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Characterization and antimicrobial resistance analysis of *Salmonella enterica* serovar Enteritidis recovered from broiler flocks and fertile eggs in Egypt

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Abstract

Salmonella species cause a significant worldwide burden of disease, morbidity, and mortality. Infections with Salmonella species in poultry and humans cause multiple clinical symptoms. Consequently, reliable information on the occurrence and prevalence of the disease is necessary to establish appropriate intervention methods to control *Salmonella* populations at the farm level. The current study aims to distinguish and identify different species of Salmonella from hatching egg chicks and explore the antimicrobial resistance pattern of the isolates. The one-day-old specific pathogen-free (SPF) chicks were used to *in vivo* assess the pathogenicity of the isolated strains of *Salmonella*. The sick chicks were obtained from localities, chicken farms, and egg batches. The species-specific multiplex PCR used to amplify the *inv-A*, *IE*-1, *flic-C* target genes for *Salmonella* genus, *S. enterica* serovar Enteritidis, and *Salmonella enterica* serovar Typhimurium, respectively, and the phenotypic characteristics of the isolated bacteria were confirmed. Seven multi-drug resistant (MDR) isolates from poultry farms, along with isolates from the hatching egg batches, were verified as *Salmonella enterica.* Despite *Salmonella enterica* having little variation in its phenotypic composition, eight of the nine detected strains of *Salmonella* had MDR strains, most of which were highly prevalent and had variable mortality rates. Interestingly, in *S. enterica* strains, the lowest MDR indices were associated with high virulence mortality (> 85%) and vice versa. The results showed a predominant *Salmonella* Enteritidis in the different farms chosen in Egypt. Therefore, uncontrolled use of antibiotics in chicken farms may be the main reason for the increased incidence of MDR *Salmonella* spp., which could hinder attempts to control *Salmonella* and jeopardize public health. Long-term antibiotic use in livestock farming leads to antibiotic residues in animal-producing foods, which can cause toxicity and low amounts of antibiotic exposure could alter the microbiota and lead to antibiotic resistance. This study found that *in vivo* pathogenicity in SPF chicks increased with decreasing MDR index.

Keywords: *Salmonella* Enteritidis, isolation, sensitivity, virulence, poultry, pathogenicity.

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1. Introduction

Salmonella is an important health concern and the source of one of the most prevalent foodborne infections in humans (He *et al*., 2023). *Salmonella* enterica is a significant zoonotic gastrointestinal infection that can cause different levels of disease (Mkangara, 2023). Foodborne illness risks are increased for consumers of contaminated food products containing *Salmonella*. Humans, frequently infected with *Salmonella*, can develop colon cancer. The *Salmonella* AvrA protein stimulates the Wnt and STAT3 signaling pathways that induce the development of colonic tumor cells (Lu *et al*., 2016). The World Health Organization (WHO) estimates that 1.3 billion cases of acute gastroenteritis and diarrhea are caused by non-typhoidal Salmonellosis. *Salmonella* Enteritidis infections affect millions of people worldwide, and each year they cause three million deaths (Fabrega and Vila, 2013; Yosef, 2024). It is believed that poultry and products derived from poultry, particularly chicken eggs and meat, are the source of human infection with this disease (Kipper *et al*., 2022). Fecal-oral transmission is a probable mode of person-to-person transfer (Khan *et al*., 2023). Long-term antibiotic usage in livestock farming contributes to *Salmonella* infections, which are a global public health concern. Along with the rise in antibiotic resistance, humans may be directly poisoned by antibiotic residues in food produced from animals. Even small amounts of antibiotic exposure may change the microbiota and result in antibiotic resistance (Procura *et al*., 2019; Xia *et al*., 2009). Antibiotic-resistant strains increase the risk of negative health outcomes in people, including prolonged sickness, increased severity of illness, extended hospitalization, or death, when compared to susceptible strains (Procura *et al*., 2019). *Salmonella* is a facultative anaerobic bacilliform bacterium from the Enterobacteriaceae family. It is gram-negative and does not produce spores; only one subspecies (*Salmonella enterica* subspecies *enterica*) is associated to disease in warm-blooded animals; the genus has six subspecies characterized by patterns of biochemical reactions; and it is made up of two genetically distinct species. *S. Bongori* and *S. enterica* (Saif *et al*., 2020). Using the Kauffmann-White approach, *Salmonella* Enteritidis is categorized into around 2600 motile and nonhost adapted serovars, such as *S. enterica* subspecies *enterica* serovar Typhimurium (ST), based on the somatic "O" and flagellar "H" antigens (Khan, 2023). Chicken is the primary source of *Salmonella* Enteritidis (SE), as it can spread the infection along the food chain while remaining asymptomatic. *Salmonella* infections in chicken flocks can cause acute and chronic clinical diseases, particularly *Salmonella* Enteritidis and ST, which impair egg production and need a significant efforts and resources for testing and management (Saif *et al*., 2020). The majority of *Salmonella* infections in chickens are caused by consuming infected food. However, a variety of variables, including as the kind of *Salmonella*, the amount of sanitation, the infection pathway, the age of the birds, and their immune status, impact how serious the illness is (Chao *et al*., 2007; Galán-Relaño *et al*., 2023).

Clinical *Salmonella* Enteritidis infections can cause depression, anorexia, diarrhea, pericarditis, necrotic foci in the liver, and indurated yolk sac remnants in chicks. Infected hens commonly have soft-shelled eggs, egg peritonitis, and malformed, stained, and/or blocked ovaries and ovules (Muna *et al*., 2016). *Salmonella* can infect eggs by colonizing the ovary and oviduct prior to the production of the shell. It can also contaminate the eggshell during the laying process (Gast *et al*., 2024). It has been challenging to identify and eradicate SE from commercial poultry flocks because infected hens seldom and without clinical symptoms produce tainted eggs (Merino *et al*., 2019). Many molecular techniques have been employed to identify Salmonella spp. in chickens. A multiplex polymerase chain reaction (M-PCR) approach targeting the *inv*-A, *IE*-1, and *flic*-C genes was developed to detect Salmonella at the genus level and identify the SE and ST (Paiao *et al*., 2013; Pui *et al*., 2011). The current work used morphological, genotypic, and *in-vivo* pathogenicity criteria to identify *Salmonella* spp. linked to poultry in hatching eggs and chicks, specifically targeting specified pathogen-free (SPF) chicks under one day of age.

2. Materials and methods

2.1 Samples collection

Samples (liver, intestinal, spleen, and heart blood) were collected from newborn chicks (1–7 days old) in duck and poultry farms in five governorates: Elminya, Beni-Suef, Ismailia, Sharkia, and Giza, which are situated in the Delta area and North Upper Egypt from December 2020 to November 2021. A sample of chicks with depression, anorexia, diarrhea, and death was taken. With an average of 15 chicks per farm, 900 liver, intestinal, spleen, and heart blood samples were taken from the chicks. Chicks from each farm were slaughtered by neck cut for the extraction of liver, intestinal, spleen, and heart blood organs under aseptic conditions in biosafety cabinet. Every organ of each bird was collected individually in sterile falcon (Manafi *et al*., 2017). The surfaces of internal organs were sterilized by heat and then a sample was obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilized surface. The sterilized surface cut with sterile scissors/scalpel and the swab or loop inserted into the cut without touching the outer surface. The sample was inoculated directly into buffered peptone water broth medium (Reid *et al*., 2018). At 0- and 7-days following incubation, 120 egg batches (40 eggs per batch) were taken from three breeder chicken farms (Mwesigwa *et al*., 2015).

2.2 Bacterial isolation

The isolation of *Salmonella* spp. was done in compliance with FDA regulations (Andrews *et al*., 2022). Samples were pre-enriched in buffered peptone water (Peptone, Sodium Chloride, Disodium Phosphate, Potassium Dihydrogen Phosphate, Distilled Water) for 16–20 h at 35–37 °C in static condition. Then, 0.1

mL of the pre-enriched inoculum was added to 10 mL of Rappaport-Vassiliadis broth (peptone, sodium chloride, potassium dihydrogen phosphate, dipotassium phosphate, magnesium chloride, malachite green) and cultured for 24 h at 42 °C to achieve selective enrichment. After complete mixing, 10 µL loopfuls of each enrichment broth were removed and streaked onto Xylose Lysine Deoxycholate (XLD) agar (xylose, lysine hydrochloride, sodium thiosulfate, phenol red, agar, deionized water). Samples were incubated for 24 h at 37 °C, and the colonies of probable *Salmonella* were then identified. Colonies with a slightly transparent red halo black center encircled by a pink-red zone on XLD agar were purified, screened, and kept at -80 °C (Marin *et al*., 2020; Tarabees *et al*., 2017). After collecting egg samples, the outside shell surface was cleaned with 70% alcohol, allowed to dry and then cracked using sterile forceps, using loop the samples were streaked onto Trypticase soy agar (TSA) for *Salmonella* isolation (Rahman *et al*., 2019). For identification, the suspicious colonies were streaked on XLD and MacConkey agar media, the purified colonies were kept at -80 °C (Xia *et al*., 2020).

2.3 Phenotypic identification of isolated Salmonella spp.

To identify the obtained *Salmonella* isolates, examination and biochemical testing methods were employed. Gram stain was applied to the dried heat-fixed smears of doubtful colonies. Biochemical tests with catalase and oxidase were used to establish the similar isolates. According to Afshari *et al.,* (2018) and Rahman *et al.,* (2019), the isolates that were found were cultured on triple sugar iron agar (TSI) (peptone, lactose, sucrose, glucose, sodium thiosulfate, phenol red, agar, distilled water), UREA medium (urea, peptone, sodium chloride, potassium phosphate, phenol red, agar, distilled water), motility indole ornithine (MIO) (peptone, sodium chloride, potassium phosphate, ornithine hydrochloride, indole reagent, agar, distilled water), citrate (sodium citrate, ammonium dihydrogen phosphate, dipotassium phosphate, agar, bromothymol blue, distilled water) and lysine decarboxylase (LIA) media (lysine hydrochloride, sodium thiosulfate, glucose, phenol red, agar, distilled water). The final identification was completed in accordance with the manufacturer's standards using the analytical profile index (API) 20E identification method (Mwang'onde *et al*., 2013).

2.4 Antimicrobial susceptibility testing

The disc diffusion method was used to assess the antibiotic susceptibility on Mueller-Hinton agar (beef extract, casein hydrolysate, agar, sodium chloride, distilled water) (CLSI, 2020). The selected antibiotic discs were applied against standardised inoculums visually similar to the 0.5 McFarland standards (1.5×10^8 CFU/mL) for 14 regularly used

antibiotics in treating or preventing *Salmonella* infection. (levofloxacin, ceftazidime, cefotaxime, ciprofloxacin, amoxicillin clavulanic acid, streptomycin, tetracycline, neomycin, gentamycin, amikacin, colistinsulphate, trimethoprimsulfa methoxazole, ampicillin, clindamycin) (Tarabees *et al*., 2017). The findings were classified as sensitive, moderate, or resistant based on the Clinical and Laboratory Standards Institute's breakpoints (CLSI, 2020). When an isolate exhibited resistance to at least one antimicrobial agent in three or more antimicrobial categories, it was classified as having a multiple drug resistance (MDR) pattern. (MDR) indices were computed using the following algorithm for bacterial isolates: MDR $Index$ = Amount of antibiotics tested × 100 total antibiotics resisted. MDR isolates were defined as those having an MDR index value greater than 0.2 isolates of *Salmonella* (Govender *et al*., 2021).

2.5 Molecular identification

The genus-level detection of *Salmonella* and the identification of the *S. enterica* serovar Enteritidis, and *S. enterica* serovar Typhimurium target *inv*-A, *IE*-1, and *flic*-C genes were achieved by the use of a multiplex polymerase chain reaction (m-PCR) assay (Pui *et al*., 2011). Bacterial colonies with phenotypic confirmation were grown over night on (TSA) and (TSB) and incubated at 37°C. The TSB tubes was subjected to DNA extraction (Jamshidi *et al*., 2010; Santos *et al*., 2020), and the Patho-Gene spin TM DNA/RNA Extraction kit (Intron Biotechnology Inc, Korea) was used to extract the bacterial DNA. A total of 25 µL was used for the PCR reactions, which included 1.5 µL of nuclease-free water, 5 µL of bacterial DNA, 2 µL of *inv*-A primers, 2 µL *IE*-1, and 2 µL *Flic*-C primers as in Table (1) (Applied Biosystem, Egypt), and 12.5 µL of 2X Easy Taq® PCR SuperMix (TransGen Biotech Co., China). The m-PCR technique (Applied biosystems, veriti) included five minutes of initial denaturation at 94 ºC, thirty seconds of 94 ºC, one minute of 58 ºC, and one minute of 72 ºC, followed by a final extension step of ten minutes at 72 ºC. 1.5% agarose gel was used for analysis of the PCR results. For the *inv*-A, *IE*-1, and *Flic*-C genes, the amplicon sizes of 796, 316, and 432 base pair were deemed positive, respectively.

Table (1): Primers to identify *Salmonella* spp in the multiplex-PCR.

Target gene	Primer sequence 5' - 3'	Annealing temp.	Product (bp)	Reference
Inv-A gene	*Inv*-A F: CGG TGG TTT TAA GCG TAC TCTT	58 °C	796	(Fratamico, 2003)
	Inv-A R: CGA ATA TGC TCC ACA AGG TTA			
S. Enteritidis	*IE*-1 F: AGT GCC ATA CTT TTA ATG AC		316	(Wang and Yeh, 2002)
	IE-1 R: ACT ATG TCG ATA CGG TGG G			
S. Typhimurium	*flic-C* F: CCCGCTTACAGGTGGACTAC		432	(Paião *et al*., 2013)
	flic-C R: AGCGGGTTTTCGGTGGTTGT			

2.6 Assessing of isolated S. enterica serovar Enteritidis strains in one-day-old SPF chicks

2.6.1 Experimental design

By using *Salmonella* standard strains including; *S. enterica* serovar Typhimurium strain "O1,4,[5],12; i;1,2"), *S. kentucky* strain "O8,20; i,2."), *S. Gallinerum* strain "O1,9,12; Hg, m; --") and *S. enterica* serovar Enteritidis strain "O1,9,12; g,m;---) were used as positive controls in the study. Eight groups (10 chicks in each group) of one-day-old SPF chicks were created. The birds were housed in Biosafety cabinet level-3 isolators and given free access to water and commercial meal free of antibiotics. By crop gavage, six groups of chicks were given 0.5 mL, which is equal to 0.5 McFarland standards (1.5×10^8 CFU/mL) of each isolate of *Salmonella* (Li *et al*., 2017). As positive controls, three groups of chicks were inoculated with *S. enterica* serovar Typhimurium, *S. kentucky*, and *S. gallinerum*, respectively. The final set of chicks served as a negative control group that received an injection of PBS.

2.6.2 Clinical observation in chicks and sample collection following infection

For five days following infection, the chicks were checked twice a day for indicators of disease and death. Dehydration, sadness, ruffled feathers, diarrhoea, and pasty vent were among the symptoms noted. Necropsies were performed on dead chicks, pathological alterations were noted, and liver and heart samples were taken in order to isolate *Salmonella* again. At the conclusion of the observation period, the surviving chickens were put to death, and samples were cultivated in order to perform bacteriological analyses (Pattison *et al*., 2007).

3. Results

3.1 Isolation of Salmonella spp.

Samples of sick chicks were provided from five separate governorates, 15 broiler chicken farms, and 120 egg batches from three breeder chicken farms. Results from the analysis of chicken farms with chicks ranging in age from one to seven days revealed that, eight of the farms were clear of *Salmonella* infection, and attempts to isolate the bacteria yielded negative results. The remaining 7 poultry farms (46.7%), however, showed varying organ detections of *Salmonella* infection. As for the 120 egg batches, only 2 (1.67%) were presumed to be infected with *Salmonella* spp. based on the morphologicl characterestics of the colonies on selective macConkey, non-selective medium (TSA) and specific XLD agar medim, as shown in Figures (1 and 2). Table (2) presents these findings.

3.2 Phenotypic identification of recovered Salmonella isolates

3.2.1 Phenotypic characterization

The presumptive 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, oxidase, indole, and urease were negative. The API-20E test confirmed the obtained biochemical profile suggestive of *S. enterica* as in Figure (3).

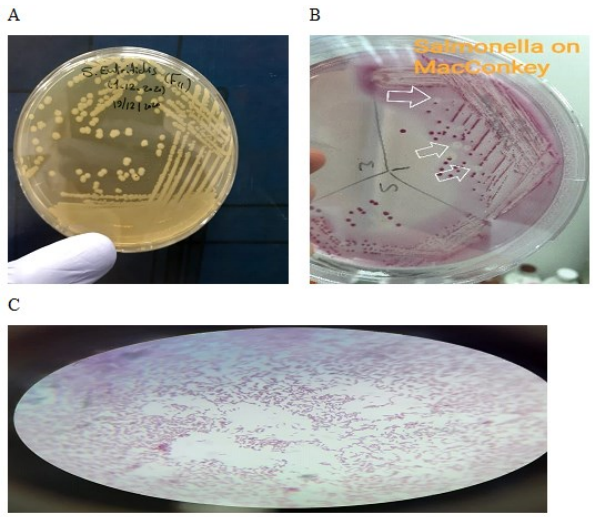

Figure (1): Cultural Growth of Salmonella isolates on TSA as a general media (A), and MacConkey agar as a selective media (B), and microscopic examination of the isolates (C).

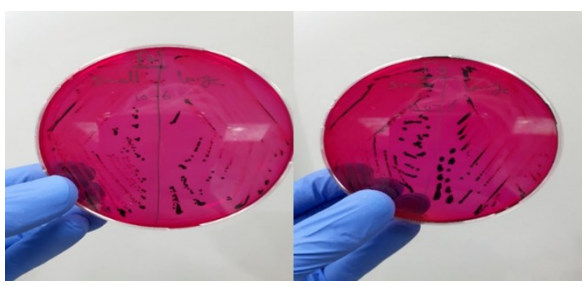

Figure (2): Cultural growth of *Salmonella* isolates on XLD.

Figure (3): Traditional biochemical analysis and API 20E for *Salmonella* Enteritidis.

Table (2): The results of isolation of SE from farms and eggs batches.

Item	Category	Location	*Salmonella* Result	Total isolates %
Farm 1	Chicken farms	Elminya	-ve*	
Farm 2	Chicken farms	Elminya	+ve*	
Farm 3	Chicken farms	Elminya	+ve	
Farm 4	Chicken farms	Beni-Suef	+ve	
Farm 5	Chicken farms	Beni-Suef	-ve	
Farm 6	Chicken farms	Beni-Suef	-ve	
Farm 7	Chicken farms	Sharkia	-ve	
Farm 8	Chicken farms	Sharkia	-ve	46.7%
Farm 9	Chicken farms	Sharkia	-ve	
Farm 10	Chicken farms	Giza	+ve	
Farm 11	Chicken farms	Giza	+ve	
Farm 12	Chicken farms	Giza	+ve	
Farm 13	Chicken farms	Ismailia	+ve	
Farm 14	Chicken farms	Ismailia	-ve	
Farm 15	Chicken farms	Ismailia	-ve	
Flock 1	Egg batches	Giza	-ve	
Flock 2	Egg batches	Giza	+ve	1.67%
Flock 3	Egg batches	Giza	+ve	

-ve*: The farm is free from *salmonella*, +ve* The farm is positive *salmonella*.

3.2.2 Antibiotic sensitivity profile

Only one isolate (SE-F2) had a 0.143 MDR index, while eight of the nine detected strains of *Salmonella* had MDR strains (0.214-0.786 MDR indices). With the exception of SE-F13, which had moderate ampicillin sensitivity, all isolates were resistant to both clindamycin and ampicillin. Most of the identified *Salmonella* were susceptible to amikacin, colistin sulphate, trimethoprim-sulfamethoxazole. cefotaxime, ciprofloxacin, ceftazidime and levofloxacin. All of which exhibited moderate sensitivity. Similar resistance patterns to gentamycin, amoxicillin-clavulanic acid, tetracycline, neomycin, and streptomycin were seen in four isolates (SE-F11, SE-F12, SE-F13, and SE-E29) (Figure 4, Table 3).

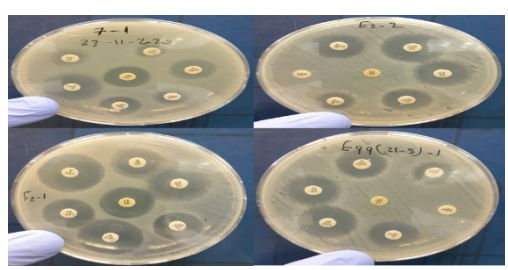

Figure (4): Antibiotic susceptibility pattern of *Salmonella* isolates against 14 antimicrobial drugs using disk diffusion method.

Table (3): Antibiotic sensitivity of *S. enterica* serovar Enteritidis isolates.

Antibiotic / SE isolates	Levofloxacin (5 µg)	Ceftazidime (30 µg)	Cefotaxime (30 µg)	Ciprofloxacin (5 µg)	Amoxicillin Clavul-anic Acid (30 µg)	Streptomycin (10 µg)	Tetracycline (30 µg)	Neomycin (30 µg)	Gentamycin (10 µg)	Amikacin (30 µg)	Colistin Sulphate (10 µg)	Trimethoprim Sulfa-methoxazole (25 µg)	Ampicillin (10 µg)	Clindamycin (2 µg)	MDR Index
SE-E29	I*	I	I	I	R*	S	R	I	S*	S	R	S	R	R	0.357
SE-E45	I	R	I	I	I	S	S	I	S	S	S	S	R	R	0.214
SE-F2	I	I	I	I	S	S	S	S	S	S	S	S	R	R	0.143
SE-F3	I	I	I	I	S	S	S	R	R	S	R	S	R	R	0.357
SE-F4	I	R	R	I	I	I	S	S	S	I	S	S	R	R	0.286
SE-F10	R	R	R	S	I	R	S	I	S	R	S	S	R	R	0.5
SE-F11	I	I	I	I	R	R	R	S	R	S	S	S	R	R	0.429
SE-F12	I	S	I	I	R	R	R	R	R	S	S	S	R	R	0.5
SE-F13	S	S	R	S	R	R	R	R	R	S	S	R	I	R	0.571

I: intermediate, R: resistant: S*: sensitive.

3.3 Molecular identification of isolated Salmonella spp.

The multiplex PCR using six sets of primer pairs, for the *Inv*-A, *IE*-1, *flic-C* genes, correctly identified *Salmonella* serovars Enteritidis and Typhimurium and differentiated the two serovars by the combinations of the different-size bands produced: two positive bands, which consist of *Inv*-A, *IE*-1 PCR products, in serovar Enteritidis and two positive bands, which consist of *flic-C* and *Inv*-A PCR products, in serovar Typhimurium (Figure 5). The *Inv*-A primers in this study reacted with both serovars Enteritidis and Typhimurium, yielding PCR products of the same size. The presence in both serovars Enteritidis and Typhimurium of the *Inv*-A gene was consistent with the findings of a previous report (Pui *et al*., 2011). The primers for *IE*-1 specifically detected the *S. enterica* serovar Enteritidis. The primers for *IE*-1 specifically detected the *S. enterica* serovar Enteritidis, the primers for *flic-C* specifically detected the *S. enterica* serovar Typhimurium.

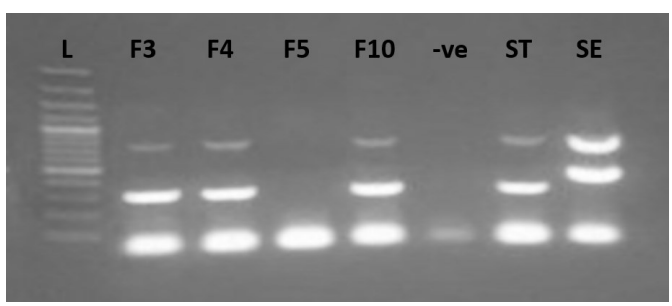

Figure (5): The amplified products for *Salmonella* Enteritidis isolates by Multiplex PCR on 1.5% agarose gel electrophoresis. L; ladder, F3; Farm 3, F4; Farm 4, F5; Farm 5, F10; Farm 10, -ve; negative control, ST; Salmonella Typhimurium SE; *Salmonella* Enteritidis.

3.4 Pathogenicity

Salmonella enterica serovar Enteritidis caused significant mortality in wild birds and gastrointestinal illness in humans (Shaji *et al*., 2023). *Salmonella* pathogenesis can be divided into several stages, including adhesion and invasion of gut epithelial cells, survival, multiplication within the host cells, and extraintestinal spread. *Salmonella*, being an enteric pathogen, reaches the intestine via oral ingestion from contaminated environments, feed, and water. Even a very low infective dose of *Salmonella* Enteritidis, as low as 1–5 bacteria cells, can lead to infection in day-old chicks, bacteremia and invasion of systemic organs such as the liver, spleen, ovary, and gallbladder (Shaji *et al*., 2023). The invasive species *S.* Enteritidis infects both young and adult birds. After the bacterial pathogen colonizes an adult bird, it may stay asymptomatic carrier, but young birds often acquire a systemic disease with high fatality rates (Velge *et al*., 2005). Non-invasive serotypes of Non-Typhoidal Salmonella are restricted to the gastrointestinal system, where they cause severe inflammation that progresses to enterocolitis. This inflammation includes diffuse and focal mononuclear cell infiltration, epithelial cell necrosis, edema, and eventually enterocolitis (Coburn *et al*., 2007). The study found that 50–100% of the SE isolates' orally injected chicks died. Figure (6) shows that the birds inoculated with SE-F4 and SE-E45 had the highest percentage of illness and mortality (100 and 87.8%, respectively), while the birds inoculated with SE-F3 and SE-F10 had the lowest mortality (50%). The most frequent gross lesions were patches of fibrinous effusion on the liver capsule, and in few cases, in the pericardium. There were additional reports of enlarged livers, occasionally with white foci and congestion. Furthermore, coagulated material, pneumonia, enteritis, pericarditis, perihepatitis, and a congested liver were found in the yolk sacs of some birds.

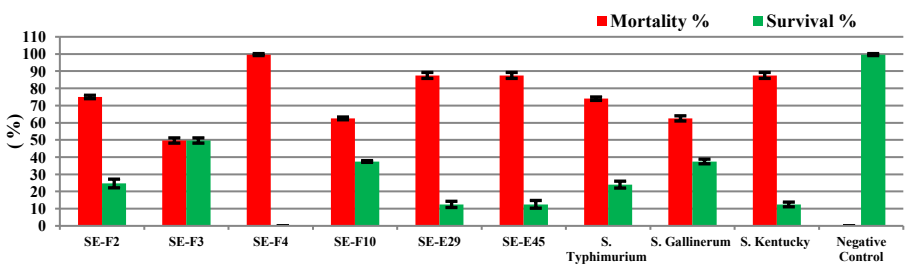

Figure (6): Pathogenicity of the day-old SPF chicks inoculated in BSL-3 isolators with different *S. enterica* serovar Enteritidis isolates (1.5×10^8 CFU/mL) and chicks were observed for 5 days. Survival and mortality rates in day-old specific pathogen-free chicks experimentally inoculated *S. enterica* serovar Enteritidis.

3.5 Statistical analysis

Survival curves were compared using several statistical tests to evaluate the Survival and mortality rates in day-old specific pathogen-free chicks (SPF) groups inoculated with different *S. enterica* serovar Enteritidis isolates (1.5×10^8 CFU/mL) and chicks were observed for 5 days. Survival curves were analyzed using GraphPad Prism and Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) to assess pathogenicity differences between groups. The Log-rank (Mantel-Cox) test indicated a significant difference in survival curves, with a chi-square value of 31.18, 9 degrees of freedom, and a p-value of 0.0003, suggesting that the survival curves are significantly different. The Log-rank test for trend also showed a significant trend with a chi-square value of 4.733, 1 degree of freedom, and a p-value of 0.0296, indicating a significant trend in survival over time. Additionally, the Gehan-Breslow-Wilcoxon test confirmed significant differences in the survival curves with a chi-square value of 29.70, 9 degrees of freedom (Team, 2020). p-value of 0.0005. Overall, the analyses demonstrate statistically significant differences in survival curves across the groups studied. Statistically significant differences ($p < 0.05$) between the curves as in Figure (7).

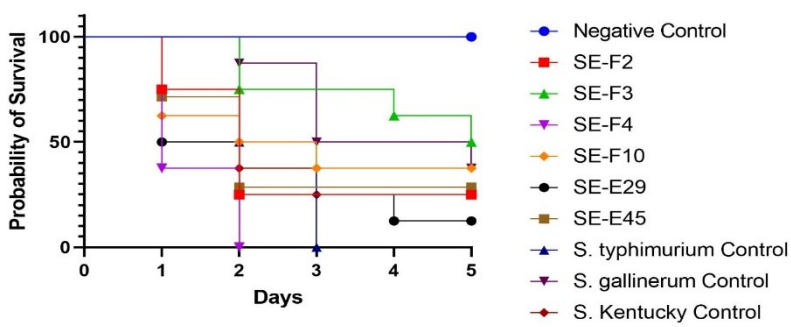

Figure (7): Survival curves analyzed using GraphPad Prism and Microsoft Excel 2016. The Log-rank (Mantel-Cox) test ($\chi^2 = 31.18$, Df = 9, $p = 0.0003$), Gehan-Breslow-Wilcoxon test ($\chi^2 = 29.70$, Df = 9, $p = 0.0005$) show significant differences in survival. The Log-rank test for trend ($\chi^2 = 4.733$, Df = 1, $p = 0.0296$) indicates a significant trend. Asterisks (*) indicate statistically significant differences ($p < 0.05$) between the curves.

4. Discussion

In this study, a high farm prevalence (46.7%) of *Salmonella* infection was observed in commercial poultry farms in Egypt. *Salmonellae* spp. was identified and isolated from hatching eggs and chick farms in five different Egyptian locations.

From 15 chicken farms, 7 had positive results for *Salmonella* spp. in animals ranging in age from one day to seven days. Among the three farms that produced eggs, two were found to be contaminated with the bacteria. The bacteria were isolated from various parts of the chicks, including the intestine, liver, heart, and spleen. The results confirm observations from other parts of Egypt by (Abdel-Maksoud *et al*., 2015) who showed 38.7% farm prevalence in commercial farms in the flesh, skin, and excrement of chickens; however, no *Salmonella* was found in the samples of raw egg yolk or eggshell (Abdel-Maksoud *et al*., 2015). A recent study found that from total 60 samples 32 (53.33%) were found positive for *Salmonella* (Paul *et al*., 2017) and the median worldwide prevalence values of *Salmonella* in broiler chickens, raw chicken meat, and in eggs and egg-laying hens were 40.5% (Castro-Vargas *et al*., 2020). The litter from thirteen farms in four southern states was tested for *Salmonella*. Samples (n = 490) from six of the thirteen (46.2%) farms tested positive (Dunn *et al*., 2022). But *Salmonella* serovars were found in 10.7% (9/84) of investigated Cloacae, litter and feed samples in Poultry farms in Sharkia Governorate, Egypt (Zaki *et al*., 2023). In another study was done to isolate *Salmonellae* from 579 birds (348 chickens, 104 ducks, 30 turkeys, 50 quail, 30 pigeons and 17 geese) from 4 Egyptian Governorates. The Samples collected from internal organs (liver, cecum, spleen and heart) were examined and *Salmonella* species were found in (10.9%) (El-Tawab *et al*., 2015). In addition, the isolation rate from different chicken shops, houses and farms in Kafr El-Sheikh Governorate comprise the examination of 100 samples of feed (40), water (36) and litter (24) collected randomly. Results of this survey revealed isolation of 14/100 (14%) *Salmonella* species (3) (12.5%) isolates from litter, (5) (13.8%) isolates from water and (6) (25%) isolates from farms (El-Tawab *et al*., 2019). Recent research indicates that just 3.3% of 120 liver samples from unhealthy 2-3-week-old grill chickens in Egypt recovered *Salmonella* (Radwan *et al*., 2021), the presence of *Salmonella* Enteritidis in poultry in Egypt (Mohamed *et al*., 2022). The uncontrolled usage of antibiotics in poultry could be the reason for the increased prevalence of MDR *Salmonella* spp., Thirty-three samples out of 490 (6.7%) were *Salmonella* positive (Dunn *et al*., 2022) and as low as 3.67%, 0.57%, and 1.95% in Bangladesh for *Salmonella* spp. that are ST, SE, and untyped, respectively (Siddiky *et al*., 2021). The high prevalence of illness in this study could be related to the participants' (chicks) young age at the time of study. *Salmonella* prevalence may fluctuate with age as a result of changing farming practices, medications, and/or hygiene standards. The extremely low frequency in hatching eggs is most likely caused by the lack of *Salmonella* infection and/or by the effective use of sanitary practices in breeding farms to prevent egg contamination. Furthermore, a recent

experimental investigation showed that, even in the presence of ambient *Salmonella* infection on the farm, the level of *Salmonella* contamination in eggs is minimal and can be further reduced by washing (McWhorter and Chousalkar, 2020). In this study, eight of the nine detected strains of *Salmonella* had MDR strains. With MDR indices ranging from 0.214 to 0.786, the majority of identified *Salmonella* bacteria were resistant to at least three antimicrobial drugs from various antimicrobial classes (Tan *et al*., 2022). In Egypt, a concerning quantity of multidrug resistant *Salmonella* isolates have been found in chicken farms and chicken eggs (Elsayed *et al*., 2024). A potential risk to human health arises from eating chicken or products containing MDR *Salmonella* isolates (Doyle, 2015). Nevertheless, elderly, infants, and immunocompromised persons infected with MDR *Salmonella* isolates develop severe complications with higher death rates (Mkangara, 2023). *Salmonella* strains in this study appeared to have very high rates of antibiotic resistance. This is most likely due to commonly used antibiotics in poultry production (Castro-Vargas *et al*., 2020), antibiotics early use in veterinary and human medicine, followed by their widespread and uncontrolled usage. Egypt has a high rate of drug resistance (Abdel-Maksoud *et al*., 2015), there could be negative effects on the treatment and prevention of diseases in poultry and diseases spread by poultry. In addition *Salmonella* can acquire resistance through mobile elements such as plasmids that account for the high rates of transfer of genes that are beneficial to the survival of the host bacteria (Heuer *et al*., 2008). Molecular techniques are frequently dependable and sensitive enough to find and identify *Salmonella* spp. in big samples (Shi *et al*., 2015). The results of the species-specific m-PCR distinguished the *S. enterica* serovar Enteritidis isolates from other *Salmonellae* in the current investigation and were in agreement with traditional phenotyping results. These results provide additional evidence of the m-PCR's robustness and sensitivity for serotyping *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium (Kim *et al*., 2006). SPF chicks that were one day old were given a dose of specific strains of *S. enterica* serovar Enteritidis to examine the virulence of the isolated strains in relation to their phenotypic standards. In chicks under two weeks old, the difference in mortality due to *S. enterica* serovar Enteritidis is clearly visible (Osman *et al*., 2010) and day-old chicks (14.5-89.5%) (Suzuki, 1994). Most of the clinical strains of *Salmonella* Enteritidis in the current investigation are highly invasive with varying mortality ranges (50–100%), despite a small amount of phenotypic variation among them. However, the lowest MDR indices were notably associated with high virulence in *Salmonella* Enteritidis strains (mortality ≥ 85% in SE-E45 and SE-F4 strains) and vice versa (mortality ≤ 60% in SE-F10 and SE-F3) (Figure 6). The Statistical analysis demonstrated significant

differences in survival curves across the groups studied ($p < 0.05$). Furthermore, there is no consistent link between most of the SE phenotypic criteria and the *in-vivo* virulence. Previous research on the association between virulence and antibiotic resistance discovered that Salmonella's virulence diminishes as it gains antibiotic resistance (Jajere, 2019), Other researchers have not established a link between antibiotic resistance and pathogenicity (Morasi *et al*., 2022). To effectively control *Salmonella* in poultry, it is crucial to implement comprehensive measures that prevent contamination of poultry products before they reach consumers. This includes enhancing food safety practices throughout the production and processing stages, such as improving hygiene and sanitation protocols. Additionally, promoting rigorous food handling practices and monitoring environmental contamination can further reduce the risk of *Salmonella* infections in humans. By addressing these areas, the likelihood of contaminated poultry products reaching the market is minimized, ultimately ensuring safer food for consumers.

5. Conclusion

The outcome demonstrated the presence of *Salmonella*, particularly *Salmonella enterica* serovar Enteritidis in poultry farms within the Egypt regions under investigation. Significant multi-drug resistant rates in *S. enterica* serovar Enteritidis strains could restrict available treatments, lead to treatment failure, raise the risk to the public's health, and increase the mortality rate of chickens. Therefore, control measures must be implemented by the poultry sector to lessen the spread of *S. enterica* serovar Enteritidis during the manufacturing process. Our study found that *in-vivo* pathogenicity in SPF chicks increased with decreasing MDR index. To find out whether virulence and drug resistance in *S. enterica* serovar Enteritidis isolates are connected, more investigation is necessary. In addition to crucial control point initiatives, consumer education campaigns are needed to lower the risk of foodborne Salmonellosis.

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