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CHARACTERIZATION OF B-LACTAMASE GENES IN *ESCHERICIA* COLI ISOLATED FROM BROILERS

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ABSTRACT

Due to intensive use of β-*lactam drugs as therapeutic agents and growth promoting the* β-*lactam resistant is developed,and it is primarily developed by* β-*lactamases . therefore ,the objective of this study was to characterize* β-*lactamase genes in E. coli isolated from broilers. A total of 105 sampels isolated from broilers from Gharbia governorates ,28 isolates were positive for E.coli (26.6%), 15 isolatas were randomly taken and they serogrouped to 7 serotypes (O 55, O78, O 125, O126, O 157, O119 :H6 ,and O1:H7) .All isolated strains showed multidrug resistance phenotypes particularly against amoxicillin, erythromycin, doxycyclin, norfloxacin, and colistin sulphate respectively, PCR for the detection of* β-*lactamase genes in isolated strains showed that , all isolates contain* β-*lactamase genes, 5 isolates contain bla TEM 5 (50%), 2 isolates contain bla CTX-M 2 (20%), 3 isolates contain bla OXA 3 (30%), 2 isolates contain bla SHV 2 (20%), 1 isolates contain both bla TEM and bla OXA, 2 isolate contain both bla TEM and bla CTX-M and 1 isolate contain three gens bla TEM , bla SHV, and bla CTX-M .*

INTRODUCTION

E. coli is a common inhabitant of the human and animal gut and it considered an indicator of fecal contamination in food. β-lactams are widely used in human and veterinary medicine to treat human and animal infections ***(Livermore, 1998).*** This widespread use of antibiotics could be associated with the selection of antibiotic resistance mechanisms in pathogenic and nonpathogenic isolates of *E. coli* ***(Sunde and Sorum, 1999).*** Resistance to β-lactams in *Enterobacteriaceae* is mainly due to the production of β-lactamases which may be encoded either chromosomally or on plasmids ***(Bradford, 2005).*** β-Lactamases are enzymes responsible for many failures of antimicrobial therapy because of the hydrolysisof β-lactam antibiotics to inert and ineffective agent ,The first of these enzymes was recognized by Abraham and Chain in 1940**.** Resistance to extended-spectrum β-lactams has been associated with the production of broad-spectrum β-lactamases such as extended-spectrum β-lactamases (ESBLs) ***(Batchelor et al., 2005a).*** Many types of *β*- lactamases have been described ,but TEM, SHV, OXA, CMY, and CTX-M type *β*- lactamases are the most common in gram-negative bacteria ***(Bradford,2001),*** Also all groups of β- lactamases were identified including bla $_{TEM-1,}$ bla $_{TEM-104,}$ bla $_{CMY-2,}$ bla $_{OXA-30,}$ bla $_{CTX-M15,}$and bla $_{SHV-2}$ ***(Ahmed, et al, 2013)***, Furthermore,bla $_{TEM,}$ bla$_{OXA,}$ and bla $_{CTX-M}$ were reported in *E.* coli strains isolated from broiler chickens and turkeys in the United Kingdom ***(Randall et al., 2011)***, while bla$_{TEM}$ and bla $_{SHV}$ were identified in APEC strains isolated from poultry in Australia ***(Obeng et al., 2012).***

MATERIAL AND METHODS

Sampling ,isolation ,and identification procedures:

A total of 105 freshly died broilers in some farms at Gharbia Governorate were transferred to bacteriology laboratory**,** the Specimens were taken from gall blader, intestin and coloacal swab. The specimens were inoculated onto Nutrient broth and incubated at 37°C for 24h. then subcultured on MacConkey agar medium ,the developed pink colonies subcultured into Eosin methylene blue medium and incubated at 37°C for 24h. Developed colonies were subjected to biochemical identification (Developed colonies were subjected to I.M.Vi.C, urease test, and triple sugar iron agar medium) ***(Baron and Finegold, 1990*)**.

Serotyping of *E. coli* isolates *(Cruickshank et al. 1975)*:

Isolates of *E. coli* were inoculated on semisolid or slope agar (incubated 24 hours at 37 ^{0} C), Isolates of *E. coli* subcultured on MacConkey agar (incubated 24 hours at 37 ^{0} C), 3-5 colonies were inoculated on to 3 ml saline and Put in water bath 100 ^{0}C for 1 hour to kill the thermo *E.coli* then centrifuged at 2000 rpm, Pour out the supernatant and kept the precipitate then added 0.5 ml saline on it. The serology Carried out as follow, added a drop from the tube on a glass slide and added one drop from the O monovalent antiserum and mix them using a wooden strike or glass rod. If agglutination occurs this indicated positive reaction.

Antimicrobial susceptibility testing(disk diffusion) *(Bauer et al. 1966):*

The antimicrobial sensitivity phenotypes of *E*.coli isolated srains were determined using Bauer disc diffusion test according to the standereds and interpretive criteria described by CLSI ***(Clinical and Laboratory Standered Institute, 2005)*** The following antibiotics were used.

Chemotherapeutic agents	Conc.
Amoxicillin/Clavulanic Acid (AMC)	30 µg
Amoxicillin (AX)	10µg
Erythromycin (E)	15 µg
Gentamicin (GN)	10 µg
Doxycyclin (DO)	30 µg
Cefotaxime (CTX)	30 µg
Colistin sulphate (CT)	10 µg
Norfloxacin (NOR)	10 µg

The disks were purchased and the results were recorded based on CLSI guidelines ***(CLSI ,2005)***. The phenomenon of multidrug resistance is defined as the ability of acell to show resistance to awide variety of structurally and functionally unrelated molecules ***(Higgins, 2007)*** We used the term multidrug-resistant *E*.coli in this work to describe any *E*.coli isolate showed resistance to more than two classes of antimicrobials.

PCR and DNA sequencing for detection of β-lactamase genes in isolated strains DNA extraction and purification:

DNA was prepared using boiled lysates, as previously described ***(Ahmed et al., 2007)*.**

1- An overnight bacterial culture(200 µl) was mixed with 800 µl of distilled water and boiled for10 min.

2- The resulting solution was centrifuged at 10.000 rpm for 15min or 12.000 rpm for 10min.

3- The supernatant was used as the DNA template. DNA template was stored at −20◦Cuntil further use.

Amplification and cycling protocol for conventional PCR:

Using of PCR 2x Reddy Mix TM Master Mix (Thermo SCIENTIFIC) with Cat. No. (AB0575/LD-A)**.**

DNA samples were amplified in a total of 50 µl as the following:

- 25 µl of PCR master mix , 2.5 µl of forward primer
- 2.5 µl of reverse primer , 10 µl of PCR grade water
- 10 µl of the template
- PCR cycling program ***(Ahamed et al. 2007).***
- Thermal profile protocol.

Target	Final extention	Cycle number	Primer extention	Annealling	Denat.	Hot start	Gene
1080Bp	72^0C/10 min	30	72^0C/1 min	50^0C/30Sec	94^0C/30Sec	94^0C/10min	TEM
795Bp	72^0C/10 min	30	72^0C/1min	50^0C/30Sec	94^0C/30Sec	94^0C/10min	SHV
591Bp	72^0C/10 min	35	72^0C/1min	56^0C/1Min	94^0C/1Min	94^0C/10min	OXA
550Bp	72^0C/5 min	30	72^0C/30sec	55^0C/30sec	95^0C/30sec	95^0C/10min	CTX-M

Detection of PCR products *(Ahamed et al. 2007).*

Aliquots of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5 % agarose gel as shown in the following steps:

1) The prepared agarose was melted using microwave and left to cool till 55°C then ethidium bromide was added by 0.5 µg/ml and poured into the assembled horizontal gel tray after proper installation of the desired comb.

2) The gel was allowed to be solidified at room temperature.

3) TBE buffers that used for preparation of the gel were added into the tank to a level 1- 2 mm above the gel layer.

4) The samples and a 100 bp DNA ladder (marker) were loaded in the wells in amount of 8µl of sample with 3µl of loading buffer and introduce 8 µl of the ladder, the tank was closed and the power supply was attached.

5) A current of 80 V for 1 hour was passed on the medi horizontal electrophoresis unit.

6)Specific amplicons were observed under ultraviolet transillumination, compared with the marker.

7) The gel was photographed by a gel documentation system and the datawasanalysed through computer software.

ESBL detection by double-disk synergy test (Ho *et al.*, 1998).

The first test specifically designed to detect ESBL production in Enterobacteriaceae was the double disk synergy test (DDST). It was initially designed to differentiate between cefotaxime resistant strains, i.e., those overproducing cephalosporinase,and those producing ESBLs. The test is performed on agar with a 30ug disk of cefotaxime(and / or ceftriaxone) and a disk of amoxicillin–clavulanate (containing 10 ug of

clavulanate) positioned at a distance of 30 mm (centre to centre) The test is considered as positive when a decreased susceptibility to cefotaxime is combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in acharacteristic shape-zone referred to as ‘champagne-cork’ or ‘keyhole’

RESULTS

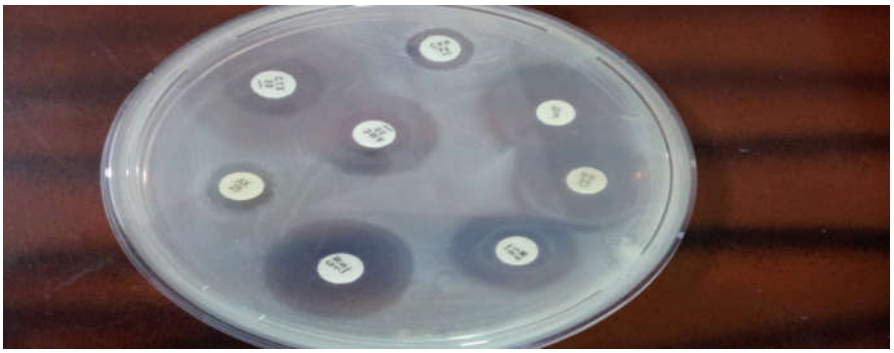
Isolation and identification of *E.*coli isolated from broilers:

From 105 samples collected from diseased briolers from Gharbia Governrate, the positive sample for *E.coli* were 28 samples with incidence 26.6%.

Serotyping of *E. coli* isolate:

The result of serological identification of randomly 15 isolates of *E.coli* from the total positive isolates revealed that, 12 isolates were tybed under 7 different O serotypes (O_{55},O_{78} ,O_{125} ,O_{1} ,O_{126} ,O_{157} ,O_{119}), while 3 isolates were untyped.

Results of antibiogram (disc diffusion) of eight chemotherapeutic agents on different *E. coli* isolate strains:



Disc diffusion test

Sensitivity percentage to each antimicrobial agent were determined (Cefotaxime ,Gentamicin ,Amoxycillin/clavulinic acid were the most effective on E. coli isolated strains with percent 80%, 70%, 60% respectively) while Colistinsulphate, Ciprofloxacin, Doxycyclin, Erythromycin were less effective with susceptibility percent 50%,40%,20%, 10% respectively, and Amoxycillin with susceptibility percent 10%.

The most effective antibiotic drug on the tested strains:

Effective antibiotic drug	Strain isolated
Cefotaxime	O 157
Cefotaxime	O 78
Cefotaxime	O 1
Colistin sulphate	O 119
Gentamicin	O 125
Cefotaxim ,Gentamicin	O 126
Gentamicin	O 55

Detection of resistance genes by PCR.

PCR were applied at Central diagnostic and research lab , faculty of veterinary medicine, kafrelsheikh Univericity.

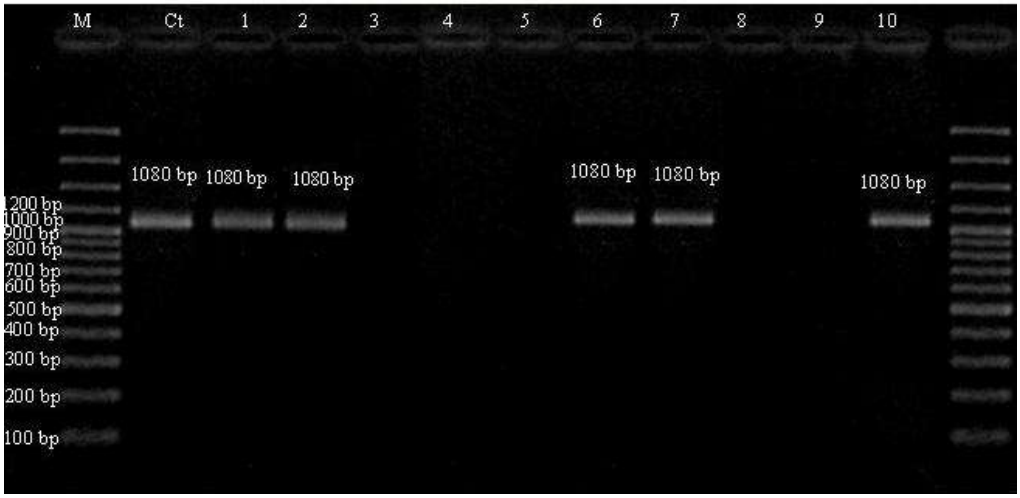
TEM, SHV, OXA and CTX-M genes were detected by PCR in *E.coli* somatic antigen serogroups.

Occurrence of TEM, SHV, OXA and CTX-M genes among *E. coli* serogroups by PCR.

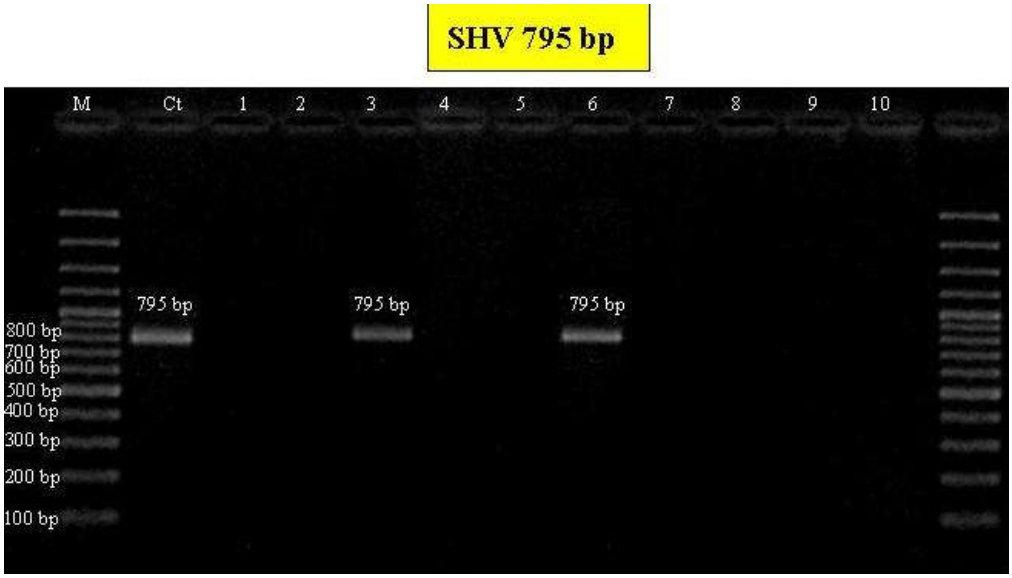
Somatic antigen	Iolates code No.	TEM	SHV	OXA	CTX-M
O 55	S 1	+	-	-	-
O 125	S 2	+	-	-	-
O 157	S 3	-	+	-	-
O 126	S 4	-	-	-	-
Untyped	S 5	-	-	+	-
Untyped	S 6	+	+	_	+
O 119	S 7	+	-	+	-
O 78	S 8	-	-	_	
O 78	S 9	-	-	+	-
O 1:H 7	S 10	+	-	-	+
Total	**10**	**5/10 (50 %)**	**2/10 (20 %)**	**3 /10 (30 %)**	**2 /10 (20 %)**

(+) +ve (-) - Ve

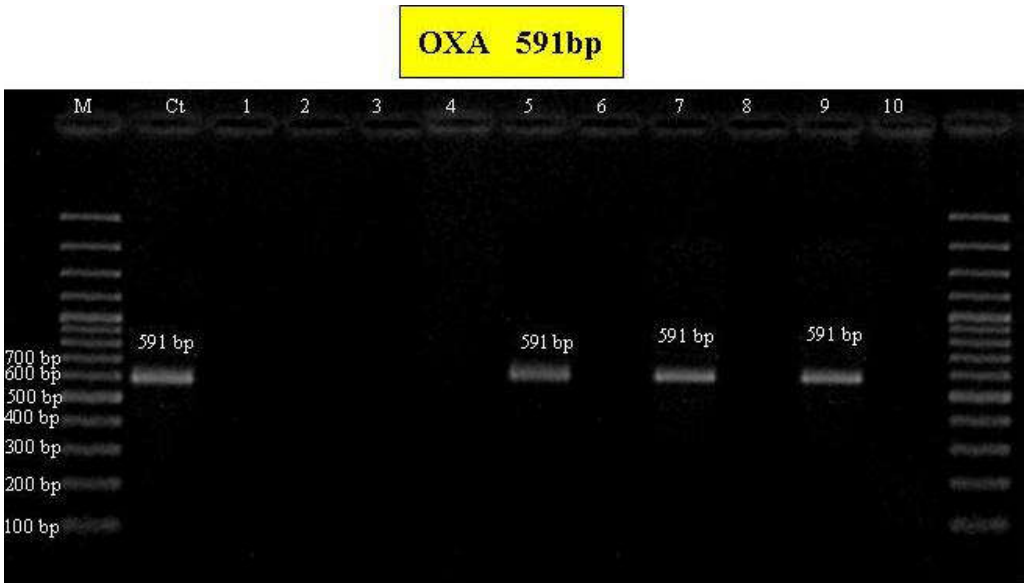
TEM 1080bp



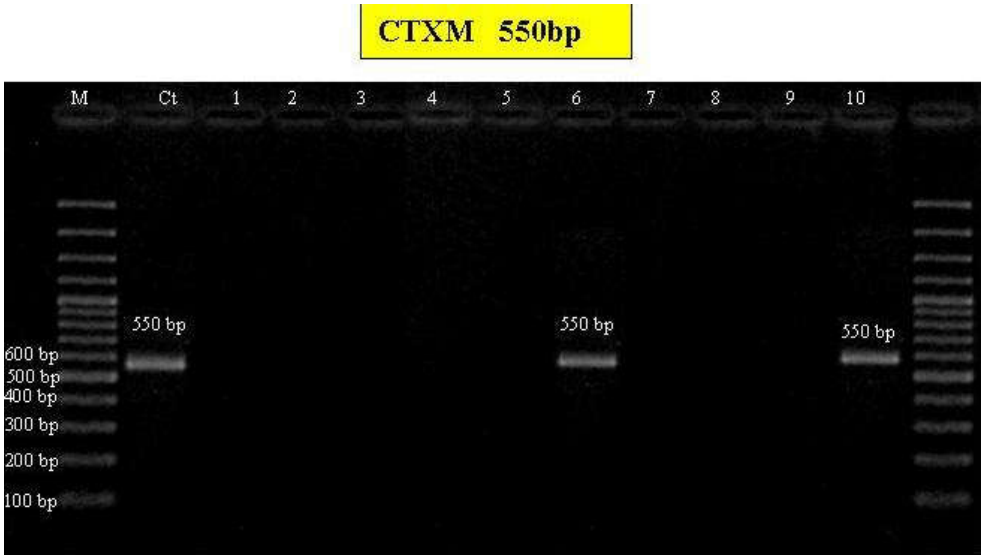
bla $_{TEM}$ (1080bp) obtained from isolates with code No S 1 ,S 2, S 6, S 7 and S 10



bla $_{SHV}$ (795 bp) obtained from isolates with code No S 3 and S6



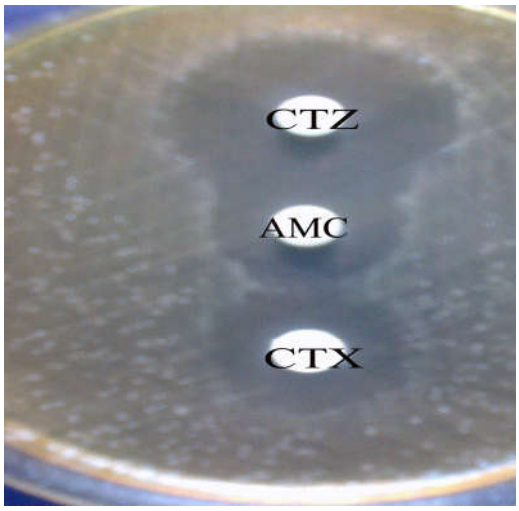
bla $_{OXA}$ (591 bp) obtained from isolates with code No S 5, S 7 and S 9



bla $_{CTX-M}$ (550 bp) obtained from isolates with code No S 6 and S 1

Results of double disc synergy test for detection of B–lactamases:

Isolates producing CTX-M were tested by DDST and they show +ve result.



ESBL detected by double Disc diffusion test for *E. coli* isolate showing synergistic inhibition between amoxicillin/ clavulanic Acid (AMC) and both ceftriaxone(CTZ) and cefotaxime (CTX).

DISCUSSION

In this study different *E.*coli strains were isolated from briolers with signs of coliseptcemia signs. Concerning the results of this study it wase showed that 105 samples collected from broilers from Gharbia governrate, the positive isolates were 28/105 (26.6%). *E.*coli isolates were serogrouped under 7 different O serotypes (O 55, O 78 ,O 125 were the most prevealent serotypes and this nearly similar to results obtained by ***(M.Alimehr et al,1999)***.

The O1 and O55 **s**erotypes are known to be pathogenic in poultry and are usually isolated from birds with colibacillosis ***(Gross et al 1988), (Gyimah & Panigrahy 1988).*** O 78 serotype is commonly associated with avian colibacillosis on a worldwide scale, and confirm its role as particularly adapted pathogen that permit involvement in extra intestinal infections ***(Dho-Moulin and Fairbrother, 1999); (La Ragione and Woodward, 2002).***

Concerning the result of antimicrobial susceptibility in this study, Cefotaxime was the most effective antimicrobial with high susceptibility percentage 80% ,that agree with ***Liu et al., (2007)*** reported that *E.* coli isolates were more sensitive to Cefotaxime, ***Wafaa .M.G (2012)*** recorded sensitivety to Cefotaxime 87.5%, that disagree with ***Sylvester et al., (2006)*** who recorded low percentage (62%). Moderate susceptibility to Gentamicin 70% were recorded and that is nearly similar to ***Momtaz .H et al., (2012)*** recorded that all E.coli isolates were susceptible to Gentamicin. *E.*coli isolates were resistant to Amoxycillin 90 %, similarly

Lambie et al., (2000) isolated *E. coli* with high resistance to amoxicillin 100%|, However ***Giurov, (1985)*** recorded sensitivity to amoxicillin was 93.8%.

In this study multi drug resistant recorded in all isolated strains. Recording the result of PCR for the detection of β-lactamase genes among 10 isolated strains, all isolates contain β-lactamase gens, 5 isolates contain *bla* $_{TEM}$ (50%), 3 isolates contain *bla* $_{CTX-M}$ (20%), 3 isolates contain *bla* $_{OXA}$ (30%), 2 isolates contain *bla* $_{SHV}$ (20%), 1 isolates contain both *bla* $_{TEM}$ and *bla* $_{OXA}$, 2 isolate contain both bla $_{TEM}$ and *bla* $_{CTX-M}$ and 1 isolate contain three gens *bla* $_{TEM}$,*bla* $_{SHV}$, and *bla* $_{CTX-M}$ Isolates producing *bla* $_{CTX-M}$ were tested by DDST and they show +ve result.

These findings demonstrate that bla $_{TEM}$ was the most dominant gen in β-lactamase producing broiler isolates ,then *bla* $_{OXA}$,*bla* $_{CTX-M}$ and finaly *bla* $_{SHV}$. Also these results indicate that TEM encoding gene play an increasing role in antibiotic resistance in broilers.

Bla $_{TEM}$ was detected in 32.4%of APEC strains isolated from chickens suffering from colibacillosis in Korea ***(Kim et al., 2007)*****,** and also from APEC strains isolatedfrom avian colibacillosis in Spain ***(Mora et al., 2012).***

Furthermore,bla$_{TEM}$, bla$_{OXA}$, and bla$_{CTX-M}$ were reported in *E.* coli strains isolated from broiler chickens and turkeys in the United Kingdom ***(Randall et al., 2011)***, while bla$_{TEM}$ and bla $_{SHV}$ were identified in APEC strains isolated from poultry in Australia ***(Obeng et al.,2012).*** Isolates producing bla $_{CTX-M}$ by PCR showed +ve result by DDST for detection

of ESBLs. ***Vercauteren et al., (1997)*** who stated that sensitivity of double disc diffusion was 96.9% . ***Paterson and Bonomo, (2005).*** Said that ESBLs but not AmpC, clavulanate inhibited the activity of ESBL, leading to enhancement of the zone of inhibition in the area between the amoxicillin / clavulanate disk and any of the third-generation cephalosporin disks. However, in organisms which produce both ESBLs and AmpC, clavulanate may induce hyperproduction of the AmpC β - lactamase, leading to hydrolysis of the third-generation cephalosporin, masking any synergy arising from inhibition of the ESBL.

CONCLUSION

It could be concluded that β-lactams antimicrobial agents are very less effective against E.coli infections in briolers and their usefulness in the treatment of infections due to presence of *β*-lactamase genes TEM, OXA , SHV and, CTX-M genes. Further investigations are required to investigate mutation type of β-lactamases by sequencing and to detect inhibitor resistant TEM genes.

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الملخص العربي

نتيجة الاستخدام المكثف لأدوية البيتالاكتام ظهرت مقاومة الميكروب القولوني عن طريق إنتاج أنزيم البيتالاكتاميز. ولذلك أجريت هذه الدراسة لتصنيف جينات البيتالاكتيميز في الميكروب القولوني المعزول من بداري التسمين حيث تم عزل الميكروب القولوني من ١٠٥ عينه من مزارع بدارى التسمين من محافظة الغربيه وكانت نتيجة العزل ٢٨ عينة إيجابية بنسبة ٢٦,٦٪. ثم تم إجراء التصنيف السيرولوجى لـ ١٥ معزولة أخذت عشوائيا حيث صنفت هذه المعزولات إلى ٧ عترات مختلفة (O 55,O 125, O 126, O157, O78, O1:H7, O119:H6). وعند إجراء اختبار حساسيه للعترات المعزولة لأنواع مختلفة من المضادات الحيوية وجد أن السيفوتاكسيم، الجينتاميسين والاموكسيسيللين وحمض كلافيولينيك أكثر المضادات الحيوية فعاليه، بينما أوضحت العزلات أكثر مقاومه ضد الاموكسيسيللين. وعند إجراء اختبارات الحامض النووي وذلك بطريقة اختبار أنزيم البلمرة المتسلسل لتحديد جينات البتلاكتاميز في الميكروب القولوني أظهرت أن جميع العزلات تحتوى على جينات البيتالاكتاميزال TEM ، OXA ، SHV وCTX-M بنسبة ٥٠٪ ،٣٠٪ ،٢٠٪ و٢٠٪ على التوالي وكانت ٢٠٪ من المعزولات تحتوى على جينات TEM ،CTX-M و١٠٪ تحتوى على جينات TEM، OXA معا و١٠٪ تحتوى على جينات TEM ، SHV ، OXA معا، وأوضحت النتائج أن جين TEM هو الأكثر وجودا حيث يسبب مقاومة ميكروب الاشريشيا القولونى للمضادات الميكروبية يليه جين الـ OXA ، CTX-M و SHV .

ومن هذه النتائج نستنتج أن المضادات الحيوية التي تحتوى على بيتالاكتام غير فعاله في علاج هذه المعزولات فهي غير مجدية في علاج الإصابة من ميكروب الاشيرشيا القولونية وذلك لوجود جينات البيتالاكتاميز.