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PARATYPHOID *SALMONELLA* SEROVARS IN CHICKENS: MOLECULAR DETECTION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES

Yousra M. NASSAR[1], Wafaa A. ABD EL-GHANY[1], Adel K. IBRAHIM[2], and Ahmed S. HAMOUDA[1]

[1]Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt
[2]Clinical Pathology Department, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt

Email: wafaa.soliman@cu.edu.eg

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ABSTRACT: Paratyphoid salmonellosis is a serious disease that threatens the poultry industry worldwide, besides its public health hazard. The aims of this study were characterization of paratyphoid *Salmonella* spp. in chicken flocks of some Egyptian governorates, demonstration of the antimicrobial susceptibility of the isolated *Salmonella* spp., and detection of some virulence genes and antibiotic resistance genes using recent molecular techniques. A total of 238 organ samples were collected from 52 broiler, layer, and breeder chicken flocks, representing 9 Egyptian governorates. Conventional characterization of *Salmonella* isolates revealed a total isolation rate of 56.3% (134/238). Moreover, the isolation rates of *Salmonella* spp. were (49/79; 62%), (47/81; 58%), (10/18; 55.5%), (9/20; 45%), (2/6; 33.3%), (2/3; 66.7%), and (15/82; 53.6%) from liver, yolk sac, heart, spleen, caecum, ovary, and dead-in-shell embryos, respectively. A total of 32/238 (13.44%) isolates of *Salmonella* were found. Serological identification revealed presence of *S. enteritidis* (21.9%), *S. kentucky* (15.6%), *S. typhimurium* (12.5%), *S. molade* (12.5%), *S. takoradi* (9.4%), *S. wingrove* (6.3%), *S. infantis* (6.3%), *S. tsevie* (6.3), *S. shangani* (3.1%), *S. bargny* (3.1%), and *S. papuana* (3.1%). All *Salmonella* strains (32/32; 100%) were resistant to streptomycin, while almost all of them (31/32; 96.9%) were susceptible to meropenem. The amplification of *16S* rRNA gene of *Salmonella* isolates using uniplex polymerase chain reaction (PCR) generated a specific *Salmonella* product of approximately 550 base pair. The multiplex PCR revealed presence of *inv*A (100%), *stn* (65.6%), and *sop*B (40.6 %) virulence-associated genes as well as *aad*A1 (100%), *bla*TEM (59.4%), *aad*B (18.75%), and *sul*1 (28.1%) antibiotic resistance genes. In conclusion, virulent paratyphoid *Salmonella* spp. are circulating in the Egyptian flocks, causing economic losses. Additionally, they became resistant to the most commonly used field antibiotics. Therefore, regular molecular surveillance studies on the circulating *Salmonella* spp. and their resistance to the used antibiotics are of significant importance.

Keywords: Antibiotic resistance genes, Chicken, Paratyphoid *Salmonella*, PCR, Serology, Virulence

INTRODUCTION

The poultry industry has grown dramatically around the world accounting for approximately 45% of global trade (Mottet and Tempio, 2017). This industry is considered as a significant source of animal protein for the Egyptians. Paratyphoid salmonellosis is one of the most important bacterial diseases affecting poultry and regarded a major source of food-borne infection in humans (Menghistu et al., 2011; Abd El-Ghany, 2020). Foodborne diseases are frequently associated with the consumption of animal-derived foods, primarily poultry products such as eggs and undercooked chicken (Castro-Vargas et al., 2020). These diseases have negative economic impacts on poultry industry as a result of the costs of investigation, surveillance, prevention, and treatment (Kuria, 2023). *Salmonella* spp. are rod-shape Gram-negative bacteria belonging to family *Enterobacteriaceae* and are known to infect many hosts (Xu et al., 2020).

Despite the European Union in 2006 prohibited using of some antibiotics as growth promotors feed additives, few countries are still using them (Gyles, 2008; Diab et al., 2019). Besides, many other antimicrobials are used haphazardly to control *Salmonella* infections either in poultry field or for human infection (Xu et al., 2020). As a result of indiscriminate use of these antibiotics, the emergence of multiple drug-resistant (MDR) *Salmonella* serotypes have significantly risen (Ammar et al., 2019). *Salmonella* spp. have developed resistance to a wide range of antimicrobials (Azizpour, 2018). The mechanisms by which the bacteria can acquire antibiotic resistance are variable and it can occur via mutations or horizontal transfer of resistance genes (Kapoor et al., 2017). However, MDR *Salmonella* spp. can be transmitted from poultry to humans through the food chain or via the direct contact (Firoozeh et al., 2012). The main threats affecting

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humans after exposure to these antibiotic-resistant bacteria are inability to treat patients successfully, along with the high risk of transmission of such resistant bacteria (Roca et al., 2015). Thus, the existence of antibiotic resistance *Salmonella* strains has become a serious global issue. In addition, presence of both antimicrobial resistance-associated genes as well as virulence associated genes in *Salmonella* strains are important indicators of pathogenicity degree (Capuano et al., 2013; Lamas et al., 2016). Among *Salmonella* spp., there are many virulence and antibiotic associated resistance genes that have been detected molecularly using polymerase chain reaction (PCR) assays. For instance, the virulence associated genes including *inv*A, *sop*B, *stn*, and *pef* are used for genetic characterization of *Salmonella* strains (Badr et al., 2021; Elshebrawy et al., 2021; Ghetas et al., 2021; Hassan et al., 2021). Moreover, the antibiotic resistance genes such as *aad*A1 (streptomycin), *aad*B (gentamicin), *sul*1 (sulphonamides), and *bla*TEM (β lactams) have been detected in many isolates of *Salmonella* spp. (Ammar et al., 2019; Alam et al., 2020; Herrera-Sánchez et al., 2020).

Therefore, the aims of this study were characterization of paratyphoid *Salmonella* spp. in chicken flocks of some Egyptian governorates, demonstration of the antibiotic sensitivity of the isolated *Salmonella* spp., and detection of some virulence genes and antibiotic resistance genes using molecular techniques.

MATERIALS AND METHODS

Sampling

From November 2020 to April 2021, a total of 238 samples were collected from 52 broiler, layer, and breeder chickens' flocks in El-Mansoura, El-Sharqia, El-Qalyubia, El-Beheira, El-Minya, El-Fayoum, El-Gharbeya, Damietta, and Giza Egyptian governorates (Table 1). Samples were taken from diseased chicks showing whitish diarrhea, pasty vent, and omphalitis, dead-in-shell embryos, and breeders with low fertility and hatchability. The post-mortem lesions of both freshly dead and scarified diseased chickens were un-absorbed yolk sac, congested liver and spleen, typhlitis, and misshapen or discolored ova. Tissue samples were collected under aseptic conditions from liver (79), yolk sac (81), cecum (6), heart (18), spleen (20), ovary (3), gallbladders (3), and organs of dead-in-shell embryos (28), and then rapidly transferred in ice box to the laboratory for further processing.

Ethical regulations

The study design and methodology were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Cairo University, Egypt, with an approval number (01122022543).

Isolation and identification

Isolation and identification of *Salmonella* spp. were done according to ISO 6579:2002 guidelines. The collected samples were inoculated in buffered peptone water and incubated at 37°C for 18-20 hr. Then, 1 ml of pre-enriched broth was transferred into tubes containing 10 ml of Selenite F broth and incubated at 37±1°C for 24 hr. A loopful from each broth culture was inoculated onto selective plating medium such as xylose lysine deoxycholate agar (XLD), brilliant green agar (BGA), and MacConkey agar media, incubated at 37°C for 24 hr, and then checked for the of growth of typical *Salmonella* colonies. Suspected colonies were stained with Gram's and examined microscopically (Quinn et al., 2011). The biochemical identification of suspected *Salmonella* colonies was performed according to ISO 6579:2002 guidelines using triple sugar iron agar (TSI), indole, methyl red, vogues proskauer (VP), citrate utilization, urea hydrolysis, and H_2S production tests. The serological identification of *Salmonella* was carried out according to Kauffman-White Scheme (Kauffman, 1974). Both somatic (O) and flagellar (H) antigens (DENKA SEIKEN Co., Japan) were demonstrated using polyvalent and monovalent O antisera according to a slide agglutination test and H antisera according to a tube agglutination test.

The antimicrobial susceptibility test

The antimicrobial susceptibility test of the isolated *Salmonella* strains was done using disc diffusion method, according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS, 2020). The used antibiotic discs (Oxoid Limited, Basingstoke, Hampshire, UK) were ciprofloxacin (CP) (5µg), tetracycline (T) (30µg), ampicillin (AM) (10µg), cefotaxime (CF) (30µg), meropenem (M) (10µg), nalidixic acid (NA) (30µg), colistin (CO) (25µg), streptomycin (S) (10µg), levofloxacin (5µg), kanamycin (K) (30µg), clindamycin (CL) (10µg), amikacin (AK) (30µg), gentamicin (G) (10µg), and sulphamethoxazole (25µg). The tested strains were evaluated as susceptible, intermediate, and resistant by measuring the inhibitory zones. Moreover, the multiple antibiotic resistance (MAR) index for each *Salmonella* strain was determined (Singh et al., 2010) as follow;

MAR index= Number of antibiotics to which an isolate was resistant / total number of the tested antibiotics.

Each strain was classified as MDR if it exhibited resistance to three or more antimicrobial classes. Besides, the strain was considered to be resistant if it expressed MAR index > 0.2 (Shehata et al., 2019).

Molecular detection of *Salmonella* spp. virulence-associated and antibiotic resistance genes

The detection of *16S* rRNA of *Salmonella* isolates genes were carried out using uniplex PCR (Borges et al., 2017). The primers sequences are listed in Table 2, and the cycling conditions of primers during PCR are shown in Table 3. The

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PCR reactions were adjusted in 25 µL reaction mixture containing 5 µL of DNA template, 12.5 µL of 2x PCR master mix, 1.25 µL each of forward and reverse primers (10 pmol/µL), and 5 µL nuclease free water. The PCR products were run through 1.5% agarose gel electrophoresis and a 100 bp DNA ladder was used as a size marker.

The multiplex PCR was used for testing the presence of both virulence associated genes and antibiotic resistance genes in the isolated *Salmonella* spp. The tested virulence associated genes were *inv*A, *sop*B, *stn*, and *pef*, while the antibiotic resistance genes were *aadA1* (streptomycin), *aad*B (gentamicin), *sul*1 (sulphonamides), and *bla*TEM (β lactams). The primers sequences of virulence associated genes and the antibiotic resistance genes are presented in Tables 4 and 5, respectively.

RESULTS

Suspected *Salmonella* isolates appeared as small, non-lactose fermenter, colorless, and transparent colonies on MacConkey agar plates. However, isolates appeared as pink colonies with black centers on XLD and pinkish white or red colonies surrounded by a red halo on BGA agar. Gram staining revealed presence of Gram-negative, medium size, and rod-shaped bacilli under the microscope. The isolates fermented glucose, but did not ferment lactose and sucrose on TSI slants, and appeared as red slant with yellow butt, with or without H_2S production. Moreover, they were negative for indole, VP, and urea hydrolysis, whereas positive for methyl red and citrate utilization.

The conventional bacteriological characterization revealed that the isolation rates of *Salmonellae* were (49/79; 62%), (47/81; 58%), (10/18; 55.5%), (9/20; 45%), (2/6; 33.3%), (2/3; 66.7%), and (15/82; 53.6%) from liver, yolk sac, heart, spleen, caecum, ovaries, and dead-in-shell embryos, respectively. However, no isolation (0/3; 0%) was obtained from gallbladder (Table 6). The total isolation rate was 134/238 (56.3%). The number of the total strains of *Salmonella* was 32/238 (13.44%). Moreover, positive *Salmonella* isolates were detected in 49 out of 76 in broilers, 4 out of 10 in layers, and 2 out of 3 in breeder chicken flocks.

By using polyvalent and monovalent O and H antiserum, the slide and tube agglutination tests confirmed that the tested *Salmonella* isolates (n= 32) belonged to 11 paratyphoid *Salmonella* serovars. Table 7 shows the types and percentages of the isolated *Salmonella* serovars. A total of 11 serovars of paratyphoid *Salmonella* were identified as *S. enteritidis* (7, 21.9%), *S. kentucky* (5, 15.6%), *S. typhimurium* (4, 12.5%), *S. molade* (4, 12.5%), *S. takoradi* (3, 9.4%), *S. wingrove* (2, 6.3%), *S. infantis* (2, 6.3%), *S. tsevie* (2, 6.3%), *S. shangani* (1, 3.1%), *S. bargny* (1, 3.1%), and *S. papuana* (1, 3.1%).

Paratyphoid *Salmonella* serovars isolated from the liver (n= 49) were *S. enteritidis* (12), *S. typhimurium* (6), *S. kentucky* (8), *S. takoradi* (7), *S. molade* (4), *S. wingrove* (3) , *S. bargny* (3) , *S. shangani* (1), *S. infantis* (2), and *S. tsevie* (3), while those isolated from the yolk sac (n= 47) were *S. enteritidis* (9), *S. typhimurium* (4), *S. kentucky* (9), *S. takoradi* (6), *S. molade* (7), *S. wingrove* (2), *S. bargny*(3), *S. shangani* (1), *S. infantis* (2), and *S. tsevie* (4). Moreover, strains of the heart (n= 10) were *S. enteritidis* (2), *S. typhimurium* (3), *S. wingrove* (1), *S. shangani* (1), *S. molade* (2), and *S. infantis* (1), but those of spleen (n= 9) were *S. enteritidis* (1), *S. typhimurium* (5), *S. kentucky* (1), *S. papuana* (1), and *S. wingrove* (1). Caecal serovars (n= 2) were *S. takoradi* (2), while ovarian serovars (n= 2) were *S. enteritidis* (1) and *S. molade* (1). Regarding serovars of the dead-in-shell embryos (n= 15), they were *S. kentucky* (3), *S. takoradi* (6), *S. molade* (3), and *S. bargny* (3).

Regarding the distribution of *Salmonella* serovars in the surveyed Egyptian governorates, the highest isolation rates were from El-Dakhlia, El-Sharqia, Damietta and El-Qalubia, followed by El-Fayoum, El-Minya, Giza, and El-Beheira. Nevertheless, there was no isolation from El-Gharbeya. The data in Table 8 reveals detection of *S. enteritidis* from El-Qalubia, El-Dakhlia, El-Beheira, El-Minya, El-Fayoum, and Damietta governorates, while *S. typhimurium* was found in El-Dakhlia, El-Fayoum, El- Sharkqia, and Giza. Moreover, *S. kentucky* was isolated from El-Dakhlia, El-Minya, and Damietta and *S. molade* from El-Beheira, El-Fayoum, and Damietta. *S. takoradi* was isolated from El-Dakhlia, El- Sharqia, and Damietta, though *S. wingrove* was demonstrated in El-Dakhlia and El-Qalubia. Both *S. shangani* and *S. bargny* were detected in El-Dakhlia, then *S. tsevie* was isolated from El-Dakhlia and El-Sharqia. *S. infantis* was demonstrated in El-Fayoum and El- Sharqia, whereas S. Papuana was found in El-Qalubia

As shown in Table 9, all strains of *Salmonella* were completely resistant to streptomycin 100% (32/32), followed by clindamycin 93.8% (30/32), nalidixic acid 75% (24/32), amikacin 65.6% (21/32), tetracycline 50% (16/32), cefotaxime 43.7% (14/32),, kanamycin 34.4% (11/32), sulphamethoxazole 28.1% (9/32), ampicillin 18.8% (6/32), ciprofloxacin 12.5% (4/32), and colistin 12.5% (4/32). However, almost all strains of *Salmonella* were susceptible to meropenem 96.9% (31/32), followed by levofloxacin 90.6% (29/32), gentamicin 87.5% (28/32), colistin 84.4% (27/32), ciprofloxacin 81.3% (26/32), ampicillin 68.8% (22/32), sulphamethoxazole 71.9% (23/32), kanamycin 59.3% (19/32), and cefotaxime 56.3% (18/32).

Additionally, a total of 24 out of 32 *Salmonella* strains were resistant to 3 or more of the tested antimicrobials with an average MAR index 0.350. Surprisingly, out of the tested (n= 14) antimicrobials, two strains of *S. enteritidis* were resistant to 14, one strain of *S. kentucky* was resistant to 13 antimicrobials, and one strain of *S. typhimurium* was resistant to 10 antimicrobials.

The results of uniplex PCR confirmed that the amplification of *16S* rRNA gene of all *Salmonella* isolates generated a product of approximate molecular size of 550 base pair (bp) (Figure 1). Concerning the virulence genes, the amplified

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products for *inv*A, *sop*B, *stn*, and *pef* genes were 284, 517, 617, and 700 bp, respectively, while those of the antibiotic resistance genes *aad*A1, *aad*B, *sul*1, and *bla*TEM were 629, 300, 591, and 608 bp, respectively.

All *Salmonella* strains (n= 32) were screened by multiplex PCR for identification of the virulence associated genes (*inv*A, *stn*, *sop*B, and *pef*) and the antibiotic resistance genes (*aad*A1, *aad*B, *sul*1, and *bla*TEM). The results indicated that *inv*A, *sop*B, and *stn* genes were harbored by 100%, 40.6%, and 65.6% of *Salmonella* strains, respectively, while none of them had *pef* gene (Table 10 and Figure 2). Further, all strains (100%) harbored *aad*A1 associated with streptomycin resistance, but 59.4%, 18.75%, and 6.3% of strains possessed *bla*TEM (ampicillin), *aad*B (gentamicin), and *sul*1 (sulphamethoxazole) resistance genes, respectively (Table 11 and Figures 3-5).

Table 1 - The source and number of the samples examined for detection of *Salmonella*

Governorates	Chickens			Dead-in-shell embryonic organs	No. of samples	No. of flocks
	Broiler	Layer	Breeder			
A	39	6	3	28	145	28
B	18	2	0	0	49	14
C	19	2	0	0	44	10
Total	76	10	3	28	238	52

A= El-Mansoura, El-Gharbeya, El-Beheira & Damietta; B= El-Qalyubia, El-Sharqia & Giza; C= El-Minya & El-Fayoum

Table 2 - Primer sequences for detection of *16S* rRNA gene of *Salmonellae*

Target gene	Primer Sequence	Gene	Reference
16S rRNA	GCA ACG CGA AGA ACC TTA CC (Forward) GGT TAC CTT GTT ACG ACT T (Reverse)	*16S* rRNA	Gopinath et al. (1998)

Table 3 - Cycling conditions of the used primers during PCR

Gene	Primary Denaturation	Secondary Denaturation	Annealing	Extension	No. of Cycles	Final Extension
16S rRNA gene and other genes	90°C for 5 min.	90°C for 1 min.	52°C for 1 min.	72°C for 1 min.	39	72°C for 7 min.

Table 4 - Primers sequences of virulence associated genes of *Salmonella* serovars

Target gene	Primer sequence	Virulence factor	Reference
*inv*A	GTGAA ATT ATC GCC ACG TTC GGG CAA (Forward) TCAT CGC ACC GTC AAA GGA ACC (Reverse)	*Salmonella* species/SPI-1	Oliveira et al. (2003)
stn	TTG TGT CGC TAT CAC TGG CAA CC (Forward) ATT CGT AAC CCG CTC TCG TCC (Reverse)	Enterotoxin/Chromosome	Murugkar et al. (2003)
*sop*B	TCA GAA GRC GTC TAA CCA CTC (Forward) TAC CGT CCT CAT GCA CAC TC (Reverse)	Translocated effector protein/SPI-5	Huehn et al. (2010)
pef	TGT TTC CGG GCT TGT GCT (Forward) CAG GGC ATT TGC TGA TTC C (Reverse)	Plasmid encoded fimbriae/Plasmid	Murugkar et al. (2003)

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Table 5 - Primers sequences of antiiotic resistance genes of *Salmonella* serovars

The antibiotic	Target gene	Primer sequence	Reference
Streptomycin	*aad*A1	CTC CGC AGT GGA TGG CGG (Forward)	Chuanchuen and Padungtod (2009)
		GAT CTG CGC GCG AGG CCA (Reverse)	
Gentamicin	*aad*B	CTAGCTGCGGCAGATGAGC (Forward)	
		CTCAGCCGCCTCTGGGCA (Reverse)	
Sulfamethoxazole	*sul*1	CGGACGCGAGGCCTGTATC (Forward)	
		GGGTGCGGACGTAGTCAGC (Reverse)	
Ampicillin	*bla*TEM	ATCAGTTGGGTGCACGAGTG (Forward)	
		ACGCTCACCGGCTCCAGA (Reverse)	

Table **6 -** The number and percentage of the positive *Salmonella* samples

Group	Liver		Yolk sac		Cecum		Heart		Spleen		Gallbladder		Ovary		Dead-in-shell embryos		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A	26/47	55.3	26/51	50.9	2/5	40	1/1	100	3/10	30	0	0	2/3	66.6	15/28	53.6	75/145	51.7
B	12/16	75	10/14	71.4	0/1	0	5/9	55.5	5/6	83.3	0/3	0	0	0	0	0	32/49	56.3
C	11/16	68.8	11/16	68.8	0	0	4/8	50	1/4	25	0	0	0	0	0	0	27/44	61.4
Total	49/79	62	47/81	58	2/6	33.3	10/18	55.5	9/20	45	0/3	0	2/3	66.7	15/28	53.6	134/238	56.3

A= El-Mansoura, El-Gharbeya, El-Beheira & Damietta; B= El-Qalyubia, El-Sharqia & Giza; C= El-Minya & El-Fayoum

Table 7 - The types and percentages of the isolated *Salmonella* serovars

Identified strains	Number	%	Antigenic structure		
			Group	O	H
S. enteritidis	7	21.9	D1	1,9,12	g,m
S. kentucky	5	15.6	C3	8,20	i : Z6
S. typhimurium	4	12.5	B	1,4,5,12	i : 1,2
S. molade	4	12.5	C2	8,20	Z10 : Z6
S. takoradi	3	9.4	C2	8,20	i : 1,5
S. wingrove	2	6.3	C2	6,8	c : 1,2
S. infantis	2	6.3	C1	6,7	r : 1,5
S. tsevie	2	6.3	B	4,5	i : e,n,z15
S. shangani	1	3.1	E1	3,10	d : 1,5
S. bargny	1	3.1	C3	8,20	i : 1,5
S. papuana	1	3.1	C1	6,7	r : e,n,Z15

Table 8 - Types of *Salmonella* serovars isolated from the Egyptian governorates

Salmonella serovar	Frequency of each isolate		Governorate
	No.	%	
S. enteritidis	7	21.9	El-Qalubia, El-Dakhlia, El-Beheira, El-Minya, El-Fayoum, & Damietta
S. kentucky	5	15.6	El-Dakhlia, El-Minya, & Damietta
S. typhimurium	4	12.5	El-Dakhlia, E-Fayoum, El-Sharqia, & Giza
S. molade	4	12.5	El-Beheira, El-Fayoum, & Damietta
S. takoradi	3	9.4	El-Dakhlia, El-Sharqia, & Damietta
S. wingrove	2	6.3	El-Dakhlia and El-Qalubia.
S. infantis	2	6.3	El-Fayoum & El-Sharqia.
S. tsevie	2	6.3	El-Dakhlia & El-Sharqia
S. shangani	1	3.1	El-Dakhlia
S. bargny	1	3.1	El-Dakhlia
S. papuana	1	3.1	El-Qalubia

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Table 9 - The antimicrobial susceptibility test of the isolated *Salmonella* strains

Salmonella serovars	No.	Antibiotic resistance													
		S	CL	NA	AK	T	CF	K	SXT	AM	CP	CO	G	L	M
S. enteritidis	7	100	100	85.7	85.7	71.4	57.1	42.9	42.9	28.5	28.5	28.5	14.3	4.3	4.3
S. kentucky	5	100	80	80	60	60	60	60	40	40	20	20	20	20	0
S.typhimurm	4	100	100	75	50	50	25	50	25	25	0	25	0	0	0
S. molade	4	100	100	75	75	50	50	25	25	25	25	0	0	0	0
S. takoradi	3	100	100	66.6	66.6	33.3	33.3	33.3	33.3	0	0	0	0	0	0
S. wingrove	2	100	100	100	50	50	50	50	50	0	0	0	0	0	0
S. infantis	2	100	100	50	50	50	50	0	0	0	0	0	0	0	0
S. tsevie	2	100	50	50	50	50	50	0	0	0	0	0	0	0	0
S. shangani	1	100	100	100	100	0	0	0	0	0	0	0	0	0	0
S. bargny	1	100	100	100	100	0	0	0	0	0	0	0	0	0	0
S. papuana	1	100	100	0	0	0	0	0	0	0	0	0	0	0	0
Total	32	32	30	24	21	16	14	11	9	6	5	4	2	2	0
Resistant %		100	93.8	75	65.6	50	43.7	34.4	28.1	18.8	12.5	12.5	6.3	6.3	3.1
Intermediate %		0	3.1	9.4	9.4	6.3	0	6.3	3.1	12.5	6.3	3.1	6.3	3.1	0
Susceptible %		0	3.1	15.6	25	43.7	56.3	59.3	68.8	68.8	78.1	81.3	87.5	90.6	96.9

S: Streptomycin; CL: Clindamycin; NA: Nalidixic acid; AK: Amikacin; T: Tetracycline; CF: Cefotaxim; K: Kanamycin; SXT: Sulphamethoxazol; AM: Ampicillin; CP: Ciprofloxacin; CO: Colistin; G: Gentamicin; L: Levofloxacin; M: Meropenem

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Table 10 - Percentages of virulence-associated genes of *Salmonella* serovars

Virulence genes / *Salmonella* serovar	No.	*16S* rRNA		*inv*A		*stn*		*sop*B		*pef*	
		No.	%	No.	%	No.	%	No.	%	No.	%
S. enteritidis	7	7	100	7	100	6	85.7	5	71.5	0	0
S. kentucky	5	5	100	5	100	4	80	2	40	0	0
S. typhimurm	4	4	100	4	100	4	100	1	25	0	0
S. molade	4	4	100	4	100	1	25	0	0	0	0
S. takoradi	3	3	100	3	100	1	33.3	1	33.3	0	0
S. wingrove	2	2	100	2	100	2	100	2	100	0	0
S. infantis	2	2	100	2	100	1	50	1	50	0	0
S. tsevie	2	2	100	2	100	1	50	1	50	0	0
S. shangani	1	1	100	1	100	1	100	0	0	0	0
S. bargny	1	1	100	1	100	0	0	0	0	0	0
S. papuana	1	1	100	1	100	0	0	0	0	0	0
Total	32	32	100	32	100	21	65.6	13	40.6	0	0

Table 11 - Percentages of antibiotic associated genes of *Salmonella* serovars

Salmonella serovar	No.	The tested antibiotic resistance genes							
		*aad*A1		*sul*1		*bla*TEM		*aad*B	
		No.	%	No.	%	No.	%	No.	%
S. enteritidis	7	7	100	3	42.8	3	42.8	0	0
S. kentucky	5	5	100	2	40	3	60	2	40
S. typhimurm	4	4	100	1	25	2	50	0	0
S. molade	4	4	100	1	25	2	50	1	25
S. takoradi	3	3	100	1	33.3	1	33.3	1	33.3
S. wingrove	2	2	100	1	33.3	2	100	0	0
S. infantis	2	2	100	0	0	2	100	1	50
S. tsevie	2	2	100	0	0	1	50	1	50
S. shangani	1	1	100	0	0	1	100	0	0
S. bargny	1	1	100	0	0	1	100	0	0
S. papuana	1	1	100	0	0	1	100	0	0
Total	32	32	100	9	28.1	19	59.4	6	18.75

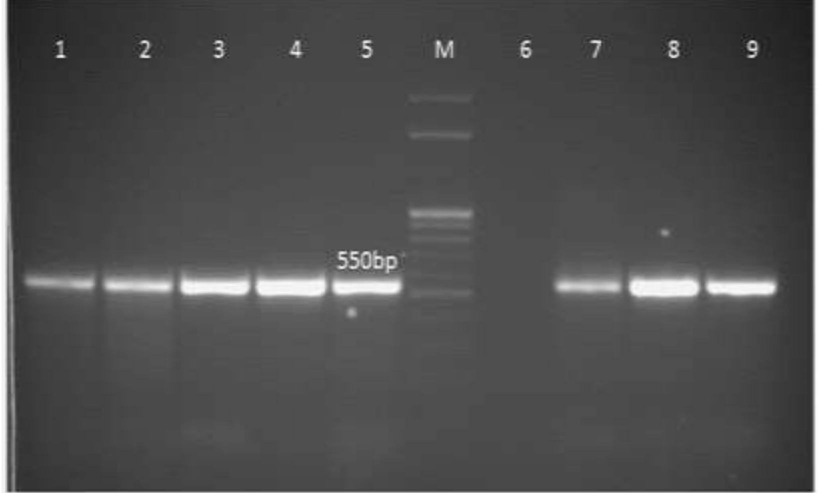

Figure 1 - Agarose gel electrophoresis for amplified samples, where Lanes 1, 2, 3, 4, 7, 8, and 9 represent the positive *Salmonella* strains, Lane 5: Positive control showing 550 bp for the amplified *16S* rRNA gene, Lane M: 100-bp DNA Marker and Lane 6: Negative control

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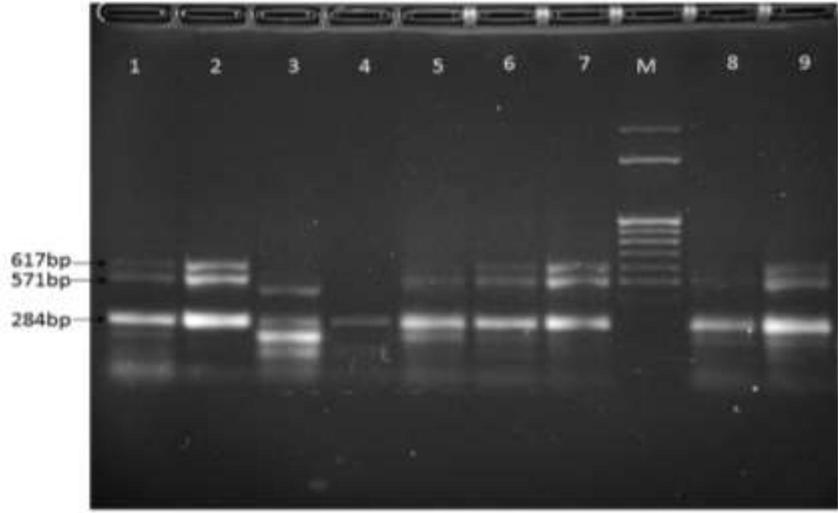

Figure 2 - Multiplex PCR products of *Salmonella* virulence genes. Lane 1: Positive control, Lanes 2, 5, 6, 7, and 8 showing amplicons of 284 bp, 517 bp, and 671 bp of *inv*A, *sop*B and *stn* genes respectively, Lanes 3 and 4 showing amplicons of 284 bp of *inv*A gene and Lane M: 100-bp DNA marker

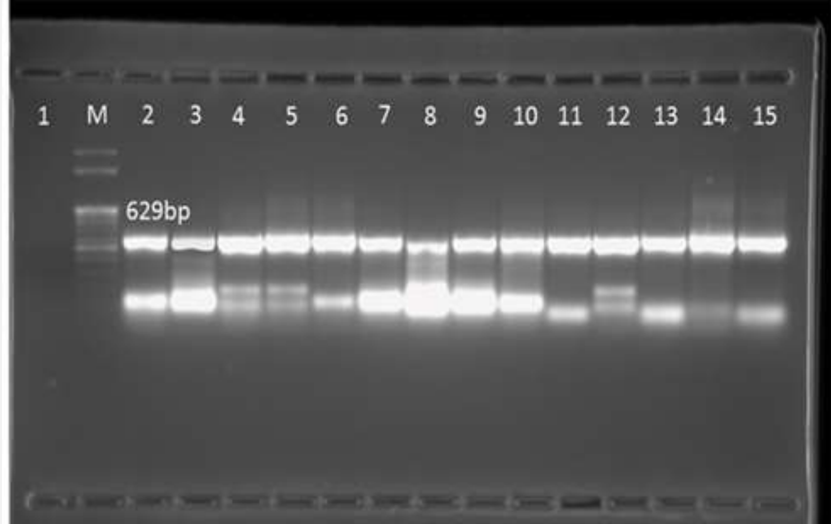

Figure 3 - Agarose gel electrophoresis for PCR products of antibiotic resistance gene (streptomycin) of *Salmonella* showing amplicon of 629 bp of *aad*A1 gene. Lane 1: Negative Control, Lane M: 100-bp DNA marker, and Lane 2: Positive control

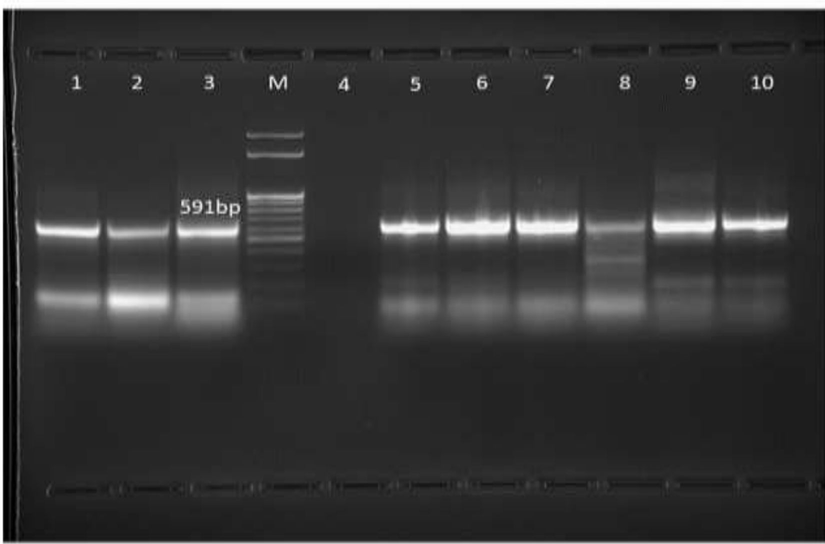

Figure 4 - Agarose gel electrophoresis for PCR products of antibiotic resistance gene (sulfamethoxazole) of *Salmonella* showing amplicon of 591 bp of *sul*1 gene. Lane M: 100-bp DNA marker, Lane 3: Positive control and Lane 4: Negative Control

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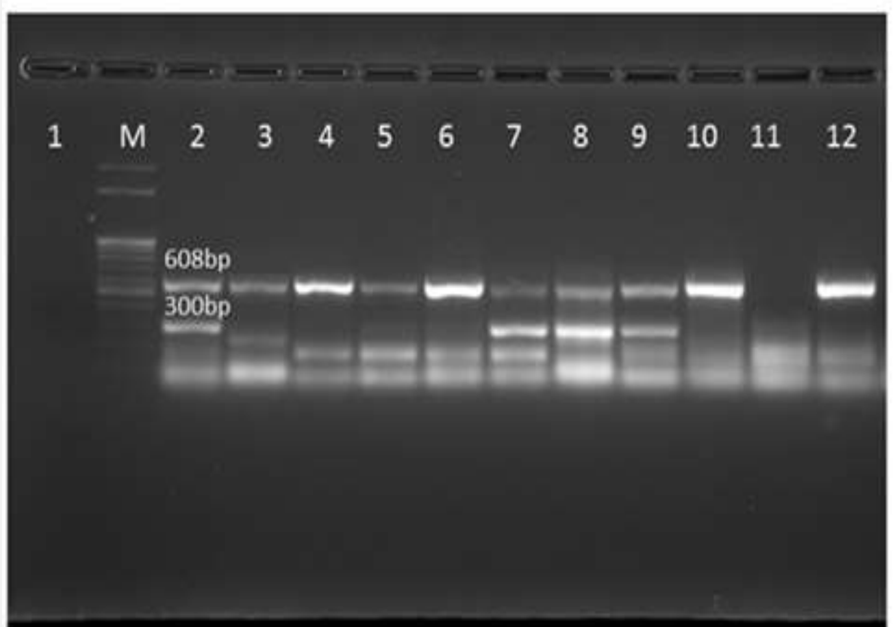

Figure 5 - Multiplex PCR products of antibiotic resistance genes (ampicillin and gentamicin) of *Salmonella* showing amplicon of 608 bp of *bla*TEM gene and 300bp of *aad*B gene, respectively. Lane 1: Negative Control, Lane M: 100-bp DNA marker and Lane 2: Positive control.

DISCUSSION

Paratyphoid salmonellosis is a prevalent foodborne disease which has a global economic and public health concern (Aziz et al., 2018). Therefore, this study characterized paratyphoid *Salmonella* spp. in chicken flocks of some Egyptian governorates. Our results indicated that *S. enteritidis* was the most prevalent serovar (21.8%), followed by *S. kentucky* (15.6%) and *S. typhimurium* (12.5 %). These results are in agreement with the findings of Elsayed et al. (2019) who detected *S. enteritidis* (38.8%), *S. kentucky* (23.3%), and *S. typhimurium* (23.3%). Further, Elshebrawy et al. (2021) detected similar observation. Many reports indicated that *S. enteritidis* is one of the predominant circulating serovars in poultry flocks, as it has been isolated in rates of 58.33% (Rabie et al., 2012), 55.6% (Moussa et al., 2010), and 37.25% (Abd El-Ghany et al., 2012). Additionally, *S. typhimurium* is another most frequently reported and prevalent serovar worldwide (WHO, 2006). The study of Ammar et al. (2016) indicated that the isolation rates of *S. typhimurium* and *S. enteritidis* were 52.94% and 11.76%, respectively (Ammar et al., 2016) and 86.6% and 9%, respectively (El-Sharkawy et al., 2017). Regarding the low isolation rates of *S. tsevie* (6.3%) and *S. papuana* (3.1%) in this study, nearly similar results were obtained by Al-baqir et al. (2019) and Abd El-Tawab et al. (2015) who reported that the isolation rate of *S. tsevie* was (2%) and *S. papuana* was 2.3%, respectively. Other serovars of *Salmonella* such as *S. wingrove* (6.3%) and *S. shangani* (3.1%) were detected here, which may be due to improper biosecurity measures within farms and the possibility of disease transmission via various reservoirs and farm workers (Elshebrawy et al., 2021).

The highest isolation rate of *Salmonella* serovars was from the liver (62%), rather than the other tissues examined. The previous reports of Menghistu et al. (2011), Abdel-Aziz (2016), Al-baqir et al. (2019), and Saleem et al. (2022) mentioned similar finding. It is important to note that the high detection of *Salmonella* in the liver live may indicate the potential of the pathogen to cause a systemic infection.

It has been noticed that the highest isolation of *Salmonella* serovars was from El-Dakhlia, El-Sharqia Damietta, and El-Qalubia followed by El-Fayoum, El-Minya, Giza, and El-Beheira governorate. These results are in accordance with the fact that the highest percentages of chicken farms allocated in El-Dakhlia, El-Sharqia, Damietta and El-Qalubia rather than other Egyptian governorates.

The development of MDR *Salmonella* strains has emerged as a major public health concern around the world (Marshall and Levy, 2011). This resistance poses a direct threat to human health when treatment is hindered without a complete course and when there are interactions with both animals and humans pathogens (Frye and Jackson, 2013).

Concerning the antimicrobial susceptibility test, the results of the current study indicated that all of the isolated *Salmonellae* strains were resistant to streptomycin (100%), which may be due to the misuse of this antibiotic in the poultry field. However, other studies reported that 100% (Habrun et al., 2012) or 89.7% (El-Sharkawy et al., 2017) of isolates were sensitive to streptomycin.

On the other side site, 96.9% of the isolated *Salmonella* strains were susceptible to meropenem. The same result was obtained by Abou Elez et al. (2021). Furthermore, Elshebrawy et al. (2021) detected a low resistance rate (7.6%) of *Salmonella enterica* serovars against meropenem. It has been reported that carbapenems could be used in the treatment

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of salmonellosis in MDR cases (Calayag et al., 2017). The low resistance rate against meropenem could be attributed to the underuse of carbapenems in veterinary medicine particularly for chicken infections.

Here, the resistance of the isolates to ciprofloxacin was 15.6%, which agrees with that of Donado-Godoy et al. (2012) (15%). In comparison, the resistance to ciprofloxacin was 50% (Abdel Rahman et al., 2014), 35% (Al-baqir et al., 2019), 33% (Badr et al., 2021), or 27.8% (Elshebrawy et al., 2021). As well, the resistance to ampicillin was 18.8%, which was much less than Diab et al. (2019) (68.2%), Ghetas et al. (2021) (78.5%), Alam et al. (2020) (82.85%), Nabil and Yonis, (2019) (94.1%), and Raji et al. (2021) (100%). Further, 50% of the isolated *Salmonellae* showed resistance to tetracycline, which nearly agreed, but lower than those stated by Raji et al. (2021) (75%) and Ibrahim et al. (2021) (62%). The maximum resistance to tetracycline (97.14%) was reported by Alam et al. (2020).

Despite conventional cultural methods offer the advantage of being able to detect live cells and evaluate large numbers of samples, they are time-consuming and laboring (Maciorowski et al., 2006; Margot et al., 2013). On the other hand, molecular techniques such as PCR are rapid, specific, and sensitive, and could replace traditional detection methods (Siddique et al., 2009; Ibrahim et al., 2014). Moreover, multiplex PCR platform provides a greater detection efficiency, allows simultaneous detection of several diseases at the same time, and saves both money and time (Lee et al., 2014; Li et al., 2020).

In the current study, the amplification of *16S* rRNA gene of all *Salmonella* isolates gave a unique specific product at 550 bp. Also, the amplified products of the virulence genes (*inv*A, *sop*B, *stn*, and *pef*) were 284, 517, 617, and 700 bp, respectively. The *inv*A protein is an inner membrane component of the *Salmonella* pathogenicity island 1 type 3 secretion system (Shah et al., 2011). The *inv*A gene allows bacteria to invade the host cells (Cha et al., 2013). This gene is only found in *Salmonella* spp. and is therefore a valuable diagnostic tool for genetic identification (O'Regan et al., 2008). Several studies have noted a high frequency of *inv*A virulence gene in *Salmonella* serovars (Karmi, 2013). All examined *Salmonella* serovars (100%) contained *inv*A gene. Many other studies agreed with ours (Shabnam and Kwai, 2010; Campioni et al., 2012; Samanta et al., 2014; Radwan et al., 2016; Amini et al., 2018; Hassan et al., 2018; ElSheikh et al., 2019; Ramatla et al., 2020; Elshebrawy et al., 2021).

The *Salmonella* enterotoxin (*stn*) gene encodes the Stn protein which causes gastroenteritis and regulates the integrity of bacterial cell membranes (Huehn et al., 2010). This gene was found in 65.6% of the strains in this study, which is similar to Elshebrawy et al. (2021) (65.8%). Other researchers have also noted high rates of *stn* gene (Zou et al., 2012; Osman et al., 2014; Ammar et al., 2019; Sabry et al., 2020; Hassan et al., 2021).

Fimbriae are essential for *Salmonella* pathogenicity, as they facilitate the attachment of the pathogen to epithelial cells. The *pef* gene encodes *pef* fimbria (Murugkar et al., 2003; Ammar et al., 2016). The examined isolates in the present study had no *pef* gene, which is similar to the results reported by Elkenany et al. (2019), Elhariri et al. (2020), and Hassan et al. (2021). Also, a low frequency of the *pef* gene was detected by Ahmed et al. (2016) (6.7%) and Ghetas et al. (2021) (21%). This could be explained by the fact that bacteria use other fimbriae to attach to the host cells. However, in other studies, various findings were noted; for example, Awad et al. (2020) identified *pef* gene in 54.8% of the isolates and highlighted the role of fimbriae in the infection.

By triggering secretory pathways, promoting inflammation or changing ion balances within cells, the *sop*B gene contributes to the development of diarrhea (Ahmed et al. 2016). The prevalence of the *sopB* gene is 40.6% which is lower than Awad et al. (2020) (87.1%), Mohamed et al. (2021) (91.3%), and Badr et al. (2021) (100%).

The amplified products of the antibiotic resistance genes (*aad*A1, *aad*B, *sul*1, and *bla*TEM) were 629, 300, 591, and 608 bp, respectively. In this study, we observed that the phenotypical resistance to ampicillin is 18.8%, but 59.4% of the isolates tested positive for the *bla*TEM gene by PCR, a gene encoding for resistance to β-lactams. This assumes that some antimicrobial resistance genes in some bacteria are inactive or “silent” *in-vitro*. However, these silent genes can spread to other bacteria or can become active *in-vivo* under antimicrobial pressure (Ma et al., 2007). In contrast, El-Sharkawy et al. (2017) found that all isolated strains of *S. enteritidis* were ampicillin-susceptible and *bla*TEM negative, suggesting that these strains had a different ampicillin resistance mechanism. In a previous work, according to Aslam et al. (2012), the *bla*TEM gene was present in 17% of *Salmonella* spp. isolated from retail meats in Canada and was the most frequently found resistance gene. According to Lu et al. (2011), 81.2 % of the 108 *S. indiana* isolates tested positive for the *bla*TEM gene. Ammar et al. (2019) noticed the *bla*TEM gene in 100% of the isolates. The genes *bla*PSE-1 and *bla*TEM, which encode β-lactamases conferring resistance to ampicillin, were found in 69.4% of the strains, according to a report by Herrera-Sánchez et al. (2020). In the present study, we found out that all isolates of *Salmonella* were completely resistant to streptomycin and the gene for streptomycin resistance, *aad*A1 was found in all the isolated strains. This rate is higher than those reported by Chuanchuen and Padungtod, (2009), who detected the resistance gene *aad*A1 in (17%), Doosti et al. (2017) (45.6%), Alam et al. (2020) (77.1%), and Herrera-Sánchez et al. (2020) (87.8%).

6.3% of the isolated *Salmonella* strains were resistant to gentamycin, however the *aad*B gene that confers resistance to gentamycin was found in 18.75% of the isolated strains, as the gene was silenced. In contrast, the *aad*B gene was not found in any of the strains in the study conducted by Herrera-Sánchez et al. (2020).

A percentage of 28.1 of the isolated strains were phenotypically resistant to sulphamethoxazole and 28.1% of isolates being positive for *sul*1 which confer sulphamethoxazole resistance. This result is lower than those reported by Adesiji et al. (2014) (100%), Mohamed and Suelam (2010) (97.3%), Aziz et al. (2018) (83.3%), and El-Sharkawy et al. (2017) (57%).

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CONCLUSION

Paratyphoid *Salmonella* spp. found in poultry flocks at high rates pose a zoonotic danger. Additionally, MDR *Salmonella* serovars with a diverse range of virulence genes have been detected in the existed *Salmonella* spp. These results indicate the importance of the constant surveillance of antibiotic resistant *Salmonella* strains, the use of alternatives instead of antimicrobials in poultry, and adoption of strong public health and food safety protocols to reduce the human health risk associated with salmonellosis.

DECLARATIONS

Corresponding author

E-mail: wafaa.soliman@cu.edu.eg

Author's contribution

This work was carried out with the contribution of all authors. Nassar YM conducted the practical part of the research and helped in data collection, analysis, and writing. Ibrahim AA conducted the molecular work and validated it. Hamouda AS, Abd El-Ghany WA, and Ibrahim AA designed the protocol, supervise the work, and approved it. Abd El-Ghany WA helped in data collection and write, formatted, and submit the draft of the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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