

## Occurrence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from chicken meat and giblets

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### SUMMARY

This study was undertaken to survey the presence of *Salmonella* in 200 chicken samples collected from Mansoura, Egypt. *Salmonella* was detected in 16% (8/50), 28% (14/50), 32% (16/50) and 60% (30/50) of whole chicken carcasses, drumsticks, livers and gizzards, respectively, with an overall prevalence of 34% (68/200) among all samples. One hundred and sixty-six isolates were identified biochemically as *Salmonella*, and confirmed genetically by PCR, based on the presence of *invA* and *stn* genes. The *spvC* gene, however, was detected in only 25·3% (42/166) of the isolates. Isolates were serotyped as *Salmonella* Enteritidis (37·3%), *S.* Typhimurium (30·1%), *S.* Kentucky (10·8%), *S.* Muenster (8·4%), *S.* Virchow (4·8%), *S.* Anatum (4·8%), *S.* Haifa (1·2%), and four were non-typable. Antimicrobial susceptibility tests of the *Salmonella* isolates revealed that 100% were resistant to each of erythromycin, penicillin, and amoxicillin, while 98·8%, 96·4%, 95·2%, and 91·6% were resistant to nalidixic acid, sulphamethoxazole, oxytetracycline, and ampicillin, respectively. Multidrug resistance was evident for 92·8% of the isolates. The high contamination level of chicken meat with multidrug-resistant *Salmonella* can constitute a problem for public health.

**Key words:** Antimicrobial resistance, chickens, PCR, *Salmonella* serovars, virulence genes.

### INTRODUCTION

*Salmonella* is one of the most important causative agents of foodborne infection in both developed and developing countries [1]. In the USA, it has been estimated that about 87% of all *Salmonella*-confirmed cases are foodborne, with 10% due to person-to-person infection, and 3% to pets [2]. Poultry and poultry products are among the main food sources most often

incriminated in outbreaks of human salmonellosis [3, 4] and infection frequently occurs as a result of cross-contamination from equipment, utensils and workers' hands with subsequent handling of raw carcasses and products, in addition to the consumption of undercooked poultry meat [5].

In recent years, there has been an increase in the incidence of human salmonellosis that is more difficult to treat due to the appearance of multidrug-resistant strains, especially *S.* Typhimurium, which have been isolated from various foods of animal origin worldwide [6]. The high prevalence of antimicrobial-resistant bacteria throughout the food industry is probably due to widespread overuse of common antimicrobials as

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therapeutics, prophylactics or growth promoters in food animals [7].

Although all the serovars of *S. enterica* are considered potentially pathogenic, there are considerable differences in their virulence to humans and this has been attributed to the absence or presence of plasmids carrying virulence-associated genes [8]. The invasion A (*invA*) gene is unique to all *Salmonella* serovars and is an internationally recognized marker for the rapid detection of *Salmonella* genus [9]. The *invA* gene is required for invasion of the organism into host cells [10], while the enterotoxin (*stn*) gene encodes a protein which mediates severe diarrhoea has also been utilized as a PCR target for the detection of *Salmonella* strains [11]. The *Salmonella* plasmid virulence gene, *spvC*, is believed to increase the growth rate of salmonellae in host cells and affect their interaction with the host immune system [12].

Due to the increased incidence of resistant or multi-resistant *Salmonella* isolates worldwide against many commonly used antimicrobials, this study set out to determine the prevalence, serotypes, presence of virulence genes, and antimicrobial resistance of *Salmonella* isolates from chicken carcasses and giblets from retail outlets in Mansoura city, Egypt.

## MATERIALS AND METHODS

### Sample collection and bacteriological analysis

In total, 200 chicken samples (50 each of whole chicken carcasses, drumsticks, gizzards and livers) were randomly collected from 25 retail shops and supermarkets, of different sanitation levels, distributed in Mansoura city, Egypt on 10 occasions during the period June–November 2012. Each of the 25 shops was visited twice for sampling. On each sampling occasion, five shops were visited, and four samples (whole chicken carcass, drumstick, gizzard and liver) were taken from each shop. Each sample was packaged individually into a sterile impermeable polyethylene bag, labelled and transferred within 1 h in an icebox at ~4 °C to the food hygiene laboratory for bacteriological analyses. The test portion for analysis was 25 g outer skin of whole chicken carcasses, skin plus muscle of drumsticks, and tissue from gizzards or livers excised aseptically with a sterile scalpel for each individual sample.

Each test portion was transferred into a sterile homogenizer flask containing 225 ml of sterile buffered peptone water (Oxoid, UK) and homogenized

for 1 min in a stomacher (Seward Medical, UK). The homogenate was incubated at 37 °C for 24 h then 0.1 and 1 ml volumes were aseptically added to 10 ml each of Rappaport Vassiliadis (RV) broth (Oxoid) and Muller–Kauffmann tetrathionate/novobiocin (MKTTn) broth (Oxoid), and incubated at 42 °C for 24 h and 37 °C for 24 h, respectively. These broths were subcultured on xylose-lysine-desoxycholate (XLD) agar (Oxoid) and Brilliant Green agar (BGA) with sulfadiazine (Neogen Corp., USA) which were incubated at 37 °C for 24 h, and at 35 °C for 24 h, respectively. Up to five typical (pink colonies with or without black centres) or suspected colonies of presumptive *Salmonella* were subcultured onto nutrient agar slopes and incubated at 37 °C for 24 h for further biochemical and serological identification.

Identification of presumptive *Salmonella* isolates was performed according to standard methods [13] and carbohydrate fermentation profile using the API Rapid 20E system (bioMérieux, France) according to the manufacturer's instructions. Biochemically confirmed *Salmonella* isolates were serotyped by slide agglutination with O and H polyvalent antisera (Wellcome Diagnostic, UK).

### Molecular analysis

Genomic DNA was prepared by a method described previously [14]. *Salmonella* Typhimurium (RIMD 1985009) and *Escherichia coli* K12DH5a were used as positive and negative control strains, respectively, for the presence and absence of *invA*, *stn* and *spvC* genes. The primers for PCR amplification of *invA* (244 bp) were as described previously [15]. For PCR amplification of *stn*, two oligonucleotide primers (forward: 5'-CTTAATCGCGCCGATGCTGTT-3'; reverse: 5'-CATGAACTGGCGCAGGTGAT-3') were constructed to produce an amplified band size of 480 bp. For amplification of *spvC*, two primers (forward: 5'-AACGGTTCCTCACGTAAAGCCTGT-3'; reverse: 5'-ACCAAATGCGGAAGATGCCGGT-AT-3') produced an amplified band size of 580 bp. PCR was performed in a 15- $\mu$ l volume comprising 1  $\mu$ l *Salmonella* DNA template, 1.6  $\mu$ l each of forward and reverse primers (3 pmol each), 3  $\mu$ l dNTPs (2 mM), 7.5  $\mu$ l of 2 $\times$ PCR buffer for KOD FX, and 0.3  $\mu$ l KOD FX DNA polymerase (Toyobo Co. Ltd, Japan). After an initial denaturation at 94 °C for 2 min, 35 cycles (98 °C for 10 s, 58 °C for 30 s, 68 °C for 30 s) were performed followed by a final extension at 68 °C for 7 min. Amplified genes were verified by

DNA sequencing with the BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied BioSystems, USA) according to the manufacturer's instructions on an ABI Prism 3100 automated sequencer (Applied Biosystems). Nucleotide sequence data were analysed with GENETYXMAC software, v. 12 (GENETYX Corp., Japan). Homology searches of the obtained sequences against the already published genes in the GenBank were performed using Standard Nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

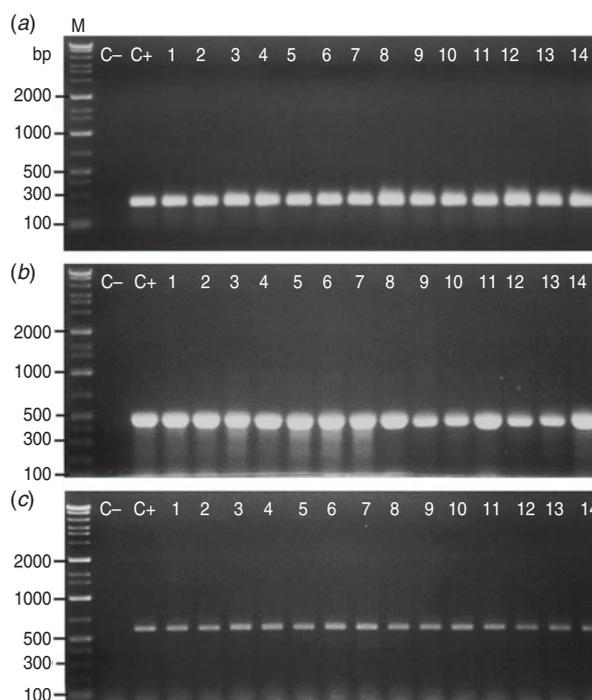
### Antimicrobial susceptibility tests

The antimicrobial susceptibility of *Salmonella* isolates was determined by an agar disk diffusion standard method [16] on Mueller–Hinton agar (Oxoid). Antibiotic discs were obtained from Difco (USA) and bioMérieux at the following drug concentrations: erythromycin (15 µg), nalidixic acid (30 µg), penicillin (10 IU), amoxicillin (30 µg), oxytetracycline (30 µg), sulphamethoxazole (25 µg), ampicillin (10 µg), streptomycin (10 µg), neomycin (30 µg), chloramphenicol (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), kanamycin (30 µg), and gentamicin (10 µg). Isolates were classified as susceptible, intermediate or resistant according to National Committee for Clinical Laboratory Standards criteria [16], with intermediate susceptibility counted as resistant. *E. coli* ATCC 25922 was used as a reference strain for antibiotic disc control. The multiple antibiotic resistance (MAR) index for each resistance pattern was calculated from the number of resistances to antimicrobials/total number of antimicrobials tested.

### RESULTS

*Salmonella* spp. were detected in 34% (68/200) of all chicken samples, distributed as 16% (8/50), 28% (14/50), 32% (16/50) and 60% (30/50) among whole chicken carcasses, drumsticks, livers, and gizzards, respectively. By PCR, all 166 isolates were positive for the *invA* and *stn* genes; while 25.3% (42/166) carried the *spvC* gene (Fig. 1) and were mainly *S. Typhimurium* and *S. Enteritidis*. The specific primers used in the present study exhibited good amplification efficiency for detection of the target genes and produced sufficient DNA for sequence analysis to confirm the identity of the amplified genes.

Seven different serovars were distinguished among the *Salmonella* isolates. *S. Enteritidis* (37.3%) was the most frequent, followed by *S. Typhimurium*



**Fig. 1.** Representative agarose gel electrophoresis for PCR products showing the proper molecular size of (a) 244 bp for the amplified *invA* gene, (b) 480 bp for the amplified enterotoxin (*stn*) gene, and (c) 580 bp for the amplified *Salmonella* plasmid virulence (*spvC*) gene in *Salmonella* isolates recovered from chicken meat and giblets. Chromosomal DNA from the *Salmonella* isolates ( $n=166$ ) was used as a template for PCR amplification using specific primer sets for *invA*, *stn* and *spvC* genes. Three microliters of the PCR product were separated by electrophoresis on 1.2% agarose gel and visualized under UV light. M, DNA marker (gene ladder 100 bp) used as a reference for fragment size; lane C-, *E. coli* K12 DH5a used as negative control strain; lane C+, *Salmonella* Typhimurium (RIMD 1985009) used as positive control strain; lanes 1–14, representative positive strains.

(30.1%), *S. Kentucky* (10.8%), *S. Muenster* (8.4%), *S. Virchow* (4.8%), *S. Anatum* (4.8%), and *S. Haifa* (1.2%). Four (2.4%) isolates could not be serotyped.

The distribution of *Salmonella* serovars among the chicken samples is shown in Table 1. *S. Enteritidis* and *S. Typhimurium* were recovered from all chicken parts, but *S. Kentucky* was not found in whole chicken carcass samples. Similarly, *S. Muenster* and the non-typable isolates were present in only liver and gizzard samples. *S. Virchow* was present in only drumstick and gizzard samples, and *S. Anatum* and *S. Haifa* were present in gizzard samples alone.

All *Salmonella* isolates were resistant to erythromycin, penicillin, and amoxicillin (Table 2), while high resistance rates (>90%) were found for nalidixic acid

Table 1. *Distribution of Salmonella serovars (n=166) among chicken samples*

Serotypes	<i>S. Enteritidis</i>	<i>S. Typhimurium</i>	<i>S. Kentucky</i>	<i>S. Muenster</i>	<i>S. Virchow</i>	<i>S. Anatum</i>	<i>S. Haifa</i>	Untyped
Whole carcasses	6	4	—	—	—	—	—	—
Drumsticks	8	8	6	—	4	—	—	—
Livers	12	6	4	2	—	—	—	2
Gizzards	36	32	8	12	4	8	2	2
Total	62	50	18	14	8	8	2	4

Table 2. *Percentages of antimicrobial susceptibility of Salmonella species isolated from chicken carcasses and products (n=166 isolates)*

Antimicrobial agent	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
Erythromycin (E)	—	—	166 (100)
Nalidixic acid (NA)	2 (1.2)	6 (3.6)	158 (95.2)
Penicillin (P)	—	12 (7.2)	154 (92.8)
Amoxicillin (AMX)	—	14 (8.4)	152 (91.6)
Oxytetracycline (T)	8 (4.8)	10 (6.0)	148 (89.2)
Sulphamethoxazole (SXT)	6 (3.6)	22 (13.2)	138 (83.1)
Ampicillin (AM)	14 (8.4)	28 (16.9)	124 (74.7)
Streptomycin (S)	30 (18.1)	24 (14.4)	112 (67.5)
Neomycin (N)	26 (15.7)	38 (22.9)	102 (61.4)
Chloramphenicol (C)	36 (21.7)	52 (31.3)	78 (47.0)
Norfloracin (NOR)	54 (32.5)	62 (37.4)	50 (30.1)
Ciprofloxacin (CP)	60 (36.1)	74 (44.6)	32 (19.3)
Kanamycin (K)	98 (59.0)	52 (31.3)	16 (9.6)
Gentamycin (G)	130 (78.3)	34 (20.5)	2 (1.2)

(98.8%), sulphamethoxazole (96.4%), oxytetracycline (95.2%), and ampicillin (91.6%). The lowest rates were recorded for ciprofloxacin (63.9%), kanamycin (41.0%), and gentamicin (21.7%). The analysis of resistance profiles and MAR indexes of isolates by serotype is shown in Table 3. The great majority (92.8%, 154/166) of isolates showed resistance to  $\geq 3$  of the 14 antimicrobials tested. Two-thirds of isolates (63.2%, 105/166) had a MAR index above the average (0.582) and within these isolates 13 resistance profiles encompassing eight to all 14 antimicrobials were identified. Among *S. Enteritidis*, 50/62 (80.6%), and all of 50 *S. Typhimurium* isolates were resistant to  $\geq 9$  antimicrobials.

Only 12 (7.2%) of the 166 isolates exhibited resistance to just one or two antimicrobials and these comprised the less frequently occurring serotypes, *S. Haifa*, *S. Muenster* and *S. Kentucky*.

## DISCUSSION

Our results of the prevalence of *Salmonella* in whole chickens and drumsticks are in agreement with the

prevalence rates of *Salmonella* in poultry meats recorded in different countries which range from 19.2% in fresh and frozen chicken carcasses in South Africa [17], 22% in Louisiana (USA) retail stores [18], and 27% in retail market broiler chicken carcasses in Colombia [19]. Higher *Salmonella* contamination rates have been reported from several studies ranging from 34% in Turkey [5] to 66% in Thailand [20]. By contrast, only 0.6% of 168 samples of meat parts of broiler chickens tested in an earlier survey in Turkey [21] were contaminated with *Salmonella*, while all of the 127 poultry carcasses tested in Brazil were negative for these organisms [22]. Similarly for chicken giblets (gizzards, liver, heart) reported rates vary depending on the survey country, notably 86% in Thailand [20], 34.5% and 41% of livers and gizzards, respectively, in Ethiopia [23] and 3% in Argentina [24].

The wide variation in *Salmonella* prevalence in chicken meat from different studies could be attributed to geographical differences, sampling techniques, bacteriological methods as well as slaughter hygiene and cross-contamination of products at different

Table 3. Antibiotic resistant profiles and multiple resistance index (MAR) of *Salmonella* isolates from chicken carcasses and products (n=166 isolates)

<i>Salmonella</i> serovars	Antimicrobial resistance profile	No. of isolates	MAR index
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR, CP, K	4	0.928
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR, CP	8	0.857
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR	8	0.785
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S, N, C	20	0.714
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S, N	10	0.642
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT, AM, S	4	0.571
<i>S. Enteritidis</i>	E, NA, P, AMX, T, SXT	2	0.428
<i>S. Enteritidis</i>	E, NA, P, AMX, T	4	0.357
<i>S. Enteritidis</i>	E, NA, P, AMX	2	0.285
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR, CP, K, G	2	1
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR, CP, K	10	0.928
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR, CP	8	0.857
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N, C, NOR	10	0.785
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N, C	8	0.714
<i>S. Typhimurium</i>	E, NA, P, AMX, T, SXT, AM, S, N	12	0.642
<i>S. Kentucky</i>	E, NA, P, AMX, T, SXT, AM, S	2	0.571
<i>S. Kentucky</i>	E, NA, P, AMX, T, SXT, AM	4	0.500
<i>S. Kentucky</i>	E, NA, P, AMX, T, SXT	4	0.428
<i>S. Kentucky</i>	E, NA, P, AMX, T	2	0.357
<i>S. Kentucky</i>	E, NA, P, AMX	2	0.285
<i>S. Kentucky</i>	E, NA, P	2	0.214
<i>S. Kentucky</i>	E	2	0.071
<i>S. Muenster</i>	E, NA, P, AMX, T, SXT, AM	6	0.500
<i>S. Muenster</i>	E, NA, P, AMX, T, SXT	2	0.428
<i>S. Muenster</i>	E, NA	2	0.142
<i>S. Muenster</i>	E	4	0.071
<i>S. Virchow</i>	E, NA, P, AMX, T, SXT, AM, S, N	2	0.642
<i>S. Virchow</i>	E, NA, P, AMX, T, SXT, AM, S	4	0.571
<i>S. Virchow</i>	E, NA, P, AMX, T, SXT, AM	2	0.500
<i>S. Anatum</i>	E, NA, P, AMX, T, SXT	6	0.428
<i>S. Anatum</i>	E, NA, P, AMX, T	2	0.357
<i>S. Haifa</i>	E	2	0.071
<i>Salmonella</i> spp.	E, NA, P, AMX, T	2	0.357
<i>Salmonella</i> spp.	E, NA	2	0.142
Average = 0.582			

E (erythromycin, 15 µg); NA (nalidixic acid, 30 µg); P (penicillin, 10 IU); AMX (amoxicillin, 30 µg); T (oxytetracycline, 30 µg); SXT (sulphamethoxazole, 25 µg); AM (ampicillin, 10 µg); S (streptomycin, 10 µg); N (neomycin, 10 µg); C (chloramphenicol, 30 µg); NOR (norfloxacin, 10 µg); CP (ciprofloxacin, 5 µg); K (kanamycin, 30 µg); G (gentamicin, 10 µg).

stages of chicken dressing and preparation. The observed greater contamination of gizzard and liver samples over other samples may reflect greater manipulation of these organs in addition to contamination from the crop and intestinal contents during evisceration. As a result of the control programme of *Salmonella* in chickens reared for meat production in the UK, the number of *S. Enteritidis*- and *S. Typhimurium*-infected breeding chicken flocks is currently very low owing to the introduction of strict control measures among which include management, cleaning and disinfection, hen vaccination, pest control, biosecurity, monitoring, and the potential use

of other aids in the control of *Salmonella* [25]. Wider application of such programmes may therefore be beneficial in reducing contamination rates in some countries.

The *invA* gene has been widely used for the detection of *Salmonella* spp. in food samples and its presence is highly associated with other virulence genes such as the *stn* gene which contributes to the pathogenicity process, primarily diarrhoea [26]. In *S. Typhimurium* and *S. Enteritidis* the virulence plasmid is known to increase the growth rate of the microorganisms at sites beyond the intestine and aid colonization of deeper tissue. Our finding of *spv* genes among these

serovars is therefore consistent with early reports of their association with highly invasive serovars.

Gizzard samples proved to be the most contaminated (30/50) of tissue samples and yielded each of the seven serovars detected from all samples. The absence of certain serovars such as *S. Virchow*, *S. Anatum*, and *S. Haifa* from some of the other sample types no doubt reflects their low overall frequency and perhaps less cross-contamination during preparation.

The predominance of *S. Enteritidis* and *S. Typhimurium* in this survey echoes the results of several other surveys of foodborne salmonellosis in the literature, albeit with differences in counties in the rates of these serovars [27]. However, low rates of *S. Enteritidis* (5.9%) were notable from Turkey [4], and 1.3% (1/73) from Brazil [22]; similarly low frequencies of *S. Typhimurium* have been reported from other surveys [17, 28]. *S. Kentucky* accounted for 10.8% of all our isolates which contrasts markedly with 59.5% and 41% in studies from the USA and Ireland, respectively [29, 30].

As expected, resistance to erythromycin, nalidixic acid and penicillin was almost universal and high resistance rates were evident for most of the antimicrobials tested which is consistent with the literature [5, 28, 31]. In the context of agents that would be considered for the treatment of diarrhoeal salmonellosis, it was surprising to find relatively poor levels of clear susceptibility to chloramphenicol (22%) norfloxacin (32.5%), and ciprofloxacin (36%). Although 78% of isolates were susceptible to gentamicin, this drug would generally be used parenterally and only for extraintestinal infections. The relatively lower rates of resistance to norfloxacin, ciprofloxacin, kanamycin and gentamicin could be attributed to their limited use in animal production. Our findings corroborate the widely held view that poultry is a major source of multidrug-resistant *Salmonella*, and underlines the value of antibiotic susceptibility surveys for selecting appropriate treatment options for salmonellosis caused by strains of poultry origin. The data also serve to highlight the need for implementation of antimicrobial stewardship programmes in developing countries, including Egypt, to optimize their use for treatment, and reduce the spread and development of antimicrobial-resistant strains.

In conclusion, we have demonstrated that a high proportion of chicken carcasses and giblets sold in Mansoura, Egypt were contaminated with *Salmonella*, predominantly *S. Typhimurium* and *S. Enteritidis*, the great majority of which were multidrug resistant.

Hence, chicken meat and their products constitute a significant problem for public health and this calls for better antimicrobial stewardship to reduce the unnecessary use of antimicrobials in the food industry.

## DECLARATION OF INTEREST

None.

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