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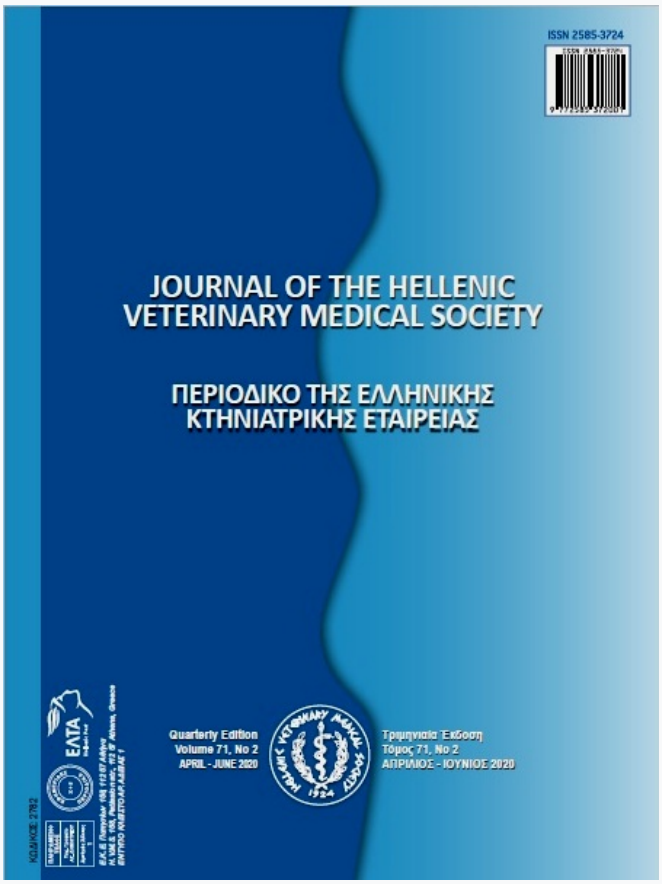

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Correlation between *ESβL Salmonella* Serovars Isolated from Broilers and their Virulence Genes

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ABSTRACT: *Salmonell*a is considered to be one of the major poultry bacterial pathogens. The poultry species are one of the main reservoirs for the human types, thus serving as public health hazards. The development of drug resistant genes and multidrug resistant types from *Salmonell*a has increased recently. This current study was undertaken to estimate the correlation between extended spectrum beta-lactamase multidrug resistant (*ESβL*) *Salmonella* serovars isolated from broilers and their virulence genes. Two hundred and forty samples were collected from clinically diseased broilers chicks (showed disorders of the intestinal tract) and examined for the presence of *Salmonella* isolates according to ISO 6579: 2002 and ISO. 6579-3:2014 Fifty *Salmonella* isolates were isolated with an incidence of 20.8%. Isolates of *Salmonella* were serotyped as follows: 25 *S.* Kentucky, 9 *S.* Infantis, 6 *S.* Enteritidis, 4 *S.* Heidelberg, and one isolates per serovars *S.* Labadie, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia. AST (antimicrobial susceptibility testing) showed that high percentage of isolates were resistant to all Ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole + Trimethoprim (82%) and Tetracycline (82%). Approximately 86% of the isolates demonstrated multiple resistance, of which 18.75% and 25% were resistant to three and four antimicrobial types, respectively. Phenotypic detection of *ESβL*s by using screening test (*Cefinase*®) and confirmatory test by using combined disk diffusion test revealed that 32% of isolates were positive for both tests with 20% similarity and 12% diversity between the two tests. Molecular characterization of some *ESβL*s genes (bla_{TEM}, bla_{CTX}, bla_{OXA}, bla_{CMY} and bla_{SHV}) and some virulence genes (*inv*A, *avr*A, *sop*B, *bcf*C, *stn* (was done using PCR. The results showed that all the *ESβL*s positive serovars were positive for amplification of all tested virulence genes and noticed that all the isolates were negative for bla_{CMY} gene. The present study suggests that virulent *ESβL Salmonella* serovars could infect broilers and should be taken into consideration as an important bacterial pathogen affecting poultry.

***Keywords*:** *Salmonella,* virulence, ESBL, Broilers, genes

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INTRODUCTION

The poultry industry is exposed to several threats such as of viral origin like avian Influenza, Newcastle disease, infectious bronchitis, infectious bursal disease, avian adenovirus, or of bacterial origin like the Salmonellae species, Escherichia coli, Clostridium spp. (Ayoub *et al.* 2019, Diab *et al.* 2019, Elhady *et al.* 2018, Fawzy *et al.* 2020, Sedeik *et al.* 2018, Sultan *et al.* 2019a, Sultan *et al.* 2019b, Sultan *et al.* 2020). Salmonella is one of the most serious bacterial pathogens as it is associated with economic losses in poultry farms in addition to the zoonotic effects. There are more than 2700 serotypes from *Salmonella* species according to the White Kauffmann classification*; Salmonella* species are responsible for a variety of acute and chronic diseases in poultry (Grimont . and Weill 2007). Avian Salmonellosis may occurs as a result of infection with poultry-specific serovars, *Salmonella* Gallinarum and *Salmonella* Pullorum, causing systemic illness in birds as well as other *Salmonella* serotypes, including *Salmonella* Typhimurium and *Salmonella* Enteritidis and many others, which contribute to paratyphoid infections (Gast *et al.* 2003)**.** *Salmonella***e** are recognized by the specific host that belongs to pathogen recognition patterns (PRR) in birds and bony fish (Abouelmaatti *et al.* 2013, Elfeil *et al.* 2012, Elfeil *et al.* 2016).Since the 1990s, several reports highlighted that some *Salmonella* strains developed resistance to a range of antimicrobials agents and the range of developed resistance increased since then; whereas, this resistance developed due to improper use of antimicrobials agents in humans and animals' husbandry**,** either using subtherapeutic doses, use using antimicrobial agents as growth promoters and incomplete antimicrobial courses...etc (Eid *et al.* 2019, Enany *et al.* 2018, M. Algamma *et al.* 2016). Multi drug resistant (MDR) bacteria (bacteria resistant to more than four different types of Antimicrobial agents' families) increased dramatically during the last decade. They have a high impact in developing several disease conditions in humans and birds and are associated with elevated levels of morbidity and mortality caused by those pathogens in addition to high economic costs of therapy (cost of medication) and real risks of the spread of resistant strains which transmit acquired Multidrug resistant (MDR) genes to other bacteria among animals and humans (Chen *et al.* 2004, Eid *et al.* 2019, Elfeil *et al.* 2020, White *et al.* 2001). Thus, the MDR bacteria have a high impact on public health issues. The rapid development of resistance to extended-spectrum Cephalosporin in different serovars of *Salmonella* subspecies enterica has been observed worldwide and is predominantly linked to plasmid-mediated production of β- lactamases-producing bacteria (*ESβL*) (Authority 2009, EFSA 2018, EFSA. 2008). The widespread of *ESβL*-producing organisms and related treatment failures may be associated with failure in detection of the complex resistant phenotypes and may reflect to uncontrolled spreading of such pathogens (Sinha *et al.* 2008).The rapid spread of *ESβL*-producing bacteria in different poultry farms provoke a serious risk to livestock and humans, especially with cross spreading of the of *ESβL*-producing bacterial strains from poultry farms to livestock farms and humans which vice versa spread the MDR pathogens that cause wide-spread population infections (Brinas *et al.* 2003, Winokur *et al.* 2000).Some bacterial virulence genes are associated with main pathogenic behavior of the bacteriał (as ability of bacteria to make adhesion to specific host cells as mucosal cell, ability for invasion cells and/ or ability to triggering fluids secretions which leads to diarrhea symptoms). Those genes are associated with the pathogenesis of *Salmonella,* as it reflects on its ability to survive and replicate inside host cells. This virulence trait is associated with the ability to develop systemic infections and a large number of genes are required to enable *Salmonella* to cope with nutritional limitations, to avoid clearance by the host defense mechanisms and immune system or overcome damage effect by antimicrobial peptides and radicals on bacterial cell (Hegazy . and Hensel 2012).

MATERIALS AND METHODS

- All methods were carried out in accordance with Cairo University (Egypt) guidelines and regulations.
- All experimental protocols were approved by Cairo University (Egypt) Ethical committee.

***Samples*:** Two-hander and forty samples were collected randomly from broilers clinically diseased chicks (showing disorders of the intestinal track.). These were 45 liver, 60 yolk sacs, 40 lung, 50 caecum and 45 spleen. The samples were then brought to the Microbiology department, faculty of Veterinary Medicine, Cairo University, in sterile wide-mouth screw capped bottles under cooling conditions and then analyzed for the presence of *Salmonella.*

***Isolation of Salmonella*:** Under complete sterile condition broilers' internal organs were examined for the presence of *Salmonellae* according to the ISO 6579: 2002/ Amd. 1: 2007 (Standardization 2007). The isolates were serotyped according to ISO 6579-3: 2014 in the Central Laboratory for Quality Control on Poultry Production

(CLQP) in Dokki, Giza, Egypt (Standardization 2014).

The Antibiogram disk diffusion technique was adapted according to CLSI manual (NCCLS 2015).

β-lactamase detection using Nitrocefin disks (Cefinase®): According to the Manufacturer's instruction, The Cefinase disc is impregnated with the chromogenic cephalosporin, Nitrocefin. Those disks exhibit quick color change from yellow to red due to hydrolysis of the amide bond in the beta-lactam ring by a beta-lactamase enzyme. When a bacterium produces certain amount of beta-lactamase enzyme the disc colour changed from yellow to red colour in the area where the isolate is smeared.

***β-lactamase detection using Combined Disc Diffusion Test (CDD)*:** The test inoculums (compared to 0.5 McFarland turbidity) were spread by sterile cotton swabs on Mueller-Hinton agar plates. The used discs were either Cefotaxime and Ceftazidime, separate and in combination with Clavulanic acid (cefotaxime 30 µg, cefotaxime/clavulanic acid 30/10 µg) and (Ceftazidime 30 µg, Ceftazidime/Clavulanic acid 30/10 µg). The plate was incubated at 35±2°C for 16-18 hours. The organisms were considered to be producing *ESβL* when A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with Clavulanic acid, versus the zone diameter of the agent when tested alone, equals *ESβL*.(NCCLS 2015).

Molecular Detection of ESβLs Salmonella serovars and its virulence genes by PCR: This was carried out by using primers for detection of *ESβL*s *Salmonella* serovars and some of its virulence genes as presented in tables (1/2).

Table 1. Primers Used for Molecular Detection of *Esβl's Salmonella Serovars and Its Virulence Genes by PCR*

Primer		Sequence	Amplified product	Reference
Stn	F	TTG TGT CGC TAT CAC TGG CAA CC	617 bp	Murugkar*et al*., (2003).
	R	ATT CGT AAC CCG CTC TCG TCC		
invA	F	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	Oliveira*et al*., (2003).
	R	TCATCGCACCGTCAAAGGAACC		
sopB	F	TCA GAA GRC GTC TAA CCA CTC	517 bp	Huehn*et al*., (2010).
	R	TAC CGT CCT CAT GCA CAC TC		
avrA	F	CCT GTA TTG TTG AGC GTC TGG	422 bp	
	R	AGA AGA GCT TCG TTG AAT GTC C		
BcfC	F	ACC AGA GAC ATT GCC TTC C	467 bp	
	R	TTC TGC TCG CCG CTA TTC G		
bla_{TEM}	F	ATCAGCAATAAACCAGC	516 bp	Colom *et al*., (2003).
	R	CCCCGAAGAACGTTTTC		
bla_{SHV}	F	AGGATTGACTGCCTTTTTG	392 bp	
	R	ATTTGCTGATTTCGCTCG		
Bla_{OXA-1}	F	ATATCTCTACTGTTGCATCTCC	619 bp	
	R	AAACCCTTCAAACCATCC		
CMY-2	F	TGG CCA GAA CTG ACA GGC AAA	462 bp	Pérez-Pérez and Hanson, (2002).
	R	TTT CTC CTG AAC GTG GCT GGC		
Bla_{CTX}	F	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	Archambault *et al*., (2006).
	R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

Table 2. Thermal cycle condition Used for Molecular Detection of *Esβl's Salmonella Serovars*

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Stn	94˚C\10 min.	94˚C\45 sec.	59˚C \45 sec.	72˚C\45 sec.	35	72˚C\10 min.
invA	94˚C\5 min.	94˚C\30 sec.	55˚C\30 sec	72˚C\30 sec	35	72˚C\5 min.
SopB	94˚C\10 min.	94˚C\30 sec.	58˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
avrA	94˚C\10 min.	94˚C\30 sec.	58˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
bcfC	94˚C\10 min.	94˚C\30 sec.	53˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
bla_{TEM}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{SHV}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{OXA-1}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
CMY-2	94˚C\10 min.	94˚C\45 sec	55˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{CTX}	94˚C\10 min.	94˚C\45 sec	60˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.

RESULTS

Results of *Salmonella* isolation from 240 samples of clinically diseased chicks revealed that 50 *Salmonellae* were isolated with an incidence of 20.8 % as shown in Table (3) with high recovery rate from liver 31% (14\240) followed with yolk sac 20% (12\240). Serotyping of the *Salmonella* isolates revealed that there were 25 *S.* Kentucky (10.4%), 9 *S.* Infantis (3.7%), 6 *S.* Enteritidis (2.5%), 4 *S.* Heidelberg (1.6%), and one isolate for the following serovars *S.* Labadi, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia in percentage (0.4%) for each serovar. Results of antimicrobial susceptibility testing (AST) in table (4), showed that there were two pan susceptible *Salmonella* isolates (i.e. susceptible to all antimicrobial agents under test). There was one *Salmonella isolate* susceptible to one type of antimicrobial agents under test and three *Salmonella* isolates were susceptible to two types of antimicrobial agents under the test. A large percentage of isolates were resistant to all antimicrobials i.e. ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole-trimethoprim (82%) and tetracycline (82%). Out of 50 *Salmonella* serovars, 13 were *ESβL* when detected phenotypically by Cefinase with a rate of 26 % while when combined with the disc diffusion test, again, 13 serovars were *ESβL* with a rate of 26 %. The comparison between the screening and confirmatory methods revealed that 10 isolates had similar response and 6 isolates responded differently. Genotypic detection of *ESβL*-producing *Salmonellae* by using PCR revealed that 16 isolates which were tested for the presence of 5 genes that are responsible for *ESβL* production, CTX (Cefotaxime), TEM (Beta-lactamase), SHV (sulfhydryl variable active site), OXA (oxacillinase) and CMY (class C carbapenemase); as showed in Table (5-6) with a comparison between results of phenotypic and genotypic detection of *ESβL*-producing *Salmonellae*. The PCR amplification result in this study shows that a total of 10 isolates of phenotypic positive isolates were positive for bla_{TEM} gene type using universal primers, 5 isolates were positive for bla_{CTX} gene type, 2 isolates were positive for bl_{OXA-1} gene type and one isolate was positive for bla_{SHV} gene type but no amplification was found encoding the gene of bla_{CMY-2} noticed that all the *ESβL*s positive serovars were positive for amplification of all tested virulence genes and also noticed that all the isolates were negative for bla_{CMY} gene.

Table 3. Result of *Salmonella* Isolation from different broilers chicks' organs

Organ	No. of samples	No. of positive	percentage	Percentage to all
Liver	45	14	31%	5.8%
Yolk Sac	60	12	20%	5%
Lung	40	4	10%	1.6%
Caecum	50	12	24%	5%
Spleen	45	8	17.7%	3.3%

Table 4. Collective resistance pattern of *Salmonella* serovars to the antibiotics used

Antimicrobial agents	Resistance patterns					
	R	%	I	%	S	%
Sulphamethaxole-Trimethoprim (SXT)	41	82%	-	0	9	18%
Amikacin 30 µg	-	0%	2	4%	48	96%
Imepenem 10 µg	-	0%	3	6%	47	94%
Tetracycline 30 µg	41	82%	1	2%	8	16%
Ampicillin 10 µg	45	90%	1	2%	4	8%
Nalidixic acid 30 µg	44	88%	1	2%	5	10%
Chloramphenicol 30 µg	21	42%	2	4%	27	54%
Gentamicin 10 µg	1	2%	3	6%	46	92%
Ciprofloxacin 5 µg	29	58%	17	34%	4	8%
Azetreonam 30 µg	8	16%	3	6%	39	78%
Ampicillin +Sulbactam	12	24%	4	8%	34	68%
Cefepem	8	16%	2	4%	40	80%
Ceftriaxone 30 µg	7	14%	5	10%	38	76%
Cephalothin 30 µg	16	32%	12	24%	22	44%
Cefotaxime 30 µg	4	8%	-	0%	46	92%
Ceftazidem 30 µg	4	8%	-	0%	46	92%

Table 5. Comparison between Cefinase® and combined disk diffusion test as phenotypic methods for detection of ESβLs.

	Cefinase®	CDD
Positive isolates	13	13
Negative isolates	37	37
Shared isolates	10	10
Different isolates	3	3
Cefinase® +ve / Genotypic -ve	(1/16) 6.25%	----------
Cefinase® -ve / Genotypic +ve	(3/15) 20%	----------
Cefinase® +ve / Genotypic +ve	(12/15) 80%	----------
CDD +ve / Genotypic -ve	----------	0
CDD –ve / Genotypic +ve	----------	(3/15) 20%
CDD +ve/ Genotypic +ve	----------	(13/15) 86.6%

Table 6. Genotypic results of the 6 isolates different in ESβLs pattern detected by phenotypic methods.

Isolate	Cefinase®	CDD	Genotypic resistance genes
S. Kentucky	Negative	Positive	bla_{CTX}
S. Kentucky	Negative	Positive	bla_{TEM}
S. Heidelberg	Negative	Positive	bla_{SHV}+bla_{OXA-1}
S. Infants	Positive	Negative	bla_{TEM}
S. Infants	Positive	Negative	bla_{TEM}
S. Agona	Positive	Negative	bla_{TEM}

DISCUSSION

Salmonellosis in poultry is a worldwide spread infection, both for poultry as a disease causative agents and as a vehicle for human infection (Mohammed *et al.* 1999). Data from current study showed; *Salmonella* different serotypes incidence rate 20.8 % with high recovery rate from liver31% (14\240) followed with yolk sac20% (12\240),the serotyping of recovered *Salmonella* isolates revealed that there were 25 *S.* Kentucky (10.4%), 9 *S.* Infantis (3.7%), 6 *S.* Enteritidis (2.5%),4 *S.* Heidelberg (1.6%), and one isolates for the following serovars *S.* Labadi, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia in percentage (0.4%) for each serovar. The emergence of antimicrobial agents' resistance is a matter of concern. People infected by antimicrobial resistant *Salmonella* spp., particularly Nalidixic acid–resistant *Salmonella* spp., are more likely to die, are more likely to be hospitalized, and are hospitalized for longer periods than patients with infections caused by susceptible to antimicrobials strains of *Salmonella* spp. (Helms *et al.* 2004, Helms *et al.* 2002, Lee *et al.* 1994). Antimicrobial susceptibility tests described in this study; show a high percentage of isolates that were resistant to ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole-trimethoprim (82%) and tetracycline (82%) and out of 50 *Salmonella* serovars, 13 were found to be *ESβL* when detected phenotypically by Cefinase with a rate of 26% while when use combined disc diffusion test 13 serovars were *ESβL* with a rate of 26%. The comparison between the screening and confirmatory methods revealed that 10 isolates expressed similar results and 6 isolates expressed different results. All *Salmonella* isolates were positive by polymerase chain reaction assay for *inv*A gene; which agreed with previous studies about the existence of the invA in *Salmonella* around the world. The detection of those genes in huge number of *Salmonella* isolates from different geographical location maybe associated with the ability of the isolates for cell invasion (Amini *et al.* 2010, Campioni *et al.* 2012, Crăciunaş *et al.* 2012). PCR is a useful and rapid tool for *Salmonella* spp detection in clinical samples., where *inv*A and the *sop*B genes may be a target gene for the detection of this genus, as both genes associated with the virulence of S*almonella* enteritis in birds (Hughes *et al.* 2008, Wood *et al.* 1998). The *avr*A gene prevalence showed 100% detection in all *salmonella* isolates similar to the detection rate found in previous report focused on *Salmonella* Enteritidis (Hopkins . and Threlfall 2004), while in other reports lower detection rate for this gene of *Salmonella* Enteritidis (Liu *et al.* 2012, Rahman *et al.* 2004, Streckel *et al.* 2004, Zou *et al.* 2010) was reported. The variation in detection rate maybe associated with the variation of recombination location of these genes (Hopkins . and Threlfall 2004). These findings are important, since variation in the repertoire of those genes, such as *avr*A and *sop*E, can be associated with the variation of these serovars ability to adapt to new

host cells and consequently provoke the emergence of novel virulent strains (Prager *et al.* 2000).The obtained data from this study showed a high percentage (100%) of *Salmonella* Enteritidis isolates that had the *avr*A gene; while, only 17.1% and 9.7% of *Salmonella* Hadar isolates possessed the *avr*A and *sop*E genes, respectively, which showed difference in the pattern of those genes among different *Salmonella* serovars. (Cesco *et al.* 2009)**.** Some reports associated the high frequency of *avr*A gene in serovars that are considered as the most pathological agents of salmonellosis in poultry farms (Ben-Barak *et al.* 2006).The obtained data from the current study showed significant association between *inv*A, *avr*A, *sop*B, *stn* and *bcf*C virulence genes and resistance to the commonly used antibiotics in Egypt (Table-4/5) as previously observed in Senegal and Gambia but with different virulence genes and antimicrobials resistant genes (Dione *et al.* 2011)**.** These correlations could be explained based on the mechanisms involved in the pathogenicity of bacteria and the acquisition of resistance genes by *Salmonella* species. In the pathogenic bacterial isolates the vast majority of molecular pathogenicity determinant fragments located specific bacterial plasmid (virulence-associated plasmids) or chromosome (Groisman . and Ochman 1996, Hacker *et al.* 1997). The MDR genes may be located on bacterial chromosome either segmented inside chromosome or located as extra chromosomal genetic elements within the chromosome that originate from other genomes (Carattoli 2003)**.** The simultaneous detection of both types of genes either different types of virulence gene or antimicrobial resistance genes has been frequently reported in different *salmonella* species (Carattoli 2003).

CONCLUSION

The data obtained from this study, suggests that broilers play a potential role as a reservoir of multi drug resistant *Salmonella* serovars with special reference to *ESβL* serovars; which contain many virulence genes that magnify the disease condition and imply failure of control.

CONFLICT OF INTEREST

All authors declare no conflict of interest

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