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Phenotypic characterization and antibiogram of extended spectrum ß-lactamase (ESBL)/AmpC-producing Escherichia coli isolated from sheep

F.R. Mahmood and 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

Abstract

This study aimed to isolate and characterize extended-spectrum ß-lactamases (ESBLs) and AmpC producing *E. coli* in sheep in Mosul city. A total of 260 milk and fecal samples were collected aseptically from healthy ewes (n=60), their respective lambs (n=60), and ewes with clinical mastitis (n=40). Standard bacterial isolation and identification on special culture media were performed to isolate ESBL/AmpC producing *E. coli*. While special antibiotic discs D68C MASTDISCS® Combi AmpC and ESBL ID set were used to characterize positive ESBL/AmpC *E. coli*. The results showed that 99/260 (38.1%) of tested samples were ESBL-*E. coli* positive and distributed as follows, 7/60 (11.٧%) and 39/60 (65%) from milk and feces of clinically healthy ewes, respectively, and 37/60 (61.7%) from feces of clinically healthy lambs, while 4/40 (10%) and 12/40 (30%) from the milk and feces of ewes with clinical mastitis, respectively. However, we could not obtain any AmpC positive isolate from all tested samples. The high recovery percentages of ESBL from feces or milk of sheep reflect the arbitrary use of the antibiotic in sheep farming. This could significantly increase the resistance of the bacterial population that might represent a potential source for transmission of antibiotic resistance to humans.

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Introduction

In Iraq, sheep farming is considered as the main livestock husbandry and provides an important source of milk, meat and wool production (1). However, sheep farming has been affected by the misuse of antibiotics that finally contribute to increase antibiotic resistance which is manifested by unresponsiveness to treatment and consequently burden the economic investment of such important industry (2). Beside the economic losses, the transfer of antibiotic resistance to human (by direct contact with animals or indirect contact through consumption of raw milk and dairy products) is considered as public health concern and a risk factor that increasing the resistance to different antibiotics (3,4). Globally, resistance to antibiotics has been increased dramatically in the last decades which can affect animals and human at the same time (4-7). Nonetheless, extended-spectrum ß-lactamases (ESBLs) and AmpC are major types of antibiotic resistance that have been reported worldwide (7,8). ESBL is an enzyme which has the ability to hydrolyze first, second, and third generation cephalosporin antibiotics including cefotaxime, ceftriaxone and cefepime in addition to aztreonam groups (5). Yet, the circulation of ESBL in different host and ecosystems complicated the scene and render the spread of resistance difficult to control (9). During the last decades, Enterobacteriaceae have emerged as possible source for spreading of ESBLs/AmpC for humans (7,9). Yet, one of the most important members of Enterobacteriacae is *Escherichia coli* that have been reported as major member species that contribute effectively in transferring the resistance (7,10). Although *E. coli* is considered as a commensal intestinal microflora for both animals and human, it can produce intestinal and extraintestinal disease conditions mainly as acute enteritis, haemorrhagic colitis and sepsis (9,11,12). The presence of *E. coli* in the milk

could be originated from a variety of sources such as feces, soil or even contaminated water (7,11,13). Nevertheless, we think that ewe feces and milk could contribute significantly in transmission and spreading of ESBL/AmpC *E. coli* to their lambs. Hence, this could be a major source for spreading antibiotic resistance to the next generations. Locally, few studies have reported the presence of ESBL *E. coli* in poultry (14), bovine milk (5) and human (12). However, detection and characterization of ESBL/AmpC sheep have not been explored yet. To focus more on the possible reasons for spreading ESBL/AmpC *E. coli* in sheep, this study was designed to isolate and characterize ESBL/AmpC *E. coli* from ewes and their lambs as a potential source for spreading antibiotic resistance.

Materials and methods

Samples collection

Milk and fecal samples/rectal swabs were collected to detect the presence of *E. coli* ESBL/AmpC in sheep from different areas of Mosul city and Veterinary Teaching Hospital, College of Veterinary Medicine, University of Mosul during the period from October 2020 to January 2021. Total of 260 samples were randomly collected from ewes (aged > 3 years) and their new born lambs (aged 1-2 months). These samples represent two main groups of animals. Group A includes clinically healthy ewes (n=60) and their respective lambs (n=60), in this group milk (n=60) and fecal samples (n=60) obtained from the same healthy ewes. Moreover, rectal swabs (n=60) were obtained from their respective lambs. Group B represent ewes with clinical mastitis (n=40), in this group mastitic milk (n=40) and fecal samples (n=40) were also obtained from the same ewe. Approximately, 15 ml of raw milk was collected aseptically from each determined ewe using sterile disposable container. At the same time, 10 g of feces was collected directly from the rectum of the same ewe using disposable gloves and kept in a sterile disposable container. Additionally, rectal swabs also were collected from the respective lambs. All samples were transported by refrigerated ice box to the Microbiology Laboratory, Department of Microbiology, College of Veterinary Medicine, University of Mosul. The specimens were kept cool at 4 °C for further processing.

Bacterial isolation and identification

Specially prepared selective MacConkey agar (Neogen, USA) plus antibiotic (MaC^+) (cefotaxime 500 mg, Tabuk, KSA) at a final concentration 1 µg/ml was prepared according to Ahmed (5). This medium has a selective property to grow the bacteria that resist cefotaxime. All the collected milk samples were transferred to a new sterile centrifuge tubes and centrifuged at 3000 x*g* for 15 min, then the supernatant was discarded and the sediment was subjected to culture on MaC^+. Whereas the fecal samples were streaked directly on MaC^+ using standard inoculating loop, and fecal swabs were cultured directly on MaC^+ plates. All the cultured plates were incubated at 37 °C for 24 h. After incubation, suspected lactose fermenters ESBL/AmpC positive colonies were selected and subculture again on MaC^+ to confirm their ability to resist cefotaxime. All produced positive colonies were purified on brain heart infusion agar (Neogen, USA) and preserved in glycerol stock at -20 °C for further identification. Suspected *E. coli* positive colonies were subcultured on eosin methylene blue (EMB) agar (Oxoid, UK.) and brilliance *E. coli*/coliform selective agar (Oxoid, UK.) for presumptive identification. Additionally, further confirmation was done using Vitek 2 Compact System (BioMerieux, France) according manufacturer instructions. Standard protocols were followed according to Ahmed (5) for molecular confirmation of *E. coli* isolated targeting 16SrRNA gene, including DNA extraction and polymerase chain reaction (PCR).

Detection of ESBL and AmpC β-Lactamase-producing *E. coli*

Total of 40 positive isolates (represent different studied groups) that showed the ability to grow on MaC^+ were selected for further antibiogram study using special discs D68C MASTDISCS® Combi AmpC and ESBL ID set (Mast Group, Germany). These discs were specially formulated to detect ESBL/AmpC β-Lactamase-producing bacteria. The discs set contain 4 types of disc cartridges (A, B, C, and D). Based on combination disc method, disc A contains 10 µg of cefpodoxime as the screening agent, disc B contains 10 µg of cefpodoxime and clavulanate as the ESBL inhibitor, disc C contains 10 µg of cefpodoxime and cloxacillin as the AmpC inhibitor, and disc D contains 10 µg of cefpodoxime in combination with both clavulanate and cloxacillin. Kirby-Bauer disc diffusion method for antibiotic sensitivity test was performed following Clinical and Laboratory Standards Institute (CLSI) (15) and Razi (16). Briefly, plates with Mueller-Hinton agar were prepared and inoculated with previously prepared bacterial suspension equivalent to 0.5 McFarland opacity standard using a sterile swab. After that, discs were placed on plates and incubated at 37 °C for 24 h. Inhibition zones were measured using digital caliper (Ingco, China). The results were interpreted by comparing A, B, C and D inhibition zone diameters according to manufacturer instructions.

Statistical analysis

Chi-square test was used for statistical analysis of the obtained data using IBM\SPSS\Statistics\Version 22.

Results

Presence of positive lactose fermenters dry colonies observed on MaC^+ plates indicating the ability of these

bacteria to resist cefotaxime and give tentative identification of *E. coli*. A total of 99/260 (38.7%) *E. coli* isolates were successfully recovered on MaC$^+$ from different samples types (Table 1) and (Figure 1-a). No significant differences were found between milk from clinically healthy ewes and ewes with clinical mastitis (P<>0.001). On the other hand, significant differences were obtained between feces from clinically healthy ewes and feces of ewes with clinical mastitis (P<<0.001).

Additionally, culture on differential EMB agar indicating characteristic metallic sheen phenomenon of the isolates (Figure 1-b), while presence of purple colonies on brilliance *E. coli*/coliform selective agar give further confirmation of *E. coli* (Figure 1-c). Vitek 2 Compact System offer fast method of identification for *E. coli* with up to 99% probability. However, final confirmation of *E. coli* isolates was obtained by PCR with 232 bp product size which indicating positive *E. coli* isolates (Figure 2).

Table 1: Recovery of *E. coli* on MaC$^+$ from difference sample types

Sample type	Animal group	No. of sample	No. of Isolates on MaC$^+$	Recovery (%)
Milk (clinically healthy ewes)	A	60	7	11.7
Feces (clinically healthy ewes)	A	60	39	65
Feces (clinically healthy lambs)	A	60	37	61.7
Milk (ewes with clinical mastitis)	B	40	4	10
Feces (ewes with clinical mastitis)	B	40	12	30
Total		260	99	38.1

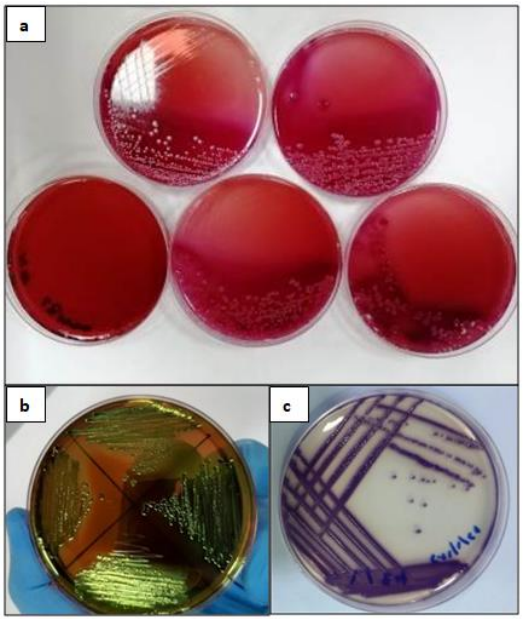

Figure 1: Culture characteristic of *E. coli* on different culture media. a: MaC$^+$; b: EMB agar; c: Brilliance *E. coli*/coliform selective agar.

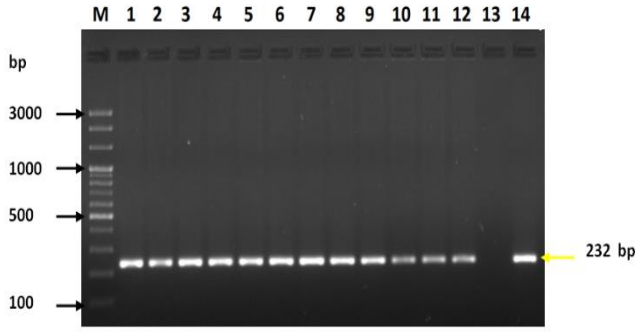

Figure 2: PCR products of *E. coli* on agarose gel electrophoresis. Lane M, 100 bp DNA ladder (Addbio, Korea); lane 1-12 positive samples; lane 13 negative control and lane 14 *E. coli* positive control.

Antibiogram results using D68C MASTDISCS® Combi AmpC and ESBL ID set showed presence of only ESBL positive isolates in different tested groups. However, we could not detect any AmpC resistance *E. coli* among the tested isolates (Table 2) and (Figure 3a and 3b).

Table 2: Recovery of ESBL *E. coli* from difference sources using D68C MASTDISCS® Combi AmpC and ESBL ID set

Sample type	Animal group	No. of tested sample	ESBL %	AmpC %
Milk (clinically healthy ewes)	A	7	17.5	0
Feces (clinically healthy ewes)	A	13	32.5	0
Feces (clinically healthy lambs)	A	12	30	0
Milk (ewes with clinical mastitis)	B	4	10	0
Feces (ewes with clinical mastitis)	B	4	10	0
Total		40	100	0

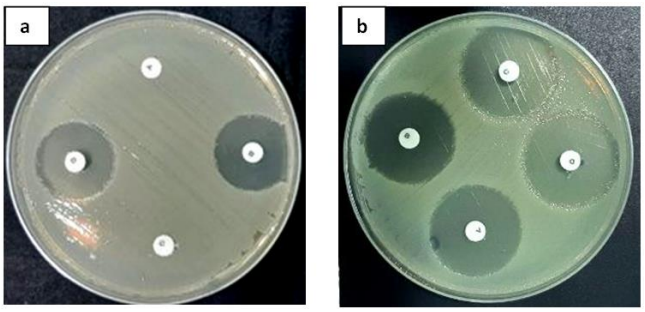

Figure 3: a: Represent only positive ESBL *E. coli* with resistance zones in A and C discs, while B and D discs show sensitive zones. b: Represent negative ESBL/AmpC *E. coli* with sensitive zones in A, B, C and D discs.

Discussion

The emergence and spread of ESBL/AmpC bacteria worldwide especially ESBL/AmpC *E. coli* increases public health concern and significantly contribute to increased morbidity and mortality rates, and consequently limit treatment options for both animal and human health (11,17,18). In Iraq and in Mosul city specifically, for best of our knowledge, there is no previously conducted study related to detect ESBL/AmpC *E. coli* in sheep. In this study, 99/260 (38.7%) of *E. coli* isolates were successfully recovered from different sample sources of sheep with ability to resist cefotaxime (as 3rd generation cephalosporin screening antibiotic anent for detection of ESBL bacteria). Our result is considering higher than reported by Pehlivanoglu *et al.* (10) who recorded presence of ESBL *E. coli* in only 3/200 (1.5%) tested sheep. Another study conducted by Loncaric *et al.* (19) on mouflons (wild sheep) who found only 1/32 (3.1%) among tested sheep. Few studies have been conducted globally related to ESBL in sheep which limit our sources. The arbitrary use of antibiotic in different ways such as growth promoter or prophylactic/mass treatment agent without any limitation gives the chance for resistant bacteria to grow and predominate susceptible normal flora population (11,13). Nevertheless, van den Brom *et al.* (3) highlighted the growing threat due to frequent treatment failure and unresponsiveness to different types of antibiotics used in veterinary practice which surly reflect treatment failure in human practice under one health concept (7,9,11,20). Fecal samples from healthy ewes 39/60 (65%) and their lambs 37/60 (61.7%) or ewes with clinical mastitis 12/40 (30%) represent the higher recovery percentages of ESBL compare to milk samples from healthy ewes 7/60 (11.7%) or ewes with clinical mastitis 4/40 (10%). These obtained results were additionally confirmed using special formulated antibiotic discs D68C MASTDISCS® Combi AmpC and ESBL ID set which indicating that all the tested isolates have the ESBL character (16). Nevertheless, we could not detect any AmpC *E. coli*. These results were in agreement with Pehlivanoglu *et al.* (17) who was unable to recover any AmpC *E. coli* from 225 tested sheep. Gastrointestinal tract represents a major reservoir of Enterobacteriaceae including *E. coli* (13,11). However, presence of high percentages of ESBL *E. coli* in lamb feces suggest potential direct oral ingestion of resistance bacteria from mother ewes milk (21,22) or by fecal contamination, indirect transmission also suggested due to contamination of the surrounding environment by feces of animals with positive ESBL bacteria (5,6,14). Presence of ESBL *E. coli* in ewes with clinical mastitis could complicate the scene due to the ability of these bacteria to transfer the resistance determinants genes to other bacterial species (3,23,24). Accordingly, the numbers of resistance bacteria become dominated and increase gradually render susceptible once eliminated, and at the same time increase the risk of unresponsive to treatment.

Conclusion

Prudent use of antibiotic in sheep farming is very important to decrease the antibiotic resistance in these animals and finally reduce spreading antibiotic resistance in food chain and human.

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Conflict of interest

The authors declare that they have no conflict of interest.

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التوصيف المظهري وصورة المقاومة للمضادات الحيوية لجراثيم الإيشيركيا القولونية المنتجة لخميرة البيتا لاكتام واسعة الطيف أو الأمبسلين والمعزولة من الضأن

فاطمة رافع محمود و إحسان منير احمد

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

الخلاصة

هدفت هذه الدراسة إلى عزل وتوصيف جراثيم الإيشريكيا القولونية المنتجة لأنزيم البيتا لاكتام أو الأمبسلين من الضأن في مدينة الموصل. تم جمع ٢٦٠ عينة حليب وبراز بطريقة معقمة من النعاج السليمة (عدد =٦٠) وحملانها (عدد= ٦٠)، كما تم جمع عينات حليب من نعاج تعاني من التهاب الضرع السريري (عدد=٤٠). تم إتباع الطرق القياسية للعزل والتوصيف الجرثومي باستخدام أوساط زرعية خاصة وذلك لعزل جراثيم الإيشريكيا القولونية المنتجة لأنزيم البيتا لاكتام أو الأمبسلين. في حين تم إستخدام أقراص خاصة لفحص الحساسية لمعرفة الجراثيم المنتجة لأنزيم البيتا لاكتام أو الأمبسلين. أظهرت النتائج إن ٢٦٠/٩٩ (٣٨,١%) من عينات الحليب والبراز موجبة لجراثيم الإيشريكيا القولونية المنتجة لأنزيم البيتا لاكتام وتوزعت كالتالي، ٦٠/٧ (١١,٧%) و ٦٠/٣٩ (٦٥%) موجبة من عينات الحليب والبراز لنعاج السليمة، على التوالي، و٦٠/٣٧ (٦١,٧%) من براز الحملان السليمة، في حين كانت ٤٠/٤ (١٠%) و٤٠/١٢ (٣٠%) موجبة من عينات الحليب والبراز للنعاج التي لديها التهاب ضرع سريري، على التوالي، على الرغم من ذلك، لم نتمكن من الحصول على أي عزلة موجبة ومقاومة للأمبسيلين من جميع العينات التي تم فحصها. إن نسبة العزل المرتفعة لجراثيم الإيشريكيا القولونية المنتجة لأنزيم البيتا لاكتام من حليب وبراز الضأن تعكس العشوائية في استخدام المضادات الحيوية في تربية الضأن. وهذا الشيء بدوره يؤدي إلى زيادة ملحوظة في المقاومة لمجاميع الجراثيم والتي قد تمثل خطرا محتملاً لنقل صفة المقاومة للمضادات الحيوية إلى الإنسان.