Phenotypic and Genotypic detection of Extended spectrum beta-lactamases (ESBLs) among patients with UTI in Misurata, Libya

Mustafa M. Drah*
Faculty of health sciences, Misurata University, Libya
Fatma M.B. Saleman
Faculty of Medical Technology, Aljufra University, Houn, Aljufra, Libya
Mohamed A. Shallouf, Eltaher A. Elshagmani
Mohamed S. Eshlak
Faculty of Medical Technology, Misurata, Libya
Esra S. Alsosi, Fatma A. Alsharif
Aisha A. Elashaikh
Faculty of sciences Misurata University
m.drah@sci.misuratau.edu.ly

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Abstract:

Antimicrobial resistance (AMR) in Enterobacteriaceae is a significant worldwide health emergency. In the last decades, many of *Escherichia coli*, and *Klebsiella pneumoniae* have developed a new resistance mechanism to extended-spectrum cephalosporins caused by Extended-spectrum beta-lactamases (ESBL), especially in the developing world. There are few reports in Libya. **Aim:** The aim of study was to determine the phenotypic and genotypic characteristics of *E.coli* and *K. pneumoniae* that produce ESBL gene isolated from urine samples in Misurata, Libya. **Methods:** Bacteria were isolated from 32 urine samples from patients with urinary tract infections (UTI) who attended Misurata Central laboratory from 01/2020 to 09/2020. Bacterial identification and biochemical tests were done. MDDST was performed to detect ESBL producers. DNA was extracted and screened for the existence of *blah-CTX-M* using PCR. **Results**: Out of 32 urine samples tested, (53.12 %) were *E.coli*, and *K. pneumonia*, (46.87%). The (40.6%) isolates were resistant to at least two cephalosporins and (12.5%) were resistant to the monobactam (aztreonam). ESBL PCR revealed the presence of ESBL gene (*bla-CTX-M*) in (53.8%) of samples, and (*bla-CTX-M*) positive sample had resistance to cefotaxime.

Conclusion: This study suggested and supported the important measure of the gene *bla-CTX* giving a very high possibility for antibiotic resistance factors.

***Keywords*:** Antibiotic resistance, *E.coli*, ESBL gene, PCR, Libya.

الكشف المظهري والجيني عن انزيم البيتا لاكتاميز واسع الطيف (ESBLs) بين المرضى الذين يعانون من التهابات المسالك البولية في مدينة مصراته، ليبيا

مصطفى محمد دراه

كلية العلوم الصحية - جامعة مصراتة

فاطمة محمد سليمان

كلية التقنية الطبيّة - جامعة الجفرة - هون

محمد عبدالسلام شلوف، الطاهر أحمد الشقماني

محمد سالم شلاك

كلية التقنية الطبيّة – مصراتة

إسراء سالم السوسي، فاطمة على الشريف

عائشة عبدالله الشيخ

كلية العلوم - جامعة مصراته

m.drah@sci.misuratau.edu.ly

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الملخص:

تعتبر مقاومة المضادات الحيوية (AMR) في البكتيريا Enterobacteriaceae بمثابة حالة طوارئ صحية في جميع أنحاء العالم. في العقود الأخيرة، طورت العديد من بكتيريا *Escherichia coli* و *Klebsiella pneumonia* آلية مقاومة جديدة للسيفالوسبورينات (cephalosporins) ذات الطيف الواسع الناتجة عن أنزيمات البيتا لاكتاميز (ESBL) وخاصة في العالم النامي. ومع ذلك، هناك القليل من التقارير الواردة من ليبيا.

الهدف من الدراسة: تهدف الدراسة إلى تحديد الخصائص المظهرية والجينية لبكتيريا *E.coli* و *K. pneumoniae* المنتجة لجين ESBL المعزول من عينات البول لمرضى التهابات المسالك البولية في مصراته، ليبيا.

المواد وطرق العمل: تم عزل البكتيريا من 32 عينة بول لمرضى مصابون بالتهابات المسالك البولية والذين ترددوا على مختبر مصراته المركزي للفترة ما بين 2020/01 إلى2020/09 وتم التعرف على البكتيريا واجراء اختبارات الكيمياء الحيوية. وتم كذلك إجراء MDDST للكشف عن أنزيم ESBL. استخراج الحمض النووي باستخدام طريقة التحلل الحراري. تم فحص العزلات البكتيرية للكشف عن وجود الجين *blah-CTX-M* باستخدام تقنية تفاعل البلمرة المتسلسل PCR.

النتائج: أظهرت النتائج أن من 32 عينة بول تم اختبارها، (53.12%) كانت *Escherichia coli* ، و (46.87%) *Klebsiella pneumonia*. وأن (40.6%) من العزلات كانت مقاومة لاثنين على الأقل من cephalosporins و (12.5%) كانت مقاومة monobactam (aztreonam) وبينت نتائج اختبار ESBL PCR عن وجود جين (*bla-CTX-M*) ESBL في (53.8%) من العينات، وكانت هذه العينات الإيجابية لـ (*bla-CTX-M*) مقاومة cefotaxime.

الاستنتاج: تقترح هذه الدراسة وتدعم أهمية الكشف عن جين *bla-CTX* الذي يعطي احتمالية عالية جدًا لعوامل مقاومة المضادات الحيوية.

الكلمات المفتاحية: مقاومة المضادات الحيوية ,*E.coli*, ESBL gene, PCR، ليبيا.

1. Introduction:

The primary cause behind 65–90% of the UTIs is the uropathogenic Escherichia coli (UPEC) (Kot, 2019; Gupta et al., 2001). Treatment of these UTIs are usually with similar the antimicrobial agents as empirical treatments. Therefore, drug resistance to the commonly used antibiotics has emerged due to the extensive use of antibiotics. Anti-microbial resistance (AMR) is a worldwide health emergency, as evidenced by the statistics figures that show resistance kill 700 000 people a year, and is expected to kill 10 million people annually by 2050, if the current situation is not improved (Ranjbar & Alam, 2024). ESBL is an enzyme that are produced by bacteria to become resistant to extended spectrum penicillins, cephalosporins, and monobactams except cephamycins and carbapenems. They can be inhibited by beta-lactamase inhibitors like clavulanic acid. There has been worrisome increasing trend on the development of resistance to extended spectrum cephalosporins caused by ESBL producing Enterobacteriaceae (Bush & Fisher, 2011; Paterson & Bonomo, 2005). Almost Gram-negative bacteria have been reported to produce ESBLs, particularly *Enterobacteriaceae* and *P. aeruginosa* (Fanaei et al., 2021). ESBLs are divided into four functional groups molecular class A (2be, 2ber, 2e), and molecular class D (2de) Bush and Jacoby (2010). bla-TEM, bla-SHV and bla-CTX-M are the most common ESBLs among members of Enterobacteriaceae. When ESBLs were first recognized in the early 1980s, they

were found to be point mutations of the TEM and SHV broad spectrum enzymes, which resulted in resistance to the β-lactam class of antibiotics. The mutation in the genes results in these enzymes having high catalytic capabilities for β-lactams due to low K m values (i.e., high affinity) for the compounds (Knothe et al., 1983; Kliebe et al., 1985). They have become a major cause of hospital acquired infection, particularly in the intensive care unit (ICU), with the majority of ESBL producers being isolated from critical care patients.

Studies on the detection and the prevalence of Extended spectrum beta-lactamases (ESBLs) among bacterial isolates obtained from Libyan patients with UTI are still poorly documented due to inadequate data available. Therefore, this study was performed to determine the phenotypic and genotypic characteristics and the prevalence of *E. coli* and *K. pneumonia* that possess ESBL genes isolated from urine samples in Misurata, Libya, using polymerase chain reaction (PCR).

2. Material and Methods:

2.1 Bacterial isolation and identification.

A total of 32 urine samples were collected from patients with urinary tract infections at Misurata Central Laboratory in period between January to September 2020. The sample were cultured on MacConkey and blood agar and incubated over night at 37^0 C. The identification of the isolates was dependent on growth characteristics, colony morphology and appearance, lactose fermentation. Gram stain and biochemical tests [Indole test, urease, TSI (triple sugar iron) and SCA (simmon citrate agar)].

2.2 Antibiotic susceptibility testing.

To determine the bacterial susceptibility to antibiotics, the antibiotic discs diffusion method was performed. Bacterial isolates cultured on nutrient agar plates using a bacterial suspension of 0.5 McFarlane standards (MF) by a sterile cotton swab. The initial screening was performed in maximum of seven antibiotic discs using a disc dispenser were dispensed on the surface of each plate. The result was interpreted as susceptible, intermediate, susceptible dose dependent or resistant according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (https://clsi.org/). Antibiotics were purchased from OFILCHEM/Italy, CONDA pronadisa/Spain, Bioanalyse/Turkey as listed in Table (1).

Table (1): Antibiotic discs were used for the initial screening for antimicrobial activity

Antibiotic disc	potency	Antibiotic disc	potency	Antibiotic disc	potency
Ceftriaxone	30µg	Tetracycline	30µg	Cefoxitin	30µg
Meropenem	10µg	Cefepime	30µg	Piperacillin	30µg
Aztreonam	30µg	Ceftazidime	30µg	Gentamicin	30µg
Ciprofloxacine	5µg	Amoxicilline-clavulanic acid	30µg	Cefotaxime	30µg
Imipenem 10 µg					

2.3 Detection of β-lactamase production.

All isolates were tested for their ability to the production of β-lactamases using rapid iodometric method according to the manufacturer's instruction (Miles et al., 1994; Hekma et al., 2020).

2.4 Phenotypic detection of ESBL.

All isolates which demonstrated reduced susceptibility to any of the third generation cephalosporins or aztreonam, were used in the initial screening (Table 2.1) to be checked for ESBL production using Modified Double Disc Synergy Test (MDDST). Briefly, a 0.5 MF of each isolate was swabbed into MHA. Plates were incubated overnight at 37^{o}C. The ESBL activity was screened according to susceptibility recommendation by (CLSI 2021), if the diameter of inhibition zone around ceftazidime is ≤ 22 mm or Cefotaxime zone ≤ 27 mm it was considered positive.

2.5 DNA extraction.

Total bacterial DNA was extracted using the heat lysis method. Briefly, one bacterial colony grown overnight on nutrient agar was picked up with a wire loop and suspended in 300µL of molecular grade water. The bacterial suspension was boiled to 100^{o}C for 10 min in a water bath. The boiled suspension was centrifuged for 5 min at 5000g and the supernatant was transferred to a sterile 1.5 Eppendorf tube. The quantity and quality of the extracted DNA was then checked using 1% agarose gel. The supernatant was used as the PCR template.

2.6 PCR detection of ESBL gene (*bla-CTX-M*).

Gene encoding *bla-CTX-M* ESBL enzymes were amplified using previously described primers as shown in Table 2 (Shallouf, 2018). Enterobacteriaces isolates were screened for the existence of *bla-CTX-M* in a PCR reaction using *bla-CTX-M* primer. Crude genomic DNA extracted by heat lysis and was used as a PCR template with AMPLIQON Taq 2X master mix (Red 1.5mM MgCl2) PCR kit. PCR reaction was performed in 12.5 µL total volume which consisted

of 0.5 µL of DNA template, 6.25 µL master mix (1X final concentration) 0.5 µL of 10 µM each primer for CTX-M and 4.75 µL of molecular grade water. Thermal cycling conditions that used for amplification of ESBL *bla CTX-M* gene is listed in Table 3. All DNA molecules were examined on 1% agarose gel in 1X TAE buffer. at 50V for 60 mins. DNA bands were visualized using gel documentation system.

Table (2): Primers and sequence used for amplification of ESBL *bla CTX-M*

Gene	Primer	Sequence (5'-3')	Size bp
bla- CTX-M	bla-CTX-M-U1	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp
	bla-CTX-M-U2	TGG GTR ARR TAR GTS ACC AGA AYC AGC GG	

Table (3): Thermal cycling conditions of (*bla-CTX-M*) gene

	Amplification condition of (*bla-CTX-M*) gene		
	PCR Steps	**Time**	**Temperature**
	Initial Denaturation	3 min	95^0C
	Number of cycles 35 cycles		
	Denaturation	15 sec	95^0C
	Annealing	30 sec	67ºC
	Extension	1 min	72ºC
	Final extension	5 min	72ºC

3. Results:

3.1 Identification of *E.coli* and *K. pneumoniae* isolates.

The total number of 32 urine samples were identified as significant microbial growth ($>10^5$ CFU/ML). The different types of microbial growing in the urine samples culture were identified Gram-negative bacilli. Isolates yielded two bacterial species and are listed in **Figure 1B**. *Escherichia coli* was the most prevalent bacterium (53.12%) which was a lactose fermenter, catalase and indole positive, and beta-haemolytic Gram stain negative bacilli; whereas the detection rate of *Klebsiella pneumoniae* was 46.87% which was a lactose fermenter, oxidase, catalase positive and indole negative, non-haemolytic (gamma-haemolytic) Gram negative bacilli.

The colonial morphology of *K. pneumoniae* was portrayed in mucoid, pink on MacConkey agar. *E. coli* colonies appeared donut and dark pink (**Fig. 1A**). The biochemical testes include Indonle test (MacWilliams, 2012), Triple Sugar Iron (TSI) (Iwade et al., 2006), Urease (Barry et al., 1969) and Simmons Citrate agar (SCA) were used to differentiate between microbial growth. The Characteristics and biochemical reactions Figures and summary of the isolates are listed in **supplementary Fig (1)**.

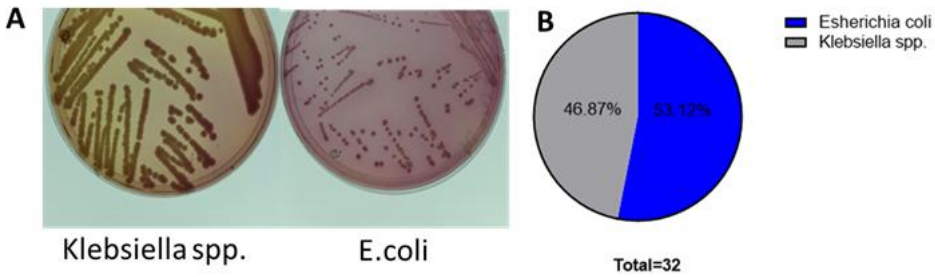

Fig (1): Prevalence and identification of *E.coli* and *K. pneumoniae* isolates
(A) Picture shows the culture morphology of gram-negative bacilli isolates of two bacterial species (*Escherichia coli* and *K. pneumoniae*). **(B)** Pie chart shows the percentage of two bacterial species (*Escherichia coli* and *K. pneumoniae*).

3.2 Antibiotic susceptibility.

The *E. coli* and *K. pneumoniae* isolates were tested for their antibiotic sensitivity profile by disc diffusion method. The antibiotic results that was obtained by disc diffusion showed 40.6% of the isolates were resistant to at least two cephalosporins (cefotaxime, ceftazidime, ceftriaxone and cefepime) (**Fig. 2A**) and 12.5% of them were resistant to the monobactam (aztreonam) (**Fig. 2B**). The highest resistance pattern of both *E. coli* and *K. pneumoniae* to tested antibiotics was noticed for Cefotaxime (CTX) (34.37%), followed by Ceftriaxone (CRO) (25%), Ceftazidime (CAZ) (18.75%), Cefepime (FEP) (12.50%) and Aztreaonam (ATM) (9.37%) shown in **Figure 2C.**

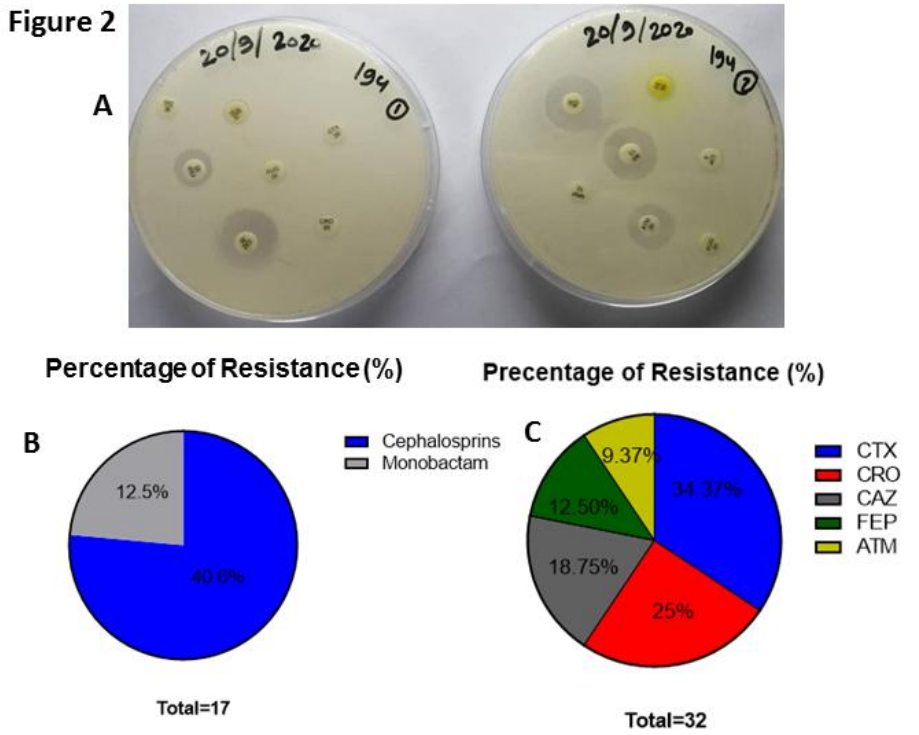

Fig (2): Inhibition zone of isolates considered as potential ESBL producer
(A) Disc diffusion susceptibility testing showing an isolates that is resistant to almost all cephalosporins. **(B)** and **(C)** Pie charts showed the numbers and percentage of isolates resistance to cephalosporins (cefotaxime, ceftazidime, ceftriaxone and cefepime) and to the monobactam (aztreonam).

3.3 Phenotypic detection.

Thirty-two Enterobacteriacea isolates (*E. coli* and *K. pneumoniae*) were subjected to ESBL screening by disc diffusion. Table 4 summaries the obtained results. Isolates which displayed susceptibility to at least one carbapenems tested in this study namely imipenem and meropenem, and showed intermediate susceptibility or resistance to at least one of the third or fourth generation cephalosporins (cefotaxime, ceftriaxone, cefepime and ceftazidime) were considered as ESBL producers. In total, thirteen isolates were presumptively identified as ESBL producers.

Table (4): Antibiotics susceptibility pattern

NO	Bacteria	Cephalosporins				Monobactams	Carbapenems	
		FEP	CRO	CAZ	CTX	ATM	MRP	IMI
76	E.coli	26mm	14mm	20mm	16mm	24mm	26mm	26mm
192	E.coli	34mm	30mm	26mm	30mm	36mm	26mm	46mm
9A	E.coli	34mm	32mm	30mm	32mm	32mm	18mm	30mm
71	E.coli	34mm	32mm	30mm	24mm	36mm	28mm	32mm
79	E.coli	38mm	36mm	34mm	36mm	38mm	32mm	32mm
78	E.coli	24mm	16mm	38mm	18mm	38mm	18mm	30mm
175	E.coli	10mm	12mm	10mm	12mm	20mm	30mm	30mm
161	E.coli	34mm	16mm	30mm	18mm	40mm	30mm	34mm
185	E.coli	40mm	38mm	36mm	40mm	40mm	30mm	40mm
48	E.coli	38mm	36mm	34mm	38mm	36mm	32mm	34mm
184	E.coli	36mm	34mm	30mm	34mm	36mm	28mm	38mm
194	E.coli	12mm	6mm	16mm	6mm	12mm	6mm	14mm
193	E.coli	34mm	28mm	22mm	26mm	26mm	22mm	20mm
26A	E.coli	36mm	20mm	30mm	34mm	36mm	30mm	44mm
26	E.coli	40mm	30mm	30mm	34mm	38mm	30mm	30mm
61	E.coli	38mm	40mm	32mm	40mm	34mm	30mm	40mm
24A	E.coli	40mm	34mm	14mm	30mm	32mm	30mm	34mm
190	Kleb	24mm	24mm	14mm	20mm	24mm	26mm	26mm
74	Kleb	23mm	12mm	21mm	14mm	20mm	28mm	30mm
169	Kleb	32mm	32mm	31mm	32mm	35mm	26mm	32mm
25A	Kleb	34mm	32mm	30mm	22mm	36mm	26mm	32mm
188	Kleb	34mm	32mm	28mm	30mm	24mm	30mm	26mm
180	Kleb	28mm	32mm	30mm	32mm	38mm	30mm	32mm
183	Kleb	30mm	32mm	28mm	34mm	34mm	27mm	26mm
6A	Kleb	32mm	28mm	28mm	30mm	30mm	30mm	34mm
70	Kleb	14mm	6mm	10mm	6mm	10mm	28mm	30mm
186	Kleb	12mm	6mm	9mm	6mm	12mm	28mm	12mm
209	Kleb	6mm	6mm	6mm	6mm	6mm	9mm	19mm
170	kleb	36mm	24mm	30mm	36mm	33mm	32mm	34mm
195	Kleb	36mm	30mm	30mm	32mm	32mm	30mm	40mm
182	Kleb	26mm	24mm	20mm	6mm	20mm	26mm	30mm
197	Kleb	32mm	32mm	28mm	34mm	32mm	30mm	38mm

3.4 Genotypic detection of *CTX-M* gene by PCR.

The genotyping results of ESBL revealed from using specific primers sequences for PCR amplification *CTX-M* gene are shown in **Figure 3A**. The ESBL PCR assay revealed that among the 13 clinical isolates identified as potential ESBL producer, only seven samples were detected by PCR for the

ESBL *bla-CTX-M* gene. Out of 6 *E.coli* isolates shown susceptible to third generation of Cephalosporins, 5 isolates were also ESBL positive as they were carrying *bla-CTX-M* gene. Only 2 out of seven *K. pneumoniae* isolates were carried out the ESBL gene *bla-CTX-M* (**Table 5**).

Fig (3): (A) PCR products of *bla CTX-M* gene run on 1% agarose gel from 13 isolates showing 7 samples that carry ESBL *CTX-M* gene.

Table (5): Distribution of susceptible to to third generation Cephalosporins and Monobactes of ESBL-positive isolates

NO	Bacteria	Cephalosporins				Monobactams	PCR/CTX-M
		FEP	CRO	CTX	CAZ	ATM	
76	E.coli	S	R	R	I	S	Positive
78	E.coli	SDD	R	R	S	S	Positive
175	E.coli	R	R	R	R	I	Positive
161	E.coli	S	R	R	S	S	Positive
194	E.coli	R	R	R	R	R	Positive
24A	E.coli	S	S	S	R	S	Negative
190	Kleb	SDD	S	R	R	S	Negative
74	Kleb	SDD	R	R	S	I	Negative
25A	Kleb	S	S	R	S	S	Negative
70	Kleb	R	R	R	R	R	Negative
186	Kleb	R	R	R	R	R	Positive
209	Kleb	R	R	R	R	R	Negative
182	Kleb	S	S	R	I	I	Positive

R(Resistant), **S**(Susceptible), **I** (Intermediate) and **SDD** (Susceptible-dose dependent).

4. Discussion:

UTIs are the second most common infections caused by bacteria. In most cases, the cause of community and hospital UTI was found to be associated with *E. coli* and *K. pneumoniae*. Increasingly, the resistance to antimicrobials among uropathogens, has limited the treatment choices and made it much more complicated. The worldwide spread of ESBL producing *E. coli* and *K. pneumoniae* has been strikingly rapid, and is an important case of invasive infection (Lopez-Cerero & Pascual, 2007; García-Hernández et al., 2011). One of the most other known factors which are also associated with ESBL are age, previous antibiotic treatment, long hospitalization, and diabetes ESBL (Silva et al., 2006). Identification of betalactamase is essential for a reliable epidemiological investigation of antimicrobial resistance. In this study, antimicrobial drug resistance was investigated, the ESBL phenotype, and detection of the gene (*bla-CTX-M*) in *E. coli* and *K. pneumoniae* strains isolated from urinary tract infections. The results showed that out of 32 isolates 17 (53.12%) were *E. coli* and 15 (46.87%) *K. pneumoniae*. This percentage agreed with the results of a Libyan study was done by Abujnah et al. (2015) showed that *E. coli* and *Klebsiella* spp. were the most predominant (55.8% and 18.5% respectively) the samples collected from patients with UTIs attending Zawiya Teaching Hospital. In addition, results obtained in the study agreed with a study was done in Iraq by Sahel and Abid (2022) showed that the UTIs were the most frequent source of the isolated gram-negative bacillus (*E. coli* and *Klebsiella pneumoniae*).

The results obtained in this study revealed that resistance pattern of both *E. coli* and *K. pneumoniae* was against Cefotaxime (CTX) (37.5%), Ceftriaxone (CRO) (28.1%), Ceftazidime (CAZ) (21.9%), Cefepime (FEP) (15,6%) and Aztreaonam (ATM) (12.5%). Moreover, 40.6% of isolates that showed antibiotics résistance to third and fourth generation cephalosporins were considered as ESBL producers. The results of ESBL–producing in this study showed that the percentage of *E. coli* isolates was higher (83.3%) compared with *K. pneumoniae* (28.5 %) by phenotypic detection and ESBL gene *bla-CTX-M*. These results were similar to the results of the Sahel and Abid (2022) who found *E. coli* isolates were significantly more frequently for ESBL-producing by both phenotypic and confirmatory tests. These results were also in agreement with a Palestinian study was conducted in Gaza that demonstrated that among the 85 entarobacteriaceae isolates collected, extended-spectrum β-lactamases were confirmed in 30 (35.3%) of the isolates. The susceptibility to imipenem (carbapenem) was 80.0% against ESBL-producing isolates. However, the resistance rate to cefotaxime was 100%, which was high among ESBL-producing isolates (Tayh et al., 2019).

Overall, this study suggested and supported that the presence of the gene *bla-CTX* gives a very high possibility for antibiotic resistance factors because all of the *bla-CTX* positive tested samples gave 71.4% of resistance to the used antibiotics while the *bla- CTX* negative tested samples gave 56.6% resistance to antibiotic, and this is a very noticeable difference that supports the resistance that is acquired with the presence of the gene with having in mind the other factors that might give the negative samples the resistance.

In addition, a study by Edelstein and colleagues reported that CTX-M beta lactamases had a more destructive effect on Cefotaxime and Cefteriaxon than Ceftazidim and this is in agreement with this study because it also shows that the bacteria have the highest resistance to these three antibiotics (Edelstein et al., 2003).

In conclusion, the *E.coli* and K. *pneumoniea* are the majority of gram-negative isolates that infected community and hospitalise patients and the most pathogens causing UTIs. Antibiotic resistance is growing concern for treatment of patients UTIs. The inadequate antibiotic prescription due to incorrect lab test results in detection of antibiotic resistance may lead to bacteria to generate new resistance genes. The challenges now are in choosing the exact and precise method to identify antibiotic resistance. It seems that the ESBL *bla-CTX-M* gene is the appropriate choices for molecular screening of ESBL positive samples.

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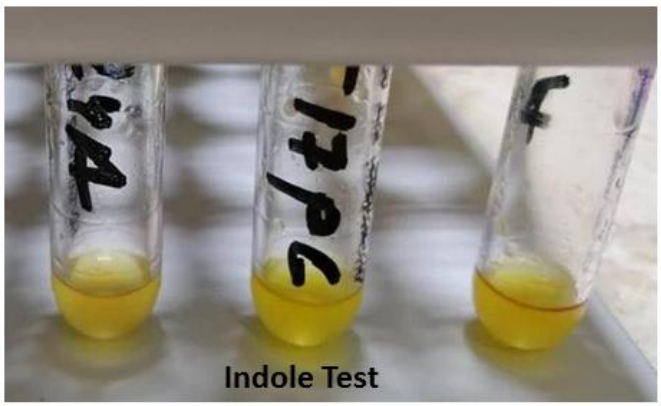
supplementary Figures:

A

B



C

Table (1): Characteristics and biochemical reactions summary of isolated

	TSI	Indole	Citrate	Urease
E.Coli	-ve	+ve	-ve	-ve
K.pneumonia	-ve	-ve	+ve	+ve

supplementary Figure (1). Characteristics and Biochemical identification of *E.coli* and *k. pneumonia* isolates

(A) Picture Shows TSI, SCA and urease tests before and after incubation **(B)** Picture shows the Indole positive with red ring and negative result remains yellow. **(C)** Table (1): summary culture characteristics and biochemical reactions of isolates.