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Prevalence of Multidrug Resistance Non-Typhoidal *Salmonellae* Isolated from Layer Farms and Humans in Egypt

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ABSTRACT

Non-Typhoidal *Salmonella* (NTS) are substantial foodborne pathogens that lead to bacteremia, gastroenteritis, and focal infection. Poultry is one of the usual provenances for the development of multidrug-resistance NTS. This problem has increased in developing countries with the indiscriminate use of antibiotics in the poultry production system. The current study aimed to determine the prevalence and tendency of antimicrobial resistance of zoonotic *Salmonella* spp. A total of 601 samples, including cloacal samples (150) eggshell (150), egg content (15 pooled samples), layer hen carcasses (150), hand swab (68) and stool samples (68) from poultry workers, were collected from five layer chicken farms. Isolation of NTS was performed by using different cultural and biochemical methods. Moreover, *Salmonella* isolates were evaluated for antimicrobial susceptibility using the disc diffusion method. The cloacal samples and stool samples showed the prevalence of *Salmonella* spp. at approximately similar rates of 4.7% and 4.4%, respectively. Chicken isolates were identified as *S.* Enteritidis, *S.* Typhimurium, and *S.* Gallinarum while the human isolates were only *S.* Typhimurium and *S.* Enteritidis. The prevalence of the NTS on the surface of the eggshells (7.3%) was higher than that in the other samples. Among 12 antimicrobials tested, 86.4% resistance was found to streptomycin and oxytetracycline followed by neomycin and erythromycin (77.3%), norfloxacin and ampicillin (68.2%) across the study sites. Kanamycin and gentamicin remained sensitive by 95.5% and 90.9%, respectively. The present study indicated that layer chickens and its products are important sources for human infection with multiple-drug resistant NTS strains.

Key words: Antibiotic sensitivity, Egg, Layer poultry, Non-typhoidal *Salmonella*, Zoonoses

INTRODUCTION

According to (Marcus et al., 2007), *Salmonella* species are the most frequent cause of foodborne gastrointestinal infections in the human community. *Salmonella* is readily transmitted through water, vegetables, fish, hamburger, pork, poultry products including eggs and chicken meat (Pires et al., 2014). Among these, chicken meat and eggs are the kinds of food that regularly cause salmonellosis (Jackson et al., 2013; Middleton et al., 2014; Phagoo and Neetoo, 2015). Therefore, the food chain, especially poultry-derived foods, can be considered as a major public health issue; if they serve as a route for the advent and dispersion of resistant bacteria including *Salmonella* species in the environment (Mossie and Dires, 2016; Oloso et al., 2018; Ramatla, et al., 2019).

The eggs might be contaminated with salmonella by two different routes, the first route is vertical transmission from an infected hen before oviposition. The second route is horizontal transmission through contaminated feces or environmental dust and then penetration of microorganisms to the egg through the eggshell. Thusly, the microorganism remains on the farm for long periods and poor eggshell quality can lead to foodborne illness (Al Momani et al., 2018).

Salmonellosis in poultry is essentially asymptomatic, however nonspecific signs, including anorexia, depression, dropping wings, diarrhea, and reduced egg production may be apparent (Gast and Beard 1992; Ezema et al., 2009; Eguale 2018).

NTS is one of the most prevalent foodborne infections around the world, causing diarrhea, fever, vomiting, and sometimes even death. The WHO has estimated that the NTS is responsible for an average number of 78.7 million foodborne diseases with more than 59000 deaths annually. Additionally, it is the most widely recognized cause for foodborne infections in the Middle East and North Africa (Johnson et al., 2014; Havelaar et al., 2015).

In the poultry industry, antibiotics have been used widely as growth promoters, prophylaxis, and therapeutic agents. the widespread and indiscriminate use of antibiotics is a contributing element for the development of antibiotic-resistant pathogens (Davis et al., 2011; Manyi-Loh et al., 2018). However, the utilization of antimicrobial for growth promotion is prohibited in the European Union but allowed in the USA, Canada and in Many countries (Gyles, 2008).

In the course of recent years, the emergence of multi-drug resistant bacteria such as salmonellae has become a major global health concern (McWhorter and Chousalkar, 2015). WHO has reported high levels of antimicrobial

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resistance involving salmonellae due to several factors such as substandard treatments, self-prescriptions, and non-adherence to medications (Dar et al., 2016). Increment in antimicrobial resistance interferes with the prevention and control of such organisms, consequently represent a danger to public health (Langata et al., 2019).

Actually, the poultry and poultry products have a great role in the dissemination of antimicrobial-resistant zoonotic pathogens. Subsequently, the objective of the present study was to isolate and identify NTS from layers, eggs, and human. Also, this study evaluated the sensitivity of the isolated salmonellae to diverse antimicrobials.

MATERIALS AND METHODS

The current study was conducted in El-Behira Governorate, Egypt.

Ethical approval

All procedures performed in this study, including the collection of human and animal samples were in accordance with the Egyptian ethical standards of the National Research Committee. All human subjects gave their informed oral consent for the collection of fecal samples, with the agreement that any identifying information of the persons should not be published.

Sample collection

Cloacal samples

To collect the cloacal samples, both wings of the birds were held with one hand, so that the tail portion remained in the upper direction. Then, the sterile swab was inserted into the cloaca of the ISA Brown hens. One hundred and fifty cloacal swab samples were collected and inoculated promptly into Buffered Peptone Water (BPW; HiMedia, India).

Egg samples

One hundred and fifty egg samples were collected from five farms (30 eggs per farm) from three sites on each farm; front, center and posterior. All eggs in individual sterile plastic bags were aseptically transported to the laboratory for the cultivation and isolation in less than six hours. Egg surface and substance were handled by the FDA's Bacteriological Analytical Manual (FDA 2012) with slight adjustments. Quickly, the whole outside surface of each egg was swabbed with a dampened swab, after that plunged in 9 ml BPW, and incubated for 18 to 24 h at 37^{o}C. After that, the eggs were submerged in disinfectant comprising of 70% alcohol and tincture of iodine (in 3:1 proportion) for 10 seconds, then permitted to air dry. Each egg was fire cleaned at the pointed end and aired out utilizing the sharp *edge* of a sterile surgical blade. The substance was filled with a sterile Whirl-Pak stomacher pack. The pooled 10 eggs were mixed with sterile tools by gloved hands until the yolk completely blended with the albumen. Then samples were kept at room temperature for 4 days after that 25 ml from each sample was added to 225 ml of tryptic soy broth supplemented with 7.8 g of ferrous sulfate (Sigma, United Kingdom) and incubated for 24±2 h at 35°C.

Laying hens carcasses samples

One hundred and fifty laying hen carcasses were collected from five farms. All the carcasses collected after slaughtering and transferred in isolated ice bags to the laboratory for further processing. All the samples were examined within 6 h after the collection. An equal amount of 25 g of each sample was added to 225 ml of BPW and mixed well by a homogenizer and incubated at 37 °C for 18–24 h (ISO6579, 2002).

Human samples

Sixty-eight stool samples, as well as 68 hand swabs, were collected from workers and visitors on the farm. Each stool specimen was received in a sterile plastic container and immediately transferred in an icebox to the laboratory where further preparation and analysis were performed. each swab of the stool was inoculated into 45 ml BPW and incubated at 37 °C for 18 h (Andoh et al., 2017).

Isolation and identification of *Salmonella* spp.

Non-selective pre-enrichment

All the swab samples and the layer hen meat samples after processing was pre-enriched in BPW in the ratio of one to nine and incubated at 37 °C for 18-24 h.

Selective enrichment

The selective enrichment medium of Rappaport-Vassiliadis (RV) (HiMedia, India) was used for the isolation of *Salmonella* spp. than bacterial colonies having similar cultural characteristics. The 0.1 ml of pre-enriched sample was transferred into a tube containing 10 ml of RV broth and incubated at 42 °C for 24 h.

Plating out and identification

After enrichment, a 10µl loopful of inoculums was streaked onto Xylose Lysine Desoxycholate (XLD) agar (HiMedia, India) and brilliant green agar (BGA; HiMedia, India) and incubated at 37 °C for 24 h. Next, the plates were

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evaluated for the presence of typical and suspect *Salmonella* colonies, according to the colony *characteristics described* in (ISO6579, 2002).

Biochemical tests

The presumptive colonies of *Salmonella* were also identified based on biochemical tests panel including urease broth, triple sugar iron, methyl red, indole, Voges-Proskauer, and Citrate test, in accordance with standard test protocol described in FDA's Bacteriological Analytical Manual (FDA, 2012).

Serotyping of isolates

The serotyping of biochemically confirmed isolates was performed by slide agglutination test according to the method described by (Kauffmann, 1974).

Antimicrobial susceptibility testing

Twelve antimicrobial agents were analyzed using concentrations detailed in table 1**.** The antimicrobial sensibility profiles of the isolates were determined using the disk diffusion method on Mueller–Hinton agar (HiMedia, India), elaborated by the Clinical and Laboratory Standards Institute (2015).

Table 1. Antimicrobial discs, concentration, and interpretation of results for isolated *Salmonella* strains.

Antimicrobial agent	**Sensitivity disc content (µg)**	**Interpretation of results** (Zone diameter; mm**)**		
		Resistant	**Intermediate**	**Susceptible**
Amoxicillin	30	14 or less	15-18	19 or more
Ampicillin	10	13 or less	14-17	18 or more
Chloramphenicol	30	12 or less	13-17	18 or more
Ciprofloxacin	5	15 or less	15-19	20 or more
Erythromycin	15	13 or less	14-22	23 or more
Gentamicin	10	12 or less	13-14	15 or more
Kanamycin	30	13 or less	14-17	18 or more
Nalidixic acid	30	13 or less	14-18	19 or more
Neomycin	30	12 or less	13-16	17 or more
Norfloxacin	10	12 or less	13-15	16 or more
Oxytetracycline	30	14 or less	15-18	19 or more
Streptomycin	10	11 or less	12-14	15 or more

RESULTS

Table 2 shows the prevalence of *Salmonella* spp. in collected samples. The highest prevalence rate (7.3%) was found in the eggshell. However, the *Salmonella* spp. not isolated from egg content and layer hen carcasses. The cloacal samples and the fecal samples showed the prevalence of *Salmonella* spp. at nearly similar rates of 4.7% and 4.4%, respectively. As shown in table 3, the poultry isolates were *S.* Enteritidis, *S*. Typhimurium and *S.* Gallinarum, while human isolates were *S*. Enteritidis and *S*. Typhimurium.

Table 4 showed that the majority of isolates were highly susceptible to kanamycin (95.5%), gentamicin (90.9%), nalidixic acid (77.3%) and ciprofloxacin (72.7%). Moreover, poor susceptibility to neomycin, amoxicillin, and erythromycin was detected among the isolated *Salmonella*. On the other hand, according to the obtained results in the current study, *Salmonella* spp. represented a high resistance rate of 86.4% to streptomycin and oxytetracycline, and 77.3% to neomycin and erythromycin. In total, 68.2% of isolates were resistant to amoxicillin, ampicillin, and norfloxacin. Moreover, 18.2% exhibited resistance to chloramphenicol and nalidixic acid. According to the results presented in table 5, in layer isolates, *S.* Enteritidis was the most resistant serovar to antibiotic followed by *S*. Typhimurium and *S.* Gallinarum. However, the data presented in table 6 indicated that human isolates were highly resistant to neomycin and norfloxacin, while they were highly susceptible to gentamicin, kanamycin, and nalidixic acid.

Table 2. Prevalence of *Salmonella* spp. in samples obtained from layer farms in Egypt

	Cloacal	**Eggshell**	**Egg content**	**Layer hen carcasses**	**Workers hand swab**	**human stool samples**
Number of samples	150	150 (30 eggs per farm)	15 pooled samples (each sample included 10 eggs)	150	68	68
Isolation rate	7 (4.7%)	11 (7.3%)	0	0	1 (1.5%)	3 (4.4%)

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Table 3. Prevalence of *Salmonella* serotypes in human and chicken samples obtained from layer farms in Egypt

Species \ Serotype	*S.* Enteritidis (n)	*S.* Typhimurium (n)	*S.* Gallinarum (n)
Chicken	8	7	3
Human	2	2	0

n: number

Table 4. Antimicrobial susceptibility profiles of *Salmonella* spp. isolated from layer farms in Egypt

Antibiotics	Number of susceptible isolates (%)	Number of intermediate isolates (%)	Number of resistant isolates (%)
Neomycin	1 (4.5)	4 (18.2)	17 (77.3)
Gentamicin	20 (90.9)	2 (9.1)	(0)
Streptomycin	1 (4.5)	2 (9.1)	19 (86.4)
Kanamycin	21 (95.5)	1 (4.5)	0
Chloramphenicol	12 (54.5)	6 (27.3)	4 (18.2)
Nalidixic acid	17 (77.3)	1 (4.5)	4 (18.2)
Ciprofloxacin	16 (72.7)	4 (18.2)	2 (9.1)
Norfloxacin	3 (13.6)	4 (18.2)	15 (68.2)
Oxytetracycline	1 (4.5)	2 (9.1)	19 (86.4)
Amoxicillin	1 (4.5)	6 (27.3)	15 (68.2)
Ampicillin	2 (9.1)	5 (22.7)	15 (68.2)
Erythromycin	1 (4.5)	4 (18.2)	17 (77.3)

Table 5. Distribution of *Salmonella* serovars in layer chicken isolates and rate of resistance to antimicrobial agents

Antimicrobial agents	Number of resistant *Salmonella* serovars (%)		
	S. Enteritidis	*S.*Typhimurium	*S.* Gallinarum
Neomycin	8 (100)	4 (57.1)	1 (33.3)
Gentamicin	0	0	0
Streptomycin	8 (100)	5 (71.4)	3 (66.7)
Kanamycin	0	0	0
Chloramphenicol	1 (12.5)	1 (14.3)	0
Nalidixic acid	3 (37.5)	1 (14.3)	0
Ciprofloxacin	0 (0)	1 (11.1)	0
Norfloxacin	7 (87.5)	3 (42.9)	1 (33.3)
Oxytetracycline	8 (100)	6(85.7)	2 (66.7)
Amoxicillin	7 (87.5)	5 (71.4)	1 (33.3)
Ampicillin	6 (75)	4 (57.1)	2 (66.7)
Erythromycin	8 (100)	4 (57.1)	3 (100)

Table 6. Distribution of *Salmonella* serovars in human isolates from layer farms and rate of resistance to antimicrobial agents

Antimicrobial agents	Number of resistant *Salmonella* serovars (%)	
	***S.* Enteritidis**	***S.*Typhimurium**
Neomycin	2 (100)	2 (100)
Gentamicin	0	0
Streptomycin	2 (100)	1 (50)
Kanamycin	0	0
Chloramphenicol	2 (100)	0 (0)
Nalidixic acid	*0* (0)	0 (0)
Ciprofloxacin	1 (50)	0 (0)
Norfloxacin	2 (100)	2 (100)
Oxytetracycline	2 (100)	1 (50)
Amoxicillin	1 (50)	1 (50)
Ampicillin	1 (50)	2 (100)
Erythromycin	1 (50)	1 (50)

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DISCUSSION

Salmonellosis is one of the most important foodborne diseases in humans, which has been increasing in recent years (Harsha et al., 2011). Salmonellosis can be transmitted through the food chain, especially poultry products in the absence of proper hygiene and infection control practices (Ifeanyichukwu et al., 2016; Ramatla et al., 2019). Contaminated feces excreted into the environment could be a source of the bacteria to naive hosts, sustaining its existence over the layer farm environment, therefore, feces played an important role in *Salmonella* dispersal (Carrique-Mas and Davies 2008; Oloso et al., 2018). The present research assessed the prevalence and antimicrobial susceptibility profile of *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, and *S.* Gallinarum) isolated from the cloacal samples, eggshell, hand swab and stool samples of workers.

The investigation of cloacal samples in the present study showed a low presence of *Salmonella* spp. (4.7%), in agreement with (Garcia et al., 2011) who reported the presence of *Salmonella* in the cloacal samples by 4%. On the contrary, higher rates of 7.33% and 14.63% were reported by (Parvej et al., 2016) and (Bordoloi et al., 2017), respectively. The possible reason for the low prevalence of *Salmonella* in the present study may be due to the reality that most of the studied poultry farms were small-scale farms. The cloaca is a significant source related to the later infection of the eggshells (El-Tras et al., 2010). In addition, the surface of the eggshell could be contaminated through the litter or even during storage, transportation, and handling (Mahmud et al., 2016).

In the current study, the *Salmonella* had a higher prevalence in eggshells (7.3%) compared to the other samples, while it was not recovered from the egg contents. These findings are consistent with obtained results in a study by El-Feky et al. (2013) who reported a similar isolation rate (7.83%) of *Salmonella* from the eggshells and also they found that *Salmonella* was not detected from egg content. However, EL-Kholy et al. (2014) reported that *Salmonella* could not be isolated from both the eggshell and the egg content. El-Prince et al. (2019) found that the prevalence of *Salmonella* from the eggshells and egg contents was 1.43%. The absence of *Salmonella* in the egg content is due to the protective barrier of the egg membranes and antibacterial constituents (Mahmud et al., 2016). It was found that the positive samples of *Salmonella* from the eggshells may be due to cross-contamination with feces, the cage or the farm environment (Harsha et al., 2011). The isolation rate of *Salmonella* from eggshells and egg contents in the present study was lower than those in reports by other studies (Akhtar et al., 2010; Ifeanyichukwu et al., 2016; Mahmud et al., 2016; Long et al., 2017). Microbial contamination of the egg has a substantial consequence in the poultry industry, particularly considering international trade. It also has severe public health importance with regard to the transmission of illness to humans, which could lead to mild symptoms or life-threatening conditions (Okorie-Kanu et al., 2016).

Chicken contamination occurs horizontally through litter, dust, food, water as well as other contaminated equipment (Tabo et al., 2013). In the current research, *Salmonella* spp. were not detected in the meat of layer hen, which is in agreement with previous reports (EFSA, 2013; Lamas et al., 2016). The rate of *Salmonella* isolation was 28.3% and 34.37% in studies by Li et al. (2013) and Djeffal et al. (2018), respectively.

Human salmonellosis is a crucial health problem in both developed and developing countries around the world. The NTS has been the major cause of secondary bacteremia accompanied by gastroenteritis. The incidence of typhoid salmonellosis is steady, although, the prevalence of non-typhoid salmonellosis is expanding worldwide (Soltan Dallal et al., 2016). In the present study, the isolation rate of *salmonella* serovars from the worker's hand and stools were 1.4 and 4.4%, respectively. Personal hand contamination is actually a result of limited personal hygiene supplies and poor handwashing habits (Abdi et al., 2017). The results obtained in the current study were nearly similar to Sousa et al. (2013) who found that the prevalence of *Salmonella* in children was 3.2% (*S.* Typhimurium 60%, *S.* Enteritidis 20% and *S. enterica* 20%). In similar, Shaaban et al. (2018) reported *Salmonella* prevalence in humans was 5%. While Farahani et al. (2018) demonstrated a higher prevalence (7%) of *Salmonella* among the children. Furthermore, Soltan Dallal et al. (2016) declared that *S.* Enteritidis was the frequent isolate among humans.

The *S.* Gallinarum is a causative agent of fowl typhoid (Kwon et al., 2000). The *S.* Typhimurium and *S.* Enteritidis cause illness in humans, usually persist sub-clinical in layer birds (Quiun et al., 2002). In the current study, the prevalence of *Salmonella* serotypes including *S.* Enteritidis, *S.* Gallinarum, and *S.* Typhimurium was 16.6%, 14.4%, and 38.8%, respectively. While the prevalence of *Salmonella* (*S*. Typhimurium, *S*. Enteritidis) in human samples was 11.11% of each serotype. The Serotyping of a total of 206 *Salmonella* isolates by Akhtar et al. (2010) showed a high prevalence of *S.* Enteritidis among poultry (75%) and human (75.86%) samples. The prevalence rates of other serovars such as *S.* Typhimurium, *S.* Paratyphi B, *S.* Pullorum and non-typable salmonellae were less than 25% of the total isolates. Although all serotypes are considered potential human pathogens, the majority of infections are due to a very limited number of serotypes, of which *S.* Enteritidis and *S.* Typhimurium are the two most common ones integrated with the gastrointestinal disease of humans (Deng et al., 2012).

Bacterial resistance to antimicrobial drugs is one of the major risks for global public health, which develops due to many reasons such as misuse of antimicrobials (Okorie-Kanu et al., 2016). The antibiotic susceptibility test carried on *Salmonella* isolated from different samples in the present study revealed that all the isolates were multidrug-resistant to

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more than 75% of the tested antibiotics. Moreover, *Salmonella* serovars showed reduced susceptibility to streptomycin, oxytetracycline, neomycin, amoxicillin, and ampicillin. This finding is consistent with the results of Okorie-Kanu et al. (2016) who reported the resistance of *Salmonella* spp. to penicillin G, oxacillin, and tetracyclines (100%), while was found to be highly susceptible to neomycin. The resistance of *salmonella* to the erythromycin, amoxicillin, oxytetracycline was reported in previous studies (Akhtar et al., 2010; Harsha et al., 2011; Phagoo and Neetoo, 2015; Islam et al., 2016; Yizengaw 2016). The general high prevalence of resistance to these antimicrobials can also be due to the uncontrolled and extensive use of these antibiotics as mainly growth promoters since the farmers have limitless access to these agents (Adesiyun et al., 1993)**.** In addition, the uncontrolled increase in the usage of prescription antibiotics frequently purchased and used by unqualified practitioners in the veterinary and public health sectors. Also, absence of compliance and monitoring of the antimicrobial drug in developing countries and the utilization of the antimicrobial drugs at sub-therapeutic or prophylactic dosage in food animals may assist in the development and spread of antimicrobial resistance genes in *Salmonella* as well as other human and animal pathogens (Abdi et al., 2017).

The most effective drugs in the current study were kanamycin, gentamicin followed by nalidixic acid, ciprofloxacin, and chloramphenicol. This may be attributed to the very limited use of these antibiotics in the layer farms in the current period. Similarly, Abdi et al. (2017) indicated that gentamicin is still effective against *Salmonella* regardless of the time or location of the study. Ramatla et al. (2019) reported that 4% of *Salmonella* isolates were resistant to gentamicin. On the other side, Abunna et al. (2016) and Akhtar et al. (2010) were detected resistant to kanamycin, nalidixic acid, and chloramphenicol. This inconsistency may be attributed to the antimicrobial drug usage pattern in their study areas, which may be varied from that in the present study area. Antibiotic sensitivity test results presented in table 6 showed that some human isolates are 100% resistant to certain antibiotics such as neomycin and chloramphenicol, despite their limited use in the human field. This finding illustrates the pivotal role of poultry farms in transmitting the infection to humans (Shang et al., 2018). The results of the antibiotic sensitivity test suggested the guidelines for both physicians and veterinarians to select the relevant antibiotics to diminish antibiotic resistance among NTS which have industrial and public health significance. This helps prevent the development of antimicrobial resistance through mutation and acquisition of resistance encoding genes (Fluit, 2005).

CONCLUSION

The findings of the present study detected the presence of multidrug-resistant salmonellae (*S.* Enteritidis, *S.* Typhimurium, and *S.* Gallinarum) in both layer and human isolates. The multidrug-resistant salmonellae with regards to the zoonotic potential of salmonellosis could be an emerging health problem. Further research on major risk factors and molecular characterization is required to identify the genes responsible for the pathogenicity and the antimicrobial resistance in *Salmonella* spp. isolated from food animals and humans.

DECLARATION

Competing interests

The authors declare that they have no competing interests

Authors' contributions

Mohamed S. Diab, Rania Samir Zaki, and Mohamed S. Abd El Hafez conceived and designed the experiments. Mohamed S. Abd El Hafez, Rania Samir Zaki performed the experiments. Mohamed S. Abd El Hafez analyzed the data. Mohamed S. Diab and Rania Samir Zaki contributed reagents, materials, and analysis tools. Mohamed S. Diab and Rania Samir Zaki wrote the paper.

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