



Molecular characterization of extended spectrum cephalosporin resistant *Escherichia coli* isolated from dogs

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Abstract

Shepherd dogs have been implemented in the transmission and distribution of many threatening pathogens. The presence of extended-spectrum-cephalosporin resistant *Escherichia coli* (ESCR *E. coli*) in dog feces can constitute a significance risk to human health due to transmission of antibiotics resistance from dogs to humans, other animals and the surrounding environment. Therefore, in this study, phenotypic and molecular characterization of fecal ESCR *E. coli* were investigated in shepherd dogs accompanied shepherders in urban areas. Sixty-seven fresh fecal samples were collected from shepherd dogs from different regions of Mosul city. Bacteriological examination of ESCR *E. coli* was done using MacConkey agar with cefotaxime followed by subsequent PCR confirmation of the CTX-M gene using specific primers and molecular characterization using specific primers directed to CTX-M-1, 2 and 9 groups. The results of bacterial examination showed successful confirmation of ESCR *E. coli* which has been isolated from fecal samples of shepherd dogs 58.2% (39/67). In addition, detection of CTX-M gene was confirmed in 53.7% (36/67) of *E. coli* isolates. Furthermore, molecular characterization of CTX-M gene revealed the presence of only one genotype belongs to CTX-M-1. However, both of CTX-M-2 and CTX-M-9 genotypes were not detected in this study. This study concluded that shepherd dogs have an essential role in carrying and spreading of ESCR *E. coli* especially in urban regions.

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Introduction

Dogs play an important role in human daily practices as they mainly kept as companion pets (1,2). In addition, they are used in many diverse activities such as animal grazing, livestock guarding, and thus it's in close contact with humans (3,4). One of the most common farming practices of sheep and goats in urban areas of Mosul city is keeping stray dogs as shepherd dogs along with these flocks to provide protection and control of the animal movement. Nevertheless, most of these dogs mainly did not receive any treatments or vaccinations which render them as potential risk for transmission of many pathogens and diseases (3-6). Recent studies approved that many bacterial species have

developed antimicrobial resistance especially in dogs (1,7,8) and this resistance might be transmitted to human (9). Spreading of beta-lactamase-producing Enterobacteriaceae is considered as one of the most important bacterial pathogens associated with high costs of health care and also unresponsiveness to treatment (7,10,11). Nevertheless, increased spreading of extended-spectrum β -lactamase (ESBL) bacteria was potentially related to the common pathogen for many infections. In addition, its treatment has become more difficult because of its resistance to antibiotics (12-14). Yet, the spreading of extended-spectrum cephalosporin resistance *E. coli* (ESCR *E. coli*) infections has complicated the scene and become a major challenge due to substantial genetic diversity, possession of virulence

factors, large and wide hosts range, multifaceted potential and various pathogenic forms and the possibility of infection and spreading among multiple hosts within the environment (12-16). A plasmid-mediated CTX-M β -lactamase has become a dominant type of ESBLs, especially ESCR *E. coli*, rather other classical β -lactamases such as SHV- and TEM-types (17-19). CTX-M type has been increasingly recorded in human and animal worldwide with most prevalent groups CTX-M-1, CTX-M-2 and CTX-M-9 (4,12,17-19). Giving the fact, there is no information about spreading of ESCR *E. coli* in the shepherd dogs in Mosul city, in addition to lack of local studies which indicate the possibility of dogs as a transporter of ESCR *E. coli*. Therefore, this study aimed to detect and molecular characterize ESCR *E. coli* in shepherd dogs.

Materials and methods

Sampling

A Total of 67 fresh fecal samples of apparently health shepherd dogs were collected in sterile containers from both sexes (male and female), ranging from 6 months to 3 years in age from different urban regions of Mosul city for the period from December 2019 to February 2020. The samples were transported immediately under cooling conditions to the Microbiology Laboratory, College of Veterinary Medicine, University of Mosul, for further microbiological examination.

Isolation and identification of ESCR *E. coli*

A loopful of the collected fecal sample was cultured on special MacConkey agar with cefotaxime (Foxime 500 mg, Tabuk, KSA) at 1 μ g/ml final concentration according to Umeda *et al.* (15). After that, all the inoculated plates were put in the incubator at 37°C for 24 h. This medium inhibits all the bacteria that are sensitive to cefotaxime and allows only resistant ones to grow. All suspected fermented ESCR *E. coli* colonies were again subculture on MacConkey agar with cefotaxime for purification and subsequent identification using standard bacteriological methods (20).

Molecular characterization

Extraction of DNA

Only ESCR *E. coli* positive colonies were subjected to DNA extraction using Bacteria DNA Preparation Cat No. PP-206S (Jena Bioscience, Germany). According to the manufacturer instructions, briefly, few fresh colonies on Brain Heart Infusion agar (Lab M, UK) were selected and suspended in an eppendorf microcentrifuge for cell lysis. Then, followed by a step of precipitation of proteins. After that the supernatant was separated in a new sterile 1.5 ml eppendorf tube with 300 μ l Isopropanol 99%. This step was necessary for DNA precipitation. The tube was centrifuged and the supernatant was discarded followed by draining of the tube. The small DNA pellet was washed by adding

washing buffer, inverted several times then centrifuged. The supernatant was eliminated and the tube was dried in air at room temperature. Hydration of DNA was done by adding 100 μ l of Hydration Solution, followed by incubation at 65 °C for 1 h, and then the DNA was kept at -20 °C for subsequent testing.

Polymerase chain reaction (PCR) assay procedure

All the primers used in this study were detailed in (Table 1). The molecular confirmation of *E. coli* was performed using species specific primers (*E. coli*: ECO223-F and ECO455-R). While confirmation of the CTX-M gene was done by CTX-M-uni-F and CTX-M-uni-R. Subsequent characterization of CTX-M groups was done using 3 sets of primers targeting G1, G2 and G9 groups of the CTX-M gene. Standard PCR reaction mixture was prepared for all protocols. Briefly, 25 μ l containing 12.5 μ l HS Prime Taq Premix (2X) (*GeNet Bio*, Korea), 1 μ M of each primer 10 mmol (IDT, USA), 2.5 μ l of DNA template final concentration (2 ng/ μ l) and 8 μ l of PCR grade water. The PCR was done using 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*, 54 °C for CTX-M-uni and 52 for CTX-M G1, G2, G3) for 30 sec, and extension at 72 °C for 45 sec). Finally, one cycle of final extension at 72 °C for 5 min. The reaction was set for cooling at 4 °C. Agarose gel electrophoresis was used to separate the PCR products using 1.2% agarose gel (Promega, USA) containing Prime safe Dye (*GeNet Bio*, Korea). The wells of agarose gel were loaded by 5 μ l of each PCR product. The electrophoresis condition was set at 75 V, 300 mA for 50 min using Wide Mini-Sub Cell GT gel electrophoresis systems and basic power supply (Bio-Rad, USA) containing 1X TBE buffer (Bio-Rad, USA). A volume of 4 μ l of DNA ladder, 100 bp (*GeNet Bio*, Korea) was used as molecular weight standard. Following electrophoresis, the gel was viewed using Gel doc Ez system (Bio-Rad-USA) to detect the specific bands.

Results

The bacterial isolation and identification showed successful recovery of ESCR *E. coli* from fecal samples 58.2% (39/67) using MacConkey agar with cefotaxime (Figure 1). However, only 53.7% (36/67) isolates were confirmed *E. coli* by PCR producing expected product size of 232 bp (Figure 2). Additionally, all the 36 positive samples of *E. coli* showed positive for the CTX-M universal gene with expected band size of 550 bp (Figure 3). Furthermore, Molecular characterization of the CTX-M gene using primers directed to CTX-M-1, 2 and 9 showed that all the isolates were belong to CTX-M-1 with product size of 415 bp. However, neither CTX-M-2 nor CTX-M-9 was confirmed in the examined samples (Figure 4).

Table1: Primers used for PCR

No.	Primer Name	Sequence 5' – 3'	Tm °C	Product size (bp)	Ref.
1	ECO223-F	ATCAACCGAGATTCCCCAGT	55	232	(21)
2	ECO 455-R	TCACTATCGGTTCAGTCAGGAG			
3	CTX-M-Uni-F	CGCTTTGCGATGTGCAG	54	550	(22)
4	CTX-M-Uni-R	ACCGCGATATCGTTGGT			
5	CTX-M G1-F	AAAAATCACTGCGCCAGTTC	52	415	(23)
6	CTX-M G1-R	AGCTTATTCATCGCCACGTT			
7	CTX-M G2-F	CGACGCTACCCCTGCTATT	52	552	(23)
8	CTX-M G2-R	CCAGCGTCAGATTTTTTCAGG			
9	CTX-M G9-F	CAAAGAGAGTGCAACGGATG	52	205	(23)
10	CTX-M G9-R	ATTGGAAAGCGTTCATCACC			

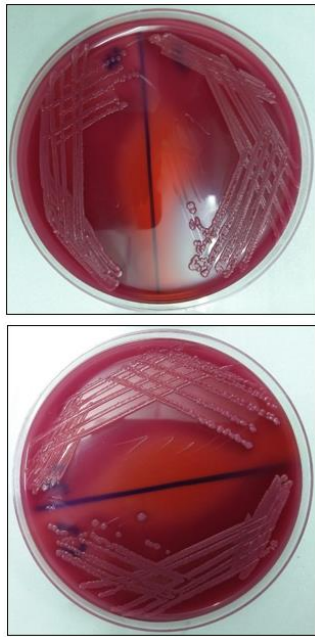


Figure 1: Isolation of ESCR *E. coli* using specific MacConkey agar with cefotaxime showing characteristic small lactose fermenting colonies.

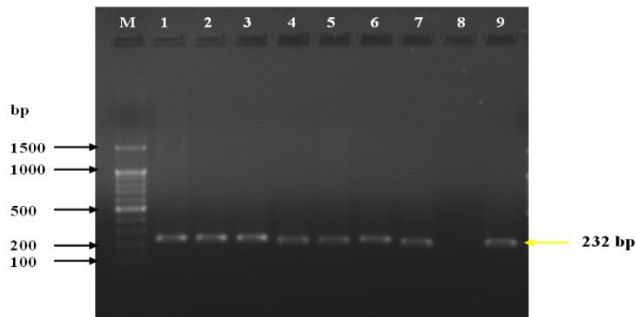


Figure 2: PCR products of *E. coli*. Lane M, DNA molecular standard; lane 1-7 positive tested samples giving 232 bp product size; lane 8, negative control and lane 9 control positive.

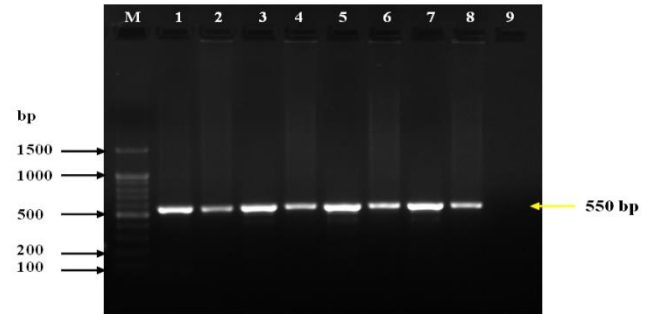


Figure 3: PCR products of CTX-M universal gene. Lane M, DNA molecular standard; lane 1-8 positive tested samples giving 550 bp product size; lane 9, control negative.

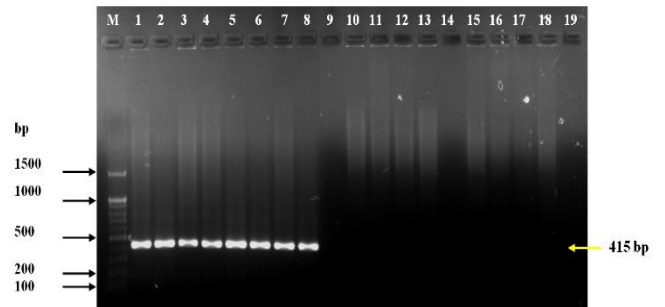


Figure 4: PCR products of CTX-M gene characterization using specific primers set. Lane M, DNA molecular standard; lane 1-8 are positive tested samples of CTX-M G1 giving 415 bp product size; lane 10-13 are negative tested samples for CTX-M G2, and lane 15-18 are samples negative tested for CTX-M G9.

Discussion

Globally, antibiotic resistance has become a serious threat and widely distributed in humans, animals and the environment (18,24-27). Nevertheless, dog feces are well known as a potential source of many zoonotic diseases and also spreading of different types of bacteria which are

resistant to antibiotics (2,5,28,29). In this study we investigated a type of stray dogs that are used commonly as shepherd dogs in our community and they are almost with contact with the animals especially sheep and goats in addition to the grazers themselves. Our results showed that 53.7% (36/67) of the shepherd dogs were confirmed positive for ESCR *E. coli* and possess CTX-M gene using standard bacteriological culture and PCR as a molecular confirmation method. This result was in consistent with the previous findings obtained by Hong *et al.* (9), they reported that 49.2% (155/315) of dogs were positive for ESCR *E. coli*. Another recent study by Abbas *et al.* (30) revealed that 69.5% (73/105) of screened fecal samples obtained from dogs, cats and their owners in addition to veterinary professionals were found positive for ESBLR *E. coli*. However, isolates among dogs were the highest number 81.8% (18/22). Other study by Zhang *et al.* (31) targeted healthy dogs in different parks in Ontario, Canada revealed that fecal carriage of ESCR Enterobacteriaceae was 26.5% of with majority belongs to ESCR *E. coli*, while ESCR *Klebsiella pneumoniae* and *Proteus mirabilis* recorded less. Also Umeda *et al.* (15) found that 22/151 (14.6%) of dogs were Cephalosporin-resistant Enterobacteriaceae with majority identifies as ESCR *E. coli*. This variation in the results may reflect the strict use of antibiotics in different countries. It is clear that the presence of ESCR Enterobacteriaceae especially ESCR *E. coli* in fecal samples of dogs is considered as a potential risk factor for spreading of antimicrobial resistance by these animals to other animal species, close contact humans and the environment (18,32-34). This mainly occurred by contamination of the ground, pasture and water by feces of roaming shepherd dogs with ESCR type of bacteria. Among the ESBL genotypes of Enterobacteriaceae, the CTX-M type ESBL genes were frequently studied in both human and veterinary fields with almost dominant of ESCR *E. coli* (9,24,25,35). In this study the CTX-M-1 was the only detected genotype, while CTX-M-2 and CTX-M-9 were not confirmed. This was in agreement with Damborg *et al.* (29), they reported isolation of ESBL with predominant CTX-M-1 genotype confirmed by multilocus sequence typing (MLST). While Haenni *et al.* (36) found that most of ESBL *E. coli* isolates belonged to CTX-M-1 and CTX-M-2. Also, Abbas *et al.* (30) reported high rates of the CTX-M genes of isolated ESBL *E. coli* with dominating CTX-M-1 genotype in all positive isolates. However, other groups of CTX-M have been studied by others such as CTX-M-2 (33,35), CTX-M-9 (13,33), CTX-M-14 (8,37), CTX-M-15 (15,29,38) or even miscellaneous CTX-M groups like CTX-M-24, CTX-M-27, CTX-M-55 (8, 15). In addition, other genes also have been reported especially, those within ESBL such as SHV, CMY groups (37-39), TEM (39), or other types rather than ESBL such as AmpC β -lactamase (pAmpC) (28,33,37) and carbapenemase (25,40). Finally, the dramatic spreading of antibiotic resistance has a great impact on human in terms of one health

concept though increasing the probability of fecal contamination by such kind of shepherd dogs, various animal species and also the environment worldwide. More studies have to be conducted to understand the relation between resistant bacteria isolates from different sources. Such information will surely help to mitigate and control the spreading of antibiotic resistance to a more dangerous level especially development of different pathogens, including multidrug-resistant once.

Conclusion

ESCR *E. coli* was successfully detected in shepherd dogs with common CTX-M G1 type. Shepherd dogs are considered as one of the most neglected sources of spreading and transmission of ESCR *E. coli*.

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Conflict of interest

The author declare that he has no conflict of interest.

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المقاومة للـسيفالوسبورين ذات الطيف الواسع المعزولة من براز كلاب الرعي المصاحبة للرعاة في المناطق المدنية. جمعت ٦٧ عينة من براز كلاب الرعي المنتشرة في مدينة الموصل. وأجريت فحوصات العزل الجرثومي والتوصيف لجراثيم الإيشيركيا القولونية المقاومة للـسيفالوسبورين ذات الطيف الواسع باستخدام وسط أكار الماكونكي المضاف له السيفوتاكسيم واتبع لاحقا باختبار تفاعل البلمرة المتسلسل لتأكيد النتائج باستخدام بادئات خاصة لجين CTX-M ومن ثم اجري التوصيف الجيني باستخدام بادئات متخصصة للمجاميع CTX-M-1, 2, 9. أظهرت نتائج الفحص الجرثومي نجاح عزل جراثيم الإيشيركيا القولونية المقاومة للـسيفالوسبورين ذات الطيف الواسع من براز كلاب الرعي وبنسبة ٥٨,٢% (٦٧/٣٩). بالإضافة إلى ذلك تم الكشف عن جين CTX-M بنسبة ٥٣,٧% (٦٧/٣٦) من عزلات الإيشيركيا القولونية. فضلا عن ذلك فقد أظهرت نتائج التوصيف الجيني وجود نمط جيني واحد يعود إلى CTX-M-1. ومع ذلك فلم يتم الكشف عن النمطين الجينيين CTX-M-2 و CTX-M-9 في هذه الدراسة. يستنتج من هذه الدراسة أن كلاب الرعي تلعب دوراً أساسياً في حمل ونشر جراثيم الإيشيركيا القولونية المقاومة للـسيفالوسبورين ذات الطيف الواسع خصوصاً في المناطق المدنية.

التوصيف الجزيئي لجراثيم الإيشيركيا القولونية المقاومة للـسيفالوسبورين ذات الطيف الواسع والمعزولة من الكلاب

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الخلاصة

تعتبر كلاب الرعي مصدراً مهماً لانتقال وانتشار العديد من العوامل المرضية. إن وجود جراثيم الإيشيركيا القولونية المقاومة للـسيفالوسبورين ذات الطيف الواسع في براز الكلاب قد يشكل عاملاً خطورة على الصحة العامة من خلال انتشار المقاومة للمضادات الحيوية من الكلاب إلى الإنسان وبقية الحيوانات والبيئة المحيطة. ولذلك تم التحري في هذه الدراسة عن الصفات المظهرية والجزيئية لجراثيم الإيشيركيا القولونية