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Research article

In-vivo characterization of multidrug-resistant *Salmonella* enterica Serovar Enteritidis (SE) recovered from fertile eggs and baby chicks

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Abstract

The present study was conducted to isolate and characterize *Salmonella* spp. from hatching eggs and baby chicks. Additionally, the pathogenicity of the isolated *Salmonella* strains was assessed in one-day-old specific pathogen-free (SPF) chicks *in-vivo*. Samples from sick baby chicks from 14 broiler chicken farms (including 1 duck farm) and 150 egg batches from three breeder chicken farms were collected from 4 different governorates. Phenotypically identified *Salmonella* isolates were confirmed using species-specific multiplex-PCR targeting the *inv*-A gene for *Salmonella* genus, *E*-1 gene for *Salmonella* Enteritidis (SE) serovar, and *Flic*-C gene for *Salmonella* Typhimurium (ST) serovar. Confirmed SE isolates were further subjected to Random Amplified Polymorphic DNA (RAPD) fingerprinting. Phenotypic, multiplex-PCR, and RAPD fingerprinting confirmed seven isolates (50%) from broiler chicken farms and two isolates from hatching egg batches (1.33%) as SE, of which eight were multidrug-resistant (MDR) strains with 0.214-0.786 MDR indices. *In-vivo* pathogenicity of selected multidrug-resistant (MDR) SE isolates was evaluated in one-day-old SPF chicks. Despite minor phenotypic diversity, most SE strains were highly invasive with variable mortality (50-100%). Interestingly, the lowest MDR indices were associated with high virulence in SE strains (mortality ≥85%) and vice versa. The study results showed the presence of SE in poultry in Egypt. The uncontrolled usage of antibiotics in poultry could be the reason for the increased prevalence of MDR *Salmonella* spp., which may limit *Salmonella* control measures and threaten public health.

Keywords: *Salmonella*, *Salmonella* Enteritidis, MDR, Multiplex-PCR, RAPD, Poultry

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Introduction

Salmonella is a substantial public health threat because it causes one of the most widespread human food-borne infections (Saif et al., 2020; Youssef et al., 2021). The World Health Organization (WHO) has estimated that 1.3 billion acute gastroenteritis and diarrhea cases are due to non-typhoid Salmonellosis (NTS). Millions of people are affected by *Salmonella* Enteritidis (SE) infection resulting in three million deaths annually (Fàbrega and Vila, 2013). Human infection with this disease appears thought to be spread by poultry and poultry-derived products, particularly meat and chicken eggs (Okamura et al., 2003). Person-to-person transmission can occur via the fecal-oral route (Christidis et al., 2020).

Salmonella, in addition to rising antibiotic resistance, is a major public health concern worldwide due to long-term antibiotic use in livestock farming. Antibiotic residues in animal-producing foods can cause direct toxicity in humans, and low amounts of antibiotic exposure could alter microbiota and lead to antibiotic resistance (Xia et al., 2020; Procura et al., 2019). Humans infected with antibiotic-resistant strains are more likely than those infected with susceptible strains to experience adverse health outcomes such as prolonged sickness, increased severity of illness, long hospi-

talization, or death (Procura et al., 2019). *Salmonella* is a Gram-negative, non-spore-forming, facultative anaerobic bacilliform bacteria of the *Enterobacteriaceae* family with a diameter of 0.7–1.5, 2-5 μm. Only one subspecies (*S. enterica* subspecies *enterica*) is associated with disease in warm-blooded animals.

The genus consists of two genetically separate species, *S. bongori*, and *S. enterica*, which encompass six subspecies identified by patterns of biochemical reactions (Schatten and Eisenstark, 2015; Saif et al., 2020). *Salmonella enterica* is categorized into about 2600 motile and non-host adapted serovars based on the somatic "O" and flagellar "H" antigens using the Kauffmann–White scheme, such as SE and *S. enterica* subspecies *enterica* serovar Typhimurium (ST) (Issenhuth-Jeanjean et al., 2014; Saif et al., 2020). The principal reservoir for SE is chicken, which can carry the disease asymptomatically and spread it along the food supply chain. *Salmonella* infections in poultry flocks can cause acute and chronic clinical illnesses, particularly SE and ST, which impair egg production in chickens and result in large losses in terms of money and resources spent on testing and control (Lee et al., 2013; Renu et al., 2020; Saif et al., 2020).

In poultry, most *Salmonella* infections arise from ingesting contaminated food. However, the severity of the disease depends on many factors, including the strain of *Salmonella*, the hygiene standard, the route of infection, age, and the immune status of birds (Chao et al., 2007). Depression, anorexia, diarrhea, pericarditis, necrotic foci in the liver, and an indurated yolk sac remnant in chicks are all indications of clinical SE infections. Deformed, discolored, and/or congested ovaries and ovules, soft-shelled eggs, and egg peritonitis are common in infected hens (Suzuki, 1994). Egg contamination can occur by *Salmonella* colonization of the ovary and oviduct before the shell is produced or eggshell contamination during egg laying (Crouch et al., 2020). Because sick chickens produce contaminated eggs infrequently and without displaying clinical signs, detecting and eliminating SE from commercial poultry flocks has proven difficult (Humphrey et al., 1991).

In chicken, numerous molecular approaches have been used to identify *Salmonella* spp. To detect *Salmonella* at the genus level and identify the SE and ST a multiplex polymerase chain reaction (m-PCR) technique targeting the *inv*-A, *E*-1, and *Flic*-C genes was developed (Paião et al., 2013). However, highly discriminative DNA-based typing methods such as plasmid profile (Foley et al., 2007), ribotyping, IS200 profile, pulsed-field gel electrophoresis (PFGE), and multilocus enzyme analysis have also been applied (Saif et al., 2020). The random amplified polymorphic DNA (RAPD) fingerprinting analysis is a simple, fast, and powerful method for subtyping SE isolates (Lin et al., 1996). The present study aimed to identify *Salmonella* spp. associated with poultry in hatching eggs and baby chicks using phenotypic, genotypic criteria, and their *in-vivo* pathogenicity in one-day-old specific pathogen-free (SPF) chicks.

Materials and methods

Samples collection

Samples were collected from newly hatched chicks (1-7 days old) in chicken and duck farms from four governorates located in North Upper Egypt and Delta region (Beni-Suef, Beheira, Minoufiya, and Sharkia). Chicks suffered from depression, anorexia, diarrhea, and mortality were sampled. A total of 840 liver, intestine, spleen, and heart blood samples were collected from chicks, with an average of 15 chicks per farm. Three breeder chicken farms were monitored between November 2019 and December 2021, yielding 150 egg batches, and 40 eggs in each batch were sampled at zero and 7 days after incubation.

Bacteriological isolation

Salmonella spp. isolation was carried out according to The United States Food and Drug Administration (FDA) guidelines (Andrews et al., 2022). For pre-enrichment, samples were pre-enriched for 16-20 hours at 35-37°C. in buffered peptone water (Merck, Darmstadt, Germany). For selective enrichment, 0.1 mL of the pre-enriched inoculum was transferred to 10 mL Rappaport-Vassiliadis broth (Difco, Le pont de claix, France) and cultured for 24 hours at 42°C. 10 μL loopful of each enrichment broth was withdrawn and streaked onto Xylose Lysine Deoxycholate (XLD) agar after thorough mixing (Merck, Darmstadt, Germany). After incubating samples at 37°C for 24 hours, the colonies of suspected *Salmonella* were identified. On XLD agar, colonies with a slightly transparent red halo black center surrounded by a pink-red zone were purified, screened, and stored at -80°C after biochemical, serotyping, and antimicrobial susceptibility testing (Tarabees et al., 2017; Marin et al., 2020). Egg samples were collected, and the outer shell surface was washed with 70% alcohol, air-dried, cracked with sterile forceps, and streaked onto Trypticase soy agar (TSA) for pathogen culture and isolation. The suspected colonies were streaked on MacConkey and XLD agar media (Merck, Darmstadt, Germany) for identification as previously described (Xia et al., 2020).

Phenotypic identification of isolated *Salmonella* spp.

Standard microscopy and biochemical testing protocols were used to identify the *Salmonella* isolates. Gram stain was used to stain dried heat-fixed smears of suspicious colonies (Difco, Le pont de claix, France). Catalase and oxidase (BD, Franklin Lakes, USA) assays were used to identify the probable isolates biochemically. The detected isolates were grown on TSI, UREA medium, motility indole ornithine (MIO), citrate (Difco, Le pont de claix, France), and lysine decarboxylase (LIA) (Oxoid, Basingstoke, Hampshire, UK) media (Afshari et al., 2018). The API 20E identification system was used to complete the final identification according to the manufacturer's specifications. (Biomerieux, Marcy-l'Etoile, France) (Marin et al., 2020).

Antibiotic susceptibility test

Antibiotics susceptibility test was performed using the disc diffusion method onto Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, UK) (CLSI, 2022). Standardized inoculums visually equivalent to the 0.5 McFarland standards (1.5×10^8 CFU/mL) were tested against the selected antibiotics discs (Oxoid, Basingstoke, Hampshire, UK) for 14 antibiotics commonly used in treating or preventing *Salmonella* infection (Tarabees et al., 2017). The results were interpreted as sensitive, intermediate, or resistant following the breakpoints of The Clinical and Laboratory Standards Institute (CLSI, 2022). Multiple drug resistance (MDR) pattern was considered when the isolate showed resistance to at least one antimicrobial compound in three or more antimicrobial categories. The MDR indices for bacterial isolates were calculated using the formula: MDR index = number of antibiotics resisted/total Number of antibiotics tested ×100. Isolates with MDR index values of more than 0.2 were considered MDR *Salmonella* isolates (Krumperman, 1983; Subramani and Vignesh, 2012).

Molecular identification

Intra-serotyping multiplex PCR genotyping

A multiplex polymerase chain reaction (m-PCR) assay was used to detect *Salmonella* at the genus level and to identify the SE and ST targeting the *inv*-A, *E*-1, and *Flic*-C genes (Table S1) (Paião et al., 2013). Phenotypically confirmed bacterial colonies were cultured overnight on Trypticase soy agar (TSA) (Jamshidi et al., 2010), and the bacterial DNA was extracted using Patho-Gene spin TM DNA/RNA Extraction kit (Intron Biotechnology Inc, Korea). PCR reactions were carried out in a total volume of 25 μL containing 12.5 μL of 2X Easy Taq® PCR SuperMix (TransGen Biotech Co., China), 2 μL *inv*-A primers, 2 μL *E*-1 and 2 μL *Flic*-C primers (Applied Biosystem, Egypt), 5 μL of bacterial DNA and 1.5 μL of nuclease-free water. The m-PCR protocol consisted of an initial denaturation step for 5 min at 94ºC followed by 35 cycles of 30 s at 94ºC, 1 min at 58ºC, and 1.5 min at 72ºC and by a final extension step for 10 min at 72ºC. The PCR products were analyzed onto 1.5% agarose gel electrophoresis. The amplicon sizes 796, 316, and 432 bp were considered positive for *inv*-A, *E*-1, and *Flic*-C genes, respectively.

Random Amplified Polymorphic DNA (RAPD) fingerprinting

Confirmed SE colonies by m-PCR assay were further subjected to RAPD-PCR as described by (Lin et al., 1996) with minor modifications. Briefly, a total reaction volume of 25 μL containing 12.5 μL of Easy 2X Taq® PCR SuperMix (TransGen Biotech Co., China), 1 μL from each RAPD oligonucleotide primer, 5 μL of bacterial DNA template, and 2.5 μL of nuclease-free water. The RAPD thermal profile consisted of 4 cycles of 94°C for 4 min, 35°C for 4 min, and 72°C for 4 min; 35 cycles of 94°C for 30 s, 35°C for 1 min, and 72°C for 2 min; and an elongation cycle at 72°C for 10 min. The PCR products were electrophoresed onto 2% agarose gel, photographed with a UV transilluminator (Biometra, Germany), and analyzed.

Evaluation of isolated SE strains in one-day-old SPF chicks

All experimental procedures were reviewed and approved by the Animal Care and Use Committee of the MEVAC Company for vaccines, Egypt (Approval no. CUC-191101E002).

Bacterial strains

Salmonella identified strains including; ST (ME/Sal.T.1/2017 strain "O1,4,[5],12; i;1,2"), *S.* Kentucky (ME/Sal.K.1/2017 strain "O8,20; i,2."), and *S.* Gallinerum (N/ME/SAL1/2018 strain "O1,9,12; Hg,m;–"), *S.* Enteritidis (ME/Sal.E.1/2017 strain "O1,9,12; g,m;–") were retrieved from the repository of the Bacteriology Laboratory at the Middle East for Vaccines company (MEVAC, Egypt) to be included in the study as positive controls.

Experimental design

Eighty one-day-old SPF chicks were divided into 8 groups (10 chicks in each group). Birds were kept in BSL-3 isolators and provided water and commercial antibiotic-free feed *ad libitum*. Six groups of chicks received 0.5 ml, equivalent to the 0.5 McFarland standards (1.5×10^8 CFU/ml) of each *Salmonella* isolate by crop gavage (Maskell, 2006). Three groups of chicks served as positive controls with ST, *S.* Gallinerum, and *S.* Kentucky, respectively. The last group of chicks was kept as a negative control group and inoculated with phosphate buffer saline (PBS).

Clinical observation and sample collection

Chicks were observed twice daily for five days post-infection for signs of illness and mortality. Signs, including diarrhea, pasty vent, ruffled feathers, depression, and dehydration, were recorded. Dead chicks were necropsied, the pathological changes were recorded, and liver and heart samples were collected for *Salmonella* re-isolation. Survived chickens were euthanized at the end of the observation period, and samples were cultured for bacteriological evaluations (Alisantosa et al., 2000).

Results

Isolation rate of *Salmonella* spp.

From 1-7 days-old chicks of the investigated 13 chicken and 1 duck farms, seven farms were free from *Salmonella* infection, and isolation failed to identify any *Salmonella*. While the remaining six chicken farms and one duck farm (50%) revealed *Salmonella* infection with variable organ detection. Meanwhile, only 2 egg batches out of 150 were infected with *Salmonella* spp., (1.33%), presumptively identified based on the morphology of *Salmonella* colonies on non-selective media (TSA agar) and selective XLD agar were summarized in Table 1.

Table 1: The results of isolation of SE from farms and egg batches.

Source	ID	Samples	*Salmonella* positivity		Total isolates (%)
			Number/total	%	
Chicken farms	Farm-1	Liver	0/15	0.00	7/14 farms (50)
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-2	Liver	9/15	60	
		Intestine	11/15	73.33	
		Spleen	9/15	60	
		Heart	5/15	33.33	
	Farm-3	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	1/15	6.67	
	Farm-4	Liver	2/15	13.33	
		Intestine	11/15	73.33	
		Spleen	8/15	53.33	
		Heart	5/15	33.33	
	Farm-5	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-6	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-7	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-8	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-9	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
	Farm-11	Liver	10/15	66.67	
		Intestine	12/15	80	
		Spleen	10/15	66.67	
		Heart	5/15	33.33	
	Farm-12	Liver	4/15	26.67	
		Intestine	8/15	53.33	
		Spleen	6/15	40	
		Heart	3/15	20	
	Farm-13	Liver	7/15	46.67	
		Intestine	8/15	53.34	
		Spleen	7/15	46.67	
		Heart	5/15	33.33	
	Farm-14	Liver	0/15	0.00	
		Intestine	0/15		
		Spleen	0/15		
		Heart	0/15		
Duck farm	Farm-10	Liver	4/15	26.67	
		Intestine	5/15	33.33	
		Spleen	4/15	26.67	
		Heart	3/15	20	
Egg batches	Flock-1		0/50	0	2/150 (1.33)
	Flock-2		1/80	1.25	
	Flock-3		1/20	5	

Phenotypic identification of recovered *Salmonella* isolates

Phenotypic characterization

The biochemical profile was suggestive of the *Salmonella* genus. The present data showed that all isolates were positive for catalase, motility, TSI, ornithine, lysine, and citrate tests. However, they were oxidase, indole, and urease negative. The API-20E test confirmed the obtained biochemical profile suggestive of *S.* enterica (Table S2).

Antibiotic susceptibility profile

Eight out of nine isolated *Salmonella* were MDR strains (0.214-0.786 MDR indices), and only one isolate (SE-F2) showed a 0.143 MDR index. All isolates were resistant to clindamycin and ampicillin except SE-F13, which showed intermediate ampicillin sensitivity. The majority of isolated *Salmonellae* showed intermediate sensitivity to cefotaxime, ciprofloxacin, ceftazidime, and levofloxacin and were also susceptible to amikacin, colistin sulfate, and Trimethoprim-sulfamethoxazole. Four isolates (SE-F11, SE-F12, SE-F13, and SE-E29) showed similar resistance patterns to streptomycin, amoxicillin-clavulanic acid, tetracycline, neomycin, and gentamycin (Table 2).

Intra-serotype and RAPD fingerprinting of isolated *Salmonella* spp.

All biochemically confirmed *Salmonella* isolates (no.= 9) were confirmed as SE using genus-specific *inv*-A gene and *E*-1. The isolated strains were further confirmed using RAPD as the tested isolates revealed the same RAPD profile (profile AAAAA) assigned by using a five-letter code referring to the pattern obtained with each primer (P1254, OPB-17, 23L, OPA-4, and OPB-15, respectively) (Betancor et al., 2004). Only two samples were negative for RAPD (Figure 1).

Pathogenicity of isolated SE in day-old SPF chicks

In this study, the mortality of SE isolates orally inoculated chicks ranged between 50-100%. Birds inoculated with SE-F4 and SE-E45 had the highest percentage of illness and mortality (100 and 87.5%, respectively), and the lowest mortality was recorded in SE-F3 and SE-F10 (62.5%) (Table 3). The most consistent gross lesions included fibrinous exudate in the pericardium and, in a few, on the liver capsule. Enlarged livers, sometimes with congestion and white foci, were also noticed. In addition, several birds retained yolk sacs containing coagulated material, pericarditis, perihepatitis, enteritis, pneumonia, and congested liver (Figure 2).

Discussion

Salmonella enterica is an important zoonotic enteric pathogen and can cause different degrees of infection (Xia et al., 2020). Therefore, developing suitable intervention strategies to control *Salmonella* populations at the farm level requires reliable data on the occurrence and prevalence of the pathogen. Hence, estimating the contamination rate of farms and eggs with *Salmonella* is the first step in infection control strategies. In the present study, *Salmonella* spp. was isolated and characterized from baby chick farms and hatching eggs in four localities in Egypt.

Salmonella spp. detection was positive in 6 out of 13 chicken farms, one duck farm in age, and two farms producing eggs out of three investigated farms. *Salmonella* spp. were isolated from different chicks organs such as spleen, intestine, liver, and heart. One representative *Salmonella* isolate was used from each farm that was positive for *Salmonella* infection with a total of 9 samples that were phenotyping and genotyping were identified; they are all defined as *Salmonella enterica* serovar Enteritidis (SE). In addition, isolated *Salmonella* spp. pathogenicity was compared in specific pathogen-free (SPF) chicks. Overall, a high prevalence in farms (50%) was observed compared to only a 1.33% prevalence in hatching eggs. A similar isolation rate of *Salmonella* (38.7%) was reported from chicken meat, chicken carcasses skin, and chicken feces; however, no *Salmonella* was isolated from raw egg yolk or eggshell samples (Abdel-Maksoud et al., 2015).

Also, the *Salmonella* spp. burden in healthy chickens was investigated in 3 poultry farms in Ethiopia. The study reported an 0.8% prevalence of *S.* Gallinerum/Pullorum in healthy birds compared to a 16.1% isolation rate from sick/dead chickens (Aragaw et al., 2011). *Salmonella* was prevalent in 14% of investigated poultry farms, and the majority of isolated *Salmonella* spp. were *S.* Enteritidis (43%) and *S.* Typhimurium (36%) (Afshari et al., 2018). A recent study reported a higher rate of *Salmonella* spp. (82%) in cloacal swabs from 50 broiler farms in the Mymensingh and Gazipur districts, Bangladesh (Talukder et al., 2021). Recent studies, however, reported as low as 3.3% *Salmonella* recovery from 120 liver samples of 2-3 weeks old diseased broiler chickens in Egypt (Radwan et al., 2022). It may have been the young age at which the study was conducted (i.e., baby chicks) that contributed to the high prevalence of disease in this study. With increasing age, there can be a change in *Salmonella* prevalence due to different hygiene practices and/or medications applied on the farms.

Additionally, a low prevalence of *Salmonella* spp. was reported at the slaughtering age. For instance, *Salmonella* was recovered in 4.8% of livers obtained from markets in Argentina (Procura et al., 2019) and as low as 3.67%, 0.57%, and 1.95% for ST, SE, and untyped *Salmonella* spp., respectively in Bangladesh (Siddiky et al., 2021). The very low prevalence in hatching eggs is probably due to the absence of *Salmonella* infection and/or due to hygienic measures applied in breeder farms efficiently preventing egg contamination. In addition, a recent experimental study demonstrated that despite *Salmonella* environmental contamination on the farm, *Salmonella* contamination of eggs is low and is further minimized by washing (McWhorter and Chousalkar, 2020).

Most isolated *Salmonella* strains were MDR to at least three antimicrobial agents belonging to different antimicrobial classes with MDR indices between 0.214-0.786 (Khan et al., 2019). An alarming number of

Table 2: The results of isolation of SE from farms and egg batches.

Antibiotic	***S.* Enteritidis isolates**[*]								
	SE-F2	SE-F3	SE-F4	SE-F10	SE-F11	SE-F12	SE-F13	SE-E29	SE-E45
Levofloxacin (5 μg)	I	I	I	R	I	I	S	I	I
Ceftazidime (30 μg)	I	I	R	R	I	S	S	I	R
Cefotaxime (30 μg)	I	I	R	R	I	I	R	I	I
Ciprofloxacin (5 μg)	I	I	I	S	I	I	S	I	I
Amoxicillin clavulanic acid (30 μg)	S	S	I	I	R	R	R	R	I
Streptomycin (10 μg)	S	S	I	R	R	R	R	S	S
Tetracycline (30 μg)	S	S	S	S	R	R	R	R	S
Neomycin (30 μg)	S	R	S	I	S	R	R	I	I
Gentamycin (10 μg)	S	R	S	S	R	R	R	S	S
Amikacin (30 μg)	S	S	I	R	S	S	S	S	S
Colistin Sulphate (10 μg)	S	R	S	S	S	S	S	R	S
Trimethoprim sulfamethoxazole (25μg)	S	S	S	S	S	S	R	S	S
Ampicillin (10 μg)	R	R	R	R	R	R	I	R	R
Clindamycin (2 μg)	R	R	R	R	R	R	R	R	R
Multi-Drug Resistance Index	0.143	0.357	0.286	0.50	0.429	0.50	0.571	0.357	0.214

[*]Abbreviations: R: resistant; S: susceptible; I: intermediate, F: farm number; SE-F: *Salmonella* Enteritidis farm.

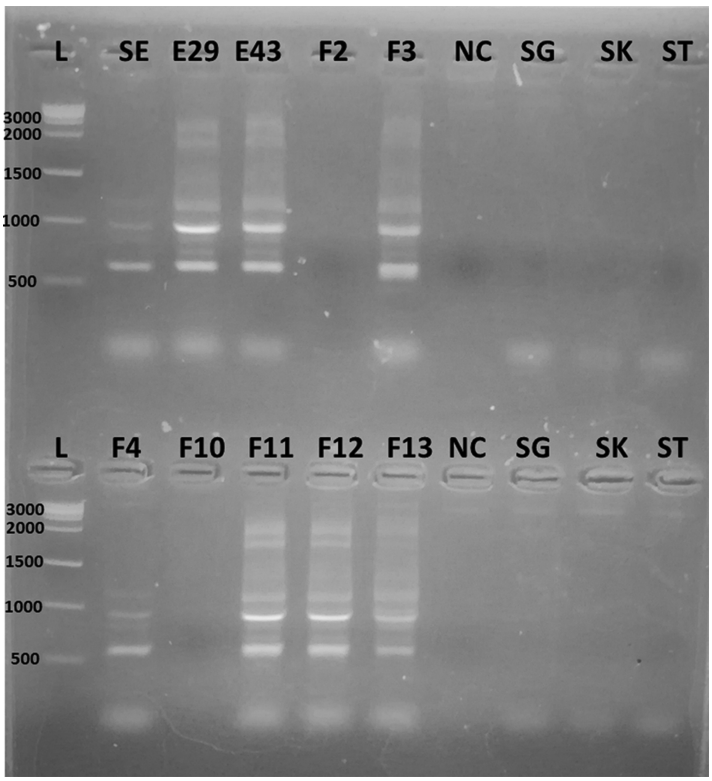

Figure 1: *Salmonella* Enteritidis RAPD typing. L; 3Kb ladder, SE; *Salmonella* Enteritidis, NC; negative control, SG; *Salmonella* Gallinerum, SK; *Salmonella* Kentucky, ST; *Salmonella* Typhimurium. *Salmonella* clinical isolates codes are indicated.

Table 3: Clinical signs and mortality rates in day-old specific pathogen-free chicks experimentally inoculated with *S.* Enteritidis.

Group (no.8)[1]	Day post inoculation										Total mortality
	1		2		3		4		5		
	Signs[2]	Death	Signs	Death	Signs	Death	Signs	Death	Signs	Death	no. (%)
SE-F2	1	2	1	4	1	0	1	0	2	0	6 (75)
SE-F3	3	0	1	2	2	0	1	1	1	1	4 (50)
SE-F4	2	3	1	4	0	1	0	0	0	0	8 (100)
SE-F10	0	1	2	1	2	1	4	0	4	2	5 (62.5)
SE-E29	1	4	1	2	1	0	1	1	1	0	7 (87.5)
SE-E45	3	2	0	3	1	2	0	0	0	0	7 (87.5)
S. Typhimurium	1	2	2	2	0	2	0	0	1	0	6 (75)
S. Gallinerum	0	0	1	1	1	3	1	0	0	1	5 (62.5)
S. Kentucky	1	3	2	2	1	1	1	1	1	0	7 (87.5)
Negative Control	0	0	0	0	0	0	0	0	0	0	0

[1]Pathogenicity of day-old SPF chicks inoculated in BSL-3 isolators with different *S.* Enteritidis isolates (1.5×10^8 CFU/ml) and chicks were observed for 5 days.

[2]Signs included diarrhea, depression, and dullness.

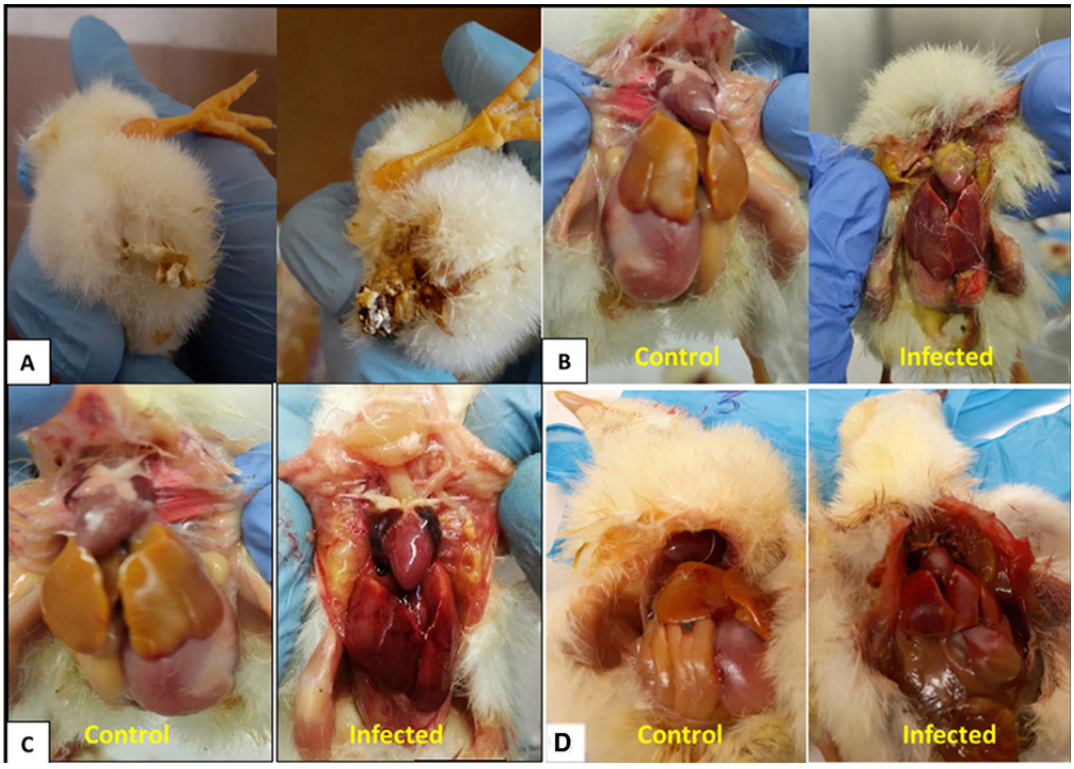

Figure 2: Gross pathology in one-day-old specific pathogen-free chicks inoculated with different *S.* Enteritidis isolates (1.5×10^8 CFU/ml). A: diarrhea and pasty vent. B: pericarditis, perihepatitis, and congested liver. C: congested liver and pneumonia. D: congested liver, retained yolk sac, and enteritis.

MDR *Salmonella* isolates in Egypt have been identified in poultry farms and chicken eggs (Abdel-Maksoud et al., 2015; Youssef et al., 2021; Radwan et al., 2022). Besides, other MDR bacterial pathogens such as avian pathogenic *E. coli* (Abd El-Mawgoud et al., 2020), MDR *Salmonella* isolates constitute a possible risk to humans from consuming poultry or its products (Sallam et al., 2014). In this study, *Salmonella* strains exhibited what appeared to be remarkably high rates of antibiotic resistance. This is likely due to the early introduction and subsequent widespread and uncontrolled use of antibiotics in veterinary and human medicine. Due to the high level of drug resistance in Egypt (Abdel-Maksoud et al., 2015), the prevention and treatment of poultry and poultry-transmitted diseases might be adversely impacted.

In many cases, molecular methods are reliable and sensitive enough to detect and identify *Salmonella* spp in large samples (Malorny et al., 2007). The species-specific m-PCR results were consistent with conventional phenotyping results and differentiated SE isolates from other *Salmonellae* in the current study. These findings further confirm the sensitivity and robust nature of the m-PCR for serotyping of SE and ST (Paião et al., 2013; Sohail et al., 2021). Compared to the classical phenotyping and m-PCR methods, the results of RAPD analysis were confirmatory in only 7 out of 9 isolates. A single major profile (profile AAAAA) was found in all positive isolates (Lin et al., 1996; Betancor et al., 2004) with no differences between the farm and hatching egg-originated isolates. This confirms that lateral dissemination is the most probable spreading method to baby chicks. The lack of other identification techniques in our laboratory (such as phage typing or PFGE) has limited our ability to further investigate the two RAPD-negative isolates.

Day-old SPF chicks were inoculated with selected SE strains to investigate the virulence of isolated SE strains concerning their phenotypic criteria. The variation in mortality caused by SE is evident in chicks less than two weeks of age (Lister, 1988) and one day-old chicks (14.5-89.5%) (Suzuki, 1994). However, in the current study, despite minor phenotypic diversity among clinical strains of SE, most strains are highly invasive with variable mortality ranges (50-100%). Furthermore, there is no consistent link between most of the SE phenotypic criteria and the *in-vivo* virulence; however, the lowest MDR indices were notably associated with high virulence in SE strains (mortality $\geq$85% in SE-F4 and SE-E45 strains) and vice versa (mortality $\leqq$60% in SE-F3 and SE-F10) (Table 3).

Previous research investigating the link between antibiotic resistance and virulence found that when *Salmonella* obtains antibiotic resistance, its virulence is reduced (Paulander et al., 2007; O'Regan et al., 2010; Higgins et al., 2020), although other researchers have found no relationship between virulence and antibiotic resistance (Nilsson et al., 2006; Andersson and Hughes, 2010) or suggested an increase of virulence upon acquiring resistance (Tamayo et al., 2002; Eswarappa et al., 2008).

Conclusion

The result showed the existence of *Salmonella*, especially SE, in poultry farms in the investigated regions of Egypt. Significant MDR rates in SE strains may limit treatment options, cause treatment failure, increase poultry mortality, and pose a public health threat. Thus, the poultry industry must implement control measures to reduce the spread of SE in the production process (Crouch et al., 2020). This study observed that the lower the MDR index, the higher the *in-vivo* pathogenicity in SPF chicks. However, further research is needed to determine whether virulence and drug resistance are related in SE isolates. In addition to critical control point programs, efforts are required to reduce the risk of food-borne Salmonellosis through consumers' awareness.

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Supplementary Data

Basuony, M. E., Hussien, H. A. M., Bakhiet, E. K., Kilany, W. H., Abu-Elala , N. M. and Ali, A. *In-vivo* characterization of multidrug-resistant *Salmonella* enterica Serovar Enteritidis (SE) recovered from fertile eggs and baby chicks. 2(3): 24-32. https://doi.org/10.51585/gjm.2022.3.0019

Table S1: Primers to identify *Salmonella* spp in the multiplex-PCR and RAPD -PCR.

Test	Target gene	Primer sequence 5'-3'	Annealing temp.	Product (bp)	Reference
Multiplex PCR	*inv*-A gene	*inv*-A F: CGG TGG TTT TAA GCG TAC TCTT *inv*-A R: CGA ATA TGC TCC ACA AGG TTA	58°C	796	Fratamico (2003)
	S. Enteritidis	*E*-1 F: AGT GCC ATA CTT TTA ATG AC *E*-1 R: ACT ATG TCG ATA CGG TGG G	58°C	316	Wang and Yeh (2002)
	S. Typhimurium	*Flic*-C F: CCCGCT-TACAGGTGGACTAC *Flic*-C R: AGCGGGTTTTCGGTG-GTTGT	58°C	432	Paião et al. (2013)
RAPD Serotyping	23L	5'-CCGAAGCTGC	35°C	NA	Lin et al. (1996)
	OPB-17	5'-AGGGAACGAG			
	OPA-4	5'-AATCGGGCTG			
	P1254	5'-CCGCAGCCAA			
	OPB-15	5'-GGAGGGTGTT			

Table S2: Biochemical molecular identification of *S.* Enteritidis isolates.

Test	**Isolate ID**								
	SE-F2[1]	SE-F3	SE-F4	SE-F10	SE-F11	SE-F12	SE-F13	SE-E29[2]	SE-E45
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-
Ornithine	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+
TSI[3]	K/A[4]	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
API-20[5]	99.9	99.9	99.9	99.8	89.6	99.9	99.9	99.9	99.9

[1]SE-F; *Salmonella* Enteritidis-farm

[2]SE-E; *Salmonella* Enteritidis-Egg.

[3]TSI; triple sugar iron agar.

[4]K/A; Alkaline /Acid.

[5]API; Analytical Profile Index.