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Molecular detection of ESBL/AmpC ß-Lactamase *Escherichia coli* isolated from sheep in Mosul city

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Abstract

Globally, extended-spectrum ß-lactamase (ESBL)/Ampicillin ß-lactamase (AmpC) producing *Escherichia coli* has become the greatest threat for distributing antibiotic resistance. Accordingly, this study was designed to detect and screen the genes that confer resistance in *E. coli* isolated from sheep as main livestock in Mosul city. Forty *E. coli* isolates previously recovered from milk and fecal samples were included in this study. These isolates were obtained from healthy ewes, their lambs, and also from ewes with clinical mastitis. Polymerase chain reaction (PCR) was used to confirm the *E. coli* isolates targeting the 16sRNA gene. Furthermore, screening of different genotypes of ESBL/AmpC was conducted using specific primers. The results showed that the CTX-M gene was predominant among ESBL genotypes and recorded 40/40 (100%). While, SHV and TEM genes recorded 7/40 (17.5%) and 5/40 (12.5%), respectively. Moreover, fecal carriage of resistance genes was more than that obtained from milk in both healthy and diseased animals. However, none of the 40 isolates showed positive results for AmpC genes. The presence of different genotypes of ESBL *E. coli* isolated from feces or milk origin may act as a potential source for transferring antibiotic resistance to humans, other animals, and the environment.

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Introduction

Antibiotic resistance has become a significant global problem that imposes negative consequences on both human and veterinary health (1,2). Bacteria that have resistant properties could potentially pass resistance genes to other bacteria (3,4). Such conditions could increase the risk for public health by transferring antibiotic resistance through animals or the food chain (5-7). Gram-negative bacteria that can produce ESBLs and AmpC β-lactamases, are become a source of worry among health sectors because of their ability to transfer resistance and spread worldwide (1,8). Antibiotics having β-lactam ring are break up in the presence of β-lactamases, which lead to induce resistance to different generations of β-lactam based antibiotics, mainly 3rd generation cephalosporins (9,10). Hence, this results in ineffective drug treatment and is also a major reason for the failure of cephalosporin therapy (11,12). ESBLs/AmpC lactamases are most commonly produced by *Escherichia coli*. However, they may be produced also by other Gram-negative bacteria (3,10). Nevertheless, *E. coli* has emerged as a leading pathogen that mediated β-lactamases type of resistance (13,14). Resistance genes are generally mediated by plasmids including TEM, SHV, and CTX-M genes (15,16). These genes are classified as ambler class A enzyme. However, changing of amino acids sequence of the lactamase active site produces several variants of each resistance gene (17). More than 200 TEM and SHV variants have been identified, and 90 different CTX-M enzymes have been described (17). On the other hand, AmpC β- lactamases, classified as Ambler class C enzymes, are cephalosporinases that are less affected by clavulanic acid inhibitors (16). They are differentiated from other ESBLs by their ability to hydrolyze cephalosporins as

well as other extended-spectrum cephalosporins (16,17). Plasmid-mediated AmpC (pAmpC) is divided into 6 families, including (CIT, FOX, MOX, DHA, EBC, and ACC) (18). In recent years, several studies have described the emergence of *E. coli* producing ESBL/AmpC type β-lactamases which are closely related to resistance against third and fourth-generation cephalosporins in both animals and humans (9,13). Due to the great importance of sheep farming in Iraq, and the shortage of related studies that cover the spreading of ESBL/AmpC type of resistance in sheep, the current study was designed to molecular characterize ESBL/AmpC *E. coli* in sheep in Iraq, specifically in Mosul city.

Materials and methods

Bacterial isolates

The study included 40 *E. coli* isolates. These isolates were obtained from our previous study (submitted for publication) targeting isolation of ESBL/AmpC producing *E. coli* from sheep. These isolates were able to grow on MacConkey agar plus cefotaxime, as third-generation cephalosporin, at a final concentration of 1µg/ml according to Ahmed (3). Also, these isolates were previously confirmed using standard microbiology methods including culture, staining, and growth on eosin methylene blue (EMB) agar (Oxoid, UK) and Vitek 2 Compact System (BioMerieux, France) according to manufacturer instructions. The obtained isolates represent milk (n=7) and fecal (n=13) samples from healthy ewes and fecal swabs (n=12) from their lambs. Additionally, milk (n=4) and fecal samples (n=4) were obtained from ewes with clinical mastitis.

DNA extraction

Few fresh overnight subculture colonies cultivated on brain heart infusion agar were selected for DNA extraction using Bacteria DNA Preparation Kit (Jena Bioscience, Germany), following the manufacturer instructions with slight modification as previously described by Ahmed (3).

Polymerase chain reaction (PCR)

Molecular confirmation of all obtained *E. coli* isolates was done using specific primers ECO223-F and ECO 455-R targeting the 16S rRNA gene. Furthermore, all the isolates that had phenotypic resistance to β-lactamases were screened by PCR for two sets of resistance genes. The first set represents the ESBL genes group, Including CTX-M, SHV, TEM genes. While, the second set represents the AmpC genes group, including CIT, MOX, DHA, ACC, and CMY2 genes. The primer sequences and amplified products (Table 1). All primers were purchased from (IDT, USA). Standard PCR protocol was followed for all primers except the annealing temperature. Briefly, 30 µl containing 15 µl Hot Start Taq Premix (2X) (Addbio, Korea), 0.5 µl of each forward and reverse primers (IDT, USA) at final concentration 10 mmol, 3 µl of extracted DNA, and 11 µl of PCR grade water. Thermocycler (BioRad, T100, Bio-Rad, USA) was used for amplification. The PCR cycling conditions were set (Table 2). PCR products were electrophorized using 1.5% agarose gel (Bio-Rad, USA) containing 3 µl of GelRed safe Dye (Addbio, Korea). Briefly, 5 µl of each PCR product was loaded in the respective well of the prepared agarose gel. Also, a volume of 4 µl of DNA standard marker, 100 bp (Addbio, Korea) was used to identify the obtained products. The gel running conditions and analysis were performed as previously described by Ahmed (3).

Table 1: PCR primers sequences used in the current study

No.	Primer Name	Sequence 5’ - 3’	Product size (bp)	Ref.
1	ECO223-F	ATCAACCGAGATTCCCCCAGT	232	(3)
2	ECO 455-R	TCACTATCGGTCAGTCAGGAG		
3	CTX-M-Uni F	CGCTTTGCGATGTGCAG	550	(3)
4	CTX-M-Uni R	ACCGCGATATCGTTGGT		
5	SHV-F	ATGCGTTATATTCGCCTGTG	763	(10)
6	SHV-R	TGCTTTGTTATTCGGGCCAA		
7	TEM-F	AAACGCTGGTGAAAGTA	822	(10)
8	TEM-R	AGCGATCTGTCTAT		
9	CIT-F	TGGCCAGAACTGACAGGCAAA	462	(13)
10	CIT-R	TTTCTCCTGAACGTGGCTGGC		
11	MOX-F	GCTGCTCAAGGAGCACAGGAT	520	(13)
12	MOX-R	CACATTGACATAGGTGTGGTGC		
13	DHA-F	AACTTTCACAGGTGTGCTGGGT	405	(13)
14	DHA-R	CCGTACGCATACTGGCTTTGC		
15	ACC-F	AACAGCCTCAGCAGCCGGTTA	346	(13)
16	ACC-R	TTCGCCGCAATCATCCCTAGC		
17	CMY2-F	CCGAAGCCTATGGCGTGAAATCC	106	(19)
18	CMY2-R	GCAATGCCCTGCTGGAGCG		

Table 2: Cycling conditions used for amplification of PCR

No.	Step	Temperature	Time	No. of Cycles
1.	Initial denaturation	94	10 min	1X
2.	Denaturation	94	45 sec	
3.	Primer annealing	*	45 sec	35X
4.	Elongation	72	1 min	
5.	Final elongation	72	10 min	1X
6.	Hold	4	4ºC	∞

*= annealing temperature at (55ºC for *E. coli*, 54ºC for CTX-M-U and 45 ºC for each SHV and TEM, 60ºC for CIT, MOX, DHA, ACC, and CMY2 genes).

Results

All 40 isolates showed positive PCR results after gel electrophoresis with product size 232 bp (Figure 1). Moreover, all the isolates showed positive results for the CTX-M gene (Table 3 and Figure 2). However, the obtained results of the SHV gene were less and recorded 0.0-30.8%, while the TEM gene recorded 0.0-23.1% (Table 3) and (Figures 3 and 4).

Additionally, fecal carriage of CTX-M, SHV, and TEM genes was higher than those present in milk (Table 3). Unfortunately, we could not detect any *E. coli* isolates with AmpC resistance genes including CIT, MOX, DHA, ACC, and CMY2 genes.

Table 3: PCR screening of ESBL *E. coli* isolated from different sources for CTX-M, SHV, and TEM genes

Sample type	No. of the tested samples	CTX-M n (%)	SHV n (%)	TEM n (%)
Milk (clinically healthy ewes)	7	7 (100)	0 (0.0)	1 (14.3)
Feces (clinically healthy ewes)	13	13 (100)	4 (30.8)	3 (23.1)
Feces (clinically healthy lambs)	12	12 (100)	2 (16.7)	1 (8.3)
Milk (ewes with clinical mastitis)	4	4 (100)	0 (0.0)	0 (0.0)
Feces (ewes with clinical mastitis)	4	4 (100)	1 (25.0)	0 (0.0)
Total	40	40 (100)	7 (17.5)	5 (12.5)

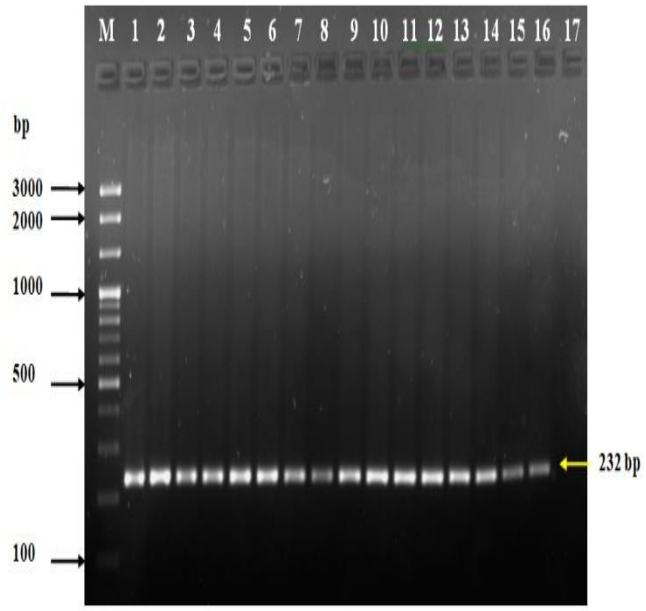

Figure 1: Electrophoresis of PCR products on the agarose gel. Lane M, DNA standard marker (100 bp); lanes 1-16 positive samples of *E. coli* giving 232 bp product size; lane 17 negative control.

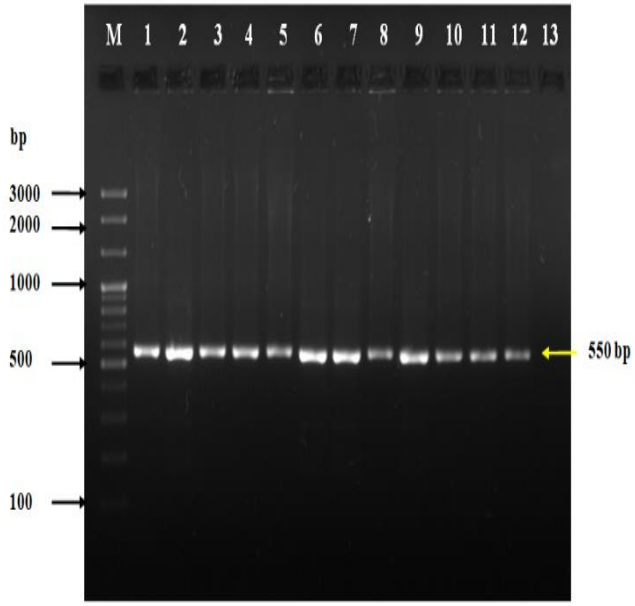

Figure 2: Electrophoresis of PCR products on the agarose gel. Lane M, DNA ladder (100 bp); lanes 1-12 positive samples of universal CTX-M gene (550 bp); lane 13 negative control.

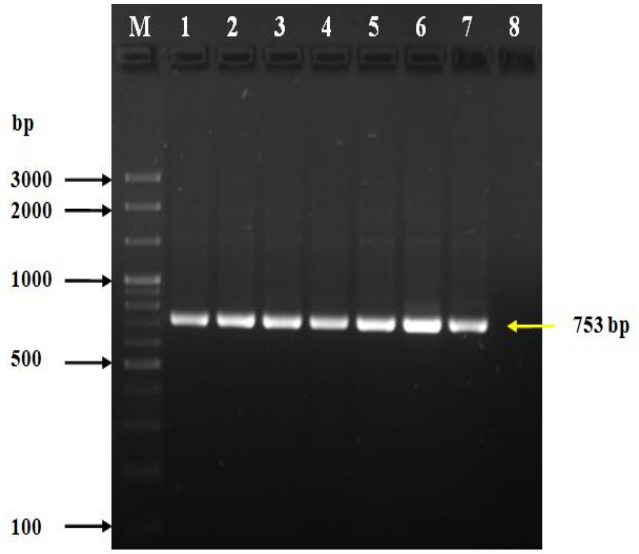

Figure 3: Electrophoresis of PCR products on the agarose gel. Lane M, DNA ladder (100 bp); lanes 1-7 positive samples of SHV gene (753 bp); lane 8 negative control.

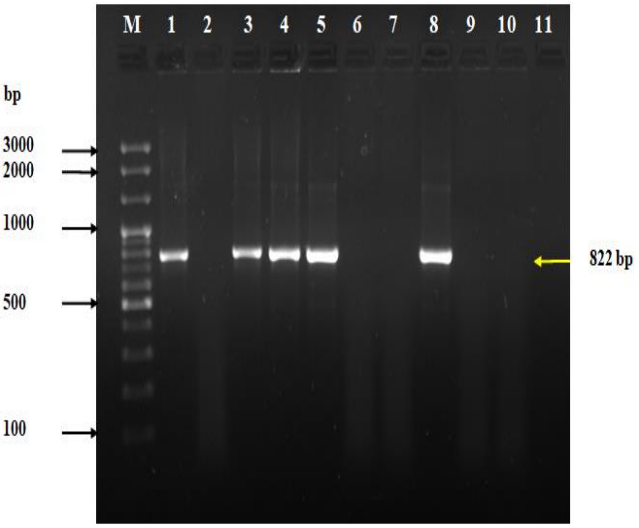

Figure 4: Electrophoresis of PCR products on the agarose gel. Lane M, DNA ladder (100 bp); lanes 1, 3, 4, 5, 8 are positive samples of TEM (822 bp); lanes 2, 6, 7, 9, 10 are negative samples; lane 11 negative control.

Discussion

The spread of ESBL producing Enterobacteriaceae of both animal and human origin is growing worldwide, which causes considerable concern among medical and veterinary practitioners (11). According to our review of previous studies in Iraq, this is the first molecular conducted study to detect the presence of ESBL/AmpC producing *E. coli* in sheep, specifically in Mosul city. Our results proved the presence of ESBL resistance genes among all studied 40 (100%) isolates, and this was expected because of the ability of these isolates to resist cefotaxime supplemented with MacConkey agar during primary isolation (14). Furthermore, among the studied ESBL genotypes, CTX-M genotype demonstrated high rates 40 (100%) followed by SHV (17.5%) 7/40 and TEM 5/40 (12.5%) genotypes, respectively. In Iraq, few studies have documented the presence of ESBL Enterobacteriaceae. However, Al-Sharook and Hassan (20) were able to detect 23.7% (9/38) of ESBL producing *E. coli* that were resistant to cefpodoxime in broilers in Erbil city, Iraq. In another study by Ahmed (3) probing CTX-M gene in ESBL-producing Enterobacteriaceae in healthy dairy cattle in Mosul city, Iraq, the study reported 28.75% (23/80) of Enterobacteriaceae isolates produced ESBL and majority of the isolates belong to *E. coli* 82.61% (19/23). Another recent study was also conducted by Ahmed *et al.* (21) on shepherd dogs accompanied by sheepherders in urban areas of Mosul city. The study focused on the essential role of shepherd dogs in carrying and spreading of extended-spectrum-cephalosporin resistant *E. coli* (ESCR *E. coli*), and 53.7% (36/67) of shepherd dogs were positive for ESCR *E. coli* with CTX-M genotype. However, the SHV and TEM genes were not covered by these previous studies, and therefore our current study considered other than CTX-M gene, including SHV, TEM genes. Nevertheless, the CTX-M genotype is appeared to be the most common type among ESBLs compared to SHV and TEM genes (9,11). Our results were inconsistent with the findings of Pehlivanoglu *et al.* (22), with predominated CTX-M gene detected in 87.1% (27/31) of the isolates in cattle and 100% (3/3) isolates of sheep. However, the same study confirms the presence of TEM 77.4% (24/31) and SHV 9.7% (3/31) of cattle isolates, and TEM 66.7% (2/3) but none SHV genes of sheep isolates. Another recent study from Malaysia, by Kamaruzzaman *et al.* (23), reported high rates of 66.7% of ESBL producing *E. coli* from cattle milk with a major combination of both CTX-M and TEM genotype. In our study, we could not able to detect any AmpC group of genes, and this might be due to the limited number of bacterial isolates used. Furthermore, our results demonstrated that the fecal load of ESBL *E. coli* was higher than those of milk samples from both healthy and diseases ewes. These results were in agreement with other previous studies (9,23). Finally, the presence of ESBL *E. coli* in sheep confer great risk due to the possibility of antibiotic resistance transmission from these animals or their product to humans.

Conclusion

Sheep are considered as a potential animal source for transmission and spreading of ESBL *E. coli* either by milk or contaminated feces, and this could increase antibiotic therapy failure among humans and animals in the future.

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Conflict of interest

No competing interests have to be declared by the authors.

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التحري الجزيئي لجراثيم الإيشيركيا القولونية المنتجة لخميرة البيتا لاكتام واسعة الطيف/الأمبسلين والمعزولة من الضأن في مدينة الموصل

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الخلاصة

على الصعيد العالمي، أصبحت جراثيم الإيشيريكيا القولونية المنتجة لأنزيم البيتا لاكتام واسعة الطيف أو الأمبسلين تشكل تهديدًا رئيسيًا لانتشار المقاومة للمضادات الحياتية. وبناءً على ذلك، فقد هدفت دراستا إلى التحري ومسح الجينات التي تمنح المقاومة لجراثيم الإيشيريكيا القولونية والمعزولة مسبقا من الضأن والتي تشكل جزأً أساسيا في مدينة

الموصل. تضمنت هذه الدراسة فحص أربعون عزلة من جراثيم جراثيم الإيشيريكيا القولونية المعزولة من عينات الحليب والبراز. حيث تم الحصول على هذه العزلات مسبقاً من النعاج السليمة وحملانها وكذلك من النعاج المصابة بالتهاب الضرع السريري. تم استخدام تفاعل البلمرة المتسلسل لتأكيد عزلات الإيشيركيا القولونية بواسطة الجين 16 sRNA. علاوة على ذلك، فقد تم فحص الأنماط الجينية المختلفة للجراثيم المنتجة لأنزيم البيتا لاكتام أو الأمبسلين باستخدام مجموعة بادئات متخصصة. أظهرت النتائج أن جين CTX-M كان سائدًا بين الأنماط الجينية للجراثيم المنتجة لأنزيم البيتا لاكتام وسجل ٤٠/٤٠ (١٠٠%). بينما سجلت الجينات SHV وTEM ٤٠/٧ (١٧,٥%) و ٤٠/٥ (١٢,٥%) على التوالي. علاوة على ذلك، فقد كان حمل الجينات المقاومة في البراز أكثر من ذلك الذي تم الحصول عليه من الحليب في كل من الحيوانات السليمة والمريضة. ومع ذلك، فلم يتم الكشف عن جينات الأمبسيلين في أي من ٤٠ عزلة. يعتبر وجود أنماط جينية مختلفة من جراثيم الإيشيريكيا القولونية المنتجة لأنزيم البيتا لاكتام واسعة الطيف المعزولة من البراز أو الحليب مصدرًا محتملًا لانتشار صفة المقاومة المضادات الحيوية للإنسان والحيوانات الأخرى والبيئة.