Detection of some antibiotic resistant genes within *Salmonella* serovars isolated from broiler chickens

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Abstract:
This study aimed to examine the sensitivity of different antibiotics against 10 *Salmonella* serovars isolated previously from broiler farms. After that, all resistant serovas were detected genetically for the presence of *qnrA*, *bla*TEM, *tet*A (A) and *aadB* resistant genes. Two *S.* Typhimurium, one *S.* Kentucky, one *S.* Newport, one *S.* Tamale, one *S.* Enteritidis, one *S.* Molade, one *S.* Takoradi, one *S.* Virchow and one *S.* Inganda were examined sensitivity against different antibiotics by disc diffusion method. The resistant serovars to quinolones, β lactams, gentamycin and doxycyclin were confirmed genetically. *S.* Kentucky, *S.* Tamale, *S.* Molade, *S.* Typhimurium and *S.* Takoradi were resistant to quinolones and carrying *qnr*A gene. *S.* Tamale, *S.* Inganda, *S.* Typhimurium, *S.* Newport, *S.* Molade, *S.* Typhimurium, *S.* Enteritidis, *S.* Takoradi and *S.* Virchow were resistant to β lactams and carrying *blaTEM* gene. *S.*Tamale, *S. Inganda*, *S.* Typhimurium, *S.* Newport, *S.* Molade, *S.* Enteritidis and *S.* Virchow were resistant to doxycyclin and carrying *tet*A (A) gene. *S.* Typhimurium, *S.* Enteritidis and *S.* Takoradi were resistant to gentamycin *and carrying aad*B gene. The results showed that emerging of multidrug resistant strains to common antibiotics in the poultry field required newly discovered herbal compounds for control the disease and prevent the resistant phenomena.

Keywords: *Salmonella* serovars, antibiotic sensitivity, broilers, multidrug resistant.

Introduction:

Poultry wealth is considered an important sector in national economy in Egypt on the one hand it represents a large part of the food security and on other hand, a source of employment in the poultry companies. Salmonellosis is an important health problem and a major challenge worldwide. *Salmonella* spp. are recognized as the most causative agents of food poisoning. ***(Gallegos et al., 2008).*** Multiple drug resistance genes have been found to be clustered on individual mobile elements, which mean that multi-resistance can be readily transferred and increase the multi-drug resistant bacterial population ***Nikaido (2009). El-Sharkawy et al. (2017)*** reported that all *S.* Enterica serovar Enteritidis isolates were susceptible to all tested antimicrobials (tetracycline, ampicillin, sulfamethoxazole/trimethoprim, gentamicin, streptomycin and chloramphenicol). The phenotypically resistant *S.* Enterica serovar Typhimurium isolates against ampicillin, tetracycline, sulphamethoxazole and chloramphenicol were harbouring *bla*TEM, (*tet*A and *tet*C), (*sul*1 and *sul*3) and (*cat*1 and *flo*R), respectively. The sensitivity rate of *S.* Enteric serovar Typhimurium to gentamycin, trimethoprim/sulphamethoxazole and streptomycin were 100, 94.8, and 89.7%, respectively. Recently, a basic role in dissemination and evolution of antimicrobial resistance in MDR *S.* Typhimurium DT104(MDR-DT104) and many other organisms has been attributed to integrons, gene expression elements that potentially account for rapid and efficient transmission of drug resistance because of their mobility and ability to collect resistance gene cassettes; ***(Tosini et al., 1998).*** So, this study aimed to detect the encoding genes responsible for antibiotic resistance by using PCR.

Material and methods:

Bacterial isolation: A total of 10 *Salmonella* serovars (2 *S.* Typhimurium, 1*S.* Kentucky, 1 *S.* Newport, 1 *S.* Tamale, 1 *S.* Enteritidis, 1 *S.* Molade, 1 *S.* Takoradi, 1 *S.* Virchow and 1 *S.* Inganda were recovered previously from broilers farms from different internal organs. All isolates were previously handled and isolated from different farms in Dakahlia Governorate and the clinically examined birds showed signs of septicemia, retarded growth, depression, profuse watery white diarrhea and accumulation of fecal matter around the vent.

Identification of recovered isolates: Each isolate was inoculated separately in selenite F broth and incubated at 37°C for not more than 18 hours and rappaport-vassiliadis soya broth incubated at 42°C for 24 hours. A loopful from the enrichment culture was streaked

onto the surface of Xylose Lysine Deoxycholate (XLD) media and *Salmonella* Shigella agar (S-S agar) then incubated at 37ºC ± 1ºC for 24hrs ± 2hrs. Each colony was identified morphologically and biochemically according to ***Quinn et al., (2002).***

Antibiotic sensitivity testing according to ISO 6579 (2002) method:

Determination of the susceptibility of the isolated strains to antimicrobial discs was adopted using the disc diffusion technique according to ***Finegold and Martin (1982)***.

PCR detection of antibiotic resistant genes

PCR assay was done to detect antibiotic resistance genes in the isolates. The isolates that showed resistance to antimicrobial agents in sensitivity tests for quinolones were examined for the presence of *qnr*A gene while the isolates that showed resistance to β lactams were examined for the presence of *bla*TEM gene. The isolates that showed resistance to doxycyclin were examined for *tet*A (A) gene. The isolates that showed resistance to gentamycin were examined for the presence of *aad*B gene. QIAamp DNA Mini Kit used for extraction of DNA. Primers used were shown in table (1). Temperature and time conditions of the primers during PCR are shown in table (2). Electrophoresis grade agarose (1 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethedium bromide was added and mixed thoroughly according to **(*Sambrook et al., 1989*).**

Table (1): *Primers of tested antibiotic resistance genes.*

Primer	Sequence	Amplified product	Reference
***bla*TEM**	ATCAGCAATAAACCAGC	516 bp	**Colom *et al*., 2003**
	CCCCGAAGAACGTTTTC		
***qnr*A**	ATTTCTCACGCCAGGATTTG	516 bp	**Robicsek *et al*., 2006**
	GATCGGCAAAGGTTAGGTCA		
***aad*B**	GAGCGAAATCTGCCGCTCTGG	319 bp	**Frana*et al*., 2001**
	CTGTTACAACGGACTGGCCGC		
***tet*A (A)**	GGTTCACTCGAACGACGTCA	576 bp	
	CTGTCCGACAAGTTGCATGA		

Table (2): *Cycling conditions of the different primers during cPCR*

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
*bla*TEM	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.
*qnr*A	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.
*aad*B	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
*tet*A(A)	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.

Results:

It was clear in table (3) that all *salmonellae* were sensitive to amikacin, norfloxacin, ciprofloxacin except *S.* Takoradi resistant to ciprofloxacin. All *salmonellae* were sensitive to enrofloxacin except *S.* Kentucky, *S.* Tamale *&* *S.* Molade. However, all *salmonellae* were resistant to flumequine except *S.* Typhimurium, *S.* Tamale, *S.* Molade *&* *S.* Inganda. All examined *salmonellae* were sensitive to amoxicillin except *S.* Typhimurium, *S.* Newport, *S.* Tamale*&* *S.* Enteritidis. However, all examined *salmonellae* were resistant to ampicillin except *S.* Typhimurium, *S.* Kentucky , *S.* Newport*&* *S.* Enteritidis. All *salmonellae* were sensitive to neomycin except *S.* Kentucky, *S.* Newport, *S.* Enteritidis*&* *S.* Inganda. Also, all *salmonellae* were sensitive to gentamycin except *S.* Typhimurium, *S.* Enteritidis*&* *S.* Takoradi. All *salmonellae* were resistant to doxycycline hydrochloride except *S.* Kentucky*&* *S.* Takoradi sensitive to doxycycline.

Five *Salmonella* isolates were examined by PCR for *qnrA* gene (a resistant gene for quinolones) while other *Salmonella* isolates not examined for this gene as they were sensitive to quinolones. All isolates were positive for this gene giving amplification of 516 bp fragments. Also, the positive control showed 516 bp fragments whereas no amplification could be observed with the negative control as shown in table (4) and figure (1).

Nine *Salmonella* isolates were examined by PCR for *bla*TEM gene (a resistant gene for β Lactamases) while other isolates not examined by PCR as they were sensitive to β Lactamas. Nine isolates (100%) were positive for this gene giving amplification of 516 bp fragments. Also the positive control showed 516 bp fragments whereas no

amplification could be observed with the negative control as in table (4) and figure (2).

Seven *Salmonella* isolates were examined by PCR for *tet*A (A) gene (a resistant gene for tetracycline) while other isolates not examined for this gene as they were sensitive to doxycycline. All isolates (100%) were positive for this gene giving amplification of 576 bp fragments. Also the positive control showed 576 bp fragments whereas no amplification could be observed with the negative control as in table (4) and figure (3).

Three *Salmonella* isolates were examined by PCR for *aad*B gene (a resistant gene for gentamycin) whiles other *Salmonella* isolates not examined for this gene as they were sensitive to gentamycin. All isolates (100%) were negative for this gene whereas no amplification at 319 bp fragments. The positive control showed 319 bp fragments whereas no amplification could be observed with the negative control as in table (4) and figure (4).

Table (3) *Results of antibiotic sensitivity tests*

Antibiotic discs / Strains	CIP	ENR	NOR	UB	AMX	AMP	N	CN	AK	DO
***2 S.* Typhimurium**	S	I	S	I	R	I	I	R	S	R
***S.* Kentucky**	I	R	S	R	I	S	R	S	S	I
***S.* Newport**	S	I	S	R	R	S	R	I	S	R
***S.* Tamale**	I	R	I	S	R	R	I	S	S	R
***S.* Enteritidis**	I	I	S	R	R	S	R	R	S	R
***S.* Molade**	I	R	S	I	S	R	S	I	I	R
***S.* Takoradi**	R	I	S	R	I	R	I	R	S	I
***S.* Virchow**	S	I	S	R	I	R	S	I	S	R
***S.* Inganda**	S	I	S	I	S	R	R	S	S	R

R= resistant, S= sensitive, I= intermediately sensitive, CIP= Ciprofloxacin, ENR= Enrofloxacin, NOR= Norfloxacin, UB= Flumequine, AMX= Amoxicillin,AMP= Ampicillin,N= Neomycin, CN= Gentamycin,AK= Amikacin, DO= Doxycycline hydrochloride

Table (4) *Results of Polymerase Chain Reaction technique for different resistant genes from the examined isolates.*

Sample	Serovar	*qnr*A	*bla*TEM	*tet*A(A)	*aad*B
1	*S.* Kentucky	+	Nd	Nd	Nd
2	*S.* Tamale	+	+	+	Nd
3	*S.* Inganda	Nd	+	+	Nd
4	*S.* Typhimurium	Nd	+	+	-
5	*S.* Newport	Nd	+	+	Nd
6	*S.* Molade	+	+	+	Nd
7	*S.*Typhimurium	+	+	Nd	Nd
8	*S.* Enteritidis	Nd	+	+	-
9	*S.* Takoradi.	+	+	Nd	-
10	*S.* Virchow	Nd	+	+	Nd

qnrA = resistant genes for quinolones, ***bla TEM***= resistant genes for B-lactamases, ***tetA (A)*** = resistant gene for doxycycline, ***aadB*=** resistant gene for gentamycin, Nd = not examined by PCR = sensitive samples.

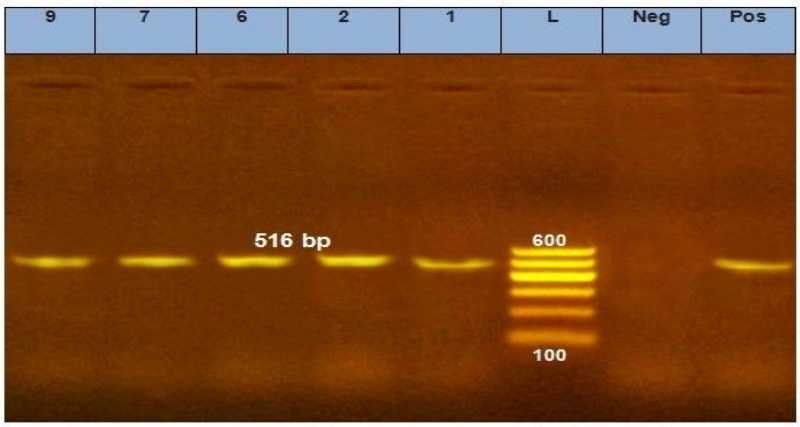

Figure (1): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *qnr*A gene (516 bp). Lane L= ladder (100-600 bp). Pos= positive control. Neg= negative control. Lane 1: *S.* Kentucky. Lane 2: *S.* Tamale. Lane 6: *S.* Molade. Lane 7: *S.* Typhimurium. Lane 9: *S.* Takoradi.

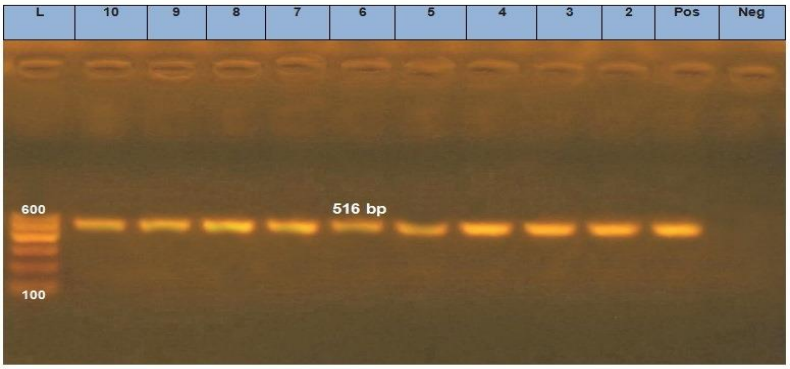

Figure (2): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *bla*TEM gene (516 bp). L: ladder (100-600 bp). Pos.: Positive control. Neg.: Negative control. Lane 2: *S.* Tamale. Lane 3: *S.* Inganda. Lane 4: *S.* Typhimurium. Lane 5: *S.* Newport. Lane 6: *S.* Molade. Lane 7: *S.* Typhimurium. Lane 8: *S.* Enteritidis. Lane 9: *S.* Takoradi. Lane10: *S.* Virchow.

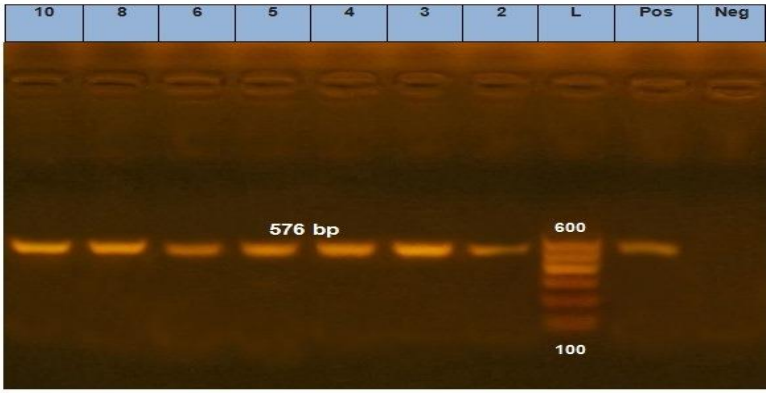

Figure (3): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *tet*A(A) gene (576 bp). L=ladder (100-600 bp).Pos.: Positive control. Neg.: Negative control. Lane 2: *S.* Tamale. Lane 3: *S.* Inganda. Lane 4: *S.* Typhimurium. Lane 5: *S.* Newport. Lane 6: *S.* Molade. Lane 8: *S.* Enteritidis. Lane10: *S.* Virchow.

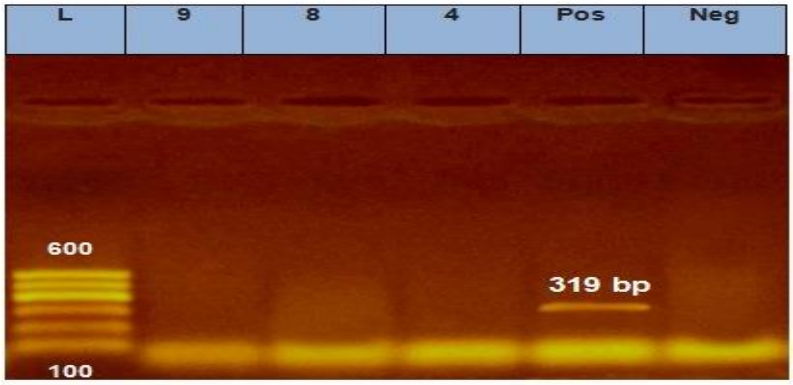

Figure (4): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *aad*B gene (319bp). L=ladder (100-600 bp). Pos.: Positive control. Neg.: Negative control. Lane 4: *S.* Typhimurium. Lane 8: *S.* Enteritidis. Lane 9: *S.* Takoradi.

Discussion:

In this study, all strains were sensitive to amikacin and norfloxacin (100%) which was the most effective chemotherapeutic agent against *Salmonella* infection which is in parallel with the result recorded by ***(Snow et al., 2007)*** who reported sensitivity to amikacin was (100%) and ***Shivhare et al. (2000)*** who recorded highest sensitivity of *Salmonella* isolated from poultry to norfloxacin was 92%. Also, higher rates of sensitivity were observed to ciprofloxacin (89%) and streptomycin (77.7%) and this nearly agree with ***Habrun et al. (2012)*** who reported that all *Salmonella* isolates were sensitive to streptomycin (100%). However, ***Hussain et al. (2010)*** and **Cardoso *et al.* (2006)** found that higher resistance to streptomycin (92.10%). The rise in resistance to gentamicin and norfloxacin is of concern, as these drugs are often considered in the treatment and control of several poultry diseases.

PCR was a perfect tool for accurate detection of *Salmonella* resistant genes and the results that *qnr*A gene a resistant gene for quinolones were reported in all examined isolates with a percentage of (100%). The results obtained for *qnr*A gene disagree with ***Kees et al. (2008).*** It is admitted that resistance to quinolones results from both chromosomal and plasmid-mediated quinolone resistance (PMQR) mechanisms. *Qnr* genes represent one of the most important PMQR mechanisms. These genes encode pentapeptide repeat proteins that block the action of ciprofloxacin (CIP) on bacterial DNA gyrase and topoisomerase IV ***Tran and Jacoby (2002).*** Three major groups of qnr

determinants have been described (*qnr*A, *qnr*B, and *qnr*S), which share between 40% and 60% similarity ***Strahilevitz et al. (2009).*** The *bla*TEM gene, a gene encoded for B- lactamases resistance was reported in the present study with a percentage of 100% and these results nearly in coordinated with ***Hur et al. (2011)*** who reported that 19 out of the 21 penicillin resistant *S.* Enteritidis in Korea carried the *bla*(TEM) gene with a percentage of (90.5%). However, ***Ahmed et al. (2009)*** found the percentage of *bla*(TEM-1) was 10% which was identified in between10 *Salmonella* isolates from retail chicken meat in Hiroshima, Japan. While, ***El-Sharkawy et al. (2017)*** reported that 65.5 % of *S.* Enterica *serovars* Typhimurium were harboured ampicillin (*Bla*TEM).

The *tet*A (A) gene, a gene encoded for tetracycline resistance was reported in the present study with a percentage of 100% these results agree with ***Yemisi et al. (2014)*** who reported that all of the 20 TET-resistant *Salmonella* isolates carried *tetA* gene (100%) and 30% (6), 35% (7), and 50% (10) of the isolates carried *tet*B, *tet*C, and *tet*G genes, respectively and these results nearly in coordinated with ***Lu et al. (2011)*** who reported that 108 *S.* Indiana possessed *tet*A gene with a percentage of 81.2% and ***Shahada et al. (2006)*** who reported that 89% of oxytetracycline-resistant *S.* Infantis from poultry in Japan carried the *tet*(A) gene. Moreover, ***El-Sharkawy et al. (2017)*** found that 84.5% of *S.* Enterica *serovars* Typhimurium and 50% of *S.* Enterica *serovars* Enteritidis isolates were harboured. The *aad*B gene, a gene encoded for gentamycin resistance was abscence in the present study with a percentage of 100% and this result agree with a study performed by ***El-Sharkawy et al. (2017)*** who reported that *aad*B and *aac*C (gentamycin resistance) were not amplified in all screened isolates.

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الكشف عن بعض الجينات المقاومة للمضادات الحيوية داخل عترات السالمونيلا المعزولة من دجاج التسمين

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تهدف هذه الدراسة إلى فحص نشاط المضادات الحيوية المختلفة ضد ١٠ أنواع من عترات السالمونيلا المعزولة سابقًا من مزارع التسمين. بعد ذلك ، تم فحص جميع العترات المقاومة لوجود جينات *qnr*A و *bla*TEM و *tet*A (A) و *aad*B. تم اجراء اختبارات الحساسيه لعترات السالمونيلا المعزوله للمضادات الحيوية: سالمونيلا تيفيميوريوم (٢) , سالمونيلا كنتاكي (١) , سالمونيلا نيوبورت (١), سالمونيلا تامالي (١), سالمونيلا انتريتيدس (١) , سالمونيلا مولادي (١), سالمونيلا تاكورادي (١), سالمونيلا اينجيدي (١) وسالمونيلا فيرشو(١). وقد تبين مقاومة سالمونيلا كنتاكي , سالمونيلا تامالي , سالمونيلا مولادي, سالمونيلا تيفيميوريوم و سالمونيلا تاكورادي للكينولونات بسبب وجود جين *qnr*A. كما تبين مقاومة سالمونيلا تامالي, سالمونيلا اينجيدي, سالمونيلا تيفيميوريوم, سالمونيلا نيوبورت, سالمونيلا مولادي, سالمونيلا انتريتيدس, سالمونيلا تاكورادي وسالمونيلا فيرشو للبيتا لاكتام بسبب وجود جين *bla*TEM. وايضا مقاومة سالمونيلا تامالي, سالمونيلا اينجيدي, سالمونيلا تيفيميوريوم, سالمونيلا نيوبورت, سالمونيلا مولادي, سالمونيلا انتريتيدس وسالمونيلا فيرشو للدوكسيسيكلين لوجود جين *tet*A (A). كما وجد مقاومة سالمونيلا تيفيميوريوم, سالمونيلا انتريتيدس و سالمونيلا تاكورادي للجنتاميسين بسبب وجود جين *aad*B. أعلنت النتائج أن ظهور عترات مقاومة للأدوية المتعددة للمضادات الحيوية الشائعة في مجال الدواجن يتطلب اكتشاف مركبات عشبية حديثًا للسيطرة على المرض ومنع ظاهرة مقاومة مضادات الحيوية.