JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Isolation and characterization of *Salmonella* Enteritidis and *Salmonella* Typhimurium from chicken meat in Egypt

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Abstract
Introduction: *Salmonella enterica* serovars Enteritidis and Typhimurium represent the major serovars associated with human salmonellosis. Contamination of meat products with these serovars is considered the main source of infection.
Methodology: In this study, 100 raw chicken meat samples were investigated for the presence of *Salmonella* spp., which were subsequently identified based on biochemical and serological tests as well as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) profile. Furthermore, the isolated serovars were examined using multiplex polymerase chain reaction (PCR) for the presence of virulence genes suspected to have a role in infection.
Results: *S.* Enteritidis was isolated from two samples (2%), while *S.* Typhimurium was isolated from three samples (3%) of chicken meat. Of the 17 examined virulence genes using multiplex PCR, the *sitC*, *sopB*, *sifA*, *lpfC*, *spaN*, *sipB*, *invA*, *spiA*, and *msgA* genes were detected in *S.* Enteritidis. However, the *sitC*, *iroN*, *sopB*, *sifA*, *lpfC*, *spaN*, *sipB*, *invA*, and *tolC* genes were successfully amplified in *S.* Typhimurium.
Conclusions: The detection of *S.* Enteritidis and *S.* Typhimurium in meat, even at low incidence, has important implications. In addition, the data presented here is the first attempt to identify a wide range of virulence genes in Egyptian *Salmonella* isolates recovered from meat products. A strict public health and food safety regime is urgently needed in order to decrease the human health hazard risk associated with salmonellosis.

Key words: *Salmonella*; virulence genes; multiplex PCR; MALDI-TOF.

J Infect Dev Ctries 2017; 11(4):314-319. doi:10.3855/jidc.8043

(Received 24 December 2015 – Accepted 26 July 2016)

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Introduction

Salmonella enterica represents the major cause of bacterial foodborne infection in the United States [1]. *Salmonella enterica* serovar Enteritidis is considered the major cause of human salmonellosis outbreaks in the United States and Europe [2-3], linked mainly to consumption of contaminated poultry products, including eggs [4-5]. Live poultry are considered the main reservoir for salmonellae; the microorganism is present in the intestinal tract, skin, and feathers of living birds. Bacterial contamination of poultry carcasses and cuts are a result of improper hygienic measures, improper cooking, and abuse of temperature. The dissemination of infection throughout plants during processing occurs in the evisceration, cooling, packaging, and transport stages [6]. Based on food standards, the presence of salmonellae in foodstuff makes food unsafe for human consumption [7-8]. The most serious form of salmonellosis for humans is typhoid fever, which is still a major problem in developing countries, mainly due to the lack of sanitation and hygiene standards [9-10]. The disease is transmitted mainly via foodstuff and water contaminated with the pathogen, and it affects more than 90 million people worldwide yearly, with variable morbidity and mortality rates [11]. In poultry, the severity of the infection depends on many factors, including the strain of *Salmonella*, the standard of hygiene, age of the bird, route of infection, and immune status of the bird [12]. Generally, the growth and multiplication of salmonellae within the host is governed by a central regulatory gene function. The virulence determinant genes of *Salmonella* spp. are associated with a combination of either chromosomal or plasmid factors [13]. These genes have a role in adhesion, invasion, and enterotoxin production [13-15]. So far, there is a scarcity of data regarding the incidence of these virulence genes in *Salmonella* from meat

products in Egypt. Therefore, the present study was undertaken to isolate *Salmonella enterica* from raw chicken meat as well as to screen the virulence genes profiles within the isolates using multiplex polymerase chain reaction (PCR).

Methodology

Isolation and identification of Salmonella

A total of 100 samples (100 g each) of chicken meat were collected from different supermarkets in Minoufiya and Cairo governorates. Samples were collected aseptically and transferred for further bacteriological examination. Samples were pre-enriched in buffered peptone water (Oxoid, Hampshire, England) for 16–20 hours at 35–37°C. One milliliter of each pre-enrichment culture was used to inoculate 10 mL of Muller-Kauffman tetrathionate (Oxoid, Hampshire, England), which was incubated at 37°C for 24 hours. After thoroughly mixing each enrichment broth, a 3 mm (10 µL) loopful was removed and streaked onto xylose lysine desoxycholate (XLD; Oxoid, Hampshire, England). Following incubation at 37°C for 24 hours, suspected *Salmonella* colonies were selected and restreaked on fresh plates of the same agar medium for purification. Identification of isolated microbial strains was based on biochemical tests [16-19]. Colonies were inoculated into triple sugar iron (TSI) agar and lysine iron agar (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours. Diagnosis was confirmed based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) profile, based on a previously developed protocol [20]. Briefly, about 10 mg of cell material of the cultured strains was suspended in 300 µL of sterile water. After the addition of 900 µL of absolute ethanol, the mixture was centrifuged at 10,000 rpm for 2 minutes. The supernatant was discarded and the pellet was suspended in 50 µL formic acid (70% v/v). After adding 50 µL of acetonitrile (AN), the mixture was centrifuged at 10,000 rpm for 2 minutes, and 1 µL of the clear supernatant was transferred to the MALDI target and allowed to dry, followed by the addition of 1 µL of α-cyano-hydroxy-cinnaminic acid (Bruker Daltonik GmbH, Bremen, Germany) in a standard organic solvent mixture (2.5% trifluoroacetic acid, 50% AN in water). All chemicals used were of the highest quality (Merck, Darmstadt, Germany, designed to be especially suitable for high performance liquid chromatography or MALDI-based techniques). Before each MALDI run, *E. coli* 1917 strain Nissle was analyzed to serve as the positive control and calibration standard. The MALDI-TOF mass spectrometry (MS) analysis was performed using a Bruker microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), and the spectra were automatically identified using the BrukerBioTyper 1.1 software (Bruker Daltonik GmbH, Bremen, Germany). Serotyping was performed based on the Kauffmann-White typing scheme using slide agglutination with different O and H antisera (Difco Laboratories, Detroit, USA).

Extraction and purification of DNA

One milliliter of freshly enriched *Salmonella* culture was transferred to a micro-centrifuge tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 minutes at 14,000 × g. The pellet was resuspended in 300 µL of DNase-RNase-free distilled water and centrifuged at 14,000 × g for 5 minutes. The supernatant was carefully discarded and the pellet was resuspended in 200 µL of DNase-RNase-free distilled water, incubated for 15 minutes at 100°C and immediately chilled on ice, then centrifuged for 5 minutes at 14,000 × g at 4°C. An aliquot of 5 µL of the supernatant was used as the template DNA in the PCR [21].

Multiplex PCR procedures

The isolated *Salmonella* strains were examined by multiplex PCR for the presence of several genes thought to be involved in virulence. Targeted genes and their primer sequences used in the amplification studies are summarized in Table 1. The primer sequence and PCR conditions were carried based on Skyberg *et al*. [22]. Three reactions were used to amplify the 17 genes; the first reaction (set 1) was used to amplify *spvB*, *spiA*, *pagC*, *cdtB*, and *msgA*; set 2 was used to amplify *invA*, *sipB*, *prgH*, *spaN*, *orgA*, and *tolC*; and set 3 was used to amplify *iroN*, *sitC*, *lpfC*, *sifA*, *sopB*, and *pefA* genes. The cycling conditions and reaction mixtures were the same for each multiplex procedure used; only the primers differed among the three reactions. The cycling conditions and reaction mixtures were the same for each multiplex procedure used; only the primers differed among the three reactions. The amplification was carried out in 50 µL reaction PCR tubes containing 5 µL master mix (10 ×, Fermentas, Leon-Rot, Germany), 5 µL of 20 Mm dTNPs mix, 0.15 µL of Taq polymerase (5 U/L µL, Fermentas, Leon-Rot, Germany), 1 µL of 0.1 mM forward and reverse primers, and 1 µL of DNA template. The reaction was subjected to the following conditions in the thermal cycler: 5 minutes at 95°C, 25 cycles of 30 seconds at 94°C, 30 seconds at 66.5°C, and 2 minutes at 72°C, with a final cycle of 10 minutes at 72°C, followed by a hold at 4°C. PCR products

Table 1. Primer sequence and targeted amplicon size used for *Salmonella* typing.

Gene	Forward	Reverse	Amplicon
spvB	CTATCAGCCCCGCACGGAGAGCAGTTTTTA	GGAGGAGGCGGTGGCGGTGGCATCATA	717
spiA	CCAGGGGTCGTTAGTGTATTGCGTGAGATG	CGCGTAACAAAGAACCCGTAGTGATGGATT	550
pagC	CGCCTTTTCCGTGGGGTATGC	GAAGCCGTTTATTTTTGTAGAGGAGATGTT	454
cdtB	ACAACTGTCGCATCTCGCCCCGTCATT	CAATTTGCGTGGGTTCTGTAGGTGCGAGT	268
msgA	GCCAGGCGCACGCGAAATCATCC	GCGACCAGCCACATATCAGCCTCTTCAAAC	189
invA	CTGGCGGTGGGTTTTGTTGTCTTCTCTATT	AGTTTCTCCCCCTCTTCATGCGTTACCC	1070
sipB	GGACGCCGCCCGGGAAAAACTCTC	ACACTCCCGTCGCCGCCTTCACAA	875
prgH	GCCCGAGCAGCCTGAGAAGTTAGAAA	TGAAATGAGCGCCCCTTGAGCCAGTC	756
spaN	AAAAGCCGTGGAATCCGTTAGTGAAGT	CAGCGCTGGGGATTACCGTTTTG	504
orgA	TTTTTGGCAATGCATCAGGGAACA	GGCGAAAGCGGGGACGGTATT	255
tolC	TACCCAGGCGCAAAAAGAGGCTATC	CCGCGTTATCCAGGTTGTTGC	161
iroN	ACTGGCACGGCTCGCTGTCGCTCTAT	CGCTTTACCGCCGTTCTGCCACTGC	1205
sitC	CAGTATATGCTCAACGCGATGTGGGTCTCC	CGGGGCGAAAATAAAGGCTGTGATGAAC	768
lpfC	GCCCCGCCTGAAGCCTGTGTTGC	AGGTCGCCGCTGTTTGAGGTTGGATA	641
sifA	TTTGCCGAACGCGCCCCCACACG	GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449
sopB	CGGACCGGCCAGCAACAAAACAAGAAGAAG	TAGTGATGCCCGTTATGCGTGAGTGTATT	220
pefA	GCGCCGCTCAGCCGAACCAG	GCAGCAGAAGCCCAGGAAACAGTG	157

obtained were subjected to horizontal gel electrophoresis in 1.5% agarose, and the size of the amplicon was determined by comparing it with the DNA marker.

Drug susceptibility testing

Salmonella isolates were assayed for their susceptibility to amoxicillin (25 µg), tetracycline (30 µg), neomycin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), ofloxacin (5 µg), doxycycline (40 µg), penicillin (10 µg), erythromycin (15 µg), and ampicillin (10 µg). The antibiotic susceptibility was assayed using disk diffusion methods based on the standards set by Clinical and Laboratory Standards Institute [23]. Media and antibiotic-impregnated disks were obtained from Difco Laboratories (Detroit, USA). The zones of inhibition were measured to assess resistance or susceptibility. The selected antimicrobials were chosen based on their common use in treating or preventing *Salmonella* infection in humans.

Results

Incidence of Salmonella spp. in the examined meat samples

Suspected *Salmonella* colonies were confirmed using biochemical tests and MALDI-TOF MS profiles. Based on the serological tests, only *S.* Enteritidis and *S.* Typhimurium could be identified. The data presented in Table 2 shows the recovered *Salmonella* spp. from the examined meat products. *S.* Enteritidis was isolated from two samples (2%) of the examined chicken meat, while in the case of *S.* Typhimurium, three samples (3%) of chicken meat were positive.

PCR typing for virulence genes

The data presented in Table 3 shows that the successfully amplified virulence genes in *S.* Enteritidis were *sitC*, *sopB*, *sifA*, *lpfC*, *spaN*, *sipB*, *invA*, *spiA*, and *msgA*. However, in the case of *S.* Typhimurium, *sitC*, *iroN*, *sopB*, *sifA*, *lpfC*, *span*, *sipB*, *invA*, and *tolC* were successfully amplified of the 17 genes examined. The sizes of *sitC*, *sopB*, *sifA*, *lpfC*, *spaN*, *sipB*, *invA*, *spiA*, *msgA*, *iroN*, and *tolC* were 768, 220, 449, 641, 504, 875, 1,070, 550, 189, 1,205, and 161 bp, respectively. The virulence genes *spvB*, *pagC*, *cdtB*, *prgH*, *orgA*, and *pefA* could not be detected in *S.* Enteritidis nor in *S.* Typhimurium.

Antibiotic sensitivity test

The three *S.* Typhimurium isolates were resistant to ampicillin, amoxicillin, penicillin, neomycin, ofloxacin, doxycycline, and chloramphenicol. However, they were sensitive to tetracycline, streptomycin, and erythromycin. On the other hand, the two *S.* Enteritidis isolates showed complete resistance to all tested antibiotics.

Table 2. Incidence and serological identification of *Salmonella* spp. isolated from chicken meat samples.

***Salmonella* isolate**	**Chicken meat**		**Antigenic structure**	
	Positive	%	O	H
S. Typhimurium	3/100	3	1.4.(5),12	i:1,2
S. Enteritidis	2/100	2	1,9,12	g,m

Discussion

Salmonella-induced foodborne illness has received more attention than have other foodborne pathogens. In this study, only 2 *S.* Enteritidis and 3 *S.* Typhimurium isolates were recovered from 100 raw chicken meat samples (Table 2). *Salmonella enterica* is highly diverse, containing over 2,500 different serovars. The representative serovars from this species are the most commonly isolated serovars during outbreaks of foodborne salmonellosis, including *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, and *S.* Infantis [24]. *S.* Enteritidis and *S.* Typhimurium are the most predominant isolated organisms in most *Salmonella* cases associated with the consumption of contaminated poultry, pork, and beef products [25]. Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, including production, processing, distribution, retail marketing, handling, and preparation [26].

The manifest of the pathogenic process of salmonellosis and the severity of infection are mainly ruled by an array of factors that work synergistically to maintain the growth of the microorganism within the host and assist the microorganism in expressing its virulence; these factors called virulence genes [27]. This study was mainly focused on 17 genes (*spvB*, *spiA*, *pagC*, *cdtB*, *msgA*, *invA*, *sipB*, *prgH*, *spaN*, *orgA*, *tolC*, *iroN*, *sitC*, *lpfC*, *sifA*, *sopB*, and *pefA*) that are known to have a role in *Salmonella* infection in poultry. Of 17 examined virulence genes, only 11 genes were successfully amplified using multiplex PCR (Table 3). The *sitC*, *sopB*, *sifA*, *lpfC*, *spaN*, *sipB*, *invA*, *spiA*, and *msgA* genes were detected in *S.* Enteritidis; however, *sitC*, *iroN*, *sopB*, *sifA*, *lpfC*, *span*, *sipB*, *invA*, and *tolC* were successfully amplified in cases of *S.* Typhimurium. These genes – *invA* [28], *orgA* [29], *prgH* [30], *tolC* [31], *sopB* [32], *lpfC* [33], *cdtC* [34], and *pefA* [35] – have a role in host recognition and invasion. Invasion gene (*invA*) exists in nearly all the *Salmonella* strains and is related to intestinal invasion [36]. The *spaN* and *sipB* genes were shown to have a role in entry into non-phagocytic cells and killing of macrophages [37]. Additionally, there are genes required for the survival within macrophages, including *pagC* [6], *msgA* [38], and *spiA* [39]. Others have a role in iron acquisition, including *sitC* [40] and *iroN* [41]. The formation of filamentous structure is the main role conducted via the *sifA* gene [42]. In addition, the survival of *Salmonella* within the host requires the presence of the *spvB* gene [43].

The Panel on Biological Hazards recommended assessing the public health risk posed by *S.* Typhimurium-like strains, as *S.* Typhimurium isolates are commonly found to be resistant to antimicrobials [44]. In the present study, the three *S.* Typhimurium isolates were resistant to ampicillin, amoxicillin, penicillin, neomycin, ofloxacin, doxycycline, and chloramphenicol. They were sensitive to tetracycline, streptomycin and erythromycin. Interestingly, the two *S.* Enteritidis isolates were resistant to all tested antibiotics. It should be noted that these antimicrobials are frequently used on Egyptian poultry farms. The increasing antimicrobial resistance in non-typhoid *Salmonella* species expresses a serious problem for public health worldwide, and the high rate of resistance hampers the use of conventional antibiotics. The growing resistance to newer antimicrobial agents is aggravating the situation as well [45,46].

Table 3. Virulence gene profile of *Salmonella* serovars.

Virulence gene	*S.* Enteritidis	*S.* Typhimurium
spvB	-	-
spiA	+	-
pagC	-	-
cdtB	-	-
msgA	+	-
invA	+	+
sipB	+	+
prgH	-	-
spaN	+	+
orgA	-	-
tolC	-	+
iroN	-	+
sitC	+	+
lpfC	+	+
sifA	+	+
sopB	+	+
pefA	-	-

Conclusions

From our study, some conclusions with potential implications for the isolation and identification of *Salmonella* from poultry meat could be drawn. First, contamination of chicken meat with *Salmonella*, even in low incidence, indicates bad microbiological quality of chicken meat. Second, the data presented is considered the first attempt to identify wide range of virulence genes of the Egyptian *Salmonella* isolates recovered from poultry meat products. This study confirms the need for strict public health and food safety regimens to decrease the human health hazard risk associated with salmonellosis.

Acknowledgements
The authors would like to acknowledge Misr Elkhair Foundation for funding this project.

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Conflict of interests: No conflict of interests is declared.