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Molecular Detection of some Antimicrobial Resistance Genes in *Salmonella* Species Isolated from Commercial Layers in Egypt

Mohammed A. Lebdah[1], Waffa M. Mohammed[2], Samah Eid [2] and Rehab I. Hamed[3*]
[1]Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt
[2] Reference Laboratory for quality control on poultry production (RLQP). Animal Health Research Institute, Dokki Lab., Giza, Egypt
[3]Reference Laboratory for quality control on poultry production (RLQP). Animal Health Research Institute, Sharkia Branch, Egypt

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Abstract

The present study was done to investigate the wide spread resistance to some antimicrobial groups among *S*almonellae isolated from replacement and layer flocks in Egypt. A total of 24 salmonellae were isolated from 200 birds (apparently healthy or diseased suffered from diarrhea, dehydration, respiratory distress and decrease of egg production) and serotyped into *S*. Enteritidis, *S*. Typhimurium, *S*. Kentucky and *S*. Newport. Twenty-one *S*almonella isolates were examined for resistance genes against different antimicrobials. The resistance pattern of all Salmonella isolates was done using antibiogram, the resistant isolates were examined for the presence of resistance coding genes using PCR technique. The investigated resistance genes were (*qnr*S, *aac (6')-ib-cr*) for quinolone resistant isolates, *bla*TEM for β-lactam resistant isolates, *aad*A1 and *aad*A2 for aminoglycosides resistant isolates and *tet*A(A) and *tet*A(B) for tetracycline resistant isolates. Resistant genes percentages for *tet*A(A), *tet*A(B), *bla*TEM, *aad*A1, *aad*A2, *aac (6')-ib-cr* and *qnr*S in the examined isolates were 70%, 20%, 93.3%, 30%, 80%, 10% and 15%, respectively. In conclusion, at the study area, antimicrobial resistance genes are widely spread in *Salmonella* isolates. Thus, minimizing the influence of antibiotics in treatment and prevention.

Keywords: *Salmonella*, Resistance Genes, Replacement Layer, Layers, Egypt

Introduction

Paratyphoid infection is a problem of economic concern to all phases of poultry industry from production to marketing. *Salmonella* spp. is gram negative, motile rods by peritrichous flagella. The genus *Salmonella* is divided into two species *Salmonella enterica* and *S. bongori*, *Salmonella enterica* itself is comprised of six subspecies, namely *S. enterica* subspecies *enteric*, *S.* enterica subspecies *salamae*, *S. enterica* subspecies Arizonae, *S.* enterica subspecies *diarizonae*, *S.* enterica subspecies *indica* and *S.*enterica subspecies *houtenae* [1]. The number of serotypes in each species and subspecies of *Salmonella* was reported to be around 2522 [2-5]. As a result of extensive use of antibiotics in human and veterinary medicine, serious increase in the spreading of multiple antibiotic resistant Salmonella has occurred [6]. Prolonged use, misusing, and overusing antibiotics led to in efficiency and enhanced the severity of the disease [7,8]. These resistant Salmonellae can be transmitted to humans through food such as poultry meat, therefore, they constitute a major public health problem [9]. Thus, this study was planned to detect antibiotic resistance genes in *Salmonella* infected replacement and layer flocks to select the highly effective antimicrobial agents for treatment and prevention.

Material and Methods

Samples collection

A total of 200 (apparently healthy (60), diseased (75), freshly dead (65) replacement and layers were collected from Sharkia Governorate and subjected to postmortem examination. The internal organs (liver, caecum, ovary, heart and lungs) were

***Corresponding author email: (rehab.44444@yahoo.com), Reference Laboratory for quality control on poultry production (RLQP). Animal Health Research Institute, Sharkia Branch.**

aseptically collected and subjected to bacterial isolation and identification.

Bacteriological examination

Isolation of *Salmonella* spp. was done according to ISO 6579 [10]. Briefly, samples were collected in buffered peptone water and incubated at 37°C for 24 h, after pre-enrichment 0.1 mL of the broth culture was transferred into a 10 mL Rappaport Vassiliadis Soya (RVS) broth and incubated at 42°C for 24-48 h. Another 1 mL of pre-enrichment broth was transferred into a tube containing 10 mL of Muller Kauffman Tetrathionate Novobiocin (MKTTN) broth and incubated at 37^{o}C for 24-48 h. A loop-full of material from the RVS broth and MKTTn was transferred and streaked separately onto the surface of Xylose Lysine Deoxycholate agar (XLD agar), Hektoen Enteric (HE agar), MacConkey's agar and S-S agar separately. Presumptive colonies were selected and purified on nutrient agar plates for further identification.

Biochemical identification

Identification of the isolates using oxidase reaction, hydrolysis of urea, H_2S production, Lysine decarboxylation, Indole test, Methyle red (MR) test, Voges Proskauer (VP) test and Simmon's Citrate agar were done according to Cruickshank *et al*. [11].

Serotyping

Biochemically suspected *Salmonella* isolates were subjected to serological identification according to Kauffman-White Scheme (Kauffman, 1974) for determination of somatic (O) and flagellar (H) antigens [10]. The antisera were kindly supplied by Prof. Dr. Mohamed Ahmed, Faculty of Veterinary Medicine, Benha University, Department of Food Control.

Antibiogram

The antimicrobial susceptibility testing was performed according to Finegold *et al*. [12] using the agar disc diffusion method on Mueller Hinton agar (Oxoid) plates**.** Few colonies of *Salmonella* were suspended in Muller broth and incubated for 4-5 hours until the turbidity was seen. Using sterile Pasteur pipette, 1 mL of the suspension was inoculated into the surface of the plate to wet the whole of its surface. Excessive fluid was discarded by its pipetting then plates were dried for up to 30 min then the chosen antibiotic discs were distributed to the surface of the plate.

The used antimicrobial agents were Florfenicol (30 µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Norfloxacin (10 µg), Amoxicillin (10 µg), Cefotaxime (30 µg), Amikacin (30 µg), Gentamycin (10 µg) Erthromycine (15 µg), Streptomycin (10 µg), Doxycyclin (30 µg) and Sulfamethaxezole-Trimethoprim (25 µg). The inhibition zone was measured to assess resistance or susceptibility according to the interpretation criteria established by Clinical Laboratory Standards Institute **(**CLSI) standard [13].

Molecular detection of resistance genes

The DNA extraction was done using QIAamp DNA Mini Kit DNA extraction kit (Catalogue no. 51304) according to manufactures' guidelines. The Oligonucleotide primers synthesized by Metabion (Germany) targeting several antibiotic resistance genes were used. Primers specific for tetracycline resistance gene *tet*A(A) and *tet*A(B) are F (5'-GGT TCA CTC GAA CGA CGT CA-3'); R (5'-CTG TCC GAC AAG TTG CAT GA-3') and F (CCT CAG CTT CTC AAC GCG TG-'3); R (5'-GCA CCT TGC TCA TGA CTC TT-'3), respectively [14]. Aminoglycosides resistance genes (*aad*A1 and *aad*A2) were detected using primers F (5'-TAT CAG AGG TAG TTG GCG TCAT-3'); R (5'- GTT CCA TAG CGT TAA GGT TTC ATT –'3) and F (5'-TGT TGG TTA CTG TGG CCG TA-3') R (5'-GAT CTC GCC TTT CAC AAA GC -3'), respectively [14,15]. β-lactams resistance gene (*bla*TEM) was detected using primers F (5'-ATC AGC AAT AAA CCA GC -3') R (5'-CCC CGA AGA ACG TTT TC -3') [16]. Quinolones resistance genes (*aac(6')-ib-cr* and *qnr*S) were detected using primers F (5'-CCC GCT TTC TCG TAG CA-3'), R (5'-TTA GGC ATC ACT GCG TCT TC-3') and F(5'-ACG ACA TTC GTC AAC TGC AA -3'),R (5'-TAA ATT GGC ACC CTG TAG GC-3'), respectively [17]. Reaction volume of 30 µL contained 12.5 µL of EmeraldAmp Max PCR Master Mix **(**Takara, Japan**)**, 1 µL of each primer of 20 pmol concentrations, 4.5 µL of nucleus free water and 6 µL of template DNA. Amplified DNA was separated by electrophoresis on 1.5% agarose gel (ABgene)

in 1x TBE buffer at room temperature. For gel analysis, 15 µl of the products was loaded in each gel slot. A 100 bp DNA Ladder (Qiagen, USA) was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software. The positive control was kindly provided by the Reference Laboratory for the Control of Poultry Production, Dokki, Giza.

Results

Cultural and biochemical characters

The cultural characters of the isolated salmonellae appeared on XLD agar are smooth pink colonies with black center (H_2S production) while, H-E agar they appeared as deep blue colored colonies. On MacConkey's agar, the colonies were pale, colorless smooth, transparent (non lactose fermenter) and on S-S agar they appeared as pale colored colonies with or without black centers.

Application of different biochemical tests revealed the following results; negative oxidase reaction, negative urea hydrolysis, positive reaction on TSI agar, positive reaction on LI agar, negative Indole reaction, positive MR reaction, negative VP reaction and positive on Simmon's Citrate agar.

Table 1: Isolation percentages of Salmonellae in organs of infected layer chickens

Samples	Positive Number	Percent
Liver	9	37.5%
Caecum	8	33.3%
Ovary	5	20.8%
Heart	2	8.3%
Lungs	0	0%

Occurrence of Salmonella spp. in different samples

Out of 200 examined birds, 24 (12%) were positive for *Salmonella* isolation**.** The isolation percentages from liver, caecum, ovaries, heart and lungs were 37.5%, 33.3%, 20.8% and 8.3%, respectively, while none of the lung samples were positive (Table1).

Serotyping of Salmonella isolates

The isolated 24 salmonellae were classified under four different serogroups including *S.* Enteritidis, *S.* Typhimurum, *S.* Kentucky and *S.* New port. The most prevalent serogroups were *S.* Enteritidis and *S.* Typhimurium with the percentage of 54.2% and 20.8%, respectively. Other serotypes including *S.* Kentucky and *S.* Newport were also recorded with the percentages of 8.3% and 4.2%, respectively. While, 3 isolates (12.5%) were untypeable

Antimicrobial susceptibility

The results in Table 2 show that *Salmonella* isolates revealed high resistance to nalidixic acid (95.2%) and Amoxycillin (71.4%). Moderate resistance to erythromycin (47.6%), streptomycin (47,6%), doxycycline (52.4%), gentamycin (47.6%), florophenicol (52.4%) and sulfamethaxezole/ trimethoprim (38.1%) was observed. Moreover, the isolates were sensitive to amikacin (80,9%); ciprofloxacin (76.2%), norofloxacine (71.2%) and cefotaxime (52.4%). Table (3) shows that multiple drug resistance to more than two antimicrobial drugs was observed in all the examined isolates.

Table 2: Antibiogram of different *Salmonellae* isolates

Antibiotic	Percentage of samples (n)		
	Sensitive	Intermediate	Resistant
Nalidixic acid	0	4.8% (1)	95.2% (20)
Amikacine	80.9% (17)	19.1% (4)	0
Ciprofloxacin	76.2% (16)	23.8% (5)	0
Norofloxacin	71.4% (15)	28.6% (6)	0
Cefotaxime	52.4% (11)	33,3 % (7)	14.3% (3)
Doxycycline	38.1% (8)	14,28% (3)	47.6% (10)
Sulfamethoxazol / trimethoprim	33.3% (7)	38.1% (8)	28.6% (6)
Streptomycine	23.8% (5)	28,58% (6)	47.6% (10)
Florophenicol	19.1%(4)	57.14% (12)	23.8%(5)
Gentamycin	19.1% (4)	33.33% (7)	47.6% (10)
Erthromycine	14.3% (3)	38,1% (8)	47.6%(10)
Amoxycillin	9.3% (2)	19.05% (4)	71.4 % (15)

The remained 3 isolates out of 24 obtained isolates were untypable.

Table 3: Antibiotic resistance pattern of *Salmonella* serotypes

Isolate No	Serotype	Resistance profile
1	*S*. Enteritidis	NA-AML-DO-E
2	*S*. Typhimurium	NA-AML-SXT
3	*S*. Kentucky	NA-AML-S-E- DO
4	*S*. Newport	NA-CN-E-SXT
5	*S*. Enteritidis	NA-CN-S-E-SXT
6	*S*. Enteritidis	NA-AML-DO-F
7	*S*. Typhimurium	NA-AML-CN-
8	*S*. Kentucky	NA-AML-CN-S-SXT
9	*S*. Enteritidis	NA-CN-DO-S-F
10	*S*. Enteritidis	NA-DO-E-F-SXT
11	*S*. Typhimurium	CN-S-E-SXT
12	*S*. Enteritidis	NA-AML-CN-S
13	*S*. Enteritidis	NA-AML-DO-SXT
14	*S*. Typhimurium	NA-DO-S-E
15	*S*. Typhimurium	NA-AML-S-E-CTX
16	*S*. Enteritidis	NA-AML-CN-DO
17	*S*. Enteritidis	NA-AML-E-F-CTX
18	*S*. Enteritidis	NA-AML-DO-S-E
19	*S*. Enteritidis	NA-AML-CN-DO
20	*S*. Enteritidis	NA-AML-CN
21	*S*. Enteritidis	NA-AML-S--F-CTX

NA=Nalidixic acid **AK**=Amikacine **CIP=**Ciprofloxacine **NOR**=Norfloxacin **AML**=Amoxycillin **CN**=Gentamycin **DO**=Doxycyclin **S**=Streptomycine **E**=Erthromycine F =Florophenicol **CTX**=Cefotaxime **SXT**=Sulfamethozol\trimethoprim

The remained 3 isolates out of 24 obtained isolates were un typable.

Molecular detection of resistance associated genes

Salmonella isolates that showed phenotypic resistance against different antimicrobial agents using antibiotic susceptibility test were examined by PCR to identify some resistance coding genes. The results revealed that *tet*A(A), *tet*A(B), *bla*TEM, *aad*A1, *aad*A2, *aac* (6')-*ib-cr* and *qnr*S were identified in 70%, 20%, 93.3%, 30% ,80 %, 10% and 15%, respectively (Table 4).

Discussion

In this study, 200 replacement and layers from different poultry farms at Sharkia Governorate were examined for the presence of *Salmonella* species. Twenty-four (12%) of the

examined birds were found positive. This was in agreement with Rehan [18] and Mohamed [19] who isolated *Salmonella* species from Dakahlia and Damietta Governorates in Egypt with an isolation rates of 12% and 12.4%, respectively. Meanwhile, these results were higher than 9.2% and 3.4% reported by AL-Abadi and Al-Mayah [20] and AL-Hakeem [21], respectively. These differences in the overall prevalence of *Salmonella* may be related to several factors such as environment, hygienic conditions of the farm and health status of the examined chicken.

Table 4: Occurrence of resistance genes among examined bacterial strains isolated from layer chickens

Resistance gene	Number of examined resistant isolates	Number of positive (%)
***qnr*S**	20	3 (15%)
acc(6)-ib-cr	20	2(10%)
***bla*TEM**	15	14(93.33)
***tet*A(A)**	10	7(70%)
***tet*A(B)**	10	2(20%)
***aad*A1**	10	3(30%)
***aad*A2**	10	8 (80%)

The prevalence of the isolated bacteria from different internal organs of the examined layers in this study showed that the highest percentage of *Salmonella* was recorded in liver (37.5%) followed by cecum (33,3%), ovary (20.8%) and heart (8.3%). These results are in agreement with Dhahar *et al*. [22] and AL-Iedani [23]. AL-Abadi and Al-Mayah [20] recorded that the highest percentage of *Salmonella* isolation was from cecum which is the primary sites of colonization of *Salmonella* due to the anatomical and structural location that allows the cecum to act as a blind sac with low content flow rate.

Salmonella strains which are resistant to a range of antimicrobials have emerged and are threatening to become a serious public health problem [8]. This resistance results from the misuse of antimicrobials both in human and poultry husbandry. In this study, 21 typable *Salmonella* isolates from replacement and layers were examined against 12 different antimicrobial agents belonged to different groups such as quinolone, phenicol, β-lactams, aminoglycosides, tetracyclines, cephlosporins and sulfonamide groups. Antibiogram of *Salmonella* isolates showed high resistance to nalidixic acid (95.2 %) and amoxicillin (71.4%). The obtained results differ from Habrun *et al*. [24] who reported that 100% *Salmonella* isolates were sensitive to florophenicol and streptomycin, while, 92 of the isolates (58%) were sensitive to nalidixic acid. In addition, Okamoto *et al*. [25] reported that florophenicol is the most effective antimicrobial for treating *Salmonella* infection as a result of lack of use of this antimicrobial in animals feed since 2003. In contrary to the obtained results, Cardoso *et al*. [26] reported that 100% of *Salmonella* isolates showed sensitivity to doxycycline, while, Shivhare *et al*. [27] reported high sensitivity of *Salmonella* spp. isolated from poultry to norfloxacin, while, all the isolates were resistant to sulfonamides trimethoprim. Moreover, our results agreed with Snow *et al*. [28] who reported that all *Salmonella* isolates from commercial layer flocks in UK were sensitive to amikacin. The results in this study differ from Zdragas *et al*. [29] who reported 5% resistance to streptomycin and 2% to nalidixic acid. Khan *et al*. [30] reported that 87.9% of salmonellae were sensitive to ciprofloxacin and amikacin. Moreover, Donado-Godoy *et al*. [31] reported that the resistance pattern of *Salmonella* isolated from chickens was 15% to ciprofloxacin, trimethoprim-sulfamethoxazole, streptomycin, and nalidixic acid. On the other hand, 8 *S*. Enteritidis isolates showed 100% sensitivity to ciprofloxacin, norfloxacin and gentamycin [26]. Also, Yah and Eghafona [32] reported that 183 *Salmonella* isolates from

chickens were highly resistant to chloramphenicol, gentamycin, trimethoprim and sulfamethoxazole and to lesser extent resistant to ciprofloxacin. Our results agreed with Threlfall [33] and Hendriksen [34] who recorded that *S.* Typhimurum isolates were resistant to amoxicillin, chloramphenicol, streptomycin and sulfonamides. In contrary to the obtained results in the current study, Asway *et al*. [35] mentioned that the isolated *Salmonella* strains were resistant to ciprofloxacin.

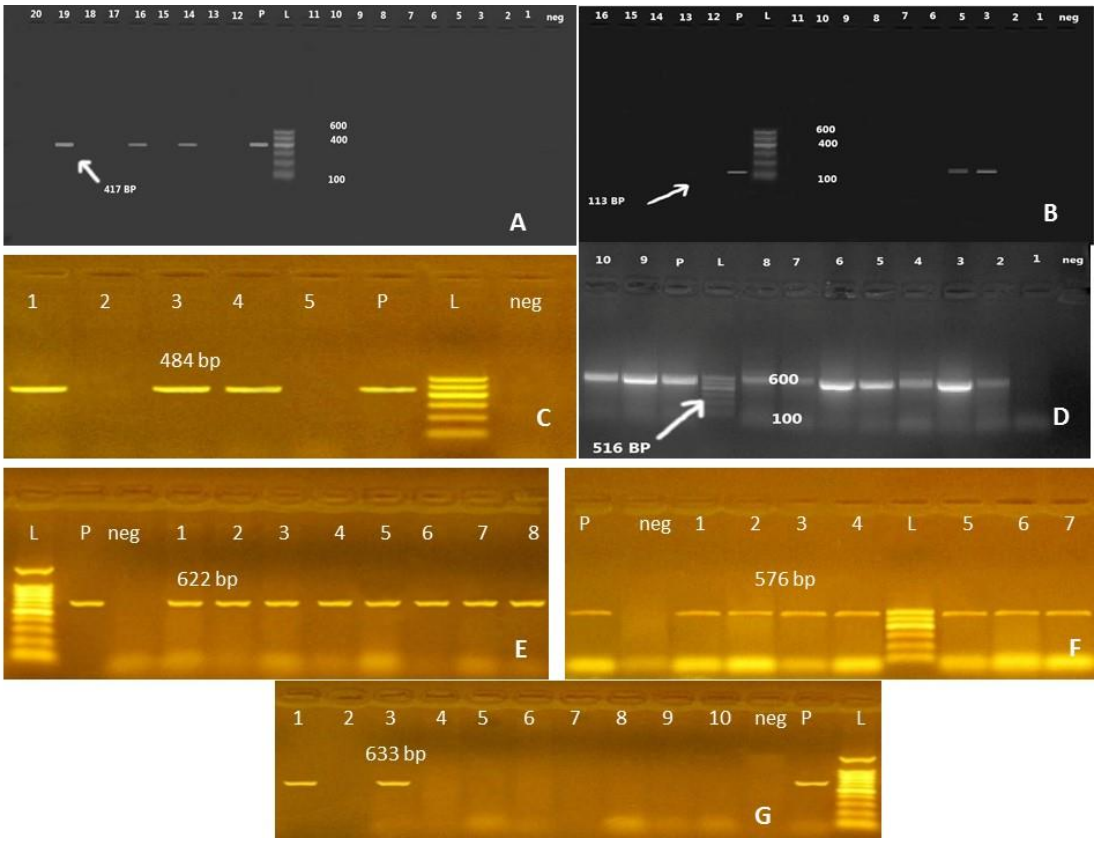

Figure 1: Exemplar of gel pictures showing amplified products of resistance associated genes; In each photo "P" stands for positive control, "neg": Negative control and numbers indicates lanes with positive and negative samples; A: *qnr*S gene (417bp); B: *acc(6)-ib-cr* gene (113bp); C: *aad*A1 gene(484bp); D: *bla*TEM gene(516 bp); E: *aad*A2 gene(622bp); F: *tet*A(A) gene(576 bp); G: *tet*A(B) gene(633 bp).

All *Salmonella* isolates from poultry origin were resistant to at more than one antimicrobial, indicating multiple drug resistance. These findings confirm that poultry is a major reservoir of multi-resistant *Salmonella* and this had no correlation with the presence of antimicrobial resistance gene indicating that other mechanisms of acquiring resistance could be present [25,36]. Molecular identification of *Salmonella* resistance genes showed that *qnr*S and *aac(6')-ib-cr* resistance genes for quinolones were reported with the percentages of 15% and 10 %, respectively. This with inconstant with Kim *et al*. [37] who investigated the prevalence and characteristics of plasmid mediated quinolone resistance genes (*qnr*, *aac(6')-ib-cr*) and found that 3.2% of the isolates contained *qnr* genes but none carried the *aac(6')-ib-cr*. While, Hao *et al*. [38] reported *aac(6')-ib-cr* and *qnr*S resistance genes in *Salmonella* isolates with the percentages of 20.2% and 1.6%, respectively.

The *bla*TEM gene, a gene encoded for β-lactamases resistance was reported in the present study with the percentage of 93.3%. This was similar to a study performed by Hur *et al*. [39] who reported that 90.5% of penicillin resistant *S.* Enteritidis carried the *bla*TEM gene. In another study, the percentage of *bla*TEM was 10% which was identified in 10 *Salmonella* isolates from retail chicken meat [40]. However, *bla*TEM gene was

detected in 51.6% resistant *Salmonella* isolates [41]. Aslam *et al*. [42] reported that the percentage of *bla*TEM gene in *Salmonella* isolated from retail meats in Canada was 17% and this gene was the most common resistance genes found. A total of 108 *S.* Indiana possessed *bla*TEM gene with a percentage of 81.2% [43]. The *aad*A2 gene, a gene encoded for streptomycin resistance was reported in the present study with the percentage of 80%. These results agree with a study performed by Shahada *et al*. [44] who reported that all streptomycin resistant *S.* Infantis from poultry in Japan carried *aad*A1 gene, while, Mohamed [19] recorded that 17 isolates possessed *aad*A1 gene with the percentage 53.1%. Sheng *et al*. [45] reported lower percentage of *aad*A2 gene in only three *Salmonella* isolates out of seventy-three isolates.

The *tet*A gene, a gene encoded for tetracycline resistance was reported in the present study with the percentage of 70%. Higher percentage was obtained by Lu *et al*. [46] who reported that 108 *Salmonella* isolates possessed *tet*A gene with the percentage of 81.2%. Moreover, Shahada *et al*. [44] reported that 89% of oxytetracycline-resistant *Salmonella* from poultry carried the *tet*(A) gene. Percentages ranging from 66.7%-100%% of *Salmonella* carriage of tetracycline resistance gene *tet*A were reported by several studies [39,47-50]. Another study by Aslam *et al*. [42] reported that the percentage of *tet*(A) gene in *Salmonella* isolates was 28%. Bacci *et al*. [51] recorded that *tet* class genes are considered the most common types in gram negative bacteria, also *tetA* and *tet*B genes are located inside non-conjugative transposons which are important way for the horizontal transfer of antibiotic resistance.

Conclusion

It could be concluded that the antimicrobial resistance genes of *Salmonella* infected chickens were extensively spread in the study area. Thus, leading to minimizing the influence of antibiotics in both treatment and prevention and increase public health significance. Therefore, other tools of prevention and treatment are important to avoid this problem.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربى

الكشف الجزيئي على بعض الجينات المقاومة لمضادات الميكروبات فى السالمونيلا المعزولة من الدجاج البياض التجاري فى مصر

محمد عبد العزيز لبده [1]،وفاء محمد محمد[2]، سماح عيد [2], رحاب اسماعيل حامد[3]
[1]قسم طب الطيور والارانب - كلية الطب البيطرى- جامعة الزقازيق
[2]المعمل المرجعي للرقابه علي الانتاج الداجنى معهد بحوث صحة الحيوان- الدقى – الجيزة
[3]المعمل المرجعي للرقابه علي الانتاج الداجنى معهد بحوث صحة الحيوان - فرع الشرقيه

تهدف هذه الدراسة الي دراسة مدي الانتشار الواسع لمقاومة عترات السالمونيلا المعزولة من قطعان الاستبدال والبياض في مصر لبعض مجموعات مضادات الميكروبات المختلفة. تم فحص 24 سالمونيلا معزولة من 200 طائر (التي تبدو سليمة واخري تعاني من اسهالات وجفاف وأعراض تنفسية وإنخفاض في معدل انتاج البيض) والتي تم تصنيفها سيرولوجيا الي Salmonella Enteritidis, S. Typhimurium, S. Kentucky and S. Newport. 21 معزولة من السالمونيلا تم اختبارها لاستبيان وجود جينات المقاومة للمضادات الميكروبية المختلفة. تم التعرف على النمط المقاوم للمضادات الحيوية بإستخدام إختبار الحساسية. كما تم اختبار المعزولات المقاومة للتأكد من وجود جينات المقاومة للميكروبات بإستخدام إختبار البلمرة المتسلسل للجينات *qnr*S, *aac(6)-ib-cr* للكينولون، *bla*TEM للبيتالاكتام، *aad*A1, *aad*A2 للامينوجليكوسايدس، *tet*A(A), *tet*A(B) للتيتراسيكلين. وقد أظهرت النتائج أن الجينات المقاومة للمضادات الحيوية *tet*A(A), *tet*A(B) *bla*TEM, *aad*A1, *aad*A2, *aac(6)-ib-cr* and *qnr*S جائت بالنسب 70%، 20%، 93.3%، 30%، 80%، 10% و15% علي التوالي. ويستخلص من هذه الدراسة مدي انتشار عزلات السالمونيلا المقاومة للمضادات الميكروبية في الدواجن بالمنطقة محل الدراسة ممايؤدي الي التقليل من تأثير المضادات الحيوية في العلاج والوقاية.