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Prevalence of Salmonellosis in Broilers and Native Breed Chicks in Beni Suef Governorate, Egypt

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Abstract

Salmonellosis is an economic burden on poultry producers' shoulders and also, it is a very critical problem to human health. Resistance of non-typhoidal *Salmonellae* to antimicrobials is a serious problem. In the current study, prevalence of avian salmonellosis in both commercial broiler and native breed farms in Beni Suef from March, 2018 through October, 2019 were investigated. Samples were collected from 31 chicken farms including broilers (N=13) and native breed (N=18). Specimens were collected from liver and caecum (5 chicks per flock) of clinically suspect cases aging 1-15 days old. Bacteriological examination revealed that 6 isolates (19.35% isolation rate) were successfully cultivated including 2/13 (15.38%) and 4/18 (22.22%) isolates for broilers and native breed farms, respectively. Serotyping of different isolates revealed the following serovars; *S. kentucky 8,20:i:z 6* in 4 farms (1 broiler and 3 native breed), *S. bonariensis 6,8:i:e,n,x* in one broiler farm and *S. rechovot 8,20:e,h:z6* in one native breed flock. Antimicrobial sensitivity testing of different *Salmonella* serovars showed 100% sensitivity to fosfomycin, 66.6% to amoxicillin/clavulinic acid, 66.6% to florfenicol, 66.6% to polymyxin- B, 33.3% to gentamycin and 16.66% sensitivity to each of kanamycin, neomycin, apramycin and enrofloxacin. *S. bonarensis* was found resistant to amoxicillin/clavulinic acid and of intermediate sensitivity to ciprofloxacin. Molecular screening of different serovars to virulence and drug resistance genes indicated 100% positivity to *invA, stn, avrA* virulence genes and also the tested antimicrobial genes except *qnrA* that was detected in only one of the tested *Salmonellae.*

Keywords

Broilers, Chicks, Native breeds, Prevalence, *Salmonella*, Resistance, Virulence Genes

1. Introduction

Salmonellosis represents an acute or chronic infection of poultry **(Osman et al., 2010; Foley et al., 2011).** Some *Salmonella* serovars may be found more frequently in particular areas of the world **(Capita et al., 2003; Liljebjelke et al., 2005).** Poultry is considered the primary reservoirs of *Salmonella* which represents an important pathogen for human and different animals as well. It can colonize the intestinal tract of mammals and reptiles beside birds which are considered the major sources of *Salmonella.* Infections of poultry with *Salmonella* is associated with severe economic losses due to reduced productivity and mortalities **(Talha et al., 2001; Haider et al., 2004)** as well as the increased costs of treatment and prevention programs. Salmonellosis can cause multi-organ systemic infection **(Ezzeldeen et al., 2013).** Thousands of *Salmonella* serovars do exist **(Grimont and Weill, 2007; Gallegos et al., 2008),** many of them have a zoonotic potential with some reports about higher prevalence of *Salmonella* serovars in poultry **(Foley et al., 2011)** than other species.

Native breeds of chickens represent a cheap and more palatable source of egg and meat for human especially in developing countries because native breeds of slow growing

chickens are more tolerant to the adverse environmental conditions than the fast growing modern poultry genotypes **(Pawar et al., 2016; Abioja and Abiona, 2020).** Household raising of poultry lacks many of the biosecurity standards; rearing of mixed species of birds of multiple ages in open or semi-closed systems all of which enhance the spread of different pathogens. Native chickens holders depend local hatcheries as the main source of one-day-old chicks where fertile eggs are collected from the surrounding villages and small farms. The absence of an efficient pathogen detection system 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 dissemination of different pathogens especially vertically transmitted ones into the hatching day old birds. During the incubation of infected eggs, the number of *Salmonella* within the egg increases with subsequent increased disease prevalence in the co-hatched progeny **(Cason et al., 1991; Hammack et al., 1993).** Infected eggs are a major source of infection in the hatchery which can serve as mechanical mean of transmission of pathogens as they facilitate cross-contamination which dramatically increases the numbers of *Salmonella*-infected chicks leaving that hatchery even with lower number of infected eggs before hatching **(Bailey et al., 1994).**

Bird immunity and nature of infecting serotype play a key role in the clinical presentation of the disease **(Jones et al., 2008).** *Salmonellae* harbor a variety virulence genes, some are located in plasmids and other are encoded by the *Salmonella* pathogenicity islands (SPI) **(Heithoff et al., 2008).** Clinical presentation of the disease is controlled by a group of virulence genes associated with adhesion and invasion **(Oliveira et al., 2003; Chuanchuen et al., 2010, Das et al., 2012).** Some of the virulence genes can help *Salmonellae* in evading the host adaptive immune system **(Wu et al., 2011).** *Salmonella* enterotoxin (*stn*) has been known as a mediator of diarrhea **(Chopra et al., 1999).** The role of *stn* as a virulence gene of *Salmonellae* is controversial; **Nakano et al. (2012)** considered *stn* as non-virulence gene while a previous report by **Chopra et al. (1999)** has linked *stn* with *Salmonella* virulence where *stn* gene inactivation reduces the ability of *S. Typhimurium* in accumulating fluids within the intestinal lumen. It is worth mentioning that many of the virulence determinants of *Salmonella* are transmissible **(Switt et al., 2012).**

The faulty application of medications in developing countries result in a rise of drug resistance **(Badr et al., 2015; Velhner et al., 2018)** which is very critical to human health due to drug residues in poultry meat and eggs as well as dissemination of drug resistant serotypes from poultry farms to poultry handlers either directly or indirectly.

This work was aimed to investigate the prevalent *Salmonella* species among poultry (broiler and native breed farms) in Beni Suef governorate during 2018 followed by investigation of the isolated *Salmonellae* for their virulence and antimicrobial sensitivity on phenotypic and genotypic bases.

2. Materials and Methods

2.1. Sampling

Samples from 31 chicken farms have been collected including broilers (N=13) and native breed farms (N=18) in Beni Suef governorate from March, 2018 through October, 2019. Specimens from liver and caecum (5 chicks per flock) of clinically suspect cases aging 1-15 days were collected. Samples were collected in sterile tubes and transported on ice with minimum delay for further examination.

2.2. Isolation of *Salmonella spp.*

Isolation of *Salmonella* was done through pre-enrichment in buffered peptone water (1:10 dilution) and aerobic incubation at 37°C/18h. To each 10ml of Rappaport Vassiliadis (RV) broth *(Oxoid, United Kingdom)* 100µl *of* the pre-enriched media were transferred and then incubated at 41.5°C/24h. A loopfull from the enriched medium was then streaked onto xylose lysine deoxycholate (XLD) agar medium *(Oxoid, United Kingdom)* to which novobiocin was added at the rate of 50µg/100 ml followed by incubation at 37ºC/24h. Suspected *Salmonella* colonies were subjected to purification on tryptone soya agar (TSA) *(Oxoid, United Kingdom)*. Biochemical identification of the isolates was applied **(Collee et al., 1996; Quinn et al., 2002).**

2.3. Serological Identification of *Salmon-ella spp.*

Serotyping of *Salmonella* was performed according to the White Kauffmann Leminor scheme **(Grimont and Weill, 2007)** at the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt.

2.4. Antimicrobial Susceptibility Testing of the Recovered Salmonella Serovars

Antimicrobial disc diffusion technique was adopted using on Mueller Hinton agar *(Oxoid, United Kingdom)* according to **(CLSI, 2019).** Seventeen antimicrobial agents were tested (as illustrated in table 3; refer to the results section). Diameters of the zones of growth inhibition were interpreted **(CLSI, 2019).** Resistance to ≥3 different classes of antimicrobials was used as evidence of multidrug resistance (MDR) **(Magiorakos et al., 2012).**

2.5. Molecular Detection of Antimicrobial Resistance and Virulence Associated Genes in Recovered Salmonella Isolates

Polymerase chain reaction was done on 5 selected *Salmonella* serovars for detection of six antimicrobial resistance genes *(bla_{TEM}, tetA, aadA1, Sul1, qnrA and floR)* and three virulence genes *(invA, stn, avrA)*. DNA was extracted according to QIAamp DNA mini kit (*Qiagen, Germany*) instruction. Sequences of the used oligonucleotide primers and amplified product size are shown in **Table (1).** PCR was performed in 25µl reactions; each reaction contained 12.5ul Emerald Amp GT PCR mastermix *(Takara Bio Inc., Japan)*, 4.5ul nuclease free water, 1ul forward

primer, 1ul reverse primer and 6ul DNA. Thermal profile followed in PCR included one cycle of initial denaturation at 94˚C/5 min., 35 cycles of amplification [each cycle included a secondary denaturation step at 94˚C/30 sec., an annealing step shown in **Table (1)** for 40sec. and an extension step at 72˚C/45 sec.] and a final extension step at 72˚C/10min. Electrophoresis in 1.5% agarose and visualization of amplified products were performed using UV illuminator.

Table 1. Oligonucleotide primers sequences

Primer	Sequence	Amplified product	Annealing temperature	Reference
bla$_{TEM}$	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	54˚C	Colom et al., 2003
tetA	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	50˚C	Randall et al., 2004
*aadA*1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484 bp	54˚C	
*sul*1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	60˚C	Ibekwe et al., 2011
qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516 bp	55˚C	Robicsek et al., 2006
floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494 bp	50˚C	Doublet et al., 2003
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	55˚C	Oliveira et al., 2003
atn	TTGTGTCGCTATCACTGGCAACC ATTCGTAACCCGCTCTCGTCC	617 bp	59˚C	Murugkar et al., 2003
avrA	CCTGTATTGTTGAGCGTCTGG AGAAGAGCTTCGTTG AATGTCC	422 bp	58˚C	Huehn et al. 2010

3. Results

3.1. Clinical Picture

Clinically the chicks in the infected flocks suffered from pasty vents, dehydration, recumbence, subnormal growth relate and variable mortalities **(Table, 2).** Grossly, dehydrated carcass, hepatic necrosis **(Fig. 1),** nephritis, distended ureters and white colored cecal contents have been observed.

3.2. Isolation and Biochemical Identifica-tion of Salmonella

Suspected colonies for *Salmonella* biovars appeared on XLD agar as red colonies with black centers **(Fig. 2),** on SS agar appeared as yellow colonies with black 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. Isolates showed positivity for 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.

3.3. Isolation Rates

Form a total of 31 chicken farms including broilers (N=13) and native breed (N=18) in Beni-Suef governorate 6 *Salmonellae* (19.35% isolation rate) were successfully isolated including 2/13 (15.38%) and 4/18 (22.22%) isolates for broilers and native breed farms, respectively. Serotyping of different isolates revealed the following serovars; *S. kentucky 8,20:i:z 6* in 4 farms (1 broiler and 3 native breed), *S. bonariensis 6,8:i:e,n,x* in one broiler farm and *S. rechovot 8,20:e,h:z6* in one native breed flock. Negative *Salmonella* isolation was evident in 25 farms representing 80.65% of the examined farms. Average percentage of daily mortality in broiler farms were (0.71%) and (0.21%) for *S. kentucky* and *S. bonariensis*, respectively. In native chicken breed farms the average percentage of daily mortality was 0.32% for *S. rechovot*, while for *S. Kentucky* the average percentages of daily mortality ranged from 0.22% to 1.43% **(Table, 2).**

3.4. Antimicrobial Susceptibility Profile

Data in **(Table, 3)** show the antimicrobial discs and their concentrations. Results of disc diffusion test applied on different *Salmonella* serovars are shown in **Table (3), Figs. (3, 4).** Summary of molecular detection of virulence and resistance associated genes among different serovars is shown in **Table (4).** All *Salmonella* proved to be sensitive to fosfomycin (100%), amoxicillin/clavulinic acid (66.6%), florfenicol (66.6%), polymyxin- B (66.6%), gentamycin (33.3%), kanamycin (16.66%), neomycin (16.66%), apramycin (16.66%) and enrofloxacin (16.66%). *S. bonarensis* was found resistant to amoxicillin + clavulinic acid and of intermediate sensitivity to ciprofloxacin compared to other tested *Salomonellae*. Moreover, one of the tested *S. kentucky* showed higher sensitivity to aminoglycosides comparable to other tested *Salmonellae*.

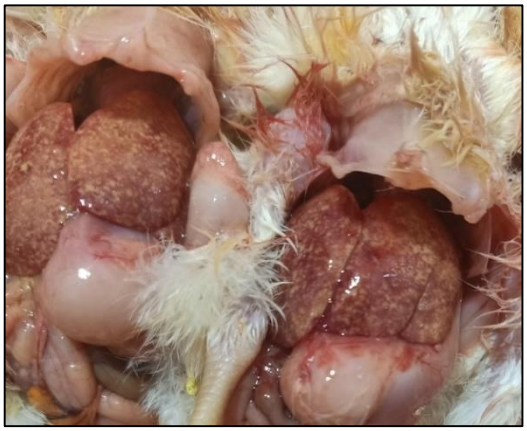

Fig.1. Hepatic necrosis in chicks infected with *Salmonella*.

Fig.2. Characteristic *Salmonella* colonies on XLD Agar

Table 2. Clinical data of the chicken flocks where positive *Salmonella* isolation was evident.

	Age	Total number of birds	Total mortality Number (%)	Average daily mortality Number (%)	*Salmonella* serovar
Broiler	3 days	16500	350 (2.12%)	117 (0.71%)	*S. kentucky 8,20:i:z 6*
	5 days	9000	93 (1.03%)	19 (0.21%)	*S. bonariensis* 6,8:i:e,n,x
Native breed	9 days	6500	125 (1.92%)	14 (0.22%)	*S. kentucky 8,20:i:z 6*
	6 days	2800	240 (8.57%)	40 (1.43%)	*S. kentucky 8,20:i:z 6*
	8 days	7500	195 (2.6%)	24 (0.32%)	*S. rechovot* 8,20:e,h:z6
	3 days	4500	145 (3.22%)	48 (1.07%)	*S. kentucky 8,20:i:z 6*

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

										No of bacterial isolates			
		Code	Conc. (µg)	*S. kentucky*	*S. bonariensis*	*S. kentucky*	*S. kentucky*	*S. rechovot*	*S. kentucky*	Resistant	Intermediate	Sensitive	Total
Antimicrobial agent	Fosfomycin	Fos	50	S	S	S	S	S	S	0	0	6	6
	Penicillin	P	10	R	R	R	R	R	R	6	0	0	6
	Amoxicillin	AML	10	R	R	R	R	R	I	5	1	0	6
	Amox/clavulnic	AMC	30	S	R	S	S	S	I	1	1	4	6
	Cefradine	CE	30	R	R	R	R	R	R	6	0	0	6
	Oxytetracycline	OT	30	R	R	R	R	R	R	6	0	0	6
	Gentamycin	CN	10	I	R	R	S	S	I	2	2	2	6
	Streptomycin	S	10	R	R	R	R	R	R	6	0	0	6
	Kanamycin	K	30	R	R	R	R	R	S	5	0	1	6
	Neomycin	N	30	R	R	R	R	R	S	5	0	1	6
	Apramycin	APR	15	R	R	R	R	I	S	4	1	1	6
	Sulfa+trimethoprime	SxT	25	R	R	R	R	R	R	6	0	0	6
	Ciprofloxacin	Cip	5	R	I	R	R	R	R	5	1	0	6
	Enrofloxacin	ENR	5	R	I	I	R	R	S	3	2	1	6
	Choramphenicol	C	30	R	R	R	R	R	I	5	1	0	6
	Florfenicol	FFC	30	S	R	R	S	S	S	2	0	4	6
	Polymyxin- B	PB	300	R	S	R	S	S	S	2	0	4	6
MDR bacteria				Yes	Yes	Yes	Yes	Yes	No				

Sulfa+trimethoprime: sulphamethoxazole-trimethoprime, 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, No: means that the microorganism is resistant to < 3 different classes of antimicrobial agents.

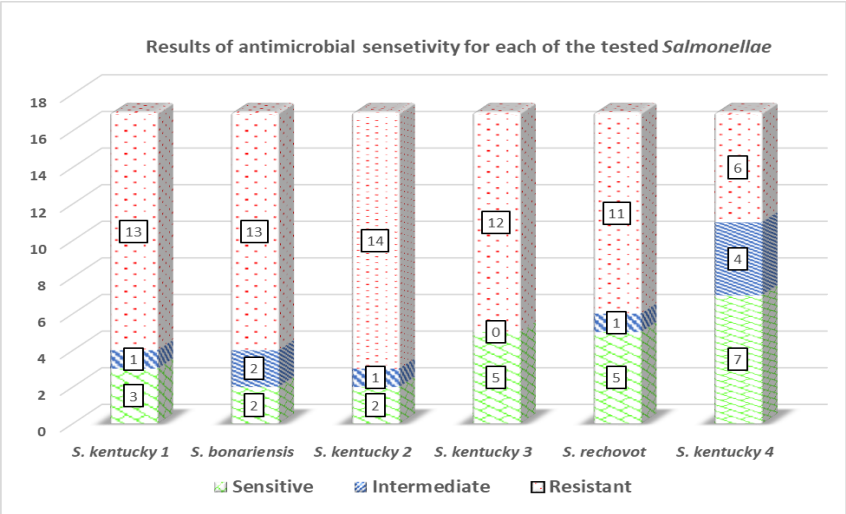

Fig.3. Results of antimicrobial sensitivity for each of the tested *Salmonellae*

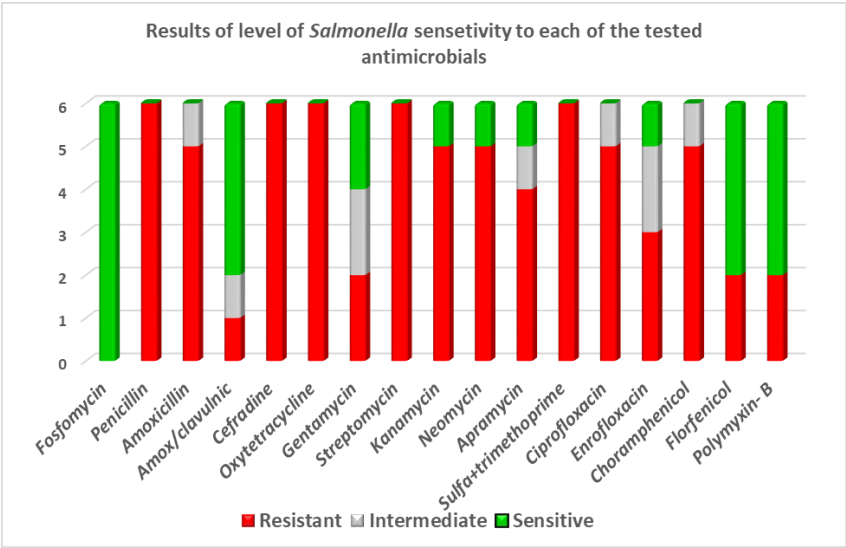

Fig.4. Results of level of *Salmonella* sensitivity to each of tested antimicrobials

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

Sample	*blaTEM*	*tetA*	*aada1*	*sul1*	*qnrA*	*floR*	*invA*	*stn*	*avrA*
S.kentucky	+	+	+	+	+	+	+	+	+
S.bonariensis	+	+	+	+	-	+	+	+	+
S.kentucky	+	+	+	+	-	+	+	+	+
S.kentucky	+	+	+	+	-	+	+	+	+
S.rechovot	+	+	+	+	-	+	+	+	+

4. Discussion

Salmonellae have the ability to cause systemic infection in chicks and adult birds followed by long period of dissemination of the microorganism in the environment **(Pedersen et al., 2002)**.

In the present study 6 *Salmonella* isolates were successfully detected in samples collected from suspect cases from the 31 broiler and native breed chicken farms in Beni Suef governorates. *Salmonella* isolation was performed by pre-enrichment on BPW then enrichment on RV broth at 41.5°C followed by plating on XLD agar medium supplemented with novobiocin at the rate of 50µg/100 ml agar was used. 19.35% total isolation rate were evident. For broilers and native breed farms, 2/13 (15.4%) and 4/18 (22.22%) isolates were detected, respectively. These results come in accordance with a previous report where 353 samples from different broiler farms at Al-Wady El-Jaded governorate were examined for the presence of *Salmonella* species. Fifty-five (15.6%) of the examined samples were found positive **(Fekry et al., 2018).** This is also nearly similar to other results obtained by **Rehan, (2004); Mohamed, (2015)** where in Dakahlia and Damietta governorates, *Salmonella* species were recovered with an isolation rates of 12 % and 12.4%, respectively. Meanwhile, our results are higher than 9.2% and 3.4% isolation rates that were previously reported by **AL-Hakeem (2003) and AL-Abadi and Al-Mayah, (2011).**

Clinically the chicks in the infected flocks suffered from pasty vents, dehydration, recumbence, subnormal growth relate and variable mortalities. Grossly, dehydrated carcass, hepatic necrosis, nephritis, distended ureters and white colored cecal contents have been observed. Similar findings were previously reported **(Dhillon et al., 2001; Kogut et al.,**

2003: Deng et al., 2008). Average percentage of daily mortality in broiler farms ranged from 0.21% to 1.43% with higher rates of mortalities were observed in flocks infected with *S. kentucky* especially in native breed chicks. It was reported that in clinical cases of salmonellosis in poultry, some variations in the clinical manifestations, mortality rates, fecal shedding and frequency of egg bacterial contamination are obvious **(Suzuki, 1994).** Lack of biosecurity in local hatcheries and poor brooding conditions could played a role in increased level of contamination of day old chicks and enhanced *Salmonella* infection virulence in native breed farms compared to commercial broilers **(Bailey et al., 1994; Kim et al., 2007; Ha et al., 2018).**

In our study, we detected *S. kentucky* (66.66%), *S. bonariensis* (16.66%) and *S. rechovot* (66.6%) in the sensitivity to fosfomycin (100%), amoxicillin/clavulinic acid (66.6%), florfenicol (66.6%), polymyxin- B (66.6%), gentamycin (33.3%), kanamycin (16.66%), neomycin (16.66%), apramycin (16.66%) and enrofloxacin (16.66%). *S. bonarensis* was found resistant to amoxicillin/clavulinic acid and of intermediate sensitivity to ciprofloxacin compared to other tested *Salomonellae*. Moreover, one of the tested *S. kentucky* showed higher sensitivity to aminoglycosides comparable to other tested *Salmonellae*. MDR phenomena was evident in 83.3% (5 out of 6) of the tested *Salmonellae* highlighting difficulty of treating infections caused by these pathogens **(Bhowmick et al., 2009).** Comparable results have been reported by **Mammina et al., (2002); Antunes et al., (2006); Frye and Fedorka-Cray, (2007) and Ahmed et al., (2009)** in Italy, Portugal, USA and Egypt, respectively. In our study, the MDR *Salmonella* isolates were further tested for their antimicrobial susceptibility to different antimicrobial agents by PCR. Interestingly, all of the tested *Salmonellae* harbored all of the tested genes except for *qnrA* that has been found lacking in all except one *S. kentucky* isolate. On genetic basis 100% of the tested *Salmonellae* are MDR although on phenotypic basis 1 out of 6 *Salmonellae* lacked the MDR phenomena. Variations in phenotypic and genotypic pattern of drug resistance could be explained by the carriage of multidrug efflux pump systems by *Salmonella* regardless their antibiotic susceptibility profile as previously reported **Dhanani et al., (2015).** The variation between the phenotypic and genotypic resistance profile for quinolones and the *qnrA* gene for one *S. kentucky* isolate in our is acceptable in the light of quinolone resistance is being mediated in three different ways including plasmid-mediated resistance genes, mutations in the quinolone resistance determining regions as well as the overexpression of efflux pumps mediated by qepA genes **(Lunn et al., 2010).**

The severity of the clinical disease is controlled by the microbial ability of production of some virulence determinants encoded by various genes. Virulence genes in *Salmonella* spp. are either chromosomal or plasmid related **(Oliveira et al. 2003).** Virulence determinants can allow the bacteria to be more virulent through adhesion, invasion, and enterotoxin production **(Oliveira et al. 2003; Chuanchuen et al. 2010, Das et al. 2012).** In our study, PCR was applied *Salmonella* positive farms similar to a previous report **(Abd El- Tawab et al., 2015). (Fekry et al., (2018)** reported wide variation in the prevalence of different *Salmonella* serotypes in broilers [*S. enteritidis* (43.3%) *S. infantis* and *S. kentucky* (16.6%), *S. maloma* and *S. bardo* (6.7%), *S. gdansk*, *S. typhimurium* and *S. blegdame* (3.3%)]. Also **Hegazy (2002),** out of 26 *Salmonella* isolates, found 50%, 30.8% and 19.2% detection rates for *S. enteritidis*, *S. typhimurium* and *S. kentuky*, respectively.

Although the beneficial effect of some antibiotics, the sub-therapeutic doses of these antibiotics may lead to the appearance of drug resistance **(Bogomazova et al., 2020; Radwan et al., 2020).** Surveillance data showed an obvious increase in *Salmonella* antimicrobial resistance **(Su et al. 2004).** In the current study, all *Salmonella* serovars showed on five MDR and one non-MDR *Salmonellae* to detect 3 different virulence related genes. The results revealed that all of the tested *Salmonellae* harbored *invA*, *stn* and *avrA* genes. These results go parallel to that obtained by **Ammar et al. (2016).** The presence or lack of some virulence genes governs *Salmonella* pathogenesis; *sopB* and *avrA* encode T3SS proteins that are essential in the inflammatory process caused by *Salmonellae*. They also have anti-apoptotic role which helps *Salmonellae* in escaping the host adaptive immune response **(Wu et al., 2011).** *Salmonella* enterotoxin (*stn*) is an important cause of diarrhea. Inactivation of this gene reduced the ability of *S. Typhimurium* to accumulate fluids within the intestinal lumen **(Chopra et al., 1999).** *stn* is encoding enterotoxin production and has been shown to be associated with diarrhea **(Huehn et al., 2010; Osman et al. 2010; Thung et al., 2018).** There is a positive relationship between the presence of variable virul.ence genes and the clinical outcome of infection by different bacteria **(Radwan et al., 2016).**

5. Conflict of Interest

The authors declare no conflict of interest.

6. References

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