





Evaluation of *Salmonella Enteritidis* Isolated from Layer Hens and Murine Fecal Pellets in Poultry Farms of Libya

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ABSTRACT

The rodents play a significant role in the transmission of *Salmonella* between farms and regions. The present study aimed to compare the virulence of *Salmonella enteritidis* isolated from fecal samples of laying hens and murine within the same poultry house but different regions in Libya using Vivo-quantitative measurement of invasiveness (chicken intestinal loop model). A total of 540 cloacal swabs from laying hens (Hy-line brown chickens) aged 36 weeks and 200 batches of murine fecal pellets were collected from the same poultry house at Gaser Bin Gisher and Furnag regions in Libya. The samples were passed on pre-enrichment broth (Buffered Peptone Water) and enrichment broths (Rappaport Vassiliadis, Selenite broth, and tetrathionate), then the samples were cultured onto Xylose Lysine Deoxycholate agar, brilliant green agar, *Salmonella* Shigella agar, and Hektoen enteric agar. Single colonies were selected and stained by gram stain and tested biochemically using analytical profile index (API) 20 tests. *Salmonella enteritidis* was isolated from all the collected samples. The invasion of *Salmonella enteritidis* isolated from laying hens and murine feces was significantly higher in the anterior inoculation position compared to the posterior position of jejunum in both regions. The account of *Salmonella enteritidis* isolated from laying feces of hens and murine at Gaser Bengasher region was significantly higher than that isolated from the AlFurnge region. In the present study, the rodents act only as mechanical transmitters without affecting *Salmonella* invasiveness capacity. Furthermore, the invasion of *Salmonella enteritidis* depends on the inoculation position in the jejunum. Moreover, the invasiveness variation of *Salmonella enteritidis* isolated from the Gaser Bengasher and AlFurnge regions could be attributed to the presence of different *Salmonella* strains in the studied area. *Salmonella enteritidis* isolated from poultry and murine in the current study was sensitive to gentamicin, ciprofloxacin, and enrofloxacin and resistant to doxycycline, chloramphenicol, sulfafurazol, and ampicillin.

Keywords: Invasiveness, Layer chicken, Murine infestation, *Salmonella enteritidis*

INTRODUCTION

Salmonella enteritidis belongs to the Enterobacteriaceae family and it is a facultative intracellular bacteria. *Salmonella* has More than 2600 different serovars, which are divided based on host adaptation into non-host-specific serovars (ubiquitous serovars) that cause potential infections in humans and animals such as *Salmonella Enteritidis* (SE) and *Typhimurium*, and host-restricted serovars, such as *Salmonella Gallinarum* (SG) and *Salmonella Pullorum* (SP, Odoch et al., 2017; Xiong et al., 2018; Sreekantapuram et al., 2021). Fowl typhoid in chickens due to infection by *Salmonella Gallinarum* (SG) and *Salmonella Pullorum* (SP) causes potential clinical disease with high mortality in all ages, and the surviving

chicken can carry the *Salmonella* for the rest of its life (Wigley et al., 2001; Eriksson et al., 2018; Berhanu and Fulasa, 2020). The factors, such as flagella, capsule, plasmids, and adhesion systems, are responsible for virulence variation of *Salmonella* pathogenesis between hosts, including adhesins, invasions, fimbriae, hemagglutinins, exotoxins, and endotoxins, type 3 secretion systems and *Salmonella* pathogenicity island system which located in chromosomes or plasmids (Daigle, 2008; Sabbagh et al., 2010). These factors control *Salmonella* colonization in the host intestine and cross host-defense-mechanisms as GIT microbial population, gastric acidity, and enzymes as proteases (Foley et al., 2008; 2013; Kaur and Jain, 2012; Yue and Schifferli, 2013). *Salmonella* is generally presented mainly in the

digestive tracts of humans, animals, and avian hosts. Therefore, the presence of *Salmonella* in water, environment, and food is due to fecal contamination (Yue and Schifferli, 2013; Mezal *et al.*, 2014). The termination of *Salmonella* from poultry farms is a difficult task in the presence of natural carriers, such as rodents, wild animals, insects, and human traffic. All those factors increase *Salmonella* persistence in animal farms (Lawson *et al.*, 2014; Brobey *et al.*, 2017; Zamora-Sanabria and Molina Alvarado, 2017). Previous studies indicated that the different pathogenicity effects of *Salmonella* serovars are related to gene mutations, gene transfer, and genome degradation (Rabsch *et al.*, 2002; Kisiela *et al.*, 2012). The present study aimed to compare the virulence of *Salmonella enteritidis* isolated from fecal samples of laying hens and murine within the same poultry house. The study considered different regions in Libya using Vivo-quantitative measurement of invasiveness (chicken intestinal loop model).

MATERIALS AND METHODS

Ethical approval

All the ethical standards for animal welfare and the experiments are done in experimental units in the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, the University of Tripoli, Libya under full-authorized staff. The Ethical Approval Committee Code Number is POU.505-2022/SA.

Sampling

Between February 2022 and June 2022, a total of 540 cloacal swabs from Hy-line brown laying hens aged 36 weeks were collected from poultry houses at Gaser Bin Gisher and Furnag regions in Libya. A total of 200 fecal pellets samples were collected from live rodents (*Meriones* spp.) by insulated Tomahawk traps inside the poultry houses as described by Kilonzo *et al.* (2013).

Isolation of bacteria from fecal samples

The fecal samples were pre-enrichment with Buffered Peptone Water (BPW; Oxoid CM0509, 1: 4) and incubated aerobically at 37°C for 24 hours. An amount of 0.1 ml of pre-enriched samples was added into Rappaport-Vassiliadis (Oxoid CM866) as the selective enrichment medium. The mixture was then incubated aerobically at a temperature of 42°C for 24 hours. The enriched samples were streaked onto Xylose- Lysine-Desoxycholate agar (XLD; Oxoid CM469) and incubated aerobically at 37°C for 24 (Aabo *et al.*, 2002, Kilonzo *et al.*, 2013, Irfan *et al.*,

2015). According to Aabo *et al.* (2000), the isolate was identified by using the analytical profile index (API) 20 (BIOMÉRIEUX, 2011- France). The experimental design was conducted on nine laying hens divided into three replicate groups.

Invasiveness

The two *Salmonella enteritidis* isolated from poultry and murine at the poultry farms and one *Salmonella* reference strain (POULVAC, *Salmonella Typhimurium* Vaccine, Live Culture, USA) were inoculated separately. Loop positions included three parts, the anterior part, the intermediate part, and the posterior part of the jejunum per chicken. After 2 hours, gentamicin was injected and left for 1 hour to kill non-invading bacteria. The bacterial counts (CFU/ ml) of homogenate mucosa tissue at diameter (42-mm²) were used to express *Salmonella* invasiveness throughout the study using log¹⁰.

Antibiotic sensitivity test

Antibiotic susceptibility of isolated bacteria against seven antibiotic substances of veterinary significance was determined by a disc diffusion test (Bauer *et al.*, 1966). *In vitro* antimicrobial susceptibility was screened on Mueller-Hinton agar (MHA- Oxoid, Hampshire, UK) which was incubated at 37°C for 24 hours. At the end of the incubation period, antibiotic inhibition zones were measured by a measuring calibrator.

Statistical analysis

The statistical analysis was done using the GraphPad Prism Version-5 software (California-USA), and one-way analysis following Tukey's Multiple Comparison Test was used (p values less than 0.05 were considered significant).

RESULTS

In the present study, the *Salmonella enterica* serovar enteritidis was isolated from feces of laying hens and murine fecal pellets in the same poultry house at Al-Furnge region and Gaser Bengasher regions in Libya in all samples (Table 1). The invasion of the reference strain (as control) *Salmonella Typhimurium* was quite similar without any significant differences between the three inoculation parts in jejunum during all experiments (p > 0.05). The prevalence of *Salmonella enteritidis* in laying hens and murine feces was significantly higher in the anterior inoculation position of the jejunum compared to the intermediate and posterior inoculation positions of the jejunum, as indicated in Table 1 (p < 0.05). Notably, the

account (\log^{10} CFU) of *Salmonella enteritidis* isolated from laying hens and murine at the Gaser Bengasher region was significantly higher than AlFurnge region during the experiment ($p < 0.05$). The accounts of *Salmonella enteritidis* isolated from poultry at Gaser Bengasher region and insulated in the jejunum were 5.3, 4.6, and 4.7 CFU/ ml in anterior, intermediate, and posterior positions, respectively. The accounts of *Salmonella enteritidis* isolated from murine were 5.7, 5.1, and 4.6 CFU/ ml in anterior, intermediate, and posterior positions, respectively (Table 1). However, at the Alfurnage region, the accounts of *Salmonella enteritidis*

isolated from poultry anterior, intermediate, and posterior positions of the jejunum, were 4.5, 4.3, and 4.2 CFU/ ml, respectively. Whereas, the account of *Salmonella enteritidis* isolated from murine at the same region in anterior, intermediate, and posterior positions were 4.5, 4.4, and 4.0 CFU/ ml, respectively (Table 1).

Salmonella enteritidis isolated from poultry and murine in the current study was sensitive to gentamicin, ciprofloxacin, and enrofloxacin and resistant to doxycycline, chloramphenicol, sulfafurazol, and ampicillin (Tables 2 and 3).

Table 1. Evaluation of two *Salmonella* isolates from the field and one *Salmonella* reference strain inoculated separately in three loop positions from the anterior part to the posterior part of the jejunum per chicken

Loop site of inoculation	Furnage region			Gaser Bengasher region		
	SEL	SEM	R.S	SEL	SEM	R.S
L1-R1	4.47	4.51	5.8	5.6	5.7	5.17
L1-R2	4.48	4.55	5.21	5.2	5.8	5.1
L1-R3	4.47	4.5	5.16	5	5.7	5.11
Average L1 \log^{10} CFU	4.5*	4.5*	5.4	5.3 ***	5.7	5.1
L2-R1	4.27	4.31	5.11	5	5	5.11
L2-R2	4.22	4.5	5.15	4.1	5.1	5.8
L2-R3	4.34	4.5	5.11	4.82	5.1	5.9
Average L2 \log^{10} CFU	4.3	4.4	5.1	4.6	5.1	5.6
L3-R1	4.19	3.9	5.2	4.68	4.7	4.92
L3-R2	4.15	4	5	4.82	4.61	5.2
L3-R3	4.12	4	4.9	4.7	4.57	4.92
Average L3 \log^{10} CFU	4.2	4.0	5	4.7	4.6	5.0
Average overall \log^{10} CFU	4.3*	4.3*	5.2	4.9**	5.2**	5.3

SEL: *Salmonella Enteritidis* (layer), SEM: *Salmonella Enteritidis* (murine), RS: Reference strain (*S. Typhimurium*), L1: Anterior loop of jejunum, L2: Intermediate loop of jejunum, L3: Posterior loop of jejunum, R: Replication. Values within a column lacking a common superscript differ at $p < 0.05$. Values within a row carrying two and three stars (**, ***) are significantly different from values carrying only one star (*) at $p < 0.05$. The bacterial counts (CFU/ ml) of homogenate mucosa tissue were expressed in \log^{10}

Table 2. The antibiotics sensitivity test for *Salmonella enteritidis* isolated from poultry in Lybia

Antibiotic	Standard inhibition zone			<i>Salmonella enteritidis</i> isolated from poultry	
	Resistant	Intermediate	Sensitive	Inhibition zone	Response
Doxycycline 30 ug	< 8	8-12	> 18	5 mm	Resistant
Enrofloxacin 5 ug	< 8	8-12	> 12	22 mm	Sensitive
Chloramphenicol 30 ug	< 16	16-21	> 20	10 mm	Resistant
Sulfafurazol 100 ug	< 11	11-15	> 15	8 mm	Resistant
Ampicillin 10 ug	< 13	14-16	> 17	9 mm	Resistant
Gentamycin 30 ug	< 11	11-15	> 15	8 mm	Sensitive
Ciprofloxacin 10 ug	< 16	16-21	> 21	25mm	Sensitive

Table 3. Antibiotics sensitivity test for *Salmonella enteritidis* isolated from murine In Lybia

Antibiotic	Standard inhibition zone			<i>Salmonella enteritidis</i> isolated from poultry	
	Resistant	Intermediate	Sensitive	Inhibition zone	Response
Doxycycline 30 ug	< 8	8-12	> 18	6 mm	Resistant
Enrofloxacin 5 ug	< 8	8-12	> 12	17 mm	Sensitive
Chloramphenicol 30ug	< 16	16-21	> 20	9 mm	Resistant
Sulfafurazol 100 ug	< 11	11-15	> 15	7 mm	Resistant
Ampicillin 10 ug	< 13	14-16	> 17	9 mm	Resistant
Gentamycin 30 ug	< 11	11-15	> 15	20 mm	Sensitive
Ciprofloxacin 10 ug	< 16	16-21	>21	24 mm	Sensitive

DISCUSSION

Throughout the study, all three inoculation sites in the jejunum indicated equal invasion results for the reference strain (*Salmonella Typhimurium*). There is a lack of data about the isolation of *Salmonella enterica* serovar *enteritidis* from the feces of laying hens and murine in Libya. However, Lawson et al. (2014), Brobey et al. (2017), and Zamora-Sanabria and Molina Alvarado (2017) isolated the *Salmonella* from intestines or feces of rodents, wild animals, and wild birds respectively. The virulence of *Salmonella* could be attenuated or strengthened depending on environmental exposure, mutation, and gastric acidity of reservoir hosts (Sabbagh et al., 2010; Yue et al., 2013; Zamora-Sanabria and Molina Alvarado, 2017). In the present study, the effects of some factors such as phage type and mutations on the virulence of *Salmonella* are not significantly obtained. However, a role in insignificant differences between the invasion of *Salmonella enteritidis* isolated from layer and murine are found. The decline in *Salmonella enteritidis* total counts between anterior to the posterior inoculation loop during the experiment in laying hens and murine isolates agrees with a previous study by Aabo et al. (2000; 2002). Aabo et al. (2000; 2002) reported an 8.5-fold decline in \log^{10} CFU of total *Salmonella* counts between the anterior and the posterior inoculation loop. The significantly high account of *Salmonella enteritidis* isolated from laying hens and murine at the Gaser Bengasher region compared to the AlFurng region could be explained by the presence of different virulence strains of *Salmonella* in the studied area. This result is compatible with the previous study by Asheg et al. (2003) that demonstrated the adhesion, colonization, and migration of *Salmonella enteritidis* in the intestinal tract of chickens depending on the dose of

the bacteria administered. According to Asheg et al. (2023), the presence of different virulence strains of *Salmonella* in the South and West of Tripoli could be due to differences in antibiotic resistance of *Salmonella* isolated from slaughterhouses in the South, West, and East of Tripoli –Libya.

Additionally, the current study considered the result of the antibiotic sensitivity test, especially after the emergence of strains resistant to multiple antibiotics as *salmonellosis* surveillance has been described all over the world, making control and treatment (Brisabois et al., 1997).

The results of the antibiotic sensitivity test in Libya by Beleid (1993) indicated that the tested isolates, including *Salmonella enteritidis*, were susceptible to ampicillin, sulfafurazol, chloramphenicol, enrofloxacin and doxycycline. However, the present result revealed that gentamycin was the most effective drug followed by enrofloxacin, and marked resistance of the isolates to ampicillin, sulfafurazol, chloramphenicol, and doxycycline. The comparison of the obtained result of the current study with Beleid's (1993) findings shows susceptibility of isolated salmonella to enrofloxacin. However, antimicrobial resistance of salmonella to specific kinds of antibiotics were recorded during the past 26 years. Recently, Asheg et al. (2023) reported resistance of *Salmonella enteritidis* isolated from broilers at slaughterhouses to sulfamethazon/trimethoprim, ciprofloxacin, trimethoprim, gentamycin, doxytetracyclin, amoxycillin/clavanic acid, and ampicillin, in percentages of 41%, 45%, 48%, 69%, 69%, 76%, and 100%, respectively.

Notably, a previous study indicated that plasmid-borne ampicillin resistance is associated with the attenuation of serovar enteritidis (Ridley et al., 1996).

The observed marked resistance of both *Salmonella enteritidis* and *Salmonella Newport* isolates in the present study is considered to be a biological indicator for the presence of multi-drug resistant bacteria. It has been reported in several countries (Arlet et al., 2006; Cobbold et al., 2006; Egorova et al., 2007; Pławińska-Czarnak et al., 2022), and it is considered a serious problem among both food animals and humans (Zhao et al., 2001; Gupta et al., 2003; Devasia et al., 2005; Poppe et al., 2006; Egorova et al., 2008). This finding is a concern for surveillance and environmental control organisms since the increase in antimicrobial resistance has limited the potential uses of antibiotics for the treatment of infections in humans and animals (Angulo et al., 2004). The total of methicillin-resistant *Staphylococcus* infections in U.S. hospitals and communities has increased from 2.4% in 1975 to 29% in 1991 (Panlilio et al., 1992). However, in 2013, the average percentage of hospitals reporting HA-MRSA in the U.S. was 61.5% (Fukunaga et al., 2016).

In addition, the recent emergence in Africa and Europe, mainly in turkey flocks of *Salmonella Kentucky* (CipR) resistant to ciprofloxacin (Le Hello et al., 2013) which is highly pathogenic and highly resistant to antibiotics reminds that the combat is never-ending.

CONCLUSION

The obtained results indicated that rodents could be active mechanical transmitters of *Salmonella* in poultry farms especially in the studied area. Furthermore, the resistance of isolated *Salmonella* to broad-spectrum antibiotics needs more attention thus further research is highly recommended to determine the extent of the problem in the suspected areas and to find the best solutions for controlling *Salmonella* isolates that resistance to broad-spectrum antibiotics from farm-to-fork.

DECLARATIONS

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Authors' contributions

Dr. Imad Benlashehr contributed to the database, data gathering, and the manuscript's preparation. Dr. Kaled Elmasry also completed the data analysis and manuscript

preparation. The primary and secondary supervisors for the study's conduct were the doctors Abdulatif Asheg and Abdulwabb Kammon. The final edition of the manuscript has been reviewed by all authors and approved for publication in the current journal.

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Availability of data and materials

The current publication contains all of the study data, and the accompanying author can provide further details upon request.

Ethical considerations

The ethical concerns of plagiarism, permission to publish, misconduct, data fabrication and falsification, double publishing, submission, and redundancy have all been reviewed by the authors.

Competing interests

The authors have proclaimed that no contending interest exists

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