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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 food-borne outbreaks and infection worldwide. The gold standard detection method of Salmonella is cultivation. With time-consuming cultivation, there is a need to investigate rapid and accurate processes. 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 for the detection of seven Salmonella serovars and a chi-square test was performed to compare the discriminatory power of the methods. Out 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 detection of 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 hours) compared to the conventional microbiological approach (3–8 days).

Introduction

Salmonella are gram-negative, rod-shaped bacteria in the family *Enterobacteriaceae*. Salmonella is the most common bacterial pathogen associated with food-borne disease in the United States (Gu, Strawn, Zheng, Reed, & Rideout, 2019). Poultry products, including meat and eggs, have been a significant source of Salmonella infections (J. Wang, Li, Liu, Cheng, & Su, 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 Typhimurian (Gantois et al., 2008). *S. enterica* serovar Enteritidis is the most reported in human outbreaks during the last two decades (Ghazalibina et al., 2019). 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 includes 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 has 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, Gordon, Feasey, & Parry, 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 & Neto, 2011). Therefore, it 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 to detect *Salmonella* spp. and their serovars is required.

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 (Khaledi & Meskini, 2020; Malorny, Huehn, Dieckmann, Krämer, & Helmuth, 2009). 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 different specific DNA sequences in the same reaction (Maciorowski, Pillai, Jones, & Ricke, 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.

1. Materials And Methods

1.1. Collection of Samples and Isolation of Salmonella

Poultry feces samples from five different areas in each poultry farm were collected. 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. Samples were collected in sterile zipper bags from January 2016 to December 2019 using sterile spoons. 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 Desoxycholate (XLD) agar (Merck, Germany) and incubated overnight at 37°C. Red colonies with a black center 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). Following the identification of the isolates, serotyping was performed by O (polyvalent), A, B, C1, C2, D1, and E antisera (MAST, Germany).

1.2. Dna Extraction And Molecular Detection Of Salmonella Genus

Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Germany) (Meskini & Esmaeili, 2018; Meskini, Ghorbani, Bahadoran, & Esmaeili, 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, Ahrens, & Rådström, 2000). The 25 µL of PCR reaction mix final volume contained 15.2 µL distilled water, 2.5 µL buffer 10× (with 15 mM $MgCl_2$), 1 µL dNTP (10mM), 1 µL $MgCl_2$ (25 mM), 10 µM of each of the primers, 0.3 µL Hot start Taq DNA polymerase (5U/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.

1.3. 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 A$D 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 1.5µL R-vi, 2.5 µL buffer 10X, 1 µL $MgCl_2$ (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 $MgCl_2$ (25 mM), 1 µL dNTP (10 mM), 0.3 µL Taq (5 U/µL) (Hot start PCR Taq plus DNA polymerase, QUIAGEN, 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 of 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 $MgCl_2$ (25mM), 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 9.2 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 1X TAE buffer. Fragment size was determined by comparison with Gene-Ruler 100 bp DNA ladder (Fermentas, EU).

Table 1
The m-PCR primers sequence, target gene, target serogroup detected, and the expected band size

Serogroups	Target gene	Primer name	Oligonucleotide sequences (5to3)	Annealing temperature (°C)	PCR product size (bp)	Reference
B	*rfbj*	F-rfbj	CCAGCACCAGTTCCAACTTGATAC	59	662	(Lim et al., 2003)
		R-rfbj	GGCTTCCGGCTTTATTGGTAAGCA			
D	*tyv*	F-tyv	GAGGAAGGGAAATGAAGCTTTT	59	614	(Hirose et al., 2002)
		R-tyv	TAGCAAACTGTCTCCCACCATAC			
C1	*wzxC1*	F-wzxC1	CAGTAGTCCGTAAAATACAGGGTGG	59	483	(Herrera-León et al., 2007)
		R-wzxC1	GGGGCTATAAATACTGTGTTAAATTCC			
E	*wzxE1*	F-wzxE1	TAAAGTATATGGTGCTGATTTAACC	59	345	(Herrera-León et al., 2007)
		R-wzxE1	GTTAAAATGACAGATTGAGCAGCAAG			
C2	*wzxC2*	F-wzxC2	ACTGAAGGTGGTATTTCATGGG	59	154	(Herrera-León et al., 2007)
		R-wzxC2	AAGACATCCCTAACTGCCCTGC			
A	A$D group	F-prt	CTTGCTATGGAAGACATAACGAACC	55	256	(Hirose et al., 2002)
		R-prt	CGTCTCCATCAAAAGCTCCATAGA			
OriC	*OriC*	P1	TTATTAGGATCGCGCCAGGC	55	163	(Widjojoatmodjo, Fluit, Torensma, Keller, & Verhoef, 1991)
		P2	AAAGAATAACCGTTGTTCAC			
Vi	Vi strain	F-vi	GTTATTCAGCATAAGGAG	55	439	(Hirose et al., 2002)
		R-vi	CTTCCATACCACTTTCCG			
H	Ha	F-H	ACTCAGGCTTCCCGTAACGC	55	423	(Levy et al., 2008)
	Hb	R-Ha	GAGGCCAGCACCATCAAGTGC		551	
	Hd	R-Hb	GCTTCATACAGACCATCTTTAGTTG		763	
	Hj	R-Hd	GGCTAGTATTGTCCTTATCGG		502	

1.4. Multiplex PCR to Serovar Typing Salmonella

The m-PCR was performed for *inv*A (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), *878–897* (*S. enterica* serovar Infantis), *had* (Salmonella serogroup C2), Cholerae-Suis *Flin C*, (Heidelberg) *heli* (predicted helicase), 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 1X TAE buffer. Fragment size was determined by comparison with Gene-Ruler 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 lwoffii* ATCC-type strain 1, and *Acinetobacter baumanii* (ATCC 19606:1113) were used as m-PCR control.

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

Target Gene (serovar)	Primer name	Oligonucleotide sequences (5to3)	Annealing temperature (°C)	PCR product size (bp)	Reference
*inv*A (Styinva-JHO-2)	invA -f	AAA CGT TGA AAA ACT GAG GA	62	199	(Barrow & 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	62	759	(McCarthy et al., 2009)
	STM4492-r	AGC AAC CGT TCG GCC TGA C			
IE-1 (*S*. Enteritidis)	IE-1-for	AGT GCC ATA CTT TTA ATG AC	58	316	(S. J. Wang & Yeh, 2002)
	IE-1-rev	ACT ATG TCG ATA CGG TGG G			
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*	878for	TTG CTT CAG ATG CTA AG	56	413	(Kardos, Farkas, Antal, Nogrady, & Kiss, 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, Pang, Hwang, & Tsen, 2005)
	Flin C-R	GAA CCC ACC ATC AAT AAC TTT G			
heli (Heidelberg) *ORF* (predicted helicase)	heli-F	ACAGCCCGCTGTTTAATGGTG	56	782	(Zhu et al., 2015)
	heli-R	CGCGTAATCGAGTAGTTGCC			
gly (Kentucky) *ORF* (putative membrane protein)	gly-F	TTCCAATTGAAACGAGTGCGG	56	170	(Mahmud, Bari, & Hossain, 2011)
	gly-R	ACTAACCGCTTGGGTTGTTGCTGT			

1.5. 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.

2. Results

2.1. 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 p*ercentage of abundance of different Salmonella serogroups are given in* Fig. 1. 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. 2) 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 were named as 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%. Also, 10.4% of the samples were not identified.

The accuracy of detecting the *fli*cs 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 3 shows the m-PCR product band to identify the subgroup H1 Salmonella genus (Ha, Hb, Hd, Hj).

2.2. Multiplex PCR to Serovar Typing Salmonella

Figure 4 depicts percentage of abundance of different Salmonella serovars among Salmonella samples and Fig. 5 shows the m-PCR identifying the Salmonella serovars. The molecular serotyping of seven serovars using nine pairs of primers was performed on samples positively identified as Salmonella. The prevalence was determined among positive samples and confirmed by molecular and cultivation approach as *S. enterica* serovar Enteritidis 50 (28.9%), *S. enterica* serovar Infantis 22 (12.7%), *S. enterica* serovar Kentucky 19 (11%), *S. enterica* serovar Hadar 15 (8.7%), *S. enterica* serovar Typhimurium 13 (7.5%), *S. enterica* serovar Choleraesuis 12 (6.9%), *S. enterica* serovar Heidelberg 5 (2.9%), and another serovar 37 (21.4%).

3. Discussion

Salmonella is a life-threatening food-borne zoonotic pathogen with more than 2,500 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 food-borne 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 (*inv*A). 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 2016–2018 (Regalado-Pineda et al., 2020). The study revealed a significantly higher prevalence ($p < 0.0001$) of *S. entertica* in supermarkets (27.2%, 158/580) than in wet markets (9.0%, 52/580) The prevalence of *S. entertica* was observed in other regions of the world and included Venezuela, the USA, Canada, Wales, Australia, Brazil, Belgium, China, Columbia, Ecuador, Portugal, and Spain, where infection levels ranged between 9.5–65% (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, Khush, Peletz, & Kumpel, 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. Additionally, the geographical locations of the studies could be another factor that influences the current findings (Shah, Sachdev, Coggon, & Hossain, 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, Ehsani, Ebrahimi-Rad, & Khaledi, 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 *fli*C genes to identify *S. enterica* serovar Typhimurium and *S. enterica* serovar Choleraesuis, respectively. These findings are 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 (Paião et al., 2013; Saeki, Alves, Bonfante, Hirooka, & de Oliveira, 2013). The *fliC* 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 (TELLI, 2018). Researchers at Konya (Turkey) used the *fli*C gene to isolate *S. enterica* serovar Typhimurium from chicken meat and giblets (Telli, Biçer, Kahraman, Telli, & Doğruer, 2018). Furthermore, studies identified *Salmonella* spp. from pediatric patients and *S. enterica* serovar Choleraesuis by targeting the *Fli*C gene (Filsner, 2018).

The *sdf* gene, a chromosome region related to invasion and infection of poultry and eggs, are used for the detection of *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. (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. (Ahmed, Younis, Ishida, & Shimamoto, 2009) in Egypt, the *had* gene was used to detect multidrug resistance in *Salmonella* spp. isolated from diarrheic calves. The P1-P2 primer pair targeting the *oriC* gene was included 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 & Ross, 2015). In our study, approximately 7.5% of Salmonella isolates were confirmed as *S. enterica* serovar Typhimurium. Similar results were found by Barua et al. (Barua, Biswas, Olsen, Shil, & Christensen, 2013), where 11% of commercial broiler chicken farm isolates were motile Salmonella and Islam et al. (Islam, Mahbub-E-Elahi, Ahmed, & Hasan, 2016) in Bangladesh, where 15.91% of isolates were *S. enterica* serovar Typhimurium. Alam et al. (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 serotypes 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 Telelkebir (8.11%) and *S. enterica* serovar Tshiongwe (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 the 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 isolate, which could contribute to understanding the mechanism of infections.

Declarations

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Consent to Participate: All authors consented to participate

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Author contributions:

RKF and AHKF were designed the study. RKF, AGL, and MM performed the laboratory procedures and experiments. Safoora. G analyzed the data. MM wrote the manuscript. SG and AHKF edited the manuscript. All authors read the latest version of the manuscript and confirmed that.

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Tables

Due to technical limitations, Table 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Percentage of abundance of different Salmonella serogroups 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.

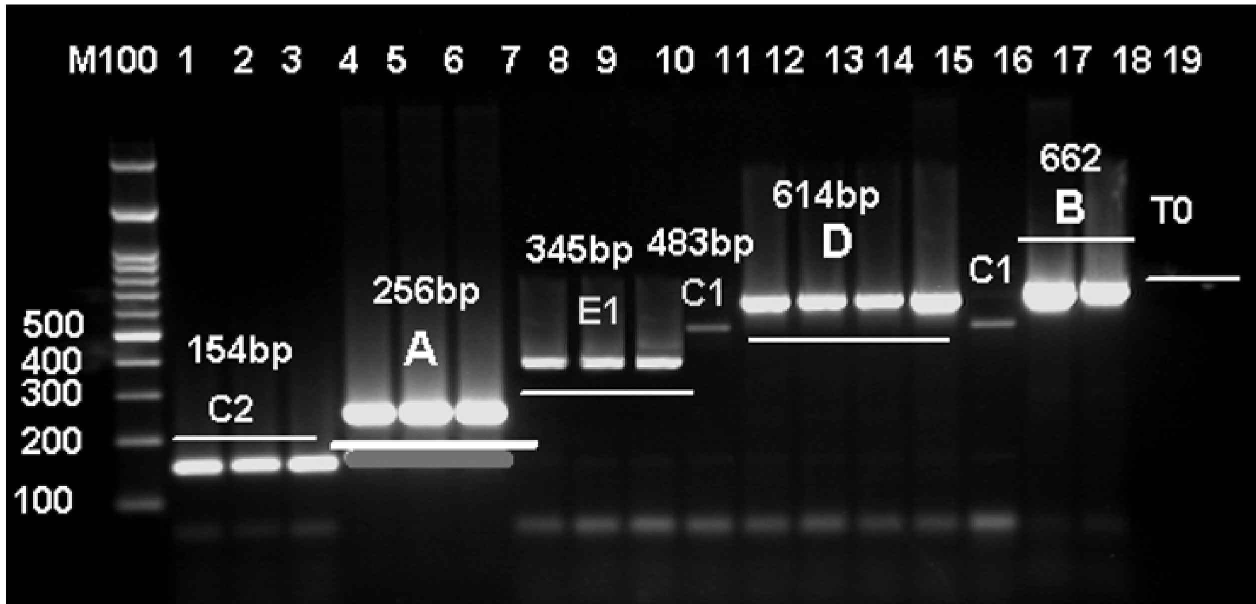

Figure 2

m-PCR to identify Salmonella serogroups. Lane 1, 2, and 3: C2, lane 4, 5, and 6: A, lane 7, 8, and 9: E1, lane 10 and 15: C1, lane 11, 12, 13, and 14: D, lane 16 and 17: B, and lane 18 and 19: negative control. Lane M100: Ladder 100bp (Invitrogen, USA).

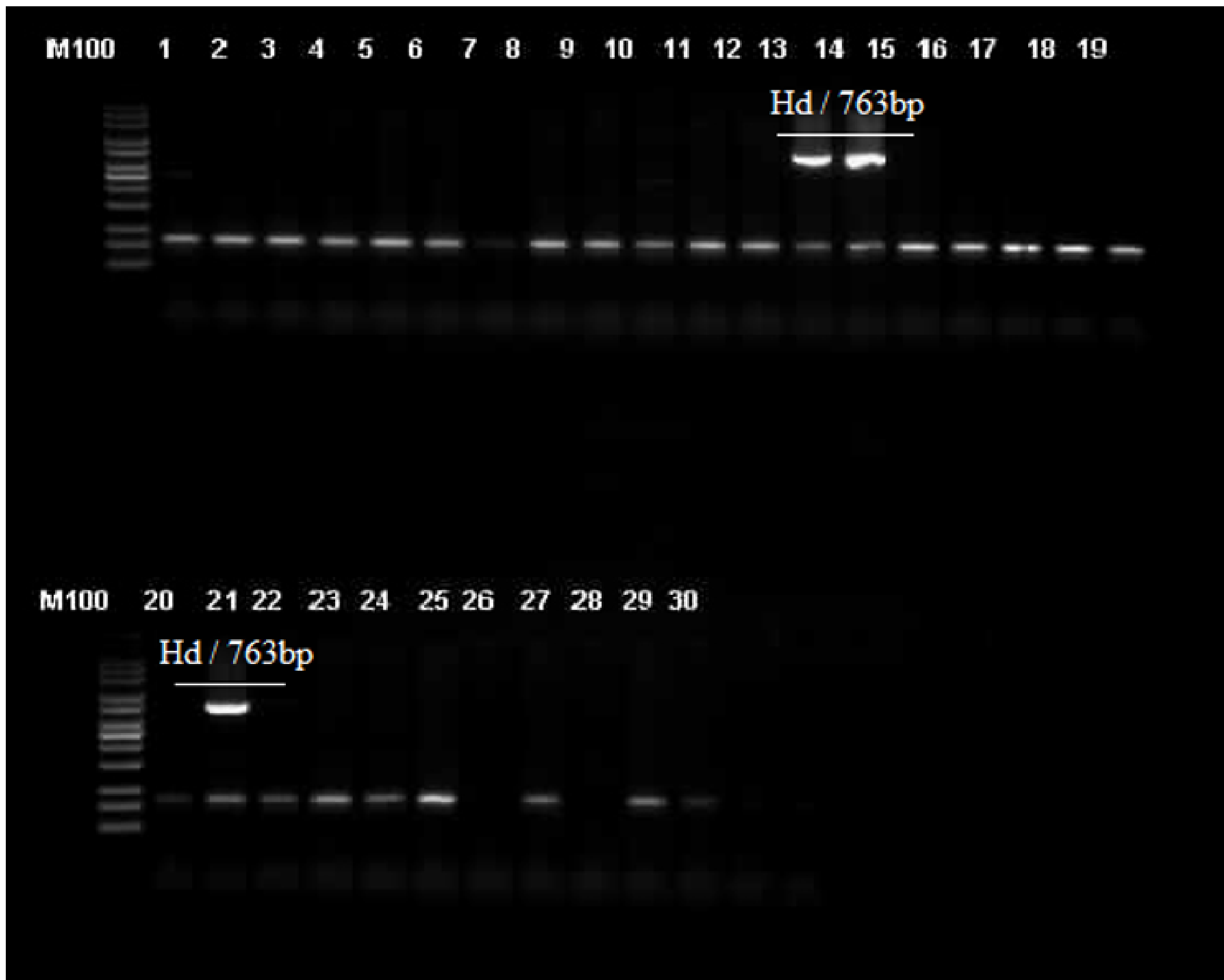

Figure 3

m-PCR to identify subgroup H1 Salmonella genus (Ha, Hb, Hd, Hj). M100: ladder 100bp (Invitrogen), Lane 14, 15, 21: subgroup H1 Salmonella genus (Hd / 763bp).

100
80
60
40
20
0
-20

Percentage

S. Typhimurium	S. Enteritidis	S.Infantis	S. Hadar	S. Chlorasuis	S. Kentucky	S. Heidelberg	Unindetified
7.5	28.9	12.7	8.7	6.9	11	2.9	21.4

Serovar

Figure 4

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

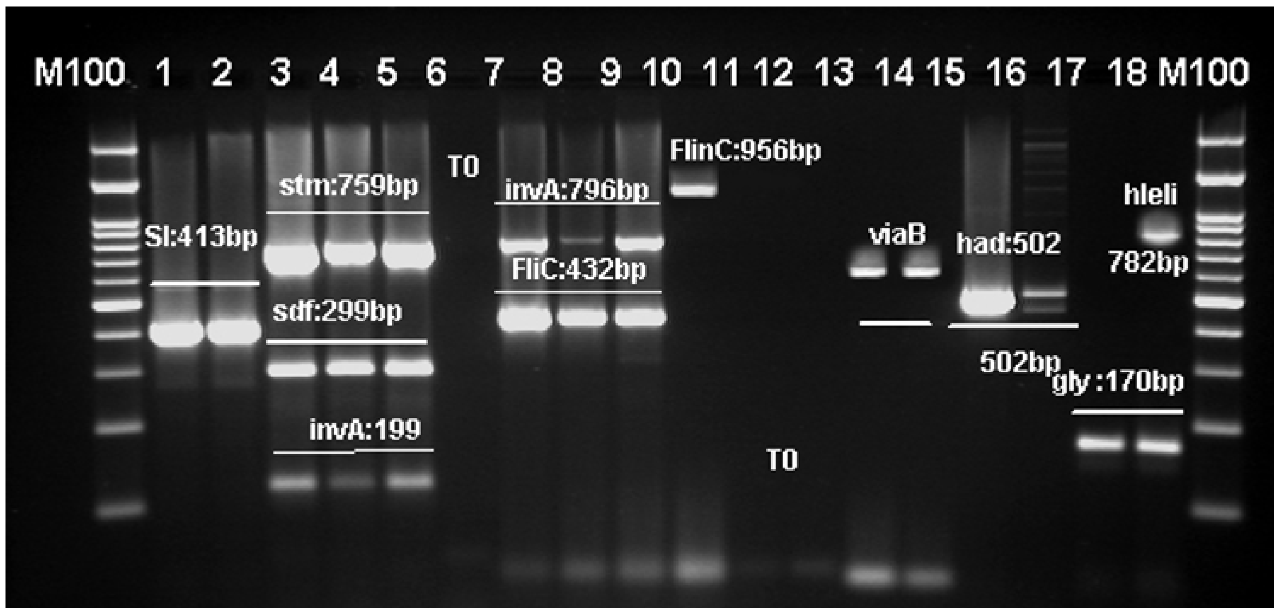

Figure 5

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

FlinC: 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:782bp), and lane 18: S. Kentucky (gly:170bp). Lane M100: Ladder 100bp (Invitrogen, USA).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.docx