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 samples isolated from broilers from Gharbia governorates, 28 isolates were positive for E. coli (26.6%), 15 isolates were randomly taken and they serogrouped to 7 serotypes (O 55, O 78, O 125, O 126, O 157, O 119: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 hydrolysis of 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°C), Isolates of E. coli subcultured on MacConkey agar (incubated 24 hours at 37°C), 3-5 colonies were inoculated on to 3 ml saline and Put in water bath 100°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 strains were determined using Bauer disc diffusion test according to the standards and interpretive criteria described by CLSI *(Clinical and Laboratory Standered Institute, 2005)*. The following antibiotics were used.

<table>
<thead>
<tr>
<th>Chemotherapeutic agents</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/Clavulanic Acid (AMC)</td>
<td>30 µg</td>
</tr>
<tr>
<td>Amoxicillin (AX)</td>
<td>10 µg</td>
</tr>
<tr>
<td>Erythromycin (E)</td>
<td>15 µg</td>
</tr>
<tr>
<td>Gentamicin (GN)</td>
<td>10 µg</td>
</tr>
<tr>
<td>Doxycyclin (DO)</td>
<td>30 µg</td>
</tr>
<tr>
<td>Cefotaxime (CTX)</td>
<td>30 µg</td>
</tr>
<tr>
<td>Colistin sulphate (CT)</td>
<td>10 µg</td>
</tr>
<tr>
<td>Norfloxacin (NOR)</td>
<td>10 µg</td>
</tr>
</tbody>
</table>

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 a cell to show resistance to a wide 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 for 10 min.
2- The resulting solution was centrifuged at 10.000 rpm for 15 min or 12.000 rpm for 10 min.

3- The supernatant was used as the DNA template. DNA template was stored at −20°C until 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 cycling protocol.

<table>
<thead>
<tr>
<th>Target</th>
<th>Final extension</th>
<th>Cycle number</th>
<th>Primer extension</th>
<th>Annealing</th>
<th>Denat.</th>
<th>Hot start</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1080Bp</td>
<td>72°C/10 min</td>
<td>30</td>
<td>72°C/1 min</td>
<td>50°C/30Sec</td>
<td>94°C/30Sec</td>
<td>94°C/10min</td>
<td>TEM</td>
</tr>
<tr>
<td>795Bp</td>
<td>72°C/10 min</td>
<td>30</td>
<td>72°C/1 min</td>
<td>50°C/30Sec</td>
<td>94°C/30Sec</td>
<td>94°C/10min</td>
<td>SHV</td>
</tr>
<tr>
<td>591Bp</td>
<td>72°C/10 min</td>
<td>35</td>
<td>72°C/1 min</td>
<td>56°C/1Min</td>
<td>94°C/1Min</td>
<td>94°C/10min</td>
<td>OXA</td>
</tr>
<tr>
<td>550Bp</td>
<td>72°C/5 min</td>
<td>30</td>
<td>72°C/30sec</td>
<td>55°C/30sec</td>
<td>95°C/30sec</td>
<td>95°C/10min</td>
<td>CTX-M</td>
</tr>
</tbody>
</table>

**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 data was analysed 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 a characteristic 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 broilers from Gharbia Governorate, 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 typed under 7 different O serotypes (O55, O78, O125, O1, O126, O157, O119), 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/clavulanic acid were the most effective on E. coli isolated strains with percent 80%, 70%, 60% respectively) while Colistin sulphate, 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:

<table>
<thead>
<tr>
<th>Effective antibiotic drug</th>
<th>Strain isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime</td>
<td>O 157</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>O 78</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>O 1</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>O 119</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>O 125</td>
</tr>
<tr>
<td>Cefotaxim ,Gentamicin</td>
<td>O 126</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>O 55</td>
</tr>
</tbody>
</table>

Detection of resistance genes by PCR.

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

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.
Characterization Of B-Lactamase Genes In …

<table>
<thead>
<tr>
<th>Somatic antigen</th>
<th>Isolates code No.</th>
<th>TEM</th>
<th>SHV</th>
<th>OXA</th>
<th>CTX-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>O 55</td>
<td>S 1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O 125</td>
<td>S 2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O 157</td>
<td>S 3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O 126</td>
<td>S 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Untyped</td>
<td>S 5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Untyped</td>
<td>S 6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>O 119</td>
<td>S 7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>O 78</td>
<td>S 8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O 78</td>
<td>S 9</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>O 1:H 7</td>
<td>S 10</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
<td><strong>5/10</strong></td>
<td><strong>2/10</strong></td>
<td><strong>3/10</strong></td>
<td><strong>2/10</strong></td>
</tr>
</tbody>
</table>

(+): +ve  
(-): -ve

**TEM 1080bp**

*bla*~TEM~ (1080bp) obtained from isolates with code No S 1 , S 2 , S 6 , S 7 and S 10

Characterization of β-Lactamase Genes In Fawzia A. Dawoud et al.

**SHV 795 bp**

bla\_shv (795 bp) obtained from isolates with code No S 3 and S 6

**OXA 591 bp**

bla\_oxa (591 bp) obtained from isolates with code No S 5, S 7 and S 9

Results of double disc synergy test for detection of β-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 broilers with signs of coliseptcemia signs. Concerning the results of this study it was 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 serotypes 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 finally 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 isolated from 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 الببتالاكتام ظهرت مقاومة الميكروب القولوني عن طريق إنتاج أنزيم الببتالاكتام. ولذلك أجريت هذه الدراسة لتصنيف جينات الببتالاكتاميز في الميكروب القولوني المعزول من بداري التسمين حيث تم عزل الميكروب القولوني من 105 عينة من مزارع بداري التسمين من محافظة الغربية وكانت نتيجة العزل 28 عينة إيجابية بنسبة 26.2٪. تم إجراء التصنيف السيرولوجي لـ 15 معزولة آخذت عشوائيا حيث صنفت هذه المعزولات إلى 7 عوامل مختلفة (O 55, O 125, O 126, O157, O78, O1:H7, O119:H6). وعند إجراء اختبار حساسية للعوامل المعزولة لأدوات مختلفة من المضادات الحيوية وجد أن السيفينتوكسين، الجيتانتيميسين والأموكسيسيللين وحمض كلاروفاينك أكثر المضادات الحيوية فعالية، بينما أوضحت العوامل أكثر مقاومة ضد الأموكسيسيللين. وعند إجراء اختبارات الحمض النووي وذلك بطريقة اختبار أنزيم البلمرة المستخدمة لتحديد جينات الببتالاكتاميز في الميكروب القولوني أظهرت أن جميع العوامل تحتوى على جينات الببتالاكتاميزال CTX-M و SHV ، OXA ، TEM تحتوى على جينات الببتالاكتاميزال CTX-M و SHV ، OXA ، TEM بنسبة 50٪، 30٪، 20٪ على التوالي وكانت 20٪ من المعزولات تحتوي على جينات TEM و 10٪ تحتوي على جينات OXA ، TEM، OXA و SHV هو الأكثر وجودا حيث يسبب مقاومة TEM ميكروب الاصابة القولوني للمضادات الميكروبية يليه جين M-CTX-M ، OXA SHV M-CTX-M ، OXA SHV M-CTX-M ، OXA SHV M-CTX-M ، OXA SHV ومن هذه النتائج نستنتج أن المضادات الحيوية التي تحتوى على ببتالاكتام غير فعالها في علاج هذه المعزولات فهي غير مجدية في علاج الإصابة من ميكروب الاصابة القولونية وذلك لوجود جينات الببتالاكتاميز. 