Antibacterial resistance and resistance gene detriments of *E.coli* isolated from chicken.

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A B S T R A C T

A total of 30 isolate of *E.coli* isolated from diseased chicken belonged to different serotypes. All isolate showed resistance to one or more antibiotics by disc diffusion methods. They were investigated against 6 antibiotics from different groups the result were, gentamicin about 46.6%, erythromycin 63.3%, tetracycline 80%, ciprofloxacin 40%, ampicellin73.3% and florfenicol 53%.3. Detection of resistance genes by PCR test relieved negative result in all isolated to detected *aac(6')-Ib-cr* gene, while 22 isolate out of 30 examined *E.coli* isolate were carried the *bla*TEM gene on anther hand *flo*R gene was detected in 29(96%) of *E.coli* isolate. Also *tetA* (A) gene was found in 22(73%) of *E.coli* isolate out of 30 examined isolate. On another hand *aad*B (gentamicin resistance gene) gene was present in 8(26%). 11(36%) of *E.coli* isolate were carried the *mph (A)* erythromycin resistance gene .Correlation between the presence of resistance gene and the resistance to antibiotics were recorded.

Keywords: *E.coli*, Resistance gene, Disc diffusion method, PCR.

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1. INTRODUCTION

Escherichia coli are a normal inhabitant of the gastrointestinal tract of humans and animals; however, some strains are known to be pathogenic. These strains induce colibacillosis in chicken, which is an important cause of economic losses for the poultry industry **(**Amara et al., 1995). It is one of the most important and frequently encountered bacterial avian pathogen causing a wide variety of disease syndrome in birds causing up to 30% of mortality in poultry (Kaul et al., 1992, Barnes and Gross 1997 and Geornaras et al., 2001). Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic Escherichia coli (APEC), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), cellulites (inflammatory process), swollen head syndrome, peritonitis, salpingitis, osteomyelitis/synovitis (turkey osteomyelitis complex), panophthalmitis and omphalitis/yolk sac infection (Saif, 2003). Concern about antibiotic resistance and its transmission to human pathogens is important because these resistant bacteria may colonize the human intestinal tract and may also contribute resistance genes to human endogenous flora. Colonization of the intestinal tract with resistant *E. coli* from chicken has been shown in human volunteers (Linton et al., 1977). The result of a study done by Van den Bogaard et al., (2001) strongly indicated that transmission of resistant clones and resistance plasmids of *E.coli* from poultry to humans commonly occurs (Van den Bogaard et al., 2001). Furthermore, in some previous studies,

spread of an antibiotic resistance plasmid, pSL222-6, in *E.coli* from chickens to human (Levy et al., 1976), direct transmission of *E. coli* resistant to streptomycin, sulphonamides and tetracycline from poultry to poultry attendants in Nigeria **(**Ojeniyi, 1985; Ojeniyi, 1989), recorded that evidence that animals were a reservoir for *E.coli* found in humans (Cooke, 1971), chickens as a source of antibiotic resistance in humans in Saudi Arabia, Morocco and northern India (Singh et al., 1992; Amara et al., 1995; and Al Ghamdi et al., 1999) was described. The aim of this work was the detection of the resistant antibiotic gene by PCR techniques and correlation with resistant to antibiotics.

2. MATERIAL AND METHODS

2.1. Chicken samples

A total of 30 isolates of *E. coli* isolated from diseased chickens was taken from the culture collection of the Central Laboratory for Veterinary Quality Control on Poultry Production, Dokki. The *E. coli* isolates were recovered from different organs (liver, lung, yolk sac and bone marrow) of chickens that suffered from colisepticaemia. The isolates belonged to different O-serogroups and showed resistance to one or more antibiotics.

2.2. Antimicrobial sensitivity

The disk diffusion technique was applied according to (Cruickshank et al., 1975).Six antibacterial sensitivity discs was used as florfenicol, ciprofloxacin, tetracycline, erythromycin, gentamicin and ampicillin.

2.3. Detection of resistance genes using Polymerase chain reaction (PCR)

Oligoneucleotide primers were designated according to Integrated DNA Technology. The primers sequences were illustrated as in Table (1).

2.3.1. DNA extraction and purification

The extraction was done by QIAamp® DNA MiniKit (Cat. No. 51304, Qiagen) used according to manufacturer's instructions.

2.3.2. Amplification and cycling protocol for conventional PCR

Using of PCR 1.1x ReddyMix TM Master Mix (Thermo SCIENTIFIC) with Cat. No. AB0575/LD-A.

2.3.3. Detection of PCR products: (Sambrooket al., 1989)

Aliquots of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel.

Table (1) Cycling conditions of the different primers during PCR

Primer	Sequence(5'- 3')	Amplified product	Reference
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	Randall et al. 2004
aadB	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	319 bp	Franaet al., 2001
bla$_{TEM}$	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom et al., 2003
aac(6′)-Ib-cr	CCCGCTTTCTCGTAGCA TTAGGCATCACTGCGTCTTC	113 bp	Lunnet al., 2010
mph(A)	GTGAGGAGGAGCTTCGCGAG TGCCGCAGGACTCGGAGGTC	403 bp	Nguyen et al., 2009
floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494 bp	Doubletet al., 2003

tetA(A): tetreacyclin, aadB: gentamicin bla$_{TEM}$: ampicilin, aac(6′)-Ib-crciprofloxacin, mph(A):erythromycin, floR: florfenicol

3. RESULTS

3.1. Antibiotic sensitivity of E.coli strains

It clears that the highest rate of resistance was shown against tetracycline group of Antibiotic ,where about80%of isolate were resistant, followed by the β-lactam antibiotic (ampicillin) 73.3% followed by erythromycin about 63.3% of tested isolate were resistant, florfenicol about 53.3% of isolate were resistance , gentamicin is about 46.6% of isolate were resistance , finally ciprofloxacin about 40%of tested isolate were resistant (Table 2).

Table (2) Result of antibiotics resistance of *E.coli* by disc diffusion method

Isolate	G	E	T	C	A	AM	FFC
Sensitive	10	7	4	10	4	5	9
Intermittent	6	4	2	8	4	3	5
Resistance	14	19	24	12	22	22	16
% *	46.6	63.3	80	40	73.3	73.3	53.3

*Resistance percent, G: gentamycin, E: erythromycin, T: tetracycline, C: ciprofloxacin, A: ampicillin FFC: florfenicol, AM: amoxcicilin

3.2. PCR for Detection of resistance Genes of E.coli

PCR using primers fragments listed in materials and methods for amplification of *tet*A(A) tetreacyclin, *aad*B gentamycin, *bla*TEM ampicilin, *aac(6')*-Ib-crciprofloxacin, *mph*(A) erythromycin, and *flo*R flourofinicol from the isolated *E.coli* strains in this study.

3.2.1. Detection of aac(6,)-Ib-cr gene of E.coli

Quinolones (ciprofloxacin) act by binding to gyrase/topoisomerase IV–DNA complex. Formation of quinolone gyrase/ topoisomerase IV-DNA complex is responsible for the inhibition of DNA replication. Our results showed in figure (1) that all isolate were negative to *aac(6)-Ib-cr* gene.

3.2.2. Detection of blaTEM gene of E.coli

The mechanism of action of β-lactams is to disrupt bacterial cell wall synthesis by linking covalently to enzymes, i.e. penicillin-binding proteins. Our results showed amplification at 516bpas showed in figure (2).

3.2.3. Detection of floR gene of E.coli

Florfenicol prevent protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. Our results showed amplification at 494bpas showed in figure (3).

Table (3): Result of PCR of different resistance gene

Sample	O group	*aac(6Ib-cr*	*mphA*	*blaTEM*	*floR*	*aadB*	*tetA(A)*
1	O91	-	+	-	+	-	-
2	o142	-	-	+	+	-	+
3	O142	-	-	-	+	+	-
4	O103	-	-	-	-	-	-
5	O1	-	-	+	+	-	+
6	O125	-	-	+	+	-	+
7	O144	-	+*	+	+	-	+
8	O28	-	-	+	+	-	+
9	O158	-	-	+	+	+	+
10	O55	-	-	+	+	+	+
11	O114	-	-	+	+	+	+
12	O158	-	-	-	+	-	+
13	O159	-	+	+	+	-	+
14	O128	-	+	-	+	-	+
15	O124	-	-	+	+	-	+
16	O125	-	+	+	+	-	+
17	O91	-	+	+	+	-	+
18	O144	-	+	+	+	-	+
19	O125	-	+	+	+	-	+
20	O166	-	+	+	+	-	-
21	O44	-	+	+	+	-	-
22	O103	-	-	+	+	+	+
23	O44	-	+	+	+	+	+
24	O26	-	-	-	+	+	-
25	O103	-	-	+	+	-	+
26	O151	-	-	+	+	+	+
27	O26	-	-	-	+	-	-
28	O63	-	-	+	+	-	+
29	O128	-	-	-	+	-	-
30	O6	-	-	+	+	-	+
Pos/ tested	-	0/30	11/30	22/30	29/30	8/30	22/30
Positive%	-	-	36%	73%	96%	26%	73%.0

tetA(A):tetreacyclin, aadB: gentamicin blaTEM: ampicilin, aac(6′)-Ib-crciprofloxacin, mph(A): erythromycin, floR: florfenicol, *strong.

3.2.4. Detection oftetA(A) gene of E.coli

Tetracycline inhibits bacterial protein synthesis. Our results showed amplification at 576 bp as showed in figure (4).

3.2.5. Detection of aad(B) gene of E.coli

Aminoglycoside antibiotics (gentamycin) target the ribosome to inhibit protein translation. Our results showed amplification at 319 bp as showed in figure (5).

3.2.6. Detection of mph(A) gene of E.coli

Macrolide-lincosamide-streptogramin (erythromycin) classes of antibiotics target the ribosome to inhibit protein translation. Our results showed amplification at 403bp as showed in figure (6).

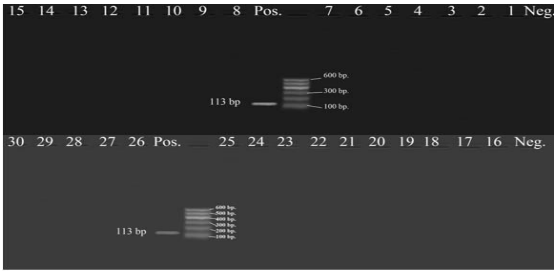
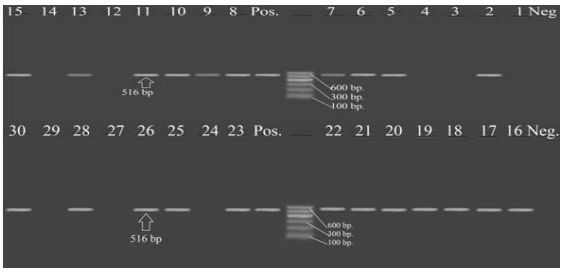
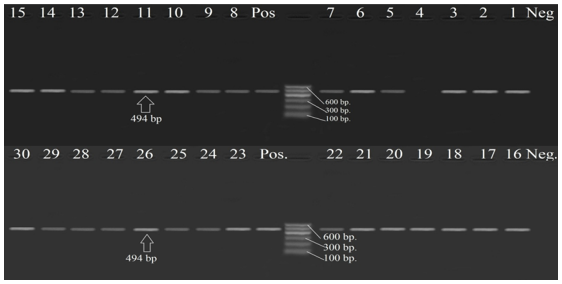


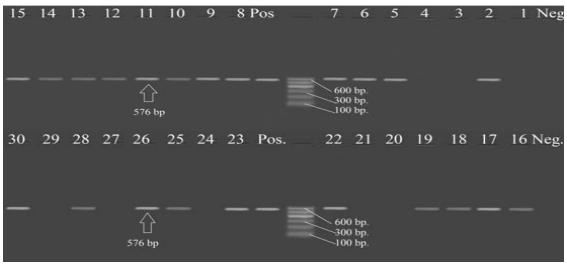
Figure (1) Agarose gel electrophoresis of products obtained by PCR for *E. coli* strains to detect gene (*aac(6,)*-Ib-cr):lane (1-30) are negative, Pos: positive Neg: negative



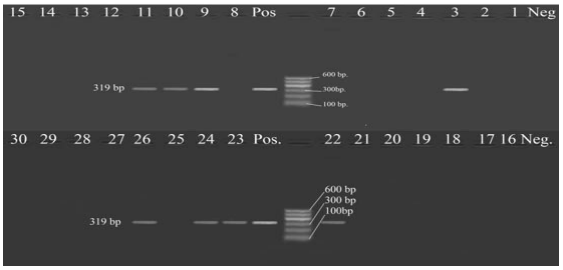
Figure(2)Agarose gel electrophoresis of products obtained by PCR for *E. coli* strains to detect gene *bla*TEM):lane no 2,5,6,7,8,9,10,11,13,15,16,17,18,19,20,21,22,23,25,26,28 ,30 were positive at 516bp. Pos: positive Neg: negative.



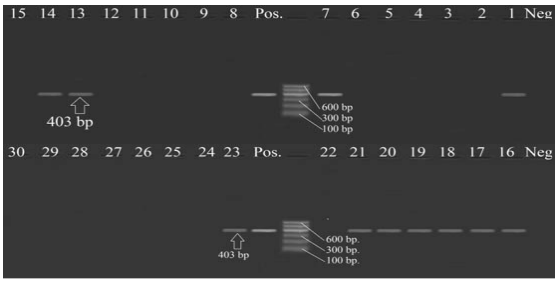
Figure(3)Agarose gel electrophoresis of products obtained by PCR for E. colistrains to detect gene(floR)all lanes were positive at 494bp except lane no(4)was negative.Pos: positive Neg: negative



Figure(4) Agarose gel electrophoresis of products obtained by PCR for *E.coli* strains to detect gene (*tet*A):lane no2,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,22,23,25,26. 28,30 were positive at 576 bp. Pos: positiveNeg: negative.



Figure(5)Agarose gel electrophoresis of products obtained by PCR for *E. coli* strains to detect gene (*aad*B):lane no 3,9,10,11,22,23,24,26 at319 bp.Pos: positiveNeg: negative.



Figure(6)Agarose gel electrophoresis of products obtained by PCR for E. coli strains to detect gene mph(A): lane no1,7.13,14,16,17,18,19,20,21,23 were positive isolate at 403 bp.Pos: positive Neg: negative.

4. DISCUSSION

In present study we used different antibiotics from different group of antibiotics as ciprofloxacin from quinolone, and ampicillin from β-lactemase group, also we used gentamycin from aminoglycosides antibiotic group, erythromycin from macrolide antibiotic group and florfenicol antibiotics from miscellanies antibiotics each type is act on different site of bacteria. Quinolones act by inhibiting the action of topoisomerases II (DNA gyrase) and topoisomerase IV. For Gram-negative bacteria the prime target of quinolones is the DNA gyrase, whereas in the Gram-positives it is the topoisomerase

IV **(**Andriole 2005). Quinolones act by binding to gyrase/topoisomerase IV–DNA complex. Formation of quinolonegyrase/ topoisomerase IV-DNA complex is responsible for the inhibition of DNA replication and the bacteriostatic action of the quinolones while their lethal action is thought to be a separate event from complex formation, and to arise from the relapse of free DNA ends from quinolone–gyrase–DNA complexes (Nordmann and Poirel, 2005).

All isolate (30) were negative to ciprofloxacin resistance gen *aac(6')-Ib-cr* fig(1) and table(3) that agree with Goswami et al., (2002) and Ahmed et al., (2014) on other hand , Xia et al., (2009) observed that 198 avian *E.coli* isolates from Shandong, China were resistant to enrofloxacin 99%, ciprofloxacin 100%, norfloxacin 100%, amoxicillin/clavulanic acid 87.4%, ampicillin 99.5%, gentamicin 97% and amikacin 27.8%. Also in present study, it was recorded that lower number of isolate was resistant to ciprofloxacin about 12(40%) of *E.coli* isolate which was nearly similar to that detected by Jiang et al., (2011). About 44.4% of isolate were resistant to ciprofloxacin a mange chicken *E.coli* strain in China. On other hand, Wang et al., (2001) found that high rates of resistance to quinolones have been reported from different parts of the world. In China, for example, more than 50% of the clinical strains of *E. coli* isolated during 1997-1999 were resistant to ciprofloxacin .Also Xia et al., (2009) observed that 198 avian *E. coli* isolates from Shandong, China were resistant to enrofloxacin 99% and ciprofloxacin100%.

Ampicillin and amoxicillin (α –amino penicillins), two penicillin derivates with greater acid stability and a better Gram-negative effect, were developed by Beecham. The β-lactamases are the collective name of enzymes that open the ß-lactam ring by adding a water molecule to the common β-lactam bond, and this inactivates the β-lactam antibiotic from penicillin to carbapenems. This hydrolyzation was first observed in 1940 by Abraham and Chain (penicillinase) in a strain of *E. coli (*Abraham et al., 1940). *bla*TEM gene was detected in about 22(73%) of *E.coli* isolate fig (2) and table (3) which similar agree with results of Jiang et al ., (2011) detected 88.9% of *bla*TEM gene among chicken *E.coli* strain isolated from Shanxi, Henan and Gansue Provinces in China. Also *bla*TEM gene were detected by Domínguez et al., (2002) about 20(48.7%) of *E.coli* isolate and Brinas et al., (2003) reported three positive *blaTEM* PCR result from five *Escherichia coli* isolates, and a positive PCR result was obtained for an additional isolate which were lower than the result of this study. Also by disc diffusion β-Lactam group (ampicillin) was 73.3% (table 2). These results agreedwithRaduet *al.* (2001) illustrated that all the ***E.coli*** isolates were identified as *E.coli* isolated from chickens. All the strains were found to be resistant to two or more of the antimicrobial agents. Resistance was observed most commonly towards bacitracin 100%., penicillin 100%., sulphafurazole 77%., ampicillin 57%., cephalothin 53%., carbenicillin 47%., ceftazidime 37% and erythromycin 30%.

Phenicols group prevent protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. Chloramphenicol (and thiamphenicol) resistance is mainly due to enzymatic modification by chloramphenicol acetyltransferases (CAT). These enzymes covalently link an acetyl group from acetylCoA to chloramphenicol, preventing it from binding to the ribosomes, and arengrouped into two types, A and B. However, these enzymes do not confer resistance to florfenicol. They are encoded by various genes, which have been found on chromosomes, plasmids, transposons, or integrin cassettes (Lambert, 2012)

The second mechanism consists of efflux pumps encoded in Gram-negative bacteria by *cml* genes, which confer cross-resistance

to chloramphenicol and florfenicol (Schwartz et al., 2004). More recently, florfenicol resistance conferred by the *floR* genes, referred to in the published literature as pp-flo, cmlA-like, floSt, flo, or *flo*R, has also been detected in *E. coli* (Bischoff et al., 2002).

By examination of 30 *E.coli* isolate recorded that29(96%) of isolate were carry florfenicol resistance against (*flo*R) gene table (3)and figure (3),agree with Jing et al., (2013**)** who detected the prevalence of*flo*R gene in chickens *E.coli* strain. It noticed that there was an increased gradually from (2007-2012). On other hand Xin et al., (2007) reported the genetic mechanisms relevant to florfenicol resistance in the chicken *E.coli* isolates were evaluated for the presence of 5 genes recognized to confer resistance to these antimicrobials: cmlA, cat-1, cat-2, cat-3, and *floR*. The total genomic DNA was found in thirteen *E. coli* isolates (18.6%) which were positive for the *floR* gene .Also 53.3%resistance to florfenciol antibiotic among 30isolate in our study by using disc diffusion. Xin et al., (2007) observed that about 29% of *E.coli* isolate was resistance to florfenicol in order to ensure the rational and effective use of these drugs. Bacteria could use three strategies to become resistant to tetracycline: limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline, and producing tetracycline-inactivating enzymes .All three types of resistance have been found in clinical isolates. With the discovery of so many tetracycline resistance genes in recent years, a classificationscheme had to be devised .The current convention is to assign a resistance gene to a particular class on the basis assign a resistance gene to a particular class on the basis of DNA-DNA hybridization with members of that class (Levey 1988). Tetracycline inhibits bacterial protein synthesis and affects bacterial cell wall (Chopra and Roberts, 2001). Tetracycline resistance due to target modification is mediated by ribosomal protection proteins (RPP) that represent a widely distributed class of resistance genes (Thaker et al., 2010). *E.coli* isolate were resistance to tetracycline (*tet*A) gene (73%). Figure (4) and table (3). Also Soufi et al., (2011) analyzed the resistance of 166 *E. coli* isolates recovered from poultry in Tunisia. High percentages of resistance were detected totetracycline 95.2%; ampicillin 65.7%; streptomycin 69.3%; nalidixic acid 72.3%; and sulphonamide 81.9 % while Moon et al., (2011) amplified *tet*A resistance gene from chicken was about 20.2%. Most of isolates was resistance to tetracycline antibiotic with 80% by disc diffusion table (2) agree with Moon et al., (2011) who studied the actual frequency of antimicrobial resistance in fecal *E.coli* isolated from chicken. One hundred and nine *E. coli* isolates were higher resistant to tetracycline (96.3%), ampicillin (68.8%) streptomycin (60.6%) and ciprofloxacin (65.1%). On other hand Radu et al., (2001) showed few strains were resistant to tetracycline 7%, streptomycin 17%., nalidixic acid 13%, chloramphenicol 10%, latamoxef 7% and kanamycin 7%, Aminoglycosides primarily act by binding to the 16S rRNA that recognizes the aminoacyl–tRNA; this action inhibits bacterial protein synthesis (Magnet and Blanchard, 2004). Target modification by ribosomal mutations or enzymatic modifications of ribosomal components inhibits the action of aminoglycosides (Davies and Wright, 1997). A number of actinomycetes that produce aminoglycosides also produce 16S rRNA methylases that protect them from the inhibitory effects of the antibiotic **(**Magnet and Blanchard, 2004). Among six antibiotics which used in these study found that 30 *E.coli* isolates showed lower percentage against (*aad*B) gentamicin resistance gene in 8(26%) as shown in figure (5) and table (3) agree with Kim et al., (2007), who found about 26.7% of *E.coli* isolate was resistance to gentamicin while Minh et al., (2009) obtained multidrug resistance isolated from Niger

Native 58.6% which were resistance to gentamicin. By disc diffusion (46.6%*)* of *E.coli* isolate resistance to gentamicin as showed in table (2) disagree with Soufi, (2009) who recorded 2% resistant to gentamicin among fifty five *E.coli* isolate. Gentamicin resistance may be due to the inclusion of this antibiotic with the Marek's vaccine that is administered to almost all poultry inovo vaccination Ricks et al., (1999). Macrolides, lincosamides and streptogramin sarecompounds that are distinct structurally but share a common mode of action and show similar antibacterial spectra, including staphylococci, streptococci, mycoplasmas and campylobacters (Leclercq, 2010). These antibiotics are produced by *Streptomycetes* by means of various types of polypeptide synthase. Macrolides are classified on the basis of the number of atoms in the ring of the macrocyclic lactone (Schwartz et al (2004), to which are attached deoxy sugars (desosamine and cladinose). Erythromycin resistance gene *mph*(A)was found in 36% of *E.coli* isolate fig(6) and table(3), while Goswami et al., (2002) showed that lower percentage of *E.coli* isolate resistance to erythromycin (7.84). By dis diffusion 63.3% of *E.coli* isolate resistance to erythromycin antibiotic among 30 *E.coli* isolate table (2) .On otherhand, Makhol et al., (2011) demonstrated that 100% of all tested isolates of *E.coli* strains isolated from poultry were resistant to erythromycin. The resistance mechanism of *E. coli* is complicated. The resistance genes mediated by plasmid can make the resistance spread among different bacteria, which make bacteria obtain resistance genes more easily and thus produce multiple resistances.

Conclusion*:* Multidrug resistant bacteria were detected by traditional and techniques and conformed by detection of gene responsible for that resistance .Great attention should begiven to use antibiotics in poultry farms.

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