



## Detection of CTX-M gene in extended spectrum $\beta$ -lactamases producing Enterobacteriaceae isolated from bovine milk

I.M. Ahmed 

Department of Microbiology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

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#### Correspondence:

I.M. Ahmed  
[ihsanahmad1@yahoo.com](mailto:ihsanahmad1@yahoo.com)

### Abstract

Extended spectrum  $\beta$ -lactamases producing *Enterobacteriaceae* (ESBL-E) have emerged recently as the main cause that facilitates the spreading of antibiotic resistance worldwide. Due to its composition and nutritive values, raw cow milk is vulnerable to bacterial contamination from different sources, especially ESBL-E. Accordingly, present study aimed to detect the ESBL-E in the raw milk of healthy cows. 80 raw cow milk samples were collected from unorganized farms and cows belong to individual owners and investigated for the presence of ESBL-E with the main focusing on CTX-M type. The bacterial isolation was performed using selective MacConkey agar plus cefotaxime (MC<sup>+</sup>), while PCR was used to confirm the species of the isolated bacteria and presence of CTX-M gene. The results showed that 28.75%(23/80) samples were ESBL-E positive and distributed as following, 82.61%(19/23) were pure *E. coli* isolates, 4.35%(1/23) was pure *K. pneumoniae* isolate and finally, 13.04%(3/23) were mixed of both *E. coli* and *K. pneumoniae* isolates. Moreover, the total number of positive ESBL-E was 26 isolates with the majority of them were belong to *E. coli* and recorded 84.61%(22/26), while *K. pneumoniae* was recorded less 15.39%(4/26). Additionally, the CTX-M gene was successfully identified in all ESBL-E positive isolates by using PCR, including *E. coli* and *K. pneumoniae* isolates. The results of this study assert the importance of raw cow milk as a potential source of ESBL-E that might be transmitted to humans.

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### Introduction

Bovine milk is considered a major source of proteins and vitamins for humans, in addition to providing energy. It is beneficial for early life and aged people due to its complete nutritional components (1). However, in many developing countries, the misuse of antibiotics in veterinary medicine and animal husbandry, especially as subtherapeutics and growth promoters, leads to development of antimicrobial resistance especially in milk and dairy products (2). Globally, drug-resistant microorganisms have become an increasingly important health problem in man and animals which results in a reduction in the efficacy of many common antibiotics (3,4). During the last decades, extended-spectrum  $\beta$ -lactamases (ESBLs) have been globally increased. They

have considered as a main source of Gram-negative bacterial pathogens that have the resistance feature to the most important antibiotics especially *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* as main members of *Enterobacteriaceae* (3,5,6). However, close contact with animals and consuming of their products is considered as a risk factor that might have a role in transferring of positive ESBL bacteria to humans, especially with the dramatic increase of commensal ESBL (3-5,7). ESBLs have the ability to hydrolyse different types of cephalosporins including, cefotaxime, ceftazidime, ceftriaxone, and cefepime (8,9). The main detected type of ESBL is CTX-M  $\beta$ -lactamase rather than other  $\beta$ -lactamases including classical TEM- and SHV-types of ESBLs. It has been reported worldwide in both human and animal populations

(6,8). In recent years, there were enormous increased of documentations that related to CTX-M-type of ESBLs produced by different members of the *Enterobacteriaceae* (7,10), and this highlighted the importance of this gene among antibiotic resistance *Enterobacteriaceae* that could transfer the resistance to other bacteria especially gut pathogens (3). Moreover, the ESBL has frequently encoded by plasmids. Yet, plasmids including ESBL may also carry other different types of antibiotic resistance genes, which make the options of treatment very limited for ESBL producing bacteria (7). In Iraq, many studies have been carried out related to the detection of ESBL mainly in humans (11,12). However, Al-Sharook and Hassan (13) referred to successful isolation of ESBL *E. coli* in broilers. Little is known about the presence of ESBL *Enterobacteriaceae* in bovine milk. Therefore, the presence of ESBL *Enterobacteriaceae* especially from milk of healthy cows is a fact that has to be addressed properly. Accordingly, this study was aimed to detect the presence of CTX-M gene in ESBL-*E* from raw cow milk as a potential marker that indicates for the presence of antibiotic resistance.

## Materials and methods

### Samples collection

Eighty samples of raw cow milk were collected randomly from unorganized smallholder dairy farms or individual owners in Mosul city, during the period of April to August 2019. Approximately, 30 ml volume of raw milk was collected aseptically from apparently healthy lactating cows in a sterile disposable container and transported using cooling box to Microbiology Laboratory-Department of Microbiology at College of Veterinary Medicine-University of Mosul. The samples were kept cooled at 4°C until being further process.

### Bacterial isolation and identification

A volume of 10 ml of milk samples was centrifuged at 3000 ×g for 20 min. Then the supernatant was removed and the pellet was mixed with 100 µl of sterile 0.9% NaCl solution and cultured on specially prepared selective MacConkey agar plus antibiotic cefotaxime (MC<sup>+</sup>) (Foxime 500 mg, Tabuk, KSA) at a final concentration 1 µg/ml according to Ali *et al.* (14). This medium has the ability to select the bacteria that resist cefotaxime. The cultured plates were put in the incubator at 37°C for 24 h. All ESBL-positive colonies were subsequently determined and subsequently subcultured on Brain Heart Infusion agar (Lab M, UK) for further bacterial identification using standard bacteriological methods, including Gram staining and biochemical tests (15).

### Extraction of DNA

Selected colonies were picked depending on their growth ability on MC<sup>+</sup> agar and subsequent phenotypic

identification, only *E. coli* and *K. pneumoniae* colonies were successfully identified as members of *Enterobacteriaceae* that showed ESBL activity. Accordingly, only ESBL positive colonies that have been isolated on MC<sup>+</sup> were subjected to DNA extraction using Bacteria DNA Preparation Kit (Jena Bioscience, Germany). Following the manufacturer instructions with slight modification, fresh overnight cultures on Brain Heart Infusion agar (Lab M, UK) were used, and few colonies were transferred into a sterile 1.5 ml microcentrifuge tube, which previously contains 300 µl of Cell Lysis Solution followed by adding 1.5 µl of RNase A Solution and mixed gently by several inverting, then the microcentrifuge was put in the incubator at 37°C for 30 min followed by cooling on ice for 1 min to precipitate the proteins. Then, 100 µl of Protein Precipitation Solution was added to each tube and mixed vigorously by vortex for 20-30 sec, then centrifuged at 15,000 ×g for 5 min. The DNA was precipitated by transferring the supernatant to a new clean 1.5 ml microcentrifuge with previously added 300 µl Isopropanol with concentration of 99% and mixed by gentle inverting for 1 min. The DNA was pellet down by centrifugation at 15,000 ×g for 1 min. The supernatant was removed out and the microcentrifuge was drained. The DNA pellet was washed by adding 500 µl of Washing Buffer followed by inverting the tube several times and centrifuged at 15,000 ×g for 1 min and the Washing Buffer was removed carefully. The microcentrifuge was dried at ambiant temperature for 20 min and the DNA was hydrated by adding 100 µl of DNA Hydration Solution to the previously dried DNA pellet and incubated in water bath at 65°C for 60 min. After hydration the DNA was kept at -20°C for subsequent assay.

### Polymerase chain reaction (PCR)

PCR was performed to confirm these bacteria using species-specific primers (*E. coli*: ECO223-F and ECO 455-R; *K. pneumoniae*: SSKP 1 F and SSKP 1 R (Table 1). The PCR cocktail mixtures were prepared in 20 µl containing 10 µl HS Prime Taq Premix (2X) (*GeNet Bio*, Korea), 1 µl of each primer 10 mmol (IDT, USA), 2 µl of DNA template final concentration (2 ng/µl) and 6 µl of PCR grade water. The PCR was done using the thermal cycler (BioRad, T100, Bio-Rad, USA). The cycling conditions include 1 cycle of initial denaturation at 94°C for 10 min, 35 cycles consisting of (initial denaturation at 94°C for 30 sec, annealing at (55°C for *E. coli* and 57°C for *K. pneumoniae*) for 30 sec and extension at 72°C for 45 sec). Then, one cycle of final extension at 72°C for 5 min. Finally, the reaction was cooled for cooling at 4°C for the next step of gel electrophoresis. Additional PCR was performed to confirm the presence of (CTX-M) using specific primers (CTX-M-Uni-F and CTX-M-Uni-R) Table 1. using the same PCR reaction and cycling conditions, except the annealing temperature that set at 54°C. The amplified products were separated using the agarose gel electrophoresis in 1.2% agarose gel (Promega, USA)

containing Prime Safe Dye (*GeNet Bio*, Korea). A volume of 5 µl of each PCR product was loaded into the well of agarose gel. The electrophoresis was carried out at 80 V, 300 mA for 1 hour using Wide Mini-Sub Cell GT gel electrophoresis systems and power supply (*Bio-Rad*, USA) containing 1X

TBE buffer (*Bio-Rad*, USA). A 100 bp DNA marker, 4 µl (*GeNet Bio*, Korea) was used as standard molecular weight marker. After electrophoresis, the gel was viewed using Gel doc Ez system (*Bio-Rad-USA*) to detect the specific bands.

Table 1: Sequences of primers used for PCR

No.	Primer Name	Primer Sequence 5' - 3'	Target gene	Annealing Temp°C	size bp	Ref.
1	ECO223-F	ATCAACCGAGATTCCTCCAGT	16SrRNA	55	232	(16)
2	ECO 455-R	TCACTATCGGTCAGTCAGGAG				
3	SSKP 1 F	ATTTGAAGAGGTTGCAAACGAT	16SrRNA	57	130	(17)
4	SSKP 1 R	TTCACTCTGAAGTTTCTTGTGTTC				
5	CTX-M-Uni-F	CGCTTTGCGATGTGCAG	<i>bla</i> CTX-M	54	550	(14)
6	CTX-M-Uni-R	ACCGCGATATCGTTGGT				

**Results**

The results of bacterial isolation on MC<sup>+</sup> and subsequent phenotypic identification methods showed that 28.75% (23/80) of raw cow milk samples were positive for ESBL-*E* as following. 82.61% (19/23) with pure *E. coli* isolates, 4.35% (1/23) with pure *K. pneumoniae* isolate and 13.04% (3/23) with mixed isolates of both *E. coli* and *K. pneumoniae* isolates. Furthermore, a total of 26 ESBL-*E* bacterial isolates were successfully obtained from these 23 samples and *E. coli* was dominated and recorded 84.61% (22/26), while *K. pneumoniae* was recorded less 15.39% (4/26). Furthermore, the results of PCR revealed the presence of expected band size at 232 bp and 130 bp, for *E. coli* (Figure 1) and *K. pneumoniae* (Figure 2), respectively. A further molecular investigation was done to detect the occurrence of CTX-M gene among the positive ESBL isolates. The results showed that all the tested isolates were confirmed positive for CTX-M gene (Figure 3).

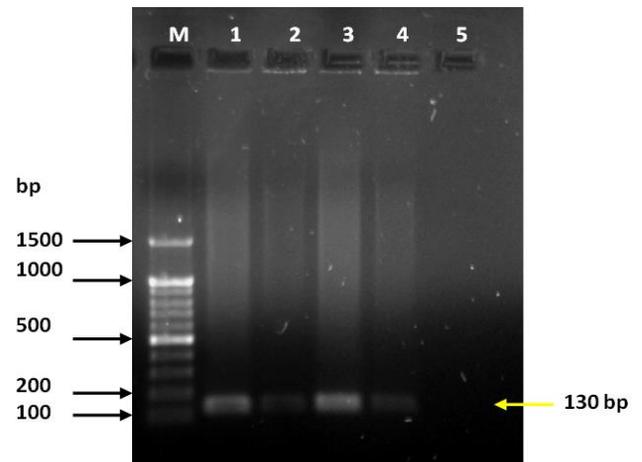


Figure 2: Represent gel electrophoresis of PCR final products of *K. pneumoniae*. Lane M, DNA marker (100 bp); lane 1-4 positive *K. pneumoniae* samples giving 130 bp product size; lane 5, control negative.

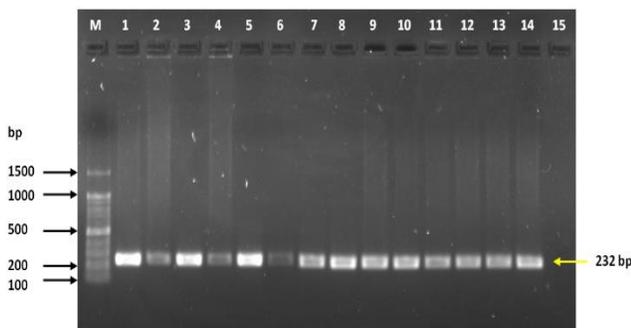


Figure 1: Represent gel electrophoresis of PCR final products of *E. coli*. Lane M, DNA marker (100 bp); lane 1-14 positive *E. coli* samples giving 232 bp product size; lane 15, control negative.

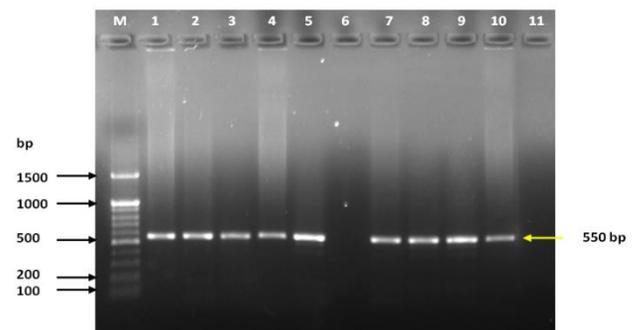


Figure 3: Represent gel electrophoresis of PCR final products of the CTX-M gene. Lane M, DNA marker (100 bp); lane 1-5 positive samples of CTX-M for *E. coli* giving 550 bp product size; lane 6 negative control, lane 7-10 positive samples of CTX-M for *K. pneumoniae* giving 550 bp product size; lane 11 control negative.

## Discussion

Increasing resistance to antibiotics became a global concern for both human and veterinary fields (8,18-20). Members of *Enterobacteriaceae* have been involved in different cases of unresponsiveness to antibiotic treatment including human infections such as community-acquired and foodborne infections. In addition, unresponsive to treatment in animals, especially production diseases such as mastitis (21-24). In this study, 28.75% (23/80) of raw milk samples were ESBL positive which belongs to apparently healthy cows. These results were higher compared with Batabyal *et al.* (5), who reported in his study in West Bengal that 12.1% (22/182) were ESBL *E. coli* positive from the collected milk samples which targeted organized dairy cow farms that appeared healthy. While Badri *et al.* (25) detect high positive ESBL-*E* with dominant CTX-M type isolated from the raw cow milk with 29.3% and 44.8% for *E. coli* and *K. pneumoniae*, respectively. The high increase of ESBL reflects the unhygienic conditions of milk handling and production in addition to the use of traditional systems for milk manufacturing and transportation that favour contamination by fecal *Enterobacteriaceae* (4,26). However, Geser *et al.* (20) reported that no ESBL bacteria were found in milk obtained from bulk storage tank in Switzerland. The authors justify that due to the high quality based system adapted for processing tank milk. It is clear that organized dairy farms that follow strict hygienic rules and controlled use of antibiotics might develop less antibiotic resistance than the farming of few cows under uncontrolled regimes. The positive isolates of ESBL-*E* were additionally identified using PCR as a molecular tool and *E. coli* and *K. pneumoniae* were confirmed. This step was necessary to confirm the species of isolated bacteria for the next step to detect CTX-M positive bacteria using molecular identification. The PCR results using species-specific primers for both *E. coli* and *K. pneumoniae* showed that the majority of isolated ESBL-*E* was belonging to *E. coli* 84.61% while *K. pneumoniae* recorded 15.39%. Many recent studies reported the implication of *E. coli* as a major source of ESBL in different circumstances (23,24,27) and to less extend *K. pneumoniae* and other species of *Enterobacteriaceae* like *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp. (8,28). Thus, the potential zoonoses and spreading of ESBL encoding bacteria between human and animals especially in farm workers are of great concern mainly in the developing countries (23). All the ESBL positive isolates on MC<sup>+</sup> were also showed a positive CTX-M gene in PCR. However other genes did not cover by this study such as SHV and TEM genes, and this might require further studies to investigate their roles. The choosing of CTX-M gene was due to its main role in antibiotics resistance among ESBLs (4,8). The presence of CTX-M was reported in human (18,29-31), in addition to different animal infections mainly related to bovine mastitis (2,20,24). The proofing of ESBL-*E* in raw cow milk in this

study highlighted the concerns and risks about the increased probability of transmission of such resistant bacteria from non-diseased to diseased cows, especially those that might develop mastitis (2,4,8). Finally, the continuous arbitrary using of the antibiotics in our human community and animal fields with the absence of standard hygienic methods for handling milk during processing and production, all could increase the risk of transmission and spreading of such ESBL-*E* in both animals and humans.

## Conclusion

In conclusion, CTX-M gene was successfully detected in ESBL-*E* from raw cow milk which could be of a major risk for the spreading of antibiotic resistance. Future studies are needed to detect other types of resistance genes in dairy cows.

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## Conflict of interest

The author declare that he has no conflict of interest.

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## الكشف عن جين CTX-M في الجراثيم المعوية المنتجة لخميرة $\beta$ -lactamases واسعة الطيف والمغزولة من حليب الأبقار

إحسان منير احمد

فرع الأحياء المجهرية، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

### الخلاصة

اعتبرت الجراثيم المعوية المنتجة لخميرة البيتا لاكتام واسعة الطيف مؤخراً من الأسباب الرئيسية لانتشار المقاومة للمضادات الحيوية عالمياً. ونظراً لتركيبية الحليب والقيمة الغذائية له فإنه يكون عرضةً للتلوث وخصوصاً من الجراثيم المعوية المنتجة لخميرة البيتا لاكتام واسعة الطيف. وعليه فقد هدفت هذه الدراسة للكشف عن وجود هذه الجراثيم في الحليب الخام من الأبقار السليمة. تم جمع ٨٠ عينة حليب من مزارع غير نظامية بالإضافة إلى عينات حليب من مربي الأبقار بشكل فردي وذلك للكشف عن الجراثيم المعوية المنتجة لخميرة البيتا لاكتام واسعة الطيف مع التركيز على النوع الجيني CTX-M. تم إجراء العزل الجرثومي باستخدام أكار الماكونكي المضاف له المضاد الحيوي سيفوتكسيم، في حين استخدم تفاعل البلمرة المتسلسل لتأكيد أنواع الجراثيم المغزولة

القولونية وسجلت ٨٤,٦١% (٢٦/٢٢)، في حين سجلت الكليبيسيلا الرئوية نسبة اقل وبلغت ٣٩.١٥% (٢٦/٤). فضلا عن ذلك فقد تم الكشف والتعرف على الجين CTX-M في جميع العزلات الموجبة للجراثيم المعوية المنتجة لخميرة البييتالاكتام واسعة الطيف باستخدام تقنية تفاعل البلمرة المتسلسل والذي شمل عزلات الإشيركيا القولونية والكليبيسيلا الرئوية. أكدت نتائج هذه الدراسة على أهمية حليب الأبقار الخام كمصدر أساسي للجراثيم المعوية المنتجة لخميرة البييتالاكتام واسعة الطيف والتي من الممكن أن تنتقل إلى الإنسان.

وكذلك احتواءها على الجين CTX-M. أظهرت نتائج العزل الجرثومي أن ٢٨,٧٥% (٨٠/٢٣) عينة كانت موجبة للجراثيم المعوية المنتجة لخميرة البييتالاكتام واسعة الطيف وقد توزعت كالتالي، أن ٨٢,٦١% (٢٣/١٩) كانت تعود وبشكل نقي إلى جرثومة الإشيركيا القولونية في حين أن ٤,٣٥% (٢٣/١) كانت تعود وبشكل نقي إلى جرثومة الكليبيسيلا الرئوية وأخيرا فإن ١٣,٠٤% (٢٣/٣) من العينات احتوت على كلتا الجرثومتين الإشيركيا القولونية والكليبيسيلا الرئوية. إضافة إلى ذلك شكل العدد الكلي للعزلات الموجبة للجراثيم المعوية المنتجة لخميرة البييتالاكتام واسعة الطيف ٢٦ عزلة كانت أغلبها تعود لجراثيم الإشيركيا