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Original Article

Virulence genotypes of clinical *Salmonella*Serovars from broilers in Egypt

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Abstract

Introduction: *Salmonella* serovars are one of the primary foodborne pathogens. Poultry consumption is responsible for the majority of disease cases worldwide. The prevalence of virulence determinants among *Salmonella* serovars appears to be lacking in Egypt. Therefore, this study investigated the occurrence, antibiotic resistance patterns, and virulence gene profiling of *Salmonella* serovars in broilers.

Methodology: Three hundred samples from broiler chickens were examined for the presence of *Salmonella* by standard microbiological techniques. All *Salmonella* isolates were tested for their sensitivity against ten antibiotics and subjected to virulence genotyping by polymerase chain reaction (PCR).

Results: The overall isolation percentage of *Salmonella* was 17%. Seven different serovars were found, with the main one being *Salmonella* Typhimurium (52.94%). *Salmonella* isolates were sensitive to most of the tested antibiotics, but they exhibited absolute resistance against amoxicillin/clavulanic acid. Nine *Salmonella* strains (52.94%) were resistant to at least three antibiotics. Further PCR investigations into 17 *Salmonella* strains revealed different distribution patterns of eight virulence determinants among the isolates. The *inv*A gene was the most prevalent one (100%), followed by *hil*A (88.24%), *stn* (58.82%), and *fli*C genes (52.94%), while each of *sop*B and *pef*A genes had a similar prevalence (41.18%), and *sef*C and *spv*C genes had the lowest prevalence (11.76 and 5.88%, respectively). PCR genotyping allowed grouping of *Salmonella* strains into ten genetic profiles.

Conclusions: These results will help in understanding the spread of virulence genotypes and antibiotic resistance among *Salmonella* serovars in broilers.

Key words: *Resistance; Salmonella Typhimurium; invA; virulence genotypes.*

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Introduction

The genus *Salmonella*, a member of the family *Enterobacteriaceae*, is a facultative intracellular pathogen that is capable of causing different disease syndromes in a wide range of hosts. To date, more than 2,541 serovars of *Salmonella* have been described (National *Salmonella* Reference Laboratory, Galway, Ireland), with new serovars being identified every year [1]. *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most frequently isolated serovars throughout the world, leading to severe economic losses.

There is substantial evidence suggesting that poultry is incriminated in many outbreaks of human salmonellosis. This is a cause for concern to public health authorities, owing to the associated risks of bacterial food poisoning [2]. Furthermore, poultry have always topped the list of foods associated with incidences of salmonellosis in many developing countries including India, Egypt, Brazil, and Zimbabwe [3].

A multitude of virulence factors controlled by an array of genes that act in tandem determine how easily humans are infected with *Salmonella* serovars and how severe the infections are. Most of the genes required for *Salmonella* virulence are clustered within five *Salmonella* pathogenicity islands (SPI-1–SPI-5), which contributes to its success as an intracellular pathogen [4]. Some virulence genes such as the chromosomally encoded *stn* (*Salmonella* enterotoxin gene) are not located on SPIs. These virulence genes act via maintenance of *Salmonella* membrane composition and integrity [5] to play an important role in the virulence of *Salmonella*.

Many *Salmonella* serovars harbor large virulence plasmids of varying sizes and genetic composition; all encode a highly conserved region of approximately 8 Kb, called the *spv* operon [6]. This operon promotes rapid growth and survival of *Salmonella* within the host cells. Moreover, *Salmonella* possesses a dedicated protein secretion system denoted as type III secretion system (TTSS), which is involved in the invasion of

intestinal epithelial cells and *Salmonella* survival in macrophages [7]. This sophisticated system has been found to contribute to the pathogenesis by directing secretion and translocation of several virulence-associated proteins (effector proteins) directly into the cytosol of host cells [8]. One such group of *Salmonella* effector proteins is *Salmonella* outer proteins (Sop), which are encoded by *sop* genes. The *hil*A gene is important for the regulation of the type III secretion apparatus, activating the expression of invasion genes [9].

Controlling *Salmonella* in poultry is problematic, and it has relied historically on a combination of farm biosecurity and the use of antibiotics [10]. The indiscriminate and injudicious use of antibiotics is an important factor in the emergence of antibiotic-resistant bacteria that subsequently can be transferred to humans through the food chain. In recent years, multidrug-resistant (MDR) phenotypes have been increasingly described among *Salmonella* species all over the world [11].

Outbreaks of human salmonellosis have been linked to antimicrobial-resistant *Salmonella* isolates [12]. Therefore, there is a need for the development of innovative methods for rapid identification of *Salmonella* foodborne pathogens. In Egypt, few studies have been conducted to investigate the occurrence of virulence determinant genes in resistant *Salmonella*.

Hence, this study was planned to estimate the occurrence of *Salmonella* serovars implicated in the majority of infections in broilers and to evaluate the resistance profile of these isolates against commonly used antibiotics. In addition, *Salmonella* isolates were further characterized by virulence gene profiling, focusing on eight virulence determinants associated with SPIs and plasmids that have been shown to be relevant for the success of *Salmonella* as an intracellular pathogen.

Methodology

Sample collection

A total of 300 samples of liver, heart, and spleen (100 each) were aseptically collected from 100 freshly dead and diseased broiler chickens from different localities in Sharkia Province, Egypt, during the period of December 2009 to May 2010. The collected samples were immediately transported to the laboratory for bacteriological analysis.

Microbiological analysis

The standard microbiological techniques for isolation and identification of *Salmonella* serovars were conducted according to the International Organization for Standardization (ISO) 6579 [13]. Briefly, 25 grams of tissue samples were aseptically weighed, minced into small pieces, pre-enriched in 225 mL of sterile buffered peptone water, and incubated at 37°C for 18 hours. After this pre-enrichment, 1 mL from this homogenate was inoculated into 10 mL of tetrathionate broth and incubated at 37°C for 18 hours. Additionally, a second transfer of 0.1 mL of the same homogenate was transferred to 10 mL of Rappaport-Vassiliadis broth and incubated at 42°C for 24 hours. Subsequently, a loopful of each broth was streaked onto *Salmonella-Shigella* agar, xylose lysine desoxycholate agar and MacConkey's agar plates, and incubated at 37°C for 24 hours. Suspected colonies of *Salmonella* were purified and then identified morphologically and biochemically according to the guidelines of the ISO 6579 [13]. The primary biochemical screening involved reactions on triple sugar iron agar and lysine iron agar, indole production in tryptone broth, carbon utilization in Simmon's citrate agar, and urea splitting ability in Christensen's urea agar. All media were supplied by Oxoid (Basingstoke, Hampshire, England, UK). Typical *Salmonella* isolates were serotyped by a standard slide and tube agglutination test using commercial polyvalent and monovalent O and H antisera (Denka-Seiken, Tokyo, Japan) to identify *Salmonella* serovars.

Antimicrobial susceptibility testing

The *in vitro* susceptibility of all *Salmonella* isolates to various routine antimicrobial drugs was tested by the Kirby-Bauer standard agar disk diffusion technique as described earlier [14], using Mueller Hinton agar and commercial antibiotic disks (Oxoid, Basingstoke, Hampshire, England, UK). The tested antibiotics and their concentrations in µg/disk were as following: amoxicillin/clavulanic acid (AMC; 20/10), streptomycin (S; 10), gentamicin (CN; 10), ceftriaxone (CRO; 30), sulfamethoxazole/trimethoprim (SXT; 25), ciprofloxacin (CIP; 5), nalidixic acid (NA; 30), doxycycline (DO; 30), chloramphenicol (C; 30), and colistin sulfate (CT; 10). The inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories in accordance with the critical breakpoints recommended by the Clinical and Laboratory Standards Institute [15].

Table 1. Oligonucleotide primer sequences used for genotyping of *Salmonella* serovars

Primer	Target gene	Specificity/location	Oligonucleotide sequence (5'-3')	Ref.
invA-1 invA-2	*inv*A	*Salmonella* species/ SPI-1	F: TTG TTA CGG CTA TTT TGA CCA R: CTG ACT GCT ACC TTG CTG ATG	[17]
fliC-1 fliC-2	*fli*C	*Salmonella* Typhimurium/ Chromosome	F: CGG TGT TGC CCA GGT TGG TAA T R: ACT GGT AAA GAT GGC T	[19]
sefC-1 sefC-2	*sef*	*Salmonella* Enteritidis/ Chromosome	F: GCG AAA ACC AAT GCG ACT GTA R: CCC ACC AGA AAC ATT CAT CCC	[18]
spvC-1 spvC-2	*spv*C	Plasmid-encoded virulence/ Plasmid	F: CGG AAA TAC CAT CTA CAA ATA R: CCC AAA CCC ATA CTT ACT CTG	[17]
stn P1 stn M13	*stn*	Enterotoxin/ Chromosome	F: TTG TGT CGC TAT CAC TGG CAA CC R: ATT CGT AAC CCG CTC TCG TCC	[18]
pefA1 pefA2	*pef*	Plasmid encoded fimbriae/ Plasmid	F: TGT TTC CGG GCT TGT GCT R: CAG GGC ATT TGC TGA TTC TTC C	[18]
hilA DS hilA US	*hil*A	Transcriptional regulator/ SPI-1	F: CGG AAG CTT ATT TGC GCC ATG CTG AGG TAG R: GCA TGG ATC CCC GCC GGC GAG ATT GTG	[20]
*sop*B PRSB1 *sop*B PRSB2	*sop*B	Translocated effector protein/ SPI-5	F: CAA CCG TTC TGG GTA AAC AAG AC R: AGG ATT GAG CTC CTC TGG CGA T	[21]

Virulence genotyping of Salmonella isolates by polymerase chain reaction (PCR)

Firstly, PCR amplification was performed with a pair of primers to indicate the *inv*A gene that is shown to be unique for the *Salmonella* genus using the same method as previously described [17]. Secondly, PCR amplifications were conducted using two primer sets targeting *sef*C and *fli*C genes for confirmation of both *S.* Enteritidis and *S.* Typhimurium serovars, respectively, according to the procedures reported previously [18,19]. Moreover, five specific primer pairs were used for PCR detection of various virulence genes located on SPIs and plasmids (*hil*A, *sop*B, *stn, pef*A, and *spv*C genes) based on the protocols of several investigators [17,18,20,21]. The primer sequences and their corresponding genes are listed in Table 1. All PCR amplification reactions were performed in a final volume of 25 µL containing 12.5 µL of DreamTaq TM Green Master Mix (2X) (Fermentas, Inc. Hanover, USA), 0.1 µL of 100 pmol of each primer (SigmaAldrich, Co., St. Louis, USA), and 2 µL of *Salmonella* DNA template; the volume of the reaction mixture was completed to 25 µL using DNase/RNase-free water. The cycling condition of each primer was carried out in a singleplex PCR using a PTC-100 TM programmable thermal cycler (MJ Research Inc., Waltham, USA). The cycling conditions and the respective molecular sizes of PCR amplified products are summarized in Table 2. The PCR products were stored in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis

An aliquot of each amplified PCR product (5 µL) was electrophoresed on 1.5% agarose gel (Sigma-Aldrich, Co., St. Louis, MO, USA) containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich, Co., St. Louis, MO, USA) using 1 X TBE buffer for 1 hour at

Table 2. Cycling conditions and expected amplified product sizes of a singleplex PCR for amplification of various virulence genes of *Salmonella* serovars

Gene	Initial denaturation	Number of cycles	Cycling conditions			Final extension	Amplified product size (bp)
			Denaturation	Annealing	Extension		
*inv*A	94°C, 5 m	30	93°C, 1 m	42°C, 1 m	72°C, 2 m	72°C, 4 m	521
*fli*C	94°C, 3 m	30	94°C, 1 m	55°C, 1 m	72°C, 1.5 m	72°C, 10 m	620
*sef*C	94°C, 5 m	25	94°C, 1 m	55°C , 1 m	72°C, 1 m	72°C, 10 m	1103
*spv*C	94°C, 5 m	30	93°C, 1 m	42°C, 1 m	72°C, 2 m	72°C, 4 m	669
stn	94°C, 5 m	25	94°C, 1 m	59°C , 1 m	72°C, 1 m	72°C, 10 m	617
*pef*A	94°C, 5 m	25	94°C, 55 s	55°C, 55 s	72°C, 55 s	72°C, 10 m	700
*hil*A	94°C, 3 m	30	94°C, 1 m	65°C, 1 m	72°C, 1 m	72ºC, 10 m	854
*sop*B	94°C, 5 m	30	94ºC, 1 m	55ºC,1 m	72ºC, 2 m	72ºC, 10 m	1,348

Table 3. Antibiograms of different *Salmonella* serovars

Serovar (No.)	Number of *Salmonella* serovars resistant to each antimicrobial agent									
	S	**CN**	**NA**	**CIP**	**SXT**	**DO**	**C**	**CT**	**AMC**	**CRO**
S. Typhimurium(9)	2	2	3	0	4	4	2	0	9	0
S. Enteritidis (2)	1	0	2	0	0	0	0	1	2	1
S. Arizona (2)	0	0	1	0	1	0	1	0	2	0
S. Birkenhead (1)	1	0	1	0	1	1	0	1	1	0
S. Virchow (1)	1	0	1	0	0	0	0	1	1	0
S. Kentucky **(1)**	0	0	0	0	0	1	0	0	1	0
S. Montevideo (1)	0	0	1	0	0	1	0	0	1	0
Total **(17)**	5	2	9	0	6	7	3	3	17	1
% Resistant*	29.41	11.76	52.94	0	35.29	41.18	17.65	17.65	100	5.88
% Intermediate	35.29	0	47.06	0	0	11.76	0	23.53	0	41.18
% Susceptible	35.29	88.24	0	100	64.71	47.06	82.35	58.82	0	52.94

S: streptomycin; CN: gentamicin; NA: nalidixic acid; CIP: ciprofloxacin; SXT: sulfamethoxazole/trimethoprim; DO: doxycycline; C: chloramphenicol; CT: colistin sulfate; AMC: amoxicillin/clavulanic acid; CRO: ceftriaxone; * The percentage of the total number of isolates resistant, intermediate, or susceptible for a particular antimicrobial is indicated in the last three rows below each antimicrobial.

100V. The separated bands were visualized and photographed under an ultraviolet transilluminator (Spectroline, Westbury, USA). A 100 bp DNA ladder (Fermentas, Inc. Hanover, USA) was used as a molecular size marker to determine the molecular weights of the PCR products.

Results

Salmonella was isolated from 17 of 100 examined broiler chickens (17%). They were identified by standard microbiological techniques. Seven serovars were identified, including *S.* Typhimurium, which accounted for 52.94% of total *Salmonella* isolates. Other serotypes isolated (47.06%) were *S.* Enteritidis, *S.* Arizona, *S.* Kentucky, *S.* Montevideo, *S.* Birkenhead, and *S.* Virchow.

All *Salmonella* serovars were tested for their susceptibility to ten different antibiotics (Table 3). The *Salmonella* isolates were sensitive to most of the tested antibiotics. Higher rates of sensitivity to ciprofloxacin, gentamicin, chloramphenicol, and sulfamethoxazole/trimethoprim (100%, 88.24%, 82.35%, and 64.71%, respectively) were found and the lowest rates of sensitivity were found to doxycycline (47.06%) and streptomycin (35.29%). All isolated *Salmonella* strains exhibited absolute resistance (100%) to amoxicillin/clavulanic acid. There were great differences in antimicrobial resistance among different *Salmonella* serovars, with high levels of resistance displayed by isolates of *S.* Birkenhead and *S.* Virchow, which showed resistance to six and four antibiotics, respectively. In addition, nine *Salmonella* strains (52.94%) were resistant to at least three of the ten antimicrobial agents tested, making them MDR, and three (17.65%) were resistant to five or more of the antibiotics under investigation. As shown in Table 4, the *Salmonella* serovars in this study demonstrated 12 different MDR patterns.

All 17 identified *Salmonella* strains representing all serovars (*S.* Typhimurium [9], *S.* Enteritidis [2], *S.* Arizona [2] and 1 of each of *S.* Kentucky, *S.* Montevideo, *S.* Birkenhead, and *S.* Virchow) were subjected to PCR genotyping for detection of some chromosomally encoded virulence determinants. It was evident that the oligonucleotide primer pairs targeting the genes under study successfully amplified the DNA extracted from tested *Salmonella* isolates, generating the specific amplicon for each primer. As expected, PCR confirmation of bacteriologically positive strains was documented by the appearance of amplified DNA fragment of 521 bp for the *inv*A gene, a target for *Salmonella* genus in all 17 *Salmonella* isolates examined (100%), irrespective of serovar or source of isolation. For identification of selected *S.* Typhimurium (n = 9) and *S.* Enteritidis (n = 2) serovars using PCR, two primer sets targeting specific fragments within *fli*C and *sef*C genes, respectively were tested individually using a panel of known serovars. Evaluation of the primer specificities for *Salmonella* species faithfully reproduced the predictable results. Only *S.* Enteritidis and *S.* Typhimurium were positive for occurrence of the given species specific genes, while other *Salmonella* serovars were negative for these genes, and no amplification could be detected with both primer pairs, indicating 100% specificity. All serologically identified *S.* Typhimurium and *S.* Enteritidis gave positive amplification of the expected 620 and 1,103 bp PCR products, respectively. Overall, both *inv*A and *fli*C

genes and the combination of *inv*A and *sef*C correctly identified *S.* Typhimurium and *S.* Enteritidis serovars, respectively. Therefore, there was a concordance in the PCR assay and traditional serotyping in the identification of both serovars. Analysis of selected *Salmonella* isolates for the prevalence of the *hil*A gene located on SPI-1 was investigated using PCR amplification technique. The results indicated a clear abundance of this virulence gene, which was detected in 15 of 17 analyzed strains (88.24%). It was widely distributed among *Salmonella* isolates regardless of their serovars, giving rise to an 854 bp PCR product. In this study, the presence of the *pef*A gene was evaluated with the help of one primer pair that encodes for the major portion of the *pef* operon. The *pef*A gene, detected by the presence of a 700 bp PCR amplicon, was present in 7 of the 17 isolates tested (41.18%). There was no serovar-specific presence or absence of the *pef*A gene; four of nine *S.* Typhimurium strains (44.44%) and only one of each *S.* Enteritidis, *S.* Kentucky, and *S.* Arizona were also positive for the presence of this gene. The isolates were also screened for another chromosomally encoded *stn* virulence gene that is not present on SPIs. Observations from the present study indicated that the *stn* gene was prevalent among *Salmonella* isolates, as evidenced by PCR results. Ten isolates (58.82%) generated an identical band of 617 bp, the predicted size for the *stn* gene. The *stn* primer set produced target size amplicons with the DNA extracted from 100% of each *S.* Enteritidis and *S.* Virchow, 66.67% of *S.* Typhimurium, and 50% of *S.* Arizona isolates. Among the genes coded by SPI-5, *sop*B was targeted in this study; it was detectable in 41.18% of the isolates, which yielded a 1,348 bp specific sequence of this gene. The results clearly demonstrated variations in the incidence of *sop*B gene among *Salmonella* serovars; four *S.* Typhimurium, just one *S.* Enteritidis, and all *S.* Birkenhead and *S.* Montevideo were positive for the occurrence of this gene. The investigation for the presence of the *spv*C virulence gene in examined isolates revealed high specific amplification with expected product size (669 bp) in only one isolate belonged to *S.* Enteritidis serovar. Specifically, *spv*C was found in only one *S.* Enteritidis serovar.

To assess the potential virulence of *Salmonella* isolates, the distribution of target virulence genes among the investigated isolates was assessed. As shown in Table 5, *inv*A was the most common gene, followed by *hil*A, *stn*, and *fli*C genes, while *sop*B and *pef*A had an equal prevalence. Overall, *Salmonella* isolates showed at least two virulence-associated genes. In general, *hil*A and *stn* genes were simultaneously present in 52.94% (9/17) of *Salmonella* isolates, demonstrating a possible expression of these virulence genes. *S.* Enteritidis isolates had a higher percentage of positives for *inv*A, *sef*C, *hil*A, and *stn* genes (100% each) and *sop*B, *pef*A, and *spv*C genes (50% each) despite the low number of isolates used for comparison purposes. Regarding the different frequencies of *stn*, *sop*B, and *pef*A genes among various serovars, a clear difference was noticed in the occurrence of these genes among the isolates; *S.* Birkenhead and *S.* Montevideo did not show the presence of *stn* and *pef*A genes. Furthermore, two *S.*

Table 4. Multiple antimicrobial resistance patterns of *Salmonella* serovars

Antimicrobial resistance pattern*	Number of resistant *Salmonella* serovars							Total (17)
	Typhimurium (9)	Enteritidis (2)	Arizona (2)	Virchow (1)	Kentucky (1)	Birkenhead (1)	Montevideo (1)	
AMC, SXT	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**2**
AMC, NA	2	-	1	-	-	-	-	3
AMC, C	1	-	-	-	-	-	-	1
AMC, DO	1	-	-	-	1	-	-	2
AMC, NA, CT	-	1	-	-	-	-	-	1
AMC, NA, DO	-	-	-	-	-	-	1	1
AMC, SXT, C	-	-	1	-	-	-	-	1
AMC, S, NA, CRO	-	1	-	-	-	-	-	1
AMC, S, NA, CT	-	-	-	1	-	-	-	1
AMC, NA, DO, C	1	-	-	-	-	-	-	1
AMC, S, CN, SXT, DO	2	-	-	-	-	-	-	2
AMC, S, NA, SXT, DO, CT	-	-	-	-	-	1	-	1
Resistance to ≥ 3 antimicrobials	3	2	1	1	-	1	1	9
Resistance to ≥ 5 antimicrobials	2	-	-	-	-	1	-	3

AMC: amoxicillin/clavulanic acid; SXT: sulfamethoxazole/trimethoprim; NA: nalidixic acid; C: chloramphenicol; CN: gentamicin; DO: doxycycline; CT: colistin sulfate; S: streptomycin; CRO: ceftriaxone; *The most common multi-drug resistance pattern observed among salmonella serovars is shown in line 1 of the table (boldface type).

Table 5. Distribution of some virulence associated genes among different avian *Salmonella* serovars

Gene	Number of *Salmonella* serovars positive for virulence genes							Total (%)
	Typhimurium (9)	Enteritidis (2)	Arizona (2)	Virchow (1)	Kentucky (1)	Birkenhead (1)	Montevideo (1)	
*inv*A	9	2	2	1	1	1	1	17 (100)
*hil*A	7	2	2	1	1	1	1	15 (88.24)
stn	6	2	1	1	0	0	0	10 (58.82)
*fli*C	9	0	0	0	0	0	0	9 (52.94)
*sop*B	4	1	0	0	0	1	1	7 (41.18)
*pef*A	4	1	1	0	1	0	0	7 (41.18)
*sef*C	0	2	0	0	0	0	0	2 (11.76)
*spv*C	0	1	0	0	0	0	0	1 (5.88)

Typhimurium and one *S*. Enteritidis were positive for these three virulence genes concurrently. Interestingly, the virulence genotyping using eight sets of virulence genes correctly differentiated the majority of *Salmonella* serovars (94.12%). Based on the different combinations of *hil*A, *stn*, *sop*B, *pef*A, and *spv*C virulence genes and apart from genus- and species-specific genes (*inv*A, *fli*C and *sef*C genes), the isolates were categorized in ten different well-defined genetic profiles (Table 6). In order to facilitate the analysis, these virulence gene profiles were named P1–P10. Analyzing the PCR profiles revealed that the variations in genotypes were limited mainly to *sop*B, *pef*A, and *spv*C genes, being highly unstable loci in the genome of *Salmonella*. P1, the most predominant profile, was observed in three isolates (17.65%).

Evidently, all *Salmonella* serovars were capable of exhibiting several virulence determinants (Table 7). Not only were the most common *Salmonella* serovars, *S*. Typhimurium and *S*. Enteritidis, incriminated in poultry outbreaks identified in our study, but other MDR and virulent *Salmonella* serovars were also recognized.

Discussion

Poultry is one of the most important reservoirs of resistant salmonellae that can be transmitted to humans through the food chain. In 2012, several outbreaks of *Salmonella* were associated with poultry (www.cdc.gov/*Salmonella*/outbreaks.html). Increasing rates of antimicrobial resistance among *Salmonella* is a growing healthcare problem that needs to be monitored continuously. For this reason, the current preliminary screening study was conducted to shed light on the antibiotic resistance profiles and the virulence genotyping of *Salmonella* serotypes isolated from broiler chickens in Egypt.

Interestingly, the overall incidence level of *Salmonella* (17%) from broilers was close to that (21.99%) reported earlier in Bangladesh [22]. Many studies showed different prevalence rates of *Salmonella* isolates in broilers worldwide: in Brazil, a low rate of 2.7% was reported [23], while in China, a high rate of 52.2% was reported [3]. These differences in prevalence rates may reflect considerable disparity in

Table 6. Virulence gene profiles of 17 *Salmonella* isolates

Profile	Virulence gene(s)	No. (%) of *Salmonella* isolates
P1	*hil*A, *stn*, *pef*A, *sop*B	3 (17.65)
P2	*hil*A, *stn*, *pef*A	2 (11.76)
P3	*hil*A, *stn*, *sop*B	1 (5.88)
P4	*hil*A, *stn*, *spv*C	1 (5.88)
P5	*hil*A, *pef*A	2 (11.76)
P6	*hil*A, *sop*B	2 (11.76)
P7	*hil*A, *stn*	2 (11.76)
P8	*hil*A	2 (11.76)
P9	*stn*	1 (5.88)
P10	*sop*B	1 (5.88)

Table 7. Molecular characterization of 17 *Salmonella* isolates belonging to different serovars

Salmonella serovar	Detected virulence genes
S. Typhimurium	*inv*A, *fli*C, *hil*A, *stn*, *sop*B
S. Typhimurium	*inv*A, *fli*C, *hil*A, *stn*, *pef*A, *sop*B
S. Typhimurium	*inv*A, *fli*C, *hil*A, *stn*
S. Typhimurium	*inv*A, *fli*C, *hil*A, *stn*, *pef*A, *sop*B
S. Typhimurium	*inv*A, *fli*C, *sop*B
S. Typhimurium	*inv*A, *fli*C, *hil*A
S. Typhimurium	*inv*A, *fli*C, *stn*
S. Typhimurium	*inv*A, *fli*C, *hil*A, *pef*A
S. Typhimurium	*inv*A, *fli*C, *hil*A, *stn*, *pef*A
S. Enteritidis	*inv*A, *sef*C, *hil*A, *stn*, *pef*A, *sop*B
S. Enteritidis	*inv*A, *sef*C, *hilA, stn, spv*C
S. Arizona	*inv*A, *hil*A, *stn, pef*A
S. Arizona	*inv*A, *hil*A
S. Virchow	*inv*A, *hil*A, *stn*
S. Kentucky	*inv*A, *hil*A, *pef*A
S. Birkenhead	*inv*A, *hil*A, *sop*B
S. Montevideo	*inv*A, *hil*A, *sop*B

the sampling scheme, sample types, *Salmonella* detection protocol, and geographic location.

Poultry are commonly infected by a wide variety of *Salmonella* serovars; one serovar may be a predominant isolate in a country for several years before it is replaced by another serovar. Serovars vary geographically, but clinically significant *S*. Typhimurium and *S*. Enteritidis were identified as the most common serovars reported globally [24]. Although, after *S*. Enteritidis, *S*. Typhimurium is the most common *Salmonella* serovars causing salmonellosis worldwide [24], the most prominent serovars identified in our study were *S*. Typhimurium (52.94%), followed by *S*. Enteritidis (11.76%). Another study in Egypt reported a predominance of *S*. Enteritidis and *S*. Typhimurium from chicken (58.33% and 41.66%, respectively [25]. In Saudi Arabia, *S*. Enteritidis and *S*. Typhimurium dominated among the recovered *Salmonella* serovars from chicken (55.56% and 22.22%, respectively) [26].

With respect to the antimicrobial susceptibility testing of *Salmonella* serovars to ten different antibiotics, *Salmonella* isolates were sensitive to most of the tested antibiotics. Higher rates of sensitivity were observed to ciprofloxacin, gentamicin, and chloramphenicol, with percentages comparable to those found in many developing countries, especially Bangladesh, Nigeria, and Pakistan [27-29].

Antimicrobial resistance of *Salmonella* is particularly worrisome in view of its potential to extend into the human food chain, posing a challenge to public health. The data from the present study indicated that all isolated *Salmonella* strains exhibited absolute resistance (100%) against amoxicillin/clavulanic acid, indicating the limited therapeutic value of this antibiotic. Of the most common antimicrobial resistance in *Salmonella*, beta-lactam resistance was cited previously in Pakistan and Brazil [29,30] and can therefore be considered a worldwide problem. This resistance may be attributed to indiscriminate use of antibiotics at recommended doses or at subtherapeutic doses as feed additives to promote growth, and as chemotherapeutic agents to control epizootic diseases on farms.

There were great differences in antimicrobial resistance among different *Salmonella* serovars, with high levels of resistance displayed by isolates of *S*. Virchow serotype, which showed resistance to four antibiotics. A recent study in Nigeria, where *S*. Virchow from chicken was the most resistant serovar with the majority of resistance to four antimicrobials, supported our results [28].

Of interest, nine *Salmonella* serovars (52.94%) were resistant to at least three of the ten tested antimicrobial agents, making them MDR. This finding was in agreement with that reported in Morocco, where 42.1% of *Salmonella* isolates showed multiple-drug resistance [32]. In addition, three (17.65%) of the *Salmonella* serovars in this study were resistant to five or more antibiotics under investigation. This is not surprising in view of the high level of resistance observed for almost all the *Salmonella* serovars in this study, representing a significant disease burden in Egypt. The emergence of multiple-antibiotic-resistant avian salmonellae had become a major concern recently in multinational studies from Nigeria, Pakistan, China, Morocco, and Egypt [28,29,31-33]. Overall, all *Salmonella* serovars displayed 12 different MDR patterns. The frequencies and patterns of antimicrobial resistance may vary depending on time, region, serovar, the particular farm, type of chicken (layer versus broiler), and antimicrobial agent.

Salmonella-specific PCR with primers for the *inv*A gene is a rapid, sensitive, and specific means for monitoring *Salmonella* at the genus level in a variety of clinical samples [34]. The present study supported the ability of these specific primer sets to confirm the isolates as *Salmonella*. The *inv*A gene encodes a protein in the inner membrane of bacteria, which is necessary for invasion of epithelial cells of the host [36]. As expected, PCR confirmation of *Salmonella* isolates identified by conventional tests were documented by the appearance of amplified DNA fragments of 521 bp for the *inv*A gene in all 17 *Salmonella* isolates examined (100%), irrespective of serovar or source of isolation. Several reports had also confirmed the successful detection of 100% of *Salmonella* isolates from poultry using specific primers for the *inv*A gene with no false positives or negatives [26,35,37].

Identifying serovars using traditional serotyping is time consuming and expensive. For these reasons, the use of PCR for identification of *Salmonella* serovars described here is an attractive alternative to the most traditional techniques. In this study, PCR used for identification of *S*. Typhimurium was very specific because it could amplify 620 bp fragments of *fli*C gene in all *S*. Typhimurium strains with no amplification detected in other *Salmonella* serovars. The *fli*C gene in the *Salmonella* chromosome encodes the phase-1 flagellin [38]. Moreover, PCR amplified 1,103 bp fragments of the *sef*C gene in all *S*. Enteritidis, whereas DNA from other *Salmonella* serovars did not produce any amplification product. The *sef*C gene encodes an outer membrane protein that contains the *sef*A subunit

and the *sef*D adhesin. These results were similar to those obtained in another study in France [39].

The *hil*A gene encodes an OmpR/ToxR transcriptional regulator that activates the expression of invasion genes and has an important role in *Salmonella* pathogenicity. *Salmonella*-specific PCR with primers for the *hil*A gene denoted a clear abundance of this virulence gene; it was detected in 15 of 17 analyzed strains (88.24%), regardless of their serovars. Compared to previous reports, *hil*A was present in all of the *Salmonella* isolates from poultry [20,40].

Fimbriae play an important role in the pathogenicity of *Salmonella* because they promote their attachment to epithelial cells. PEF fimbria is encoded by the *pef* operon. Among the isolates analyzed in this study, the *pef*A gene was found to be present in 41.18% of the isolates with no serovar-specific presence or absence of this gene. The obtained percentage was lower than that recorded in India (89.47%), with inter-serovar variation in the presence of the *pef* gene [18].

With respect to another chromosomally encoded *stn* virulence gene, a wide distribution of this gene (100%) had also been recorded earlier among *Salmonella* isolates, irrespective of their serovars [41]. This strengthens the finding of the present study, where the *stn* gene was prevalent among *Salmonella* isolates as evidenced by PCR (58.82%).

Interestingly, some recognized proteins have some relevance to bacterial virulence (e.g., *Salmonella* pathogenicity island effector protein) [42]. We screened the isolates for the *sop*B gene coded by SPI-5, and the results revealed that it was detected in 41.18% of the isolates as compared with 100% of the isolates tested in India [21].

Genetic analysis indicated that the *spv*C gene is required for the virulence phenotype of *Salmonella* [43]. In the present study, 94.12% of the isolates were negative for the presence of the *spv*C gene. In a study carried out with 245 *Salmonella* isolates in Athens, USA, 84.9% of the isolates were negative for the *spv*C gene [17]. This gene was present in only one of our *S.* Enteritidis strains (50%). A previous study in Iran suggested that the *spv* gene for *S.* Enteritidis is on the increase in recent years [44].

The PCR profiles of *Salmonella* isolates revealed that the variations in genotypes were limited to mainly *sop*B, *pef*A, and *spv*C genes. In a Brazilian study, *Salmonella* isolates were classified into four genetic profiles based on the distribution of *spv*C, *sef*C, and *pef*A genes [45].

The present study shows that resistant *Salmonella* serotypes are also capable of exhibiting several virulence determinants. This finding is significant with respect to public health and had been previously reported in India [46] and Egypt [47].

Conclusions

This first report in Egypt about the phenotypic antimicrobial results and genotypic detection of some virulence genes among different *Salmonella* serovars could be effective in providing a more accurate profile for understanding the dangerous spread of virulence genotypes and antibiotic resistance in *Salmonella* species. Further novel studies including, for example, bacteriophage therapy are needed as a biocontrol tool for salmonellosis in broilers, and this therapy will be a preventive measure against multiple-antibiotic resistance of avian *Salmonella* serovars.

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