette, France) in a partial WKL scheme using a limited number of antisera available in the routine laboratory. This procedure was developed to identify the most concerning poultry *Salmonella* serovars (such as *S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium) by testing all main antigens that identify them [16]. Furthermore, all bacterial isolates were stored in brain–heart infusion (BHI) broth (Laborclin, Pinhais, Brazil) with 15% sterile glycerol at −20 °C until other procedures were conducted.

Eight other *Salmonella* isolates from the culture collection of the Molecular Diagnostic Laboratory at ULBRA, with whole-genome sequence (WGS) information, were also included in the study to implement the multiplex PCR. This sampling included isolates from the serovars Enteritidis (n = 2; ULBRA-SA068, ULBRA-SA078), Gallinarum (n = 2; ULBRA-SA090, ULBRA-SA146), Heidelberg (n = 2; ULBRA-SA358, ULBRA-SA388), and Typhimurium (n = 2; ULBRA-SA065, ULBRA-SA209) (BioSamples accession numbers in GenBank: SAMN08387173, SAMN08387194, SAMN08387188, SAMN08387182, SAMN10393207, SAMN10393206, SAMN08387149, SAMN08387181, respectively).

2.2. Clinical/Environmental Poultry Flock Samples

A total of 34 clinical (tissues and organs from necropsies) and environmental (drag and boot swabs, feces, and poultry litter) samples were obtained from Brazilian poultry flocks with clinical suspicion of infection with *Salmonella*. These samples were pre-enriched in 225 mL of 1% of BPW (Laborclin, Pinhais, Brazil), homogenized in a stomacher for 2 min, and then incubated at 36 °C ± 1 °C for 16 ± 2 h. Then, 1 mL and 0.1 mL of BPW were added, respectively, to 10 mL of Tetrathionate (TT) broth (Difco, Detroit, USA) and 10 mL of Rappaport–Vassiliadis (RV) broth (Difco, Detroit, USA), and both were incubated at 42–43 °C for 18–24 h. Aliquots of the samples pre-enriched in BPW were also stored at −20 °C for molecular procedures. TT and RV broths were plated in MacConkey and Hektoen Enteric Agar (Difco, Detroit, USA). Complete bacteriological *Salmonella* isolation was further performed according to the standard PNSA methods [24]. *Salmonella* isolates were also serotyped according to the reduced WKL scheme described above.

2.3. DNA Extraction and Multiplex Real-Time PCRs

Nucleic acids from bacterial isolates were extracted by the boiling method [25]. DNA from the pre-enriched field samples was extracted using NewGene Prep and Preamp reagents according to the manufacturer's instructions (Simbios Biotecnologia, Cachoerinha, Brazil). Briefly, 100 µL of the sample was added to 400 µL of lysis buffer and incubated at 60 °C for 10 min. After centrifugation (9410× *g*, 1 min), the supernatant was transferred to a tube containing 20 µL of silica suspension. After vortex and centrifugation (9410× *g*, 1 min), the pellet was washed with 300 µL GuSCN-Tris buffer, followed by washing with 75% ethanol and absolute ethanol. The silica suspension was dried and DNA was eluted with 50 µL of elution buffer. The DNA was stored at −20 °C until use in PCR assays. Real-time PCR for *Salmonella* detection was carried out using the commercial reagent NewGene SALAmp (Simbios Biotecnologia) [17].

The real-time multiplex PCR (SHTAmp) was designed to simultaneously detect *S.* Typhimurium and *S.* Heidelberg, with the use of primers and probes targeting these serovars' specific genetic regions (fliA-IS200 and ACF69659, respectively), as previously demonstrated [17,20]. All assays were adjusted to a total volume of 30 µL with 1.5 mM of $MgCl_2$, 1U of Taq DNA Polymerase (4G P&D, Porto Alegre, Brazil), 0.1 mM of dNTPs (Promega, Madison, WI, USA), 0.125 µM of each probe (IDT, Coralville, IA, USA), and 0.1 to 0.5 µM of the respective primers (IDT). All SHTAmp PCRs were carried out with 2.0 µL of extracted DNA in StepOnePlus™ or Applied Biosystems 7300 Real-Time PCR systems (Thermo Fisher Scientific, Waltham, MA, USA). The thermocycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Amplification plots were created with the analysis of the fluorescent signal from all samples and controls. The cycle threshold (Ct) was determined for each sample in the real-time PCR runs. Only samples producing a cycle threshold (Ct) of <36 were considered positive. Positive (DNA from *S.* Typhimurium ULBRA-SA065 or ULBRA-SA209 and *S.* Heidelberg ULBRA-SA358 or ULBRA-SA388) and negative (DNA from *Escherichia coli*) controls were used in the PCR runs.

3. Results

3.1. Analysis of Salmonella Isolates

All 147 bacterial isolates were classified as *Salmonella* by standard microbiological analysis and they presented positive results for this bacterial genus after analysis with the SALAmp assay. In the serological analysis, isolates with complete serological profiles (n = 83) were classified as *S.* Heidelberg (n = 57), *S.* Typhimurium (n = 21), *S.* Hadar (n = 1), *S.* Minnesota (n = 2), *S.* Sandiego (n = 1), and *S.* Schwarzengrund (n = 1). The remaining 64 isolates had only been partially analyzed because of technical limitations in the serological analyses. Since they had incomplete antigenic formulae, they were identified as *Salmonella* spp. (Table 1).

To implement the SHTAmp PCR multiplex assay, DNA from eight previously characterized *Salmonella* isolates belonging to four serovars (Gallinarum, Enteritidis, Heidelberg, and Typhimurium) were initially analyzed. The DNA from the *S.* Typhimurium isolates ULBRA-SA065 and ULBRA-SA209 was positive in the PCR targeting the fliA-IS200 genetic region and negative in the PCR using the gene ACF69659 as the target, while the DNA from the *S.* Heidelberg isolates ULBRA-SA358 and ULBRA-SA388 was positive in the PCR targeting the gene ACF69659 and negative in the PCR amplifying fliA-IS200. The DNA from the other isolates was negative in both PCRs.

After this, all *Salmonella* isolates from the culture collection were analyzed with the SHTAmp assay (Table 1). All isolates identified as *S.* Heidelberg (n = 57) by the partial WKL scheme presented positive results for the specific *S.* Heidelberg ACF69659 target and negative results for fliA-IS200. Also, all isolates identified as *S.* Typhimurium (n = 21) by the partial WKL scheme presented positive results for the specific *S.* Typhimurium DNA fragment fliA-IS200 and negative results for ACF69659.

Table 1. *Salmonella enterica* isolates (n = 147) from Brazilian poultry flocks with clinical suspicion of infection and results according to partial WKL scheme and qPCR assays.

Serological Profile (n)	Antigenic Formula	*S. enterica* qPCR	Heidelberg qPCR	Typhimurium qPCR	Partial WKL [1] Scheme (n)	SHTAmp qPCR (n)
Poli O; OMA; O:4; O:5; O:12; H:r and H:2 (57)	1,4,[5]12:r:1,2	+	+	-	Heidelberg (57)	Heidelberg (57)
Poli O; OMA; O:4; O:5; O:12; H:i and H:2 (21)	1,4,[5]12:i:1,2	+	-	+	Typhimurium (21)	Typhimurium (21)
Poli O, OMB; O:8; H:z10 e H:enx (1)	6,8:z10:e,n,x	+	-	-	Hadar (1)	Negative (1)
Poli O; OMA; O:21; H:e,n,x; H:b (2)	21:b:e,n,x	+	-	-	Minnesota (2)	Negative (2)
Poli O; OMA; O:4; H:E; H:h e H:enz15 (1)	1,4,[5]12:e,h:e,n,z15	+	-	-	Sandiego (1)	Negative (1)
Poli O; OMA; O:4; O:12; O:27; H:d and H:7 (1)	1,4,12,27:d:1,7	+	-	-	Schwarzengrund (1)	Negative (1)
Poli O; OMA; O:4; O:5; O:12; H:i (4)	1,4,[5],12:i:-	+	-	+	*Salmonella* spp. (4)	Typhimurium (4)
Poli O; OMA; O:4; O:5; O:12; H:2 (5)	1,4,[5],12:-:1,2	+	-	+	*Salmonella* spp. (5)	Typhimurium (5)
Poli O; OMA; O:4; O:5; O:12; H:2 (2)	1,4,[5],12:-:1,2	+	-	-	*Salmonella* spp. (2)	Negative (2)
Poli O; OMA; O:4; O:5; O:12; H:2 (9)	1,4,[5],12:-:1,2	+	+	-	*Salmonella* spp. (9)	Heidelberg (9)
Poli O; OMA; O:4; H:2 (4)	1,4:-:1,2	+	+	-	*Salmonella* spp. (4)	Heidelberg (4)
Poli O; OMA; O:4; H:2, H:r (3)	1,4:r:1,2	+	+	-	*Salmonella* spp. (3)	Heidelberg (3)
Poli O; OMA; O:4; H:r (2)	1,4:r:-	+	+	-	*Salmonella* spp. (2)	Heidelberg (2)
Poli O; OMA; O:4; O:12 (5)	1,4,12:-:-	+	+	-	*Salmonella* spp. (5)	Heidelberg (5)
Poli O; OMA; O:4; O:5; O:12 (7)	1,4,[5]12:-:-	+	+	-	*Salmonella* spp. (7)	Heidelberg (7)
Poli O; OMA; O:4 (3)	1,4:-:-	+	+	-	*Salmonella* spp. (3)	Heidelberg (3)
Poli O, O:4 (1)	1,4:-:-	+	+	-	*Salmonella* spp. (1)	Heidelberg (1)
Poli O, O:5 (1)	1,[5]:-:-	+	+	-	*Salmonella* spp. (1)	Heidelberg (1)
Poli O (9)	-:-:-	+	+	-	*Salmonella* spp. (9)	Heidelberg (9)
Poli O (3)	-:-:-	+	-	-	*Salmonella* spp. (3)	Negative (3)
Poli O; OMA; O:4; O:5; O:12; H:r (2)	1,4,[5],12:r:-	+	-	-	*Salmonella* spp. (2)	Negative (2)
Poli O; OMA; O:4; H:e (1)	1,4:e:-	+	-	-	*Salmonella* spp. (1)	Negative (1)
Poli O; OMA; O:4; O:12; O:27 (2)	1,4,12,27:-:-	+	-	-	*Salmonella* spp. (2)	Negative (2)
Poli O; OMA; O:4; O:12; O:27, H:r (1)	1,4,12,27:r:-	+	-	-	*Salmonella* spp. (1)	Negative (1)

[1] WKLM = White–Kaufmann–Le Minor scheme.

Moreover, 53 *Salmonella* isolates with incomplete antigenic formulae, all identified as *Salmonella* spp. by serological diagnosis, could be identified as *S.* Heidelberg (n = 44) or *S.* Typhimurium (n = 9) using SHTAmp. Importantly, the four isolates with the antigenic formula 1,4,[5],12:i:- (without the second flagellar antigen) were positive for fliA-IS200 and negative for ACF69659, demonstrating that they should be classified as *S.* Typhimurium. In opposition, the 16 isolates with the antigenic formula 1,4,[5],12:-:1,2 (without the first flagellar antigen) presented three different results in SHTAmp: positive for ACF69659 and negative for fliA-IS200 (n = 9), thus being classified as *S.* Heidelberg; positive for fliA-IS200 and negative for ACF69659 (n = 5), thus being classified as *S.* Typhimurium; and negative for both of these targets (n = 2), thus being classified as *Salmonella* spp. It is noteworthy that the two isolates with the antigenic formula 1,4,[5],12:r:- (without the second flagellar antigen) were negative for both targets, thus being classified as *Salmonella* spp. No sample tested positive for the two serovar-specific genetic targets (ACF69659 and fliA-IS200).

The remaining 16 isolates with other antigenic formulae, including all isolates of the serovars *S.* Hadar, *S.* Minnesota, *S.* Sandiego, and *S.* Schwarzengrund, presented negative results for both targets, so they were classified as *Salmonella* spp. by the SHTAmp assay (Table 1).

3.2. Analysis of Clinical/Environmental Poultry Flock Samples

The clinical/environmental poultry flock samples were analyzed for *Salmonella* detection. *Salmonella* spp. was detected in all 34 poultry samples using conventional PNSA methodology. In addition, they were also evaluated using the SALAmp assay, exhibiting positive results for *Salmonella*. In the serological analysis, 30 isolates were classified into

the serovars *S.* Heidelberg (*n* = 6), *S.* Typhimurium (*n* = 3), *S.* Anatum (*n* = 1), *S.* Bredeney (*n* = 1), *S.* Enteritidis (*n* = 8), *S.* Gallinarum (*n* = 8), *S.* Schwarzengrund (*n* = 2), and *S.* Tennessee (*n* = 1). The remaining four isolates presented a partial serological profile and the serovar could not be identified, so they were classified as *Salmonella* spp. (Table 2).

Table 2. Results of the 34 field poultry samples pre-enriched with BPW and results according to WKLM scheme and qPCR assays.

Antigenic Formula	*S. enterica* qPCR	Heidelberg qPCR	Typhimurium qPCR	WKLM [1] Scheme Result (n)	SHTAmp qPCR (n)
3,10[15][15,34]:e,h:1,6	+	-	-	Anatum (1)	Negative (1)
1,4,12,27:l,v:1,7	+	-	-	Bredeney (1)	Negative (1)
1,9,12:g,m:-	+	-	-	Enteritidis (8)	Negative (8)
1,9,12:-:-	+	-	-	Gallinarum (8)	Negative (8)
1,4,[5]12:r:1,2	+	+	-	Heidelberg (6)	Heidelberg (6)
1,4,12,27:d:1,7	+	-	-	Schwarzengrund (2)	Negative (2)
6,7,14:z29:[1,2,7]	+	-	-	Tennessee (1)	Negative (1)
1,4,[5]12:i:1,2	+	-	+	Typhimurium (3)	Typhimurium (3)
Missing data	+	-	-	*Salmonella* spp. (4)	Negative (4)

[1] WKLM = White–Kaufmann–Le Minor scheme.

These same poultry flock samples were also analyzed with the SHTAmp assay after DNA extraction from an aliquot of the pre-enriched broth (BPW). All isolates previously defined as *S.* Heidelberg (*n* = 6) by the partial WKL scheme presented positive results for the specific *S.* Heidelberg ACF69659 target and negative results for fliA-IS200. In opposition, all samples detected by serological analysis as *S.* Typhimurium (*n* = 3) presented positive results for the specific *S.* Typhimurium DNA fragment fliA-IS200 and negative results for ACF69659. The remaining 25 samples, including four partially analyzed isolates, presented negative results for both targets, so they were classified as *Salmonella* spp. (Table 2). No sample tested positive for the two serovar-specific genetic targets (ACF69659 and fliA-IS200).

4. Discussion

Salmonella enterica is a cause for concern in poultry farming around the world. Traditionally, the classification into serovars has made it possible to monitor the emergence of pathogenic lineages of this bacterial species that represent a microbiological danger to poultry farming. Among all serovars, *S.* Typhimurium and *S.* Heidelberg have been frequently associated with many foodborne infection outbreaks. Furthermore, some of their main disseminated lineages present multidrug-resistant profiles worldwide [3–6,9,26]. Besides being detected in poultry farms, *S.* Typhimurium is an important cause of human gastroenteritis in different continents [27]. Moreover, *S.* 1,4,[5],12:i:- (monophasic variant of *S.* Typhimurium) has emerged in poultry and swine industries more recently, often associated with salmonellosis and human outbreaks [7]. In Brazil, *S.* Typhimurium and *S.* 1,4,[5],12:i:- have been isolated in human samples as well as in those from swine and poultry farms [5,28–30]. On the contrary, *S.* Heidelberg has emerged mainly in poultry farms over recent decades [8,9]. In North America, this serovar has been commonly isolated in broilers, layers, turkeys, and even pigs [31–33]. In Brazil, it has been frequently found in poultry farms since the year 2000 [8,9,34–37].

These two serovars present very similar antigenic formulae (1,4,[5],12:i:1,2 for *S.* Typhimurium and 1,4,[5],12:r:1,2 for *S.* Heidelberg). Often, the identification and differentiation of these two serovars are inconclusive using the classic serological method, since the reactions of antigens "i" and "r" may not be clearly evident using specific antisera in the laboratory. The precise identification of these two serovars has been a particular challenge in many poultry microbiological analysis laboratories due to the antigenic similarity associated with the possibility of the occurrence of monophasic and aphasic isolates. This situation has led to the release of several misleading serovar identification

results. Therefore, other laboratory methods are necessary to differentiate these similar antigenic serovars.

Molecular methods have become an important alternative for the complex procedure based on the microbiological method combined with complete serological analysis [17,20]. The WKL scheme is time-consuming, expensive, and laborious, requiring many antisera and agglutination tests to obtain the complete antigenic formula of the isolates. Therefore, most veterinary laboratories use partial serology to detect the most frequent and concerning serovars, frequently resulting in partial serological characterization [3,16,17].

The present study focused on the development and effective application of a multiplex real-time PCR to detect two of the most concerning *Salmonella* serovars for public health that are frequently identified in poultry farms in Brazil [6]. The developed method (SHTAmp) was evaluated in the analysis of 147 bacterial isolates obtained from Brazilian poultry flocks. It demonstrated 100% inclusivity, which means all isolates of *S.* Typhimurium and *S.* Heidelberg previously detected with the partial WKL scheme presented positive results in the SHTAmp. This performance is in agreement with previously published results [17,20].

In addition, the method was very useful to precisely identify these two serovars in isolates with incomplete antigenic formulas. In a total of 64 isolates classified as *Salmonella* spp. by the partial WKL scheme used in one veterinary laboratory, SHTAmp was able to identify 53 (53/64; 82.8%) of these samples as Typhimurium or Heidelberg. It was also possible to identify the serovar in isolates lacking one of the flagellar antigens (1,4,[5],12:i:- and 1,4,[5],12:-:1,2). As expected, all isolates with the antigenic formula 1,4,[5],12:i:-, known in the literature as *S.* Typhimurium monophasic variant 1,4,[5],12:i:-, were classified as *S.* Typhimurium, since the genetic region fliA-IS200 is present in the monophasic and biphasic isolates of *S.* Typhimurium [20,38]. Isolates with the antigenic formula 1,4,[5],12:-:1,2 cannot be considered monophasic variants of a specific serovar, since the results described here showed that they can be classified as *S.* Typhimurium, *S.* Heidelberg, or even other serovars. Similarly, isolates with the antigenic formula 1,4,[5],12:r:- are not necessarily monophasic variants of *S.* Heidelberg lacking flagellar phase 2, as demonstrated in this study. In these cases, the two isolates were probably the partially serotyped serovar *S.* Bochum (complete antigenic formula 1,4,[5],12:r:l,w) or another serovar with a misinterpretation of agglutination in the serological analysis [16]. This situation has become common in some poultry laboratory analyses, since *S.* Typhimurium and *S.* Heidelberg are frequently detected as monophasic variants in the poultry production chain. The effort to detect these serovars in the poultry industry is necessary because they are widespread in many countries and cause serious illness in humans [39,40].

The SHTAmp assay was also tested with DNA extracted directly from field poultry samples pre-enriched in BPW, since recent studies concluded that it is a suitable media for use before molecular methods to detect *Salmonella* and provides the results within 24 h, as there is no need for bacterial isolation with other microbiological media, saving cost and time of response [17,41]. Other previous studies have also developed serovar-specific PCR methods to detect *S.* Typhimurium and *S.* Heidelberg from different matrices, including food, poultry, and clinical samples [17,38,42–44]. However, this is the first report to include the detection of these two important serovars in the same assay, as well as to demonstrate the utility in identifying their monophasic variants. All field poultry samples with *S.* Typhimurium and *S.* Heidelberg assessed using the bacteriological procedure were also positive for the respective serovar in the multiplex real-time PCR developed here, demonstrating the application of SHTAmp to obtain fast results in poultry diagnostic laboratories.

5. Conclusions

In conclusion, the multiplex PCR assay described here is a fast and less complex procedure to detect the DNA of *S.* Typhimurium, *S.* Heidelberg, and their monophasic variants, besides being able to identify isolates of serovars partially analyzed by serological procedures. This method could be applied in public and private diagnostic laboratories.

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