

ORIGINAL ARTICLE

Prevalence of Antimicrobials and Virulence-Related Genes in *Salmonellae* Detected in Local Hatcheries in Northern Upper Egypt

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Abstract

Salmonellosis represents a critical problem not only to the poultry producers owing to its economic impact, but also for poultry consumers due to its zoonotic health problems. In the present study avian *Salmonellae* were isolated from local hatcheries in 2 Egyptian governorates, Beni Suef and El Fayoum, during the period from May, 2018 until November, 2019. Samples were collected from 16 local chicken hatcheries in Beni Suef (N=12) and El Fayoum (N=4) governorates. Specimens were collected from liver of dead in shell embryos. *Salmonella* isolation was successful in 31.25% of the samples. Serotyping revealed detection of *S. kentucky*, *S. sinchew*, *S. infantis*, *S. larochelle*, and *S. colindale*. *Salmonellae* examination for antimicrobial susceptibility by disc diffusion method showed resistance to several antimicrobial drugs including [penicillin, amoxicillin, cefradine and streptomycin (no sensitivity at all) followed by apramycin and tetracyclines (25% sensitivity for each)]. On the other hand, *Salmonellae* were sensitive to enrofloxacin (100%) fosfomycin (75%) and sulphamethoxazole-trimethoprim (75%). Molecular screening of both virulence and drug resistance genes revealed the harboring of *invA*, *stn*, *avrA* virulence genes by all isolates. Phenotypic and genotypic variation in drug resistance was observed for different serovars and different classes of antimicrobial agents; *S. infantis* and *S. larochelle* were positive for PCR targeting *qnrA* gene although phenotypically they were sensitive to enrofloxacin. All of the tested serovars (except *S. colindale*) showed sensitivity to sulphamethoxazole-trimethoprim although they harbored *sul1* gene. At the same time *S. colindale* harbored *tetA* gene and it was sensitive to oxytetracycline by disc diffusion. Similar discrepancy was observed for florfenicol and some aminoglycosides.

Keywords

Antimicrobial, Hatcheries, Poultry, Prevalence, Resistance, Salmonella, Virulence Genes

1. Introduction

Beside the health hazard exemplified by *Salmonellae*, infection with *Salmonellae* is a critical problem to poultry farms due to reduced bird productivity or mortality (Talha et al., 2001; Haider et al., 2004). *Salmonella* is a Gram-negative, non-capsulated, small rod shaped, non-spore forming, and facultative anaerobic bacteria (Denise et al., 2015). *Salmonella* causes reduction in egg production, as well as reduced fertility and hatchability of infected eggs (Hameed et al., 2014). Both young and adult birds are susceptible to infection with *Salmonella* spp. (Habrun et al., 2006). The microorganism can be transmitted by horizontal

and/or vertical routes (Berchieri et al., 2001). Infected hens play an important role in the infection cycle to the chicks either directly from an infected ovary, oviduct or indirectly by egg shell contamination during egg passage through the cloaca of clinically diseased or carrier hens (Saif et al., 2008; Gantois et al., 2009). Infection of chicks post-hatching by feco-oral and nasal routes is considered another important mode of transmission. After infection chicks develop complete in-appetence, severe weakness, thirstiness, drooped wings, ruffled feathers and diarrhea especially in acute cases.

Mild respiratory distress could be also observed (Nazir et al., 2012). Damage in different organs including liver, intestine, pancreas, heart, ovary, lung, kidneys, foot pad, joints, anterior chamber of the eye and muscles of the gizzard in chicks and chickens infected salmonellosis (Johnson et al., 1992; Salem et al., 1992; Nazir et al., 2012) approves the extensive dissemination of the pathogen by the infected flocks. Shedding the pathogen into the environment and poultry byproducts constitute a great risk for human infection.

Native breeds of chickens represent a good and cheap source of protein (egg and/or meat) for human especially in villages and rural areas in developing countries. They are generally preferred to raise by household poultry producers because of their palatability as well as their tolerance to the adverse environmental conditions which are comparable to the modern poultry genotypes that require more comfortable system of housing (Pawar et al., 2016; Abioja and Abiona, 2020). Household poultry producers depend on local hatcheries as the major source of hatchlings. Fertile eggs in those low capacity hatcheries are collected from the surrounding villages and small farms. The absence of an efficient pathogen detection systems or carrier birds culling as well as the lack of biosecurity in these egg producing holdings beside the inefficient egg or hatchery cleaning before hatching allow the shedding of different pathogens especially vertically transmitted ones into the hatching day old birds. *Salmonella* increases in number during the incubation of a contaminated egg (Cason et al., 1991; Hammack et al., 1993). This will result in cross contamination between infected and non-infected eggs and chicks with subsequent higher prevalence of the disease (Bailey et al., 1994). This highlights the role of hatcheries as a *Salmonella* reservoir.

In the present study we are aiming to detect and identify *Salmonella* in local chick hatcheries in Beni Suef and El Fayoum governorates. Phenotypic screening of the antimicrobial resistance pattern and molecular detection of virulence and antimicrobial resistance genes in *Salmonella* will be performed.

2. Materials and Methods

2.1. Sampling

Samples were collected from 16 local chicken hatcheries (a single visit per hatchery) in Beni Suef (N=12) and El Fayoum (N=4) governorates, Egypt from May, 2018 through November, 2019. Specimens were collected from liver of embryos being dead in shell (10 embryos per hatchery). Samples were collected and transported in sterile tubes under cold condition for further investigation.

2.2. Isolation and Identification of *Salmonella* spp.

Salmonella isolation was accomplished according to Collee et al., (1996). Samples were pre-enriched in buffered peptone water (1:10 dilution) at 37°C/18 hr. 100µl were then mixed with 10 ml of the Rappaport Vassiliadis (RV) broth (Oxoid, United Kingdom) and incubated at 41.5°C/24 hr. A loopfull was then cultivated onto xylose lysine deoxycholate (XLD) agar medium (supplemented with novobiocin at the rate of 50µg/100 ml agar), *Salmonella Shigella* (SS) agar medium (Oxoid, United Kingdom) and brilliant green (BG) agar medium (Oxoid, United Kingdom), then the inoculated plates were incubated aerobically at 37°C for 24 h. From each plate one of the suspicious colonies of typical appearance for *Salmonella* was picked up for further purification on tryptone soya agar (TSA) (Oxoid, United Kingdom). Biochemical identification of the isolates was done according to Collee et al. (1996); Quinn et al. (2002).

2.3. Serological Identification

Salmonella serotyping was carried out according to White Kauffmann Leminor scheme as described by Grimont and Weill (2007). The first screening was conducted using polyvalent O antisera. Positive strains were further examined with poly H (phase 1 and 2) antisera.

2.4. Antimicrobial Susceptibility

It was done on Mueller Hinton agar (Oxoid, United Kingdom) following the instruction of CLSI (2019). 17 discs (Oxoid, United Kingdom) (refer to table 3) were used. The diameters of the inhibition zones was measured and evaluated according to CLSI (2019). The lack of sensitivity to more than two groups of antibiotics was used as an evidence of multidrug resistance (MDR) (Magiorakos et al., 2012).

2.5. Molecular Detection of Antimicrobial Resistance and Virulence Associated Genes in Recovered *Salmonellae*

Polymerase chain reaction was performed to detect *avrA*, *stn* and *invA* virulence genes as well as different antimicrobial associated genes. DNA was extracted using QIAamp DNA mini kit (Qiagen, Germany). Primers used for each gene are shown in Table (1). Each reaction was prepared by mixing 12.5ul Emerald Amp GT PCR mastermix (Takara Bio Inc., Japan), 4.5ul PCR grade water, 1ul forward primer, 1ul reverse primer and 6ul template DNA. Thermal profile followed in PCR included one cycle of initial denaturation at 94°C/5 min. followed by 35 cycles of amplification each cycle included a secondary denaturation step at 94°C/30 sec., an annealing step (refer to table 1) for 40 sec. and an extension step at 72°C/45 sec. and the last cycle was the final extension at 72°C/10 min. PCR products were then separated by electrophoresis in 1.5% agarose.

Table 1. Oligonucleotide primers sequences

Primer	Sequence	Amplified product	Annealing temperature	Reference
<i>bla_{TEM}</i>	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTTC	516 bp	54°C	Colom et al., 2003
<i>TetA</i>	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	50°C	Randall et al., 2004
<i>aadA1</i>	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484 bp	54°C	
<i>Sul1</i>	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	60°C	Ibekwe et al., 2011
<i>QnrA</i>	ATTTCTCACGCCAGGATTTG GATCGGCAAAGTTAGGTCA	516 bp	55°C	Robicsek et al., 2006
<i>FloR</i>	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494 bp	50°C	Doublet et al., 2003
<i>Stn</i>	TTGTGTCGTATCACTGGCAACC ATTCGTAACCCGCTCTCGTCC	617 bp	59°C	Murugkar et al., 2003
<i>InvA</i>	GTGAAATTATCGCCACGTTCCGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	55°C	Oliveira et al., 2003
<i>AvrA</i>	CCTGTATTGTTGAGCGTCTGG AGAAGAGCTTCGTTG AATGTCC	422 bp	58°C	Huehn et al., 2010

3. Results

3.1. Isolation and Biochemical Identification of Salmonella: Suspected Colonies for Salmonella biovars

Appeared on XLD agar as red colonies with black centers, on SS agar appeared as yellow with black colored centers and on BG agar appeared as red colonies. Microscopical examination of Gram's stained smears of the suspected

colonies showed Gram negative short bacilli. *Salmonella* isolates showed positivity to citrate utilization, methyl red, and hydrogen sulphide production in TSI. On the other hand, they were negative for indole production, Voges- Proskauer, lactose fermentation and urea hydrolysis tests.



Fig.1. Characteristic *Salmonella* colonies on SS agar (left) and XLD agar (right). *Salmonella* colonies appear yellow with black center on SS agar and appear black on XLD agar.

3.2. Isolation rates

From a total of 16 hatcheries 5 *Salmonella* isolates were obtained (31.25% detection rate) (Table, 2). Two isolates were obtained from four hatcheries tested at El Fayoum (50%) and 3 isolates out of 12 hatcheries from Beni Suef (25%). Serotyping of different isolates revealed the following serovars; *S. larochelle* 6,7:e,h:1,2 and *S. infantis* 6,7,1y:r:1,5 for isolates from Fayoum governorate while isolates from Beni Suef governorate were *S. sinchew* 3,10:1,v:z35, *S. kentucky* 8,20:i:z 6 and *S. colindale* 6,7:r:1,7.

3.3. Antimicrobial susceptibility profile

Data in (Table, 3) demonstrate the antimicrobial discs, concentration and results of antimicrobial disc diffusion test applied on different *Salmonella* serovars. Summary of molecular detection of resistance and virulence-related genes among different serovars is shown in (Table, 4).

Table 2. Summary of isolation of *Salmonella* from different examined hatcheries

	Location	
	Fayoum	Beni-Suef
Number of hatcheries	4	12
Number of isolates	2 (50%)	3 (25%)
Serovars	<i>S. larochelle</i> 6,7:e,h:1,2 <i>S. infantis</i> 6,7,1y : r: 1,5	<i>S. sinchew</i> 3,10:1,v:z35 <i>S. kentucky</i> 8,20:i:z 6 <i>S. colindale</i> 6,7:r:1,7.

Table 3. Antimicrobial discs, concentration and interpretation of the results of antimicrobial susceptibility among different *Salmonella* serovars.

Antimicrobial agent	Code	Conc. (µg)	<i>Salmonella</i> serovars					No of bacterial isolates			
			<i>S. larochelle</i>	<i>S. sinchew</i>	<i>S. infantis</i>	<i>S. kentucky</i>	<i>S. colindale</i>	Resistant	Intermediate	Sensitive	Total
Fosfomycin	Fos	50	S	S	S	I	S	0	1	4	5
Penicillin	P	10	R	R	R	R	R	5	0	0	5
Amoxicillin	AML	10	R	R	R	R	R	5	0	0	5
Amox+clavulnic	AMC	30	S	R	S	R	I	2	1	2	5
Cefradine	CE	30	R	R	R	R	R	5	0	0	5
Oxytetracycline	OT	30	R	R	R	R	S	4	0	1	5
Gentamycin	CN	10	R	S	R	R	S	3	0	2	5
Streptomycin	S	10	R	R	R	R	R	5	0	0	5
Kanamycin	K	30	R	I	R	S	S	2	1	2	5
Neomycin	N	30	I	I	R	I	S	1	3	1	5
Apramycin	APR	15	R	R	R	R	I	4	1	0	5
Sulfa+trimethoprim	SxT	25	S	S	S	S	R	1	0	4	5
Ciprofloxacin	Cip	5	I	I	R	R	S	2	2	1	5
Enrofloxacin	ENR	5	S	S	S	S	S	0	0	5	5
Choramphenicol	C	30	R	S	R	R	S	3	0	2	5
Florfenicol	FFC	30	R	S	R	S	S	2	0	3	5
Polymyxin- B	PB	300	S	S	S	R	R	2	0	3	5
MDR bacteria			Yes	Yes	Yes	Yes	Yes				

Sulfa+trimethoprim: sulphamethoxazole-trimethoprim, S: mean sensitive, R: mean resistant, I: intermediate sensitivity, MDR: mean multi drug resistant, Yes: means that the microorganism is resistant to ≥ 3 different classes of antimicrobial agents.

Table 4. Summary of molecular detection of antimicrobial resistance and virulence associated genes among different *Salmonella* serovars.

Sample	<i>bla</i> _{TEM}	<i>tetA</i>	<i>aadA1</i>	<i>sul1</i>	<i>qnrA</i>	<i>floR</i>	<i>invA</i>	<i>stn</i>	<i>avrA</i>
<i>S. larochelle</i>	+	+	+	+	+	+	+	+	+
<i>S. sinchew</i>	+	+	-	+	-	-	+	+	+
<i>S. infantis</i>	+	+	-	+	+	-	+	+	+
<i>S. kentucky</i>	+	-	+	+	-	+	+	+	+
<i>S. colindale</i>	+	+	-	+	-	-	+	+	+

4. Discussion

Salmonella is a critical food borne pathogen representing a risk to humans and poultry producers (Lynne et al., 2009). Subclinical *Salmonella* infections in healthy carriers allows easy transmission of the pathogen not only from bird to bird but also from farm to farm with subsequent dissemination to table eggs or poultry meat (Hugas and Beloeil, 2014). In poultry production, the hatchery is linking the breeder flock and hatchlings thus; contaminated hatcheries have a vital role in disease epidemiology (Wideman, 2016). In the present study 5 *Salmonella* isolates were obtained from the 16 local hatcheries in Beni Suef and El Fayoum governorates. *Salmonella* isolation was successfully performed by pre-enrichment on BPW then enrichment on RV broth at 41.5°C. XLD agar medium supplemented with novobiocin at the rate of 50µg/100 ml agar was used. Five isolates were obtained out of 16 tested hatcheries in both governorates (31.3% positivity) which differs than those reported by Basuony et al., (2022) who detected *Salmonella* infection in 2/3 hatcheries (2 out of 150

egg batches and 40 eggs). In the current study we targeted dead in shell embryos in which the rate of bacterial infection is high. Also the use of Novobiocin improves the efficiency of culturing *Salmonella* (Hoben et al., 1973; Restaino et al., 1977; Moats, 1978; Komatsu and Restaino, 1981). Serotyping revealed the presence of *S. infantis*, *S. kentucky*, *S. larochelle*, *S. sinchew*, *S. colindale* in the positive hatcheries. High prevalence of *S. infantis* and *S. kentucky* was reported by many authors (Barua et al., 2013; Nabil, 2015; Andoh et al., 2016). El-Sheikh, (2019) showed the isolation of seven *Salmonella* serovars from chickens; *S. enteritidis*, *S. infantis*, *S. newlands*, *S. kentucky*, *S. weybridge*, *S. naestved*, and *S. ferruch*. Sharma et al., (2019) demonstrated that *S. kentucky*, *S. virchow*, and *S. typhimurium* are more prevalent. Although the prevalent serovars differ according to the geographic locations, it seems that certain serovars of *Salmonella* are of higher prevalence in poultry compared to live stock animals (Hegazy, 2002; Foley et al., 2011; Djeflal et al., 2018). The absence of an efficient pathogen detection systems or carrier birds culling as well as the lack of

biosecurity and low levels of hatchery hygiene in local hatcheries allow further spread of different pathogens (Cason et al., 1991; Hammack et al., 1993).

Isolation of *S. infantis* highlights the risk of hatcheries in infection as possible source of infection to human. *S. infantis* is one of the main causes of gastroenteritis in human worldwide (Najjar et al., 2012). Most *S. enterica* serotypes can cause self-limiting gastroenteritis but non treated infections can be life threatening (Rhoades et al., 2009).

Improper use of some antibiotics in poultry and animal farms is threatening the human health (WHO, 2016). Therefore the antibiotics should be selected properly on phenotypic or genotypic bases (Seifi et al., 2015; Radwan et al., 2016; Khaltabadi Farahani et al., 2018). In the present study, 100% of tested isolates (n=5) although of being sensitive to some antimicrobials, they have MDR phenomena. They showed high sensitivity to enrofloxacin (100%) fosfomycin (75%) and sulphamethoxazole-trimethoprim (75%). The least effective antimicrobials against the tested *Salmonella* serovars were penicillin, amoxicillin, cefradine and streptomycin (no sensitivity at all) followed by apramycin and tetracyclines (25% sensitivity for each). These results come in accordance with previous reports (Radwan et al., 2016; Asif et al., 2017; Hassan et al., 2018; Uddin and Ahn 2018). In our study, MDR pattern in 100% of the isolates which is more or less similar to the findings of Radwan et al., (2016); Hassan et al., (2018) reveals the bad infection control practices within the poultry farms and the surrounding environment. Antimicrobial resistance is uncommon among *Salmonellae* obtained from poultry hatchling although Habing et al. (2015) detected resistance in 19% of the isolates to more than one group of antimicrobials. The haphazard application of antimicrobial medication in different farms specially the traditional and well known antimicrobials as beta lactams and aminoglycosides has resulted in higher rates of drug resistance. The study area targeted local hatcheries where bad hygiene is common. Also samples were collected from dead in shell embryos (the late stage of hatching) where the humidity, temperature and the organic matters are available allowing the replication of bacterial members *Enterobacteriaceae* even non-pathogenic ones.

The correlation of MDR phenomena to the presence of mobile genetic element in *Salmonella* (Dhanani et al., 2015; Radwan et al., 2020) could explain the variations in phenotypic and genotypic pattern of drug resistance in our study. Phenotypic and genotypic variation in drug resistance was observed for different serovars and different classes of antimicrobial agents; *S. infantis* and *S. larochelle* were positive for PCR targeting *qnrA* gene although phenotypically they were sensitive to enrofloxacin. All of the tested serovars (except *S. colindale*) showed sensitivity to sulphamethoxazole-trimethoprim although they harbored *sulI* gene. In the same time *S. colindale* harbored *tetA* gene and it was sensitive to oxytetracycline by disc diffusion. Similar discrepancy was observed for florfenicol and some aminoglycosides. The presence of clavulanic acid and

trimethoprim in combination with amoxicillin and sulphonamide, respectively could enhance the efficacy of the antimicrobials and reduce resistance of the target bacteria. The variation between the phenotypic and genotypic resistance pattern for quinolones among *S. infantis* and *S. larochelle* in the current study could be explained by the presence of 3 different ways for quinolone resistance (Lunn et al., 2010). At the same time *S. colindale* harbored *tetA* gene and they were sensitive to oxytetracycline by disc diffusion. Similar discrepancy was observed for florfenicol and some aminoglycosides. Tetracycline resistance is mediated by *tetA* as well as *tetB* genes and this explains the possible variation in genotypic and phenotypic pattern of resistance to tetracycline (Adesiji et al., 2014).

In the current work, the results illustrated in Table (4) revealed that all the tested *Salmonella* isolates harbored *invA*, *stn* and *avrA* genes which come in accordance with Ammar et al., (2016). Although the enterotoxin (*stn*) is a diarrheic mediator in *Salmonella* (Chopra et al., 1999; Huehn et al., 2010; Osman et al., 2010; Thung et al., 2018), the ability of *stn* deletion mutant *Salmonella* to maintain its virulence led some researchers to consider *stn* as non-virulence factor (Nakano et al., 2012). In contrast, inactivation of this gene reduced the accumulation of the intra-intestinal fluids after infection with *Salmonella* Typhimurium (Chopra et al., 1999).

5. Conclusion

In the present study high rate of prevalence of *Salmonella* and the presence of multidrug resistance were observed. Improvement of hatchery hygiene, application of an efficient pathogen detection and disease control strategies as well as the proper application of antimicrobial agents and the research for novel antimicrobial agents would improve *Salmonella* control.

6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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