Evaluation of the different methods to detect *Salmonella* in poultry feces samples

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Abstract

Salmonella is one of the most common causes of foodborne outbreaks and infection worldwide. The gold-standard detection method of Salmonella is cultivation. There is a need to investigate rapid and accurate processes with time-consuming cultivation. The study evaluated different approaches to detect Salmonella in poultry feces samples. Poultry farm feces samples from 21 cities in Iran were collected from January 2016 to December 2019. Microbiological cultures, serological assays, and multiplex PCR (m-PCR) were used to detect and characterize *Salmonella* spp. isolates. Serological assays and m-PCR were used to determine the serogroups A, B, C1, C2, D1, E, H, and FliC. The m-PCR was used to detect seven Salmonella serovars, and a Chi-square test was performed to compare the discriminatory power of the methods. Of 2300 poultry feces samples, 173 (7.5%) and 166 (7.2%) samples were detected as *Salmonella* spp. by cultivation and m-PCR, respectively. The sensitivity of the molecular method was equal to cultivation at 0.96 (CI = 95%). Assessment of H antigenic subgroups showed the same for both m-PCR and serological tests. Therefore, the matching rate of the two methods for detecting all H antigenic subgroups was 100%. Thus, the relationship between the results obtained from both methods was significant in the contingency table test ($P < 0.01$). The PCR-based approach confirmed the detection of Salmonella in a shorter period (24–36 h) compared to the conventional microbiological approach (3–8 days).

Keywords  *Salmonella* · Poultry · Feces · Multiplex PCR · Culture

Introduction

Salmonella is gram-negative, rod-shaped bacteria in the family *Enterobacteriaceae*. *Salmonella* is the most common bacterial pathogen associated with foodborne disease in the United States (Gu et al. 2019). Poultry products, including meat and eggs, have been a significant source of *Salmonella* contamination. Therefore, it is crucial to develop rapid and accurate methods for detecting *Salmonella* in poultry feces samples.
of Salmonella infections (Wang et al. 2020). Salmonella infections account for 93.8 million cases and 155,000 deaths per year worldwide, with several serotypes involved, such as *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Senftenberg, *S. enterica* serovar Hadar, *S. enterica* serovar Agona, and *S. enterica* serovar Typhimurium (Gantois et al. 2008). *S. enterica* serovar Enteritidis is the most reported in human outbreaks during the last 2 decades (Ghazalibina et al. 2019). There is limited information on the prevalence of foodborne diseases in Iran. Studies have considered small populations and did not show the status of foodborne diseases in Iran. The Centers for Disease Control developed a national guideline for foodborne diseases in 2006 and launched it in the same year (Asl et al. 2015). The country's technical committee has approved the guideline. Its first step is to identify the five most common foodborne illnesses caused by *Salmonella*, *Shigella*, *E. coli*, *Staphylococcus* toxin and Botulism (Asl et al. 2015). Therefore, *Salmonella* spp. infection prevention is crucial for poultry health and food processing industries.

Accordingly, the diagnosis and serotyping of *Salmonella* spp. are critical subjects. Conventional Salmonella detection methods include culturing in a selective medium, followed by colony characterization using biochemical and serological tests (Kasturi 2020). Most laboratories use serotyping as the main phenotyping method for subspecies *Salmonella* spp. typing and approximately 2600 serotypes have been described according to the Kauffman–White–LeMinor scheme. Included is the somatic antigen (O) determining the group, flagellar antigen (H) determining the serotype, and capsular antigen (K) (Kariuki et al. 2015).

Conventional detection methods are laborious and time-consuming. Serotyping methods are often ineffective as epidemiological tools because of their low discriminatory capacity for strains with the same serotype or similar biochemical characteristics (Barrow and Neto 2011). Therefore, it is not often possible for research laboratories to detect *Salmonella* in-house, and isolates are sent to commercial or expert laboratories, which may delay the results (Diep et al. 2019). Consequently, a rapid and sensitive method is required to detect *Salmonella* spp. and their serovars.

Some molecular techniques are widely used to detect *Salmonella* spp. and serovars. Molecular techniques are used instead of conventional methods because of their reduced time for diagnosis with similar or higher efficiency, increased discriminatory power, simplicity, better standardization, reproducibility, and higher sensitivity and specificity (Malorny et al. 2009; Khaledi and Meskini 2020). The diagnosis time of Salmonella in samples is very critical because, in some situations, especially the clearance of heavy shipments is very important in several respects: first, to observe the cold chain to prevent their spoilage (for example, shipments of several thousand tons of imported meat); secondly, the cost of keeping them in proper conditions at customs is very high, and thirdly, the late clearance of such shipments is important in terms of cargo fluctuations. By shortening the detection time to less than 2 days by molecular method, all the above problems can be overcome (Barrow 1994; Roy et al. 2002).

Molecular techniques based on the amplification of DNA, such as multiplex polymerase chain reaction (m-PCR), have been used to detect Salmonella serotypes (Du et al. 2020). The m-PCR uses pairs of primers that allow the simultaneous detection and identification of specific DNA sequences in the same reaction (Maciorowski et al. 2005). To the best of our knowledge, the comparison of traditional versus m-PCR techniques to detect *Salmonella* genus, serogroups, and serovars in Iran has not been conducted. Therefore, this study compared the discriminatory power of several methods such as cultivation, serological, and m-PCR to detect *Salmonella* genus, serogroups and serovar in farm poultry feces samples.

**Materials and methods**

**Collection of samples and isolation of Salmonella**

Poultry feces samples from five different areas in each poultry farm were collected (Fig. 1). Farms were located in Semnan, Fars, Qazvin, Qom, Yazd, Khorasan Razavi, Mazandaran, Kerman, Alborz, South Khorasan, Kurdistan, Markazi, Isfahan, Kohkiluyeh, Boyerahmad, West Azerbaijan, Golestan, Zanjan, Hamedan, Kermanshah, Khuzestan, and Lorestan, of Iran. Using sterile spoons, samples were collected in sterile zipper bags from January 2016 to December 2019. All samples were transported immediately to the Department of Molecular Microbiology, Pasteur Institute of Iran while maintaining sterile and cold chain conditions.

A 25 g of each sample was added to 225 mL 0.1% peptone water, and the mixture was incubated overnight at 37 °C. After incubation, 0.1 mL of each sample was transferred to 10 mL Rappaport–Vassiliadis Soy Peptone (RVS) Broth (Merck, Germany) and incubated overnight at 41.5 °C. Following the incubation, samples were cultured on Xylose Lysine Deoxycholate (XLD) agar (Merck, Germany) and incubated overnight at 37 °C. Red colonies with a black center (Fig. 2) were subcultured in nutrient agar (NA) (Merck, Germany) to perform Gram staining and biochemical tests (Sobur et al. 2019). Gram staining identified morphological characteristics and biochemical identification included sugar fermentation, Voges Proskauer (VP) test, indole, and methyl red (MR) test (Alam et al. 2020). Each sample with TSI (+), urease (−), indole (−),
LDC (+), MR (+), VP (−), citrate (±), and motility (+) was considered for further analysis. After identifying the isolates, serotyping was performed by O (polyvalent), A, B, C1, C2, D1, and E antisera (MAST, Germany).

DNA extraction and molecular detection of Salmonella genus

Genomic DNA was extracted using a High-Pure PCR Template Preparation Kit (Roche, Germany) (Meskini and Esmaeil 2018; Meskini et al. 2020). Following the extraction, genomic DNA was subjected to polymerized chain reaction (PCR) amplification of the invA gene using oligonucleotide primers, Fw 5′-AAA CGT TGA AAA ACT GAG GA-3′ and Rv 5′-TCG TCA TTC CAT TAC CTA CC-3′ (MWG, Germany) (Hoorfar et al. 2000). The 25 μL of PCR reaction mix final volume contained 15.2 μL distilled water, 2.5 μL buffer 10× (with 15 mM MgCl₂), 1 μL dNTP (10 mM), 1 μL MgCl₂ (25 mM), 10 μM of each of the primers, 0.3 μL Hot start Taq DNA polymerase (5 U/μL) (QIAGEN, Germany), and 3 μL DNA template. Thermal cycling condition consisted of one cycle of initial denaturation (95 °C, 10 min), 35 cycles of denaturation (94 °C at 60 s), annealing (62 °C at 90 s) extension step (72 °C at 60 s), and a final extension cycle (72 °C at 10 min). The PCR products were run on a 2% (w/v) agarose gel containing 1 μg/mL ethidium bromide. A 100 bp ladder was used. The PCR primers, GeneAmp™ PCR Core Kit, and DNA molecular marker were procured from MWG (Germany), Perkin Elmer Cetus (Norwalk CT), and Bethesda Research Laboratories (Inc. Burlington, Ontario), respectively.

Multiplex PCR to serogroup typing Salmonella

The m-PCR was performed with a final volume of 25 μL in a gradient thermal cycler (Eppendorf, Germany). The optimized PCR mixture for ASD group, OriC, and Vi strains consisted of 1.5 μL F-prt (10 μM), 1.5 μL R-prt, 1.5 μL P1 (10 μM), 1.5 μL P2, 1.5 μL F-vi (10 μM), and 1.5 μL R-vi, 2.5 μL buffer 10×, 1 μL MgCl₂ (25 mM), 1 μL
dNTP (10 mM), 0.3 µL Taq (5 U/µL) (Hot start PCR Taq plus DNA polymerase, QIAGEN, Germany), 5 µL sample (300–500 ng/µL), and 3.2 µL distilled water. The optimized cycling parameters of the m-PCR consisted of pre-denaturation at 95 °C for 10 min, followed by 35 cycles of 94 °C for 60 s, 56 °C for 90 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. Inside, the P1–P2 primer pair targeting the oriC gene was included as an internal control in all m-PCR reactions.

The optimized PCR mixture for B, C1, C2, D, and E strains consisted of 1.5 µL F-rfbj (10 µM), 1.5 µL R-rfbj, 1.5 µL F-tyv (10 µM), 1.5 µL R-tyv, 1.5 µL F-wzxC1 (10 µM), 1.5 µL R-wzxC1, 1.5 µL F-wzxE1 (10 µM), 1.5 µL R-wzxE1, 1.5 µL F-wzxC2 (10 µM), and 1.5 µL R-wzxC2, 2.5 µL buffer 10X, 1 µL MgCl₂ (25 mM), 1 µL dNTP (10 mM), 0.3 µL Taq (5 U/µL) (Hot start PCR Taq plus DNA polymerase, QIAGEN, Germany), 5 µL of the sample (300–500 ng/µL), and 3.2 µL DW. The optimized cycling parameters of the m-PCR consisted of pre-denaturation at 95 °C for 10 min, followed by 35 cycles 94 °C for 60 s, 59 °C for 90 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min.

The optimized PCR mixture for Ha, Hb, Hd, Hj, and oriC strains consisted of 1 µL F–H (10 µM), 1 µL R-Ha, 1 µL R-Hb (10 µM), 1 µL R-Hd, 1 µL P1, 1 µL P2, 2.5 µL buffer 10X, 1 µL MgCl₂ (25 mM), 1 µL dNTP (10 mM), 0.3 µL Taq (5 U/µL) (Hot start PCR Taq plus DNA polymerase, QIAGEN, Germany), 5 µL sample (300–500 ng/µL), and 3.2 µL DW. The optimized cycling parameters of the m-PCR consisted of pre-denaturation at 95 °C for 10 min, followed by 35 cycles 94 °C for 60 s, 56 °C for 90 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min.

The PCR was performed in a thermocycler (Eppendorf Thermomixer comfort, Germany). The oligonucleotide sequences used in this study, annealing temperature, and the expected band size are listed in Table 1. The PCR product fragments were analyzed in 2% (w/v) agarose gel by electrophoresis using a 1× TAE buffer. Fragment size was determined by comparison with Gene-Ruler 100 bp DNA ladder (Fermentas, EU).

**Multiplex PCR to serovar typing Salmonella**

The m-PCR was performed for invA (Styinva-JHO-2), sdf (S. enterica serovar Enteritidis), STM4492 (S. enterica serovar Typhimurium), IE-1 (S. enterica serovar Enteritidis), Flic-C (S. enterica serovar Typhimurium), had (Salmonella serogroup C2), Cholerae-Suis Flinc, (Heidelberg) heli (predicted helicase),

| Serogroups | Target gene | Primer name | Oligonucleotide sequences (5–3) | Annealing temperature (°C) | PCR product size (bp) | References |
|------------|-------------|-------------|---------------------------------|-----------------------------|------------------------|------------|
| B           | rfbj        | F-rfbj      | CCAGCACCATCCTCCAATTTGATAC       | 59                          | 662                    | Lim et al. (2003) |
|             |             | R-rfbj      | GGCATTCCGCTATTGTGTAACCA         |                             |                        |            |
| D           | tyv         | F-tyv       | TAGGAGGCAGTTAGAAGCTTTTT         | 59                          | 614                    | Hirose et al. (2002) |
|             |             | R-tyv       | TGCAGTACAACTCCCCACATATC         |                             |                        |            |
| C1          | wzxC1       | F-wzxC1     | CAGTGTCCGTGTTAATACTACGGTTG     | 59                          | 483                    | Herrera-León et al. (2007) |
|             |             | R-wzxC1     | GGCTGTTAATAATCTGTGTTAATC       |                             |                        |            |
| E           | wzxE1       | F-wzxE1     | TAAATGATATGGTGTCTGTTAACC       | 59                          | 345                    | Herrera-León et al. (2007) |
|             |             | R-wzxE1     | GTAAAAATGACAGATTGAACGACAGCAAG  |                             |                        |            |
| C2          | wzxC2       | F-wzxC2     | ACTGAAGTTGATTATTTTACTGTTG      | 59                          | 154                    | Herrera-León et al. (2007) |
|             |             | R-wzxC2     | AAGACATCCCTAAACTGCCCCGTCG      |                             |                        |            |
| A           | ASD group   | F-prt       | CTTGCTATGGAAGACATAACAGGACC     | 55                          | 256                    | Hirose et al. (2002) |
|             |             | R-prt       | GTCTCCCATCAAAGCTCCATAG         |                             |                        |            |
| OriC        | OriC        | P1          | TTATAGGATTCCGCGCCAGGC          | 55                          | 163                    | Widjojoatmodjo et al. (1991) |
|             |             | P2          | AAAGAATAACCGTTGTTCAC           |                             |                        |            |
| Vi          | Vi strain   | F-vi        | GTTATTACGATACAAAGGA            | 55                          | 439                    | Hirose et al. (2002) |
|             |             | R-vi        | CTCCTACCATACCTTCCG            |                             |                        |            |
| H           | Ha          | F-H         | ACTCAGGGTTTCCGGTAAAGGC        | 55                          | 423                    | Levy et al. (2008) |
|             | Hb          |            | GAGGCCAGCAGCTACAGTGC          |                             |                        |            |
|             | Hd          | R-Hb        | GCTTACAGACACATCTTGTGTT        |                             |                        |            |
|             | Hj          | R-Hd        | GGCTAGATTGTGCTTATCGG          |                             |                        |            |
and (S. enterica serovar Kentucky) gly (putative membrane protein) genes in a final volume of 25 µL in a gradient thermal cycler (Eppendorf™, Mastercycler Pro, Germany). The optimized PCR mixture and cycling parameters consisted of pre-denaturation, denaturation, annealing, extension, and the final extension for the mentioned genes (Tables 2 and 3). The PCR product fragments were analyzed in 2% (w/v) agarose gel by electrophoresis using a 1× TAE buffer. Fragment size was determined by comparison with GeneRuler 100 bp DNA ladder (Fermentas, EU). Type strain S. enterica serovar Enteritidis (ATCC 13076), S. enterica serovar Typhimurium (ATCC 14028), S enterica serovar Typhimurium (ATCC1730), S. enterica serovar Infantis (ATCC BAA-1675), S. enterica serovar Hadar (ATCC 51956), S. enterica serovar Dublin (ATCC 15480), Enterococcus faecalis (ATCC® 51299™), Citrobacter freundii (ATCC 8090), Escherichia coli (ATCC 25952), Klebsiella pneumoniae (ATCC 13883), Acinetobacter Iwoffii ATCC-type strain 1, and Acinetobacter baumannii (ATCC 19606:1113) were used as m-PCR control.

Figure 3 shows all summarized steps performed in the present study.

**Statistical analysis**

The statistical analysis of data was conducted using IBM SPSS version 16.00 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to compare different methods, and P<0.05 was considered statistically significant.

**Results**

**Salmonella genus and serogroup detection**

A total of 2300 poultry feces samples from farms located in 21 cities of Iran were collected from January 2016 to December 2019. The percentage of abundance of different Salmonella serogroups is given in Fig. 4. Among them, 173 (7.5%) samples were detected as Salmonella by cultivation, and 166 (7.2%) samples were detected as Salmonella by amplification of invA gene. Thus, the sensitivity of molecular detection and microbiological cultivation was equal to 0.96 (CI = 95%). Molecular serotyping gave the same results as the antisera approach with A (prt), C1 (wzxC1), and E (wzxE1) serogroups. The concordance of molecular detection of serogroups (Fig. 5) and the serological method for all samples were 100%. Other samples were in different groups, and the matching results of the two molecular and serological serotypes were higher than 93% for all samples. The detection of B (rfb), C2 (wzxC2), and D (tyv) showed that the concordance of molecular serotyping and antisera method was 93.3%, 94.9%, and 94.2%, respectively.
Eighteen samples were identified by neither serological nor molecular methods and named unidentified. The D and C2 Salmonella serogroups were the most abundant. The match between molecular serotyping and serology methods for unidentified samples was 94.4%. In addition, 10.4% of the samples were not identified.

The accuracy of detecting the flics gene by m-PCR and antigen H by the serological method significantly correlated with the contingency table test ($P < 0.01$). In the Receiver-Operating Characteristic (ROC) diagram, the sensitivity and specificity of the m-PCR were 92.3% and 95.2%, respectively, compared to the serological method (as gold standard). In evaluating H antigenic subgroups (including Ha, Hb, Hc, and Hj), the same results were obtained with both m-PCR and serological methods for a matching rate of 100%. Therefore, the relationship between methods was significant in the contingency table test ($P < 0.01$). Figure 6 shows the m-PCR product band to identify the subgroup H1 Salmonella genus (Ha, Hb, Hd, Hj).

### Table 3 The m-PCR primers sequence, target gene, target serovar detected, and the band size

| Target gene (serovar) | Primer name | Oligonucleotide sequences (5–3) | Annealing temperature ($^\circ$C) | PCR product size (bp) | References |
|-----------------------|-------------|---------------------------------|----------------------------------|-----------------------|------------|
| invA (Styinv-JHO-2)  | invA -f | AAA CGT TGA AAA ACT GAG GA | 62 | 199 | Barrow and Neto (2011) |
|                       | invA -r | TCG TCA TTC CAT TAC CTA CC | | | |
| Sdf (S. Enteritidis)  | Sdf-f | AAA TGT GTT TTA TCT GAT GCA AGA GG | 62 | 299 | O'Regan et al. (2008) |
|                       | Sdf-r | GTT CGT TCT GGT ACT TAC GAT GAC | | | |
| STM4492 (S. Typhimurium) | STM4492-f | ACA GCT TGG CCT ACG CGA G AGC AAC GFT TCG GCC TGA C | 62 | 759 | McCarthy et al. (2009) |
|                       | STM4492-r | | | | |
| IE-1 (S. Enteritidis) | IE-1-for | AGT GCC ATA CTT TTA ATG AC | 58 | 316 | Wang and Yeh (2002) |
|                       | IE-1-rev | ACT ATG TCG ATA CGG TGG G AC | | | |
| Flic-C (S. Typhimurium) | Flic-C-for | CCC GCT TAC AGG TGG ACT AC | 58 | 432 | Paião et al. (2013) |
|                       | Flic-C-fev | AGC GGG TTT TCG GTG GTT GT | | | |
| S. Infantis 878–897   | 878f | TTG CTT CAG ATG CTA AG | 56 | 413 | Kardos et al. (2007) |
|                       | 1275rev | TTG CTT CAG ATG CTA AG | | | |
| S. Hadar (Salmonella serogroup C2) | HAD-For | ACC GAG CCA ACG ATT ATC AA | 57 | 502 | Ahmed et al. (2009) |
|                       | HAD-rev | AAT AGG CCG AAA CAA CAT CG | | | |
| Cholerae-Suis Flin C  | Flin C-F | AAG GAA AAG ATC ATG GCA CAA | 53 | 956 | Chiu et al. (2005) |
|                       | Flin C-R | GAA CCC ACC ATC AAT AAC TTT G | | | |
| heli (Heidelberg) ORF (predicted helicase) | heli-F | ACAGCCCGCTGTATTTAATGTTG CGCGTAAATCGAGTGTTGCC | 56 | 782 | Zhu et al. (2015) |
|                       | heli-R | | | | |
| gly (Kentucky) ORF (putative membrane protein) | gly-F | TTCCAAATGAAACGAGTGCCCG ACTAACCAGCTTGTTGTCG TGT | 56 | 170 | Mahmud et al. (2011) |
|                       | gly-R | | | | |

![Fig. 3 The piktochart of whole methods which were performed in this study](image-url)
Multiplex PCR to serovar typing Salmonella

Figure 7 depicts the percentage of the abundance of different Salmonella serovars among Salmonella samples. The D, C2, and C1 serogroups were the three most abundant serogroup, and “A” serogroup was the least abundant serogroup among Salmonella samples. The “U” showed unidentified samples.

Discussion

Salmonella is a life-threatening foodborne zoonotic pathogen with more than 2500 serotypes. Over 95% of the strains cause infections in humans and animals to belong to serogroups A to D (Diep et al. 2019). Identification of Salmonella is necessary for the prevention, surveillance, and control of foodborne diseases. Therefore, there is a need for rapid detection, identification of sources, control of outbreaks, and identification of emerging serotypes of Salmonella. In this study, traditional (culture and serology) and molecular methods were used to detect Salmonella isolates.
from poultry farms in Iran. Serogroups and serovars were compared to determine the best fast and valid method.

In this study, 7.5% (173/2300) of the isolates were identified as Salmonella by culturing and 7.2% (166/2300) were identified by PCR (invA). The current study exhibited a lower prevalence of Salmonella than broiler poultry farms in Bangladesh where prevalence ranged from 23 – 38% (35/100; 36/123; 106/503) (Alam et al. 2020). A longitudinal Salmonella surveillance study was conducted in raw chicken meat in Mexico on 1160 samples collected between 2016 and 2018 (Regalado-Pineda et al. 2020). The study revealed a significantly higher prevalence (P < 0.0001) of S. enterica in supermarkets (27.2%, 158/580) than in wet markets (9.0%, 52/580). The prevalence of S. enterica was observed in other regions of the world, it included Venezuela, the USA, Canada, Wales, Australia, Brazil, Belgium, China, Columbia, Ecuador, Portugal, and Spain, where infection levels ranged between 9.5 and 65% (Regalado-Pineda et al. 2020). The lower prevalence of Salmonella observed in the current study could be attributed to the sample size (Persoons et al. 2011), where larger samples were compared in previous studies. The sampling sources could be another factor (Taylor et al. 2018) as the previous studies included various sampling locations and sources such as cloacal swabs, litter, chicken meat, feed. In comparison, the current study only included fecal samples from poultry farms. In addition, the geographical locations of the studies could be another factor that influences the current findings (Shah et al. 2011).

Target genes used in our study were previously validated in several studies using PCR and m-PCR assays to detect Salmonella serogroup A–E (Farahani et al. 2018). The present study implemented m-PCR of 878–897 gene to identify S. enterica serovar Infantis following previous studies where m-PCR was used on the same gene to identify S. enterica serovar Infantis in spiked chicken feces and meat samples. Furthermore, the current study used STM4492 and fliC genes to identify S. enterica serovar Typhimurium and S. enterica serovar Choleraesuis, respectively. These findings are

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**Fig. 7** Percentage of the abundance of different Salmonella serovars among Salmonella samples. The highest serotypes of S. Enteritidis, S. Infantis, and S. Kentucky were most commonly identified.

**Fig. 8** m-PCR to identify Salmonella serovars. Lane 1 and 2: S. Infantis, lane 3, 4, and 5: Salmonella (invA: 199 bp), S. Typhimurium (STM: 759 bp), S. Enteritidis (sdf: 299 bp), lane 6, 11, and 12: negative control, lane 10: S. Choleraesuis (with FliC: 956 bp), lane 13 and 14: S. Choleraesuis (with viaB 600 bp), lane 15 and 16: S. Hadar (had: 502 bp), lane 17: S. Heidelberg (hli: 782 bp), and lane 18: S. Kentucky (gly: 170 bp). Lane M100: Ladder 100 bp (Invitrogen, USA)
consistent with previous reports where STM4492 was used as a target marker gene to identify *S. enterica* serovar Typhimurium and exhibited high specificity and differentiation between the Salmonella serovars (McCarthy et al. 2009). Studies have shown that the STM4492 gene discriminated *S. enterica* serovar Typhimurium from *S. enterica* serovar Enteritidis in broiler and chicken meat samples (Païão et al. 2013; Saeki et al. 2013). The *flaC* gene is the other target gene for *S. enterica* serovar Typhimurium and *S. enterica* serovar Choleraesuis detection that encodes the phase 1 flagellin protein (H1) which is the most frequently used gene to differentiate Typhimurium serovar from the others (Gürbüz et al. 2018). Researchers at Konya (Turkey) used the *flaC* gene to isolate *S. enterica* serovar Typhimurium from chicken meat and giblets (Telli et al. 2018). Furthermore, studies identified *Salmonella* spp. from pediatric patients and *S. enterica* serovar Choleraesuis by targeting the *FlaC* gene (Filsner 2018).

The *sdf* gene, a chromosome region related to invasion and infection of poultry and eggs, is used to detect *S. enterica* serovar Enteritidis in humans and animals (Del Serrone 2019). To detect *S. enterica* serovar Hadar, *S. enterica* serovar Kentucky, and *S. enterica* serovar Heidelberg, *had*, *gly*, and *heli* genes were used, respectively. Martínez-Ballesteros et al. (2012) detected *had* gene by an improved m-PCR method to detected *S. enterica* serovar Hadar and typing them as *S. enterica* serovar Hadar. Furthermore, in another study by Ahmed et al. (2009) in Egypt, the *had* gene was used to detect multidrug resistance in *Salmonella* spp. isolated from diarrheic calves. The P1-P2 primer pair targeted the *oriC* gene as an internal control in all m-PCR reactions.

The present study found the highest prevalence of *S. enterica* serovar Enteritidis in fecal samples from poultry in Iran. The lowest prevalence was associated with *S. Heidelberg*, indicating that live poultry was the source of *S. enterica* serovar Enteritidis for contamination of raw chicken meat in the primary part of the chain production. The motile *Salmonella* spp. are mainly associated with food products, and they are the significant causes of salmonellosis in humans (Whiley and Ross 2015). Approximately 7.5% of *Salmonella* isolates were confirmed as *S. enterica* serovar Typhimurium in our study. Similar results were found by Barua et al. (2013), where 11% of commercial broiler chicken farm isolates were motile Salmonella and Islam et al. (2016) in Bangladesh, where 15.91% of isolates were *S. enterica* serovar Typhimurium. Alam et al. (2020) showed that 85.7% of the isolates from Bangladesh were confirmed as motile Salmonella, which is higher than our results. In another study conducted from 154 commercial poultry layer farms in the Southern part of India, a total of 1215 samples containing poultry meat, tissues, egg, and environmental samples were screened for non-typhoidal Salmonella (NTS) serovars. Multiplex-PCR, allele-specific PCR, enterobacterial repetitive intergenic consensus (ERIC) PCR, and pulse field gel electrophoresis (PFGE) revealed 21/1215 (1.73%) samples positive for NTS (Saravanan et al. 2015). Similarly, during disease outbreaks (40–80% mortality) in poultry farms in Lagos, Ogun and Oyo states, Nigeria, PCR and serotyping conducted on chicken organ samples collected at postmortem examinations identified motile Salmonella serotyping primarily represented by *S. enterica* serovar Zega (34.14%), *S. enterica* serovar Kentucky (24.32%), *S. enterica* serovar Herston (16.22%), *S. enterica* serovar Nima (10.81%), *S. enterica* serovar Colindale (2.70%), *S. enterica* serovar Telelkебir (8.11%) and *S. enterica* serovar Tshiongwе (2.70%) (Mshelbwala et al. 2017).

Bacterial culture-based techniques are time-consuming, laborious, and have a lower discriminatory capacity. Simultaneously, molecular methods such as m-PCR are crucial in detecting, typing, speciating, and classifying Salmonella at the genus level, serogroups, and serovars. The m-PCR assay is a sensitive, reliable, specific, and highly effective diagnostic test for the simultaneous identification of Salmonella and its serogroups and serovars. However, the cultivation-based PCR-dependent technique has certain limitations, such as the less abundant microbes could not be grown easily, and uncultivable microorganisms are not retrieved, resulting in the wrong interpretation of the result. Conversely, the cultivation-independent PCR-dependent technique is more reliable as it involves the PCR of the metagenome directly retrieved from the environment, devoid of any prior cultivation (Ghosh 2015). This system could significantly reduce reliance on tedious conventional serotyping. However, the main issues to be considered are the cost scale-up of these advanced methods and the regulatory necessities. Although the present results are preliminary, the m-PCR assay could offer a valuable alternative to traditional typing methods (culture and serological) to identify and differentiate the most *Salmonella* spp. in diverse samples. Further investigations should embark on the whole genome sequencing, functional genomics, extraction, and purification of the bioactive compounds from these isolates, which could contribute to understanding the mechanism of infections.

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References

Ahmed AM, Younis EE, Ishida Y, Shimamoto T (2009) Genetic basis of multidrug resistance in Salmonella enterica serovars Enteritidis and Typhimurium isolated from diarrheic calves in Egypt. Acta Trop 111:144–149

Alam SB et al (2020) Molecular detection of multidrug resistant Salmonella species isolated from broiler farm in Bangladesh. Pathogens 9:201

Asl HM, Gouya MM, Soltan-Dallal MM, Aghili N (2015) Surveillance for foodborne disease outbreaks in Iran, 2006–2011. Med J Islam Repub Iran 29:285

Barrow P (1994) Serological diagnosis of Salmonella serotype enteritidis infections in poultry by ELISA and other tests. Int J Food Microbiol 21:55–68

Barrow P, Neto OF (2011) Pullorum disease and fowl typhoid—new thoughts on old diseases: a review. Avian Pathol 40:1–13

Barua H, Biswas PK, Olsen KE, Shil SK, Christensen JP (2013) Molecular characterization of motile serovars of Salmonella from breeder and commercial broiler poultry farms in Bangladesh. PLoS One 8:e57811

Chiu T-H, Pang J-C, Hwang W-Z, Tsen H-Y (2005) Development of PCR primers for the detection of Salmonella enterica serovar Choleraesuis based on the flaC gene. J Food Prot 68:1575–1580

Del Serrone P (2019) Cocoa vs pathogenic bacteria of human and animal concern. EC Microbiol 15:50–60

Diep B et al (2019) Salmonella serotyping; comparison of the traditional method to a microarray-based method and an in silico platform using whole genome sequencing data. Front Microbiol 10:2554. https://doi.org/10.3389/fmicb.2019.02554

Du J, Wu S, Niu L, Li J, Zhao D, Bai Y (2020) A gold nanoparticle-assisted multiplex PCR assay for simultaneous detection of Salmonella typhimurium, Listeria monocytogenes and Escherichia coli. J. Anal Methods 12:212–217

Farahani RK, Ehsani P, Ebrahim-Rad M, Khaledi A (2018) Molecular detection, virulence genes, biofilm formation, and antibiotic resistance of Salmonella enterica serotype enteritidis isolated from poultry and clinical samples. Jundishapur J Microbiol 11:e69504

Filisner PHNDL (2018) Caracterização de Salmonella enterica subespécie enterica sorovar Cholerasuis provenientes de suínos no Brasil. Universidade de São Paulo, São Paulo

Gantois I, Eeckhaut V, Pasmans F, Haesebrouck F, Ducatelle R, Van Immerseel F (2008) A comparative study on the pathogenesis of egg contamination by different serotypes of Salmonella. Avian Pathol 37:399–406. https://doi.org/10.1080/03079450802216611

Ghazalbina M, Farahani RK, Mansouri S, Meskini M, Farahani AHK, Khaledi A (2019) Molecular detection of antibiotic resistance genes, and class I and II inteegrons in Salmonella enteritidis isolated from Iranian one-day-old chicks. Gene Rep 16:100441

Gu G, Strawn LK, Zheng J, Reed EA, Rideout SL (2019) Diversity and dynamics of Salmonella enterica in water sources, poultry litters, and field soils amended with poultry litter in a major agricultural area of Virginia. Front Microbiol 10:2868

Gürbüz Ü, Telli AE, Kahraman HA, Balpetekükülçü D, Yalçın S (2018) Determination of microbial contamination, pH and temperature changes in sheep and cattle carcasses during the slaughter and pre-cooling processes in Konya, Turkey. Ital J Food Sci 30(4)

Herrera-León S, Ramiro R, Arroyo M, Diez R, Usea MA, Echeita MA (2007) Blind comparison of traditional serotyping with three multiplex PCR assays for the identification of Salmonella serotypes. Res Microbiol 158:122–127

Hirose K et al (2002) Selective amplification of tyv (rfbE), pte (rfbS), viaB, and fliC genes by multiplex PCR for identification of Salmonella enterica serovars Typhi and Paratyphi A. J Clin Microbiol 40:633–636

Hoofar J, Ahrens P, Rådström P (2000) Automated 5′ nuclease PCR assay for identification of Salmonella enterica. J Clin Microbiol 38:3429–3435

Islam MJ, Mahbub-E-Elahi A, Ahmed T, Hasan MK (2016) Isolation and identification of Salmonella spp. from broiler and their antibiogram study in Sylhet, Bangladesh. J Appl Biol Biotechnol 4:046–051

Kardos G, Farkas T, Antal M, Nagyadny N, Kiss I (2007) Novel PCR assay for identification of Salmonella enterica serovar Infantis. Lett Appl Microbiol 45:421–425

Kariuki S, Gordon MA, Feasey N, Parry CM (2015) Antimicrobial resistance and management of invasive Salmonella disease. Vaccine 33:C21–C29

Kasturi KN (2020) A real-time PCR for rapid identification of Salmonella enterica Gaminara serovar. J Microbiol Methods 169:105729

Khaledi A, Meskini M (2020) A systematic review of the effects of Satureja khuzestanica Jamzad and Zataria multiflora Boiss against Pseudomonas aeruginosa. Iran J Med Sci 45:83

Levy H et al (2008) PCR method to identify Salmonella enterica serovars Typhi, Paratyphi A, and Paratyphi B among Salmonella isolates from the blood of patients with clinical enteric fever. J Clin Microbiol 46:1861–1866

Lim Y-H et al (2003) Multiplex polymerase chain reaction assay for selective detection of Salmonella enterica serovar Typhimurium. Jpn J Infect Dis 56:151–155

Maciorowski K, Pillai S, Jones F, Rickes S (2005) Polymerase chain reaction detection of foodborne Salmonella spp. in animal feeds. Crit Rev Food Sci 31:45–53

Mahmud MS, Bari ML, Hossain MA (2011) Prevalence of Salmonella serovars and antimicrobial resistance profiles in poultry of Savar area, Bangladesh. Foodborne Pathog Dis 8:1111–1118

Malorny B, Huehn S, Dieckmann R, Krämer N, Helmuth R (2007) Novel PCR based on vfaB, and fliC genes by multiplex PCR for identification of Salmonella enterica serovars Typhimurium and Heidelberg. J Food Prot 70:318–322

Martínez-Ballesteros I et al (2012) Intra-and inter-laboratory evaluation of an improved multiplex-PCR method for detection and typing of Salmonella. J Infect Dev Ctries 6:443–451

McCarthy N, Reen FJ, Buckley JF, Frye JG, Boyd EF, Gilroy D (2009) Sensitive and rapid molecular detection assays for Salmonella enterica serovars Typhimurium and Heidelberg. J Food Prot 72:2350–2357

Meskini M, Esmaeili D (2018) The study of formulated Zoush ointment against wound infection and gene expression of virulence factors Pseudomonas aeruginosa. BMC Complement Altern Med 18:1–10
Meskini M, Ghorbani M, Bahadoran H, Esmaeili D (2020) ZOUSH ointment with the properties of antibacterial moreover, burn wound healing. Int J Pept Res Ther 26:349–355

Mshelbwala FM et al (2017) Motile Salmonella serotypes causing high mortality in poultry farms in three South-Western States of Nigeria. Vet Rec Open 4:e000247. https://doi.org/10.1136/vetreco-2017-000247

O’Regan E et al (2008) Development of a real-time multiplex PCR assay for the detection of multiple Salmonella serotypes in chicken samples. BMC Microbiol 8:156

Paião F, Arisitides L, Murate L, Vilas-Bôas G, Vilas-Boas L, Shimokomaki M (2013) Detection of Salmonella spp, Salmonella Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay. Braz J Microbiol 44:37–42

Persoons D et al (2011) The importance of sample size in the determination of a flock-level antimicrobial resistance profile for Escherichia coli in broilers. Microb Drug Resist 17:513–519. https://doi.org/10.1089/mdr.2011.0048

Regalado-Pineda ID, Rodarte-Medina R, Resendiz-Nava CN, Saenz-Garcia CE, Castaneda-Serrano P, Nava GM (2020) Three-year longitudinal study: prevalence of Salmonella Enterica in chicken meat is higher in supermarkets than wet markets from Mexico. Foods. https://doi.org/10.3390/foods9030264

Roy P, Dhillon A, Lauerman LH, Schaberg D, Bandli D, Johnson S (2002) Results of Salmonella isolation from poultry products, poultry, poultry environment, and other characteristics. Avian Dis 46:17–24

Saeki EK, Alves J, Bonfante RC, Hirooka EY, de Oliveira TCRM (2013) Multiplex PCR (mPCR) for the detection of Salmonella spp. and the differentiation of the Typhimurium and Enteritidis Serovars in chicken meat. J Food Saf 33:25–29

Saravanan S et al (2015) Molecular epidemiology of nontyphoidal Salmonella in poultry and poultry products in India: implications for human health. Indian J Microbiol 55:319–326. https://doi.org/10.1007/s12088-015-0530-z

Shah A, Sachdev A, Coggon D, Hossain P (2011) Geographic variations in microbial keratitis: an analysis of the peer-reviewed literature. Br J Ophthalmol 95:762–767. https://doi.org/10.1136/bjo.2009.169607

Sobur MA, Sabuj AAM, Sarker R, Rahman AT, Kabir SL, Rahman MT (2019) Antibiotic-resistant Escherichia coli and Salmonella spp. associated with dairy cattle and farm environment having public health significance. Vet World 12:984

Taylor DDJ, Khush R, Peletz R, Kumpel E (2018) Efficacy of microbial sampling recommendations and practices in sub-Saharan Africa. Water Res 134:115–125. https://doi.org/10.1016/j.watres.2018.01.054

Telli A, Biçer Y, Kahraman HA, Telli N, Doğruer Y (2018) Presence and antibiotic resistance of Salmonella spp isolated from chicken meat and giblets consumed in Konya, Turkey. Eurasian J Vet Sci 34:164–170

Wang SJ, Yeh DB (2002) Designing of polymerase chain reaction primers for the detection of Salmonella enteritidis in foods and faecal samples. Lett Appl Microbiol 34:422–427

Wang J, Li J, Liu F, Cheng Y, Su J (2020) Characterization of Salmonella enterica isolates from diseased poultry in Northern China between 2014 and 2018. Pathogens 9:95

Whiley H, Ross K (2015) Salmonella and eggs: from production to plate. Int J Environ Res Public Health 12:2543–2556

Widjojoatmodjo M, Fluit A, Torensma R, Keller B, Verhoef J (1991) Evaluation of the magnetic immuno PCR assay for rapid detection of Salmonella. Eur J Clin Microbiol Infect Dis 10:935–938

Zhu C, Yue M, Rankin S, Weill F-X, Frey J, Schifferli DM (2015) One-step identification of five prominent chicken Salmonella serovars and biotypes. J Clin Microbiol 53:3881–3883

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