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Research Paper

MOLECULAR IDENTIFICATION OF PLASMID MEDIATED ANTIMICROBIAL RESISTANCE DETERMINANTS AMONG SALMONELLA ENTERICA IN ILL-CHICKEN

Gamal A Younis[1], Rasha M Elkenany[1*] and Samar A Asfor[1]

*Corresponding Author: Rasha M Elkenany, ✉ dr_rashavet22@mans.edu.eg

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Salmonella enterica is a prominent cause of salmonellosis throughout the world. In this study, 17 (9.4%) out of 180 samples from ill-chicken were positive for *Salmonella* enterica. *S. Typhimurium* and *S. Kentucky* were the most common serotypes 4 (23.52% each), followed by *S. Enteritidis* (3, 17.6%), *S. Labadi* (2, 11.8%), *S. Larochelle*, *S. Tamale*, *S. Takoradi* and *S. Papuana* (1, 5.9% each). Concerning the occurrence of *Salmonella enterica* in ill-chicken viscera, it was clear that liver (41.2%) was the most site of isolation followed by yolk sac (23.5%) then, spleen and heart (17.6% each). Overall, the antimicrobial resistance testing showed higher resistance rates to tetracycline (94.11%), penicillin (94.11%), erythromycin (94.11%), doxycycline (88.23%), streptomycin (88.23%) and nalidixic acid (82.35%). On the contrary, lower rates of resistance were observed for ceftriaxone, ciprofloxacin, cephalothin (5.88%, each), norfloxacin, amoxicillin-clavulonic acid (11.76%), cefotaxime (17.64%) and gentamicin (23.52%). A number of antimicrobial resistance genes including [bla_{TEM}, bla_{CTX}, *tetA(A)* and *ere(A)*] in the most common *Salmonella enterica* serovars (*S. Typhimurium*, *S. Enteritidis* and *S. Kentucky*) were determined on plasmids and chromosomal DNA of five isolates using PCR. The bla_{TEM} genes, that responsible for the extended spectrum β-lactamase phenotype, were present on plasmids and bacterial chromosome of all examined isolates of S. enterica. Moreover, one isolate harboring the bla_{TEM} gene also contained bla_{CTX} on plasmid only. Also, *tet(A)* and *ere(A)* genes were identified in all tested Salmonella isolates on plasmids and bacterial chromosome. This investigation indicates the importance of plasmid in spreading of antimicrobial resistance. This research is one of the little data that reported on plasmid mediated antimicrobial resistance determinants in Egypt.

Keywords: Salmonella enterica, Antimicrobial resistance, Plasmid, Ill-chicken

INTRODUCTION

Salmonella enterica has emerged as a primary cause of salmonellosis in poultry and foodborne outbreaks in human worldwide. Most *Salmonella*,

[1] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.

except *S. Pullorum* and *S. Gallinarum* and possibly other strains, e.g., *S. Kentucky*, are capable of asymptomatically residing in the intestinal tracts of poultry (Buission *et al.*, 1992; Wilson *et al.*, 2000; and Ogunleye and Carlson, 2012). Many *Salmonella* serotyps can be acquired by the fecal-oral route and then shed into the feces (Traub-Dargatz *et al.*, 2006). Many birds can be infected since the ingestion, colonization, and shedding events typically cause no harm to the bird and since *Salmonella* is global in the environment. *Salmonella* can therefore contaminate poultry meat prior to (from fecal shedding) or during processing (from intestinal leakage), resulting in one of the leading causes of *Salmonella* infections in humans (CDC, 2015).

During the past few decades, the emergence of multidrug resistant (MDR) *Salmonella* isolates has increased. The most important concern regarding the increase of MDR *Salmonella* isolates is the ineffective treatment of salmonellosis by several antibiotics (Bacci *et al.*, 2012; and Ke *et al.*, 2014). Resistant determinants are often placed on extra-chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes. Not only the genetic transformation is responsible for acquisition of a new gene, but also the conjugative transfer can mobilize resistance genes that are located on plasmids-self-replicating double-strand circles of DNA (Carattoli *et al.*, 2003).

Consequently, the objective of this study was to characterize antimicrobial resistance among different *Salmonella* serotypes recovered from ill-chicken, and determine the genetic factors contributing to antibiotic resistance using PCR reactions on plasmid and chromosomal DNA of *Salmonella* isolates in Egypt.

MATERIALS AND METHODS

Sampling

A total of 180 samples (liver, spleen, heart and yolk sac) were collected from moribund chickens showing diarrhea and respiratory manifestation from different localities at Damietta province, Egypt from January 2016. All samples were exposed to bacteriological analysis.

Bacteriological Analysis

Salmonella isolates were enriched in selenite F-broth and Rappaport-vassiliadis soya broth at 37 °C for 18 h and then, subculutered on MacConkey's agar, XLD agar, S-S agar and brilliant green agar at 37 °C for 24-48 h (Oxoid). Pure cultures of the isolates were morphologically and biochemically identified using the following conventional tests; Triple Sugar Iron agar (TSI) test, urease test, indole and methyl red test.

Serological Identification of *Salmonellae*

It was performed consistent with Kauffman- Le Minor scheme (Grimont and Weill, 2007) for the identification of somatic (O) and flagellar (H) antigens by slide agglutination test using polyvalent and monovalent *Salmonella* antisera (DENKA SEIKEN Co., Japan).

Antimicrobial Resistance

It was determined by the standardized disk diffusion technique and interpreted according to (NCCLS, 2002). The strains were screened for their resistance to the following antibiotics (Oxoid); erythromycin (15 µg), tetracycline (30 µg), penicillin (10 µg), doxycycline (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), aztreonam (30 µg), chloramphenicol (30 µg), azithromycin (15 µg), gentamicin (10 µg), cefotaxime (30 µg), amoxicillin-clavulonic acid (30

µg), norfloxacin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg) and ceftriaxone (30 µg). The diameter of inhibitory zone was measured and compared with antibiotic susceptibility testing sheet.

Plasmid Analysis

The QLA prep spin Mini prep kits handbook was used for extraction of plasmid DNA from cultured cells. Processing of the *Salmonella* strains was done by using gel electrophoresis for determination of the number of plasmid copies present in different isolates according to Sambrook and Russel (2001). The DNA fragments were stained using ethidium bromide and they were visualized by UV-Trans illumination.

Molecular Determination of Antimicrobial Resistance Genes

Phenotypically resistant *Salmonella* isolates were screened for the presence of genes coding for drug resistance on plasmid and bacterial chromosome using PCR. The QIAamp DNA Mini kit was used for DNA extraction and purification from *Salmonella* isolates grown in BHI (Qiagen, Germany, GmbH) with some modifications from the manufacturer's recommendations. Specific primers (Metabion, Germany) were used for PCR amplification with specific profiles (Table 1). PCR products were subjected to electrophoresis grade agarose (1.5%). A gel documentation system was used for photography of the gel and the data was analyzed through computer software.

RESULTS

Incidence of *Salmonella* Species

In this study, 17 (9.4%) out of 180 samples from ill chicken were positive for *Salmonella* species. Regarding to the serotyping, there were 8 serotypes of *Salmonella*. *S. Typhimurium* and *S.*

Table 1: Oligonucleotide Primers Sequences, Target Genes and Cycling Conditions

Primer	Sequence	Amplified Product (bp)	Primary Denaturation	Secondary Denaturation	Annealing	Extension	No. of Cycles	Final Extension	Ref
TetA(A)	GGTTCACTCGAACGACGTCA	576	94 24 °C	94 °C	50 °C	72 °C	35	72 °C	Randall *et al.* (2004)
	CTGTCCGACAAGTTGCATGA		5 min.	30 sec.	45 sec.	45 sec.		10 min.	
blaTEM	ATCAGCAATAAACCAGC	516	94 °C	94 °C	54 °C	72 °C	35	72 °C	Colom *et al.* (2003)
	CCCCGAAGAACGTTTTC		5 min.	30 sec	45 sec	45 sec		10 min.	
ereA	GCCGGTGCTCATGAACTTGAG	420	94 °C	94 °C	60 °C	72 °C	35	72 °C	Nguyen *et al.* (2009)
	CGACTCTATTCGATCAGAGGC		5 min.	30 sec	45 sec	45 sec		10 min.	
blaCTX	ATGTGCAGYACCAGTAARGTKATGGC	593	94 °C	94 °C	60 °C	72 °C	35	72 °C	Archambaul *et al.* (2006)
	TGGGTRAARTARGTSACCAGAAYCAGCGG		5 min.	30 sec	45 sec	45 sec		10 min.	

Kentucky were the most common serotypes 4 (23.52% each), followed by *S. Enteritidis* (3, 17.6%), *S. Labadi* (2, 11.8%), *S. Larochelle*, *S.* Tamale, *S. Takoradi* and *S. Papuana* (1, 5.9% each) (Table 2).

Concerning the occurrence of *Salmonella* spp in ill chicken viscera, it was cleared from Table 2 that liver (41.2%) was the most organ of isolation followed by yolk sac (23.5%) then, spleen and heart (17.6% each).

Antimicrobial Resistance Patterns of *Salmonella* spp

From Table 3, the antimicrobial resistance testing showed higher resistance rates to tetracycline (94.11%), penicillin (94.11%), erythromycin (94.11%), doxycycline (88.23%), streptomycin (88.23%) and nalidixic acid (82.35%). On the contrary, lower rates of resistance were observed for ceftriaxone, ciprofloxacin, cephalothin (5.88%, each), norfloxacin, amoxicillin-clavulonic acid (11.76%), cefotaxime (17.64%) and gentamicin (23.52%).

Incidence of Antibiotic Resistant Genes and Plasmid-Mediated Antimicrobial Resistance

To determine the genetic factors contributing to antimicrobial resistance in five isolates representing the most common *Salmonella* serovars (*S. Typhimurium*, *S. Enteritidis* and *S. Kentucky*), the PCR reactions were applied to identify resistance genes bla_{TEM} , bla_{CTX} , *tetA(A) and ere(A)* on plasmids and chromosomal DNA (Table 4). In the current study, five cephalosporin-resistant isolates were subjected to screening of expanded-spectrum β-lactamase genes (bla_{TEM} *and* bla_{CTX}) on plasmids and chromosomal DNA. The bla_{TEM}, that confers resistance to penicillins and first-generation cephalosporins, was identified on plasmids and bacterial chromosome of all examined isolates of *S.* enterica by 100% (Photo 1).

In addition, resistant genes bla_{CTX}, that confers resistance to third-generation cephalosporins, located only in one isolate on plasmids with 20% not on chromosomal DNA (Photo 2). Moreover, one isolates harboring the bla_{TEM} gene also contained $bla_{CTX.}$

Table 2: Prevalence of Salmonella Serotypes in Ill Chicken Viscera

Serotypes	Antigenic Structure	Samples n (%)				Total (%)
		Spleen	Liver	Heart	Yolk Sac	
S. Typhimurium	O:1,4,5,12; H_1I;H:$_2$1,2:	1	1	1	1	4 (23.5%)
S. Kentucky	O:8,20; H_1I; H:$_2$Z:$_6$	1	1	1	1	4 (23.5%)
S. Enteritidis	O:1,9,12; H_1g,m; H:$_2$-:	1	1	1	0	3 (17.6%)
S. Labadi	O:8,20;H_1d;H:$_2$Z:$_6$	0	2	0	0	2 (11.8%)
S. Takoradi	O:8,20;H_1I;H:$_2$1,5:	0	0	0	1	1 (5.9%)
S. Tamale	O:8,20;H1:Z_{29}H;$_2$e,n,Z:$_{15}$	0	1	0	0	1 (5.9%)
S. Larochelle	O:6,7;H1:e,h H2:1,2	0	0	0	1	1 (5.9%)
S. Papuana	O:6,7;H:r : e,n,Z15	0	1	0	0	1 (5.9%)
Total (%)	8	3 (17.6%)	7 (41.2%)	3 (17.6%)	4 (23.5%)	17 (9.4%)

Table 3: Antimicrobial Susceptibility Pattern of Salmonella Isolates as Determined by Disk Diffusion Assay

Antibiotic(s) Testeda	Sympol	Sensitive		Resistant	
Name		(No.)	(%)	(No.)	(%)
Erythromycin	E_{15}	1	5.88	16	94.11
Tetracycline	TET_{30}	1	5.88	16	94.11
Penicillin	P_{10}	1	5.88	16	94.11
Doxycycline	DO_{30}	2	11.76	15	88.23
Streptomycin	STR_{10}	2	11.76	15	88.23
Nalidixic acid	NAL_{30}	3	17.64	14	82.35
Aztreonam	ATM_{30}	9	52.94	8	47.05
Chloramphenicol	CHL_{30}	10	58.82	7	41.17
Azithromycin	AZM_{15}	12	70.58	5	29.41
Gentamicin	GM_{10}	13	76.47	4	23.52
Cefotaxime	CTX_{30}	14	82.35	3	17.64
Amoxicillin-clavulonic acid	AMC_{30}	15	88.23	2	11.76
Norfloxacin	NOR_{10}	15	88.23	2	11.76
Cephalothin	CEF_{30}	16	94.11	1	5.88
Ciprofloxacin	CIP_5	16	94.11	1	5.88
Ceftriaxone	CRO_{30}	16	94.11	1	5.88

In this work, *tet(A)* gene was identified in all tested *Salmonella* isolates (100%) on bacterial chromosome and also presented on plasmid (100%) (Photo 3).

Furthermore, the erythromycin belonging to the macrolides class is the most public drug used for treatment of gram-negative bacteria. In this research, polymerase chain reaction identified *ere(A)* conferring resistance to erythromycin in all examined *Salmonella* isolates (100%) and also presented in plasmids (100%) (Photo 4).

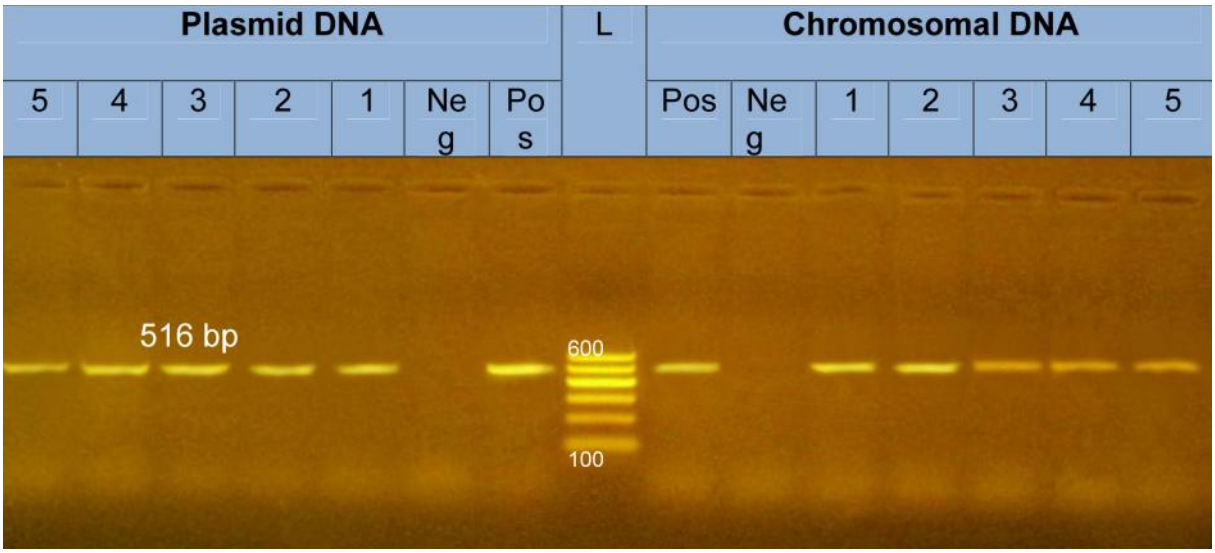
DISCUSSION

Salmonella species are the most common pathogens responsible for economic losses in poultry farms and food-borne illness in humans. Therefore, the identification of *Salmonella*e is important for epidemiological surveillance and investigations of outbreak. In the present study, incidence of *Salmonellae* in ill chicken samples was (9.4%). Similar observations were detected by Gharib *et al.* (2015) and Soliman *et al.* (2016) who isolated *Salmonella* spp from chickens with

Table 4: Antibiotic Resistance Pattern and Sites of Antibiotic Resistance Genes (n = 5)

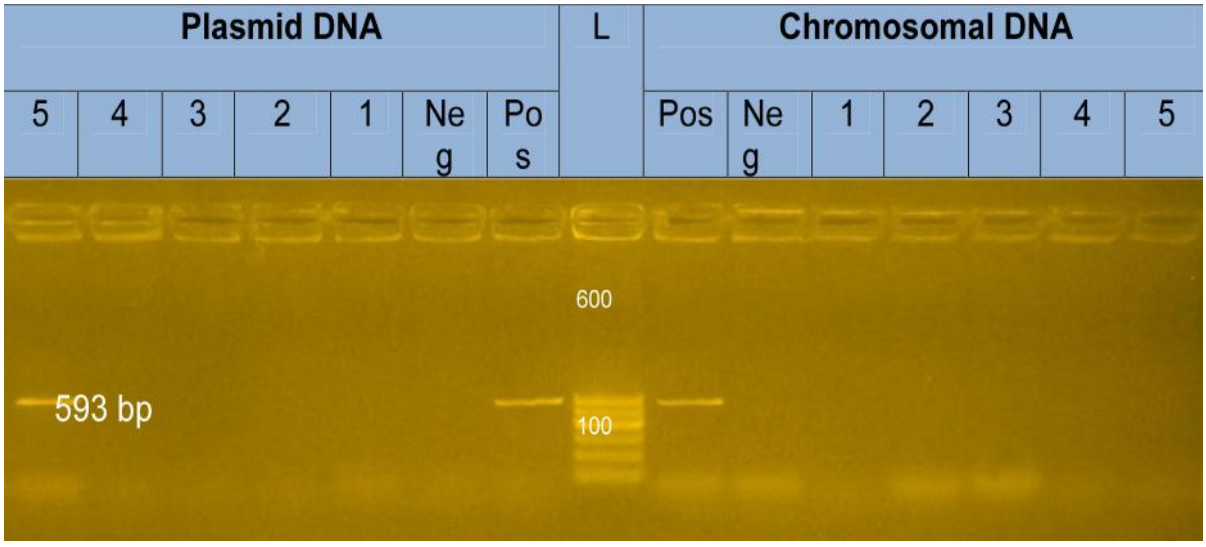
Strain No.	Serovar	Resistance Phenotypes	Resistance Genes							
			On Chromosome				On Plasmid			
			(A)ere	*bla* $_{TEM-1}$	*bla* $_{CTX}$	*(A)tet*	*(A)ere*	*bla* $_{TEM-1}$	*bla* $_{CTX}$	*(A)tet*
1	*S. Typhimurium*	P, TET, STR, DO, E, GEN, NAL	+	+	-	+	+	+	-	+
2	*S. Typhimurium*	P, CHL, GEN, STR, TET	+	+	-	+	+	+	-	+
3	*S. Enteritidis*	P, CHL, NAL, STR, TET, DO	+	+	-	+	+	+	-	+
4	*S. Enteritidis*	TET, E, GEN, DO, NAL, CHL	+	+	-	+	+	+	+	+
5	*S. Kentucky*	CHL, NAL, STR, TET, E	+	+	-	+	+	+	-	+
Total (%)			5 (100%)	5 (100%)	0	5 (100%)	5 (100%)	5 (100%)	1 (20%)	5 (100%)

Photo 1: Agarose Gel Electrophoreiss Showing an Amplification of bla_{TEM} Gene (516 bp) in Salmonella Isolates from Ill Chicken



Note: Lane L: DNA ladder (100 bp), lane Pos: Positive control, Lane Neg: negative control, lanes 1, 7: bla_{TEM} negative, lanes 1, 2, 3, 4, 5: bla_{TEM} positive.

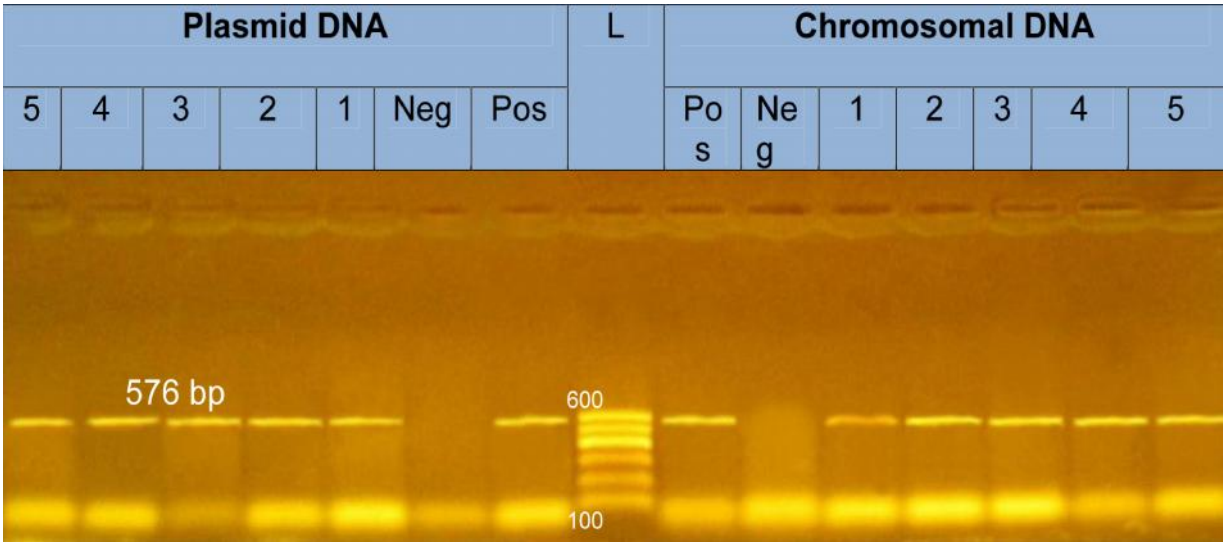
Photo. 2: Agarose Gel Electrophoreiss Showing an Amplification of bla_{CTX} Gene (593 bp) in Salmonella Isolates from Ill Chicken



Note: Lane L: DNA ladder (100 bp), lane Pos: Positive control, Lane Neg: negative control, lanes 1, 7: bla_{CTX} negative, lanes 1, 2, 3, 4, 5: bla_{CTX} positive.

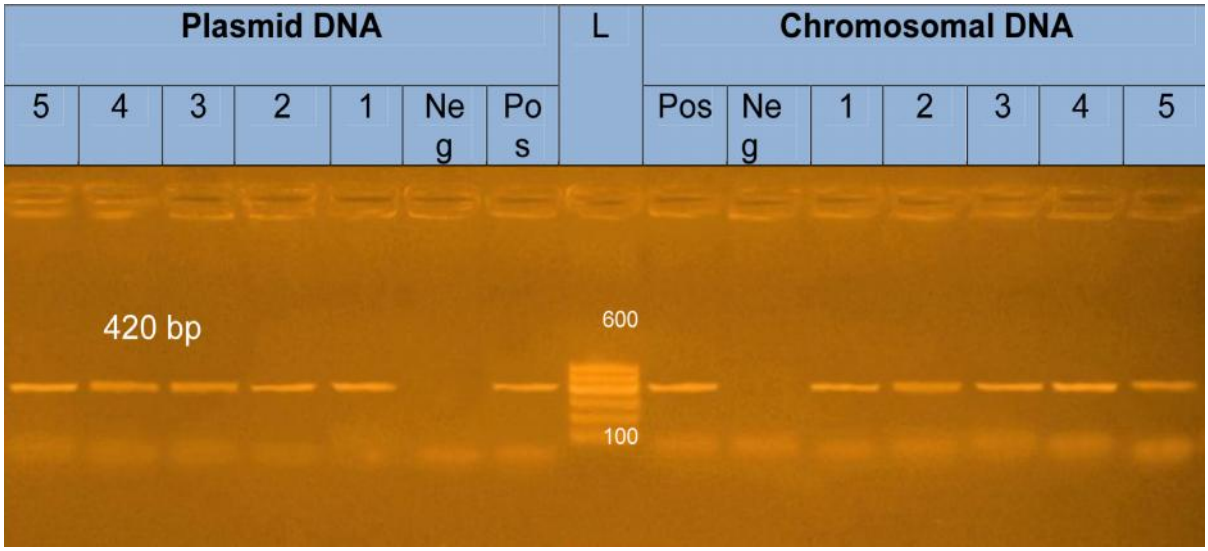
an incidence 10% and 10.37%, respectively in Egypt. Other investigators isolated *Salmonella* spp from broiler farms with percentages 29% in lithuania, 20% in Italy and 11% in Netherlands (Van Overbeke *et al.*, 2006; and Pieskus *et al.*, 2008). On the other hand, Osman (1992) detected 30% *Salmonella* strains in broiler farms.

Photo 3: Agarose Gel Electrophoreiss Showing an Amplification of tet(A) Gene (576 bp) in Salmonella Isolates from Ill Chicken



Note: Lane L: DNA ladder (100 bp), lane Pos: Positive control, Lane Neg: negative control, lanes 1, 7: *tet(A)* negative, lanes 1, 2, 3, 4, 5: *tet(A)* positive.

Photo 4: Agarose Gel Electrophoreiss Showing an Amplification of ere(A) Gene (420 bp) in Salmonella Isolates from Ill Chicken



Note: Lane L: DNA ladder (100 bp), lane Pos: Positive control, Lane Neg: negative control, lanes 1, 7: *ere(A)* negative, lanes 1, 2, 3, 4, 5: *ere(A)* positive.

Regarding to the serotyping, these results were nearly similar to Verma and Gupta (1995) who isolated *S. Typhimurium* with a percentage of (18%). On the other hand, the previous studies detected the predominance of *S. Enteritidis* (65%), *S. Kentucky* (16%) and *S. Typhimurium* (4%) as recorded by Soliman *et al.* (2016).

It was clear from this work that liver was the most organ of isolation followed by yolk sac then, spleen and heart. In contrast, Soliman *et al.* (2016) reported that intestine (17.39%) and yolk sac (13.64%) were the most common sites of *Salmonella* isolation. Also, Kapondorah and Sebunya (2007) revealed that 35% of the chicken livers were positive to *Salmonella.*

An increasing rate of *Salmonella* antimicrobial resistance has been recorded all over the world (Ashtiani *et al.*, 2009). In this work, these resistant antibiotics are commonly used for salmonellosis treatment in different poultry farms in Egypt. Nearly similar resistance rates to tetracycline, erythromycin, streptomycin and nalidixic acid were observed in *Salmonella* species recovered from chicken meat in Pakistan and Egypt (Soomro *et al.*, 2010; and Gharib *et al.*, 2015). On the other hand, the reduced susceptibility rates to ceftriaxone and ciprofloxacin corroborate with other researchers in India (Kaushik *et al.*, 2014).

This is not surprising because there is overuse of antibiotics in poultry farms with farmers not only for treatment and prevention of diseases without veterinary guidance but also for growth promotion, leading to the enteric bacterial flora resistance in poultry. Thus, the MDR *Salmonella*e strain may be developed from the enteric flora and transferred to human through food chain. The emergence of resistant *Salmonella* strains to fluoroquinolones (as ciprofloxacin) and third generation of cephalosporins (as ceftriaxone) is of great clinical significance because they are the front line therapeutic drugs for the treatment of non typhoidal *Salmonella* infection in hospitals (Bradford, 2001; and Hohmann, 2001). Thus, monitoring the resistance among bacteria isolated from clinical sources is necessary to plan effective antimicrobial treatments in severe cases and to inform about the consequences of antibiotic overuse.

The production of extended-spectrum β-lactameses (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) is the main mechanism of resistance to extended-spectrum β-lactam (ESBL) antibiotics in *Salmonella* isolates (Livermore *et al.*, 2006; and Eller *et al.*, 2013). Moreover, the most important concern regarding the spreading of MDR *Salmonella* isolates is the carrying of antibiotic resistance genes on mobile genetic elements such as plasmids. The plasmid is self-replicating double-strand circles of DNA that has an essential consideration in the acquirement of antibiotic resistance by conjugative transfer and so evolution and dissemination of MDR isolates (Carattoli, 2003).

TEM β-lactamase has been previously detected in *Salmonella* serovars recovered from animals in Korea and Japan as well as broilers in Egypt (Yang *et al.*, 2002; Ahmed *et al.*, 2009; and Ahmed and Shimamoto, 2012). In addition, resistant genes bla_{CTX}, that confers resistance to third-generation cephalosporins, located only in one isolate on plasmids with 20% not on chromosomal DNA (Photo 2) Moreover, one isolates harboring the bla_{TEM} gene also contained bla_{CTX} . In another study, only two *Salmonella* isolates harboring bla_{CTX} genes were identified on chromosome in Taiwan (Kao *et al.*, 2015).

Though there are different bacterial tetracycline resistance genes identified, one of the most common types is the *tetA(A)* in Gram-negative bacteria (Ng *et al.*, 2001). In this work, *tet(A)* gene was identified in all tested *Salmonella* isolates by 100% on bacterial chromosome and also presented on plasmid by 100%. This result

is compatible with other researchers (Riano *et al.*, 2006; and Ahmed and Shimamoto, 2012). Furthermore, the erythromycin belonging to the macrolides class is the most public drug used for treatment of gram-negative bacteria. In this study, polymerase chain reaction identified *ere(A)* conferring resistance to erythromycin in all examined *Salmonella* isolates by 100% and also present in plasmids by 100% on plasmid and bacterial chromosome of all examined *Salmonella* isolates by 100%. The erythromycin resistant genes (*ere(A*)) were previously detected in gram-negative bacteria (Nguyen *et al.*, 2009).

The more recently recognized structure among *Salmonella* strains is plasmids that have been linked to extended-spectrum beta-lactamase (ESBL) genes, tetracycline resistance genes and macrolides resistance genes (Garcia-Fernandez *et al.*, 2008; and Cain and Hall, 2012). As stated above, a variety of resistance-associated genes are transmitted by plasmids, including bla_{TEM}, bla_{CTX}, *tetA(A)* and *ere(A)* encoding resistance to ESBL antibiotics, tetracycline and erythromycin (Fricke *et al.*, 2009; and Fernandez-Alarcon *et al.*, 2011). This has indicated that the spread of multiple plasmids has been responsible for the dissemination of this resistance among Enterobacteriaceae around the world. This investigation supports the suggestion that the spreading of antimicrobial resistance is usually associated with the versatility of plasmids as well as the usage of antimicrobials in animal husbandry. Previous studies emphasized the correlation between the rapid development of resistance to antibiotics particularly β-lactams and plasmids in unrelated *Salmonella* strains (Eller *et al.*, 2013). These notes suggest that there is a worldwide problem associated with plasmid-mediated antimicrobial resistance that does not placed any limitations, either between animals and humans, or bacterial species and genera, indicative of the strong ability of plasmids to transmit horizontally. Although the occurrence of bla_{TEM}, bla_{CTX}, *tetA(A)* and *ere(A)* in *Salmonella* isolates were previously identified in Egypt (Ahmed and Shimamoto, 2012; and Abdel-Maksoud *et al.*, 2015), this is one of the little studies that demonstrate plasmid mediated antimicrobial resistance in *Salmonella* in Egypt.

CONCLUSION

In many instances, *Salmonella enterica* was isolated from different organs of ill-chicken. Data presented provide the indication that plasmid-mediated antibiotic resistance, including bla_{TEM}, bla_{CTX}, *tetA(A) and ere(A)*, are involved in MDR *Salmonella*. Thus the increase in MDR *Salmonella* serotypes is related to the high prevalence of transportable genetic elements as plasmid which causes serious problems for public health; hence, mandatory laws should be set by the authorities aiming at making a more careful use of antibiotics in veterinary medicine. Importantly, this is one of the little studies that characterize plasmid-mediated antibiotic resistance of *Salmonella* in Egypt. Further researches should be applied for understanding the extension of drug resistance, genetic diversity and opportunity of cross species transmission of the organism.

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