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Detection of Carbapenemase-Producing *Klebsiella pneumoniae* and *Escherichia coli* Recovered from Clinical Specimens in Erbil City Kurdistan Region of Iraq

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Article Info	Abstract
Submitted 04/02/2019	<p>Background: Carbapenems are usually the choice of antimicrobial agents in infections produced by Enterobacteriaceae bacteria-producing ESBL (extended spectrum β-lactamases). Carbapenemase production among clinical isolates of Enterobacteriaceae has been widely reported and Resistance to carbapenems group is generally due to production of Carbapenemases. Phenotypic determination and distinction of Carbapenemases in drug-resistant gram-negative is crucial for appropriate infection control.</p> <p>Materials and Methods: Carbapenemase production among Enterobacteriaceae isolates was identified phenotypically using a commercially available EDTA-combined disc diffusion test containing inhibitors to the various carbapenemase classes and Modified Hodge test (MHT).</p> <p>Results: A total of 98 Enterobacteriaceae isolates were included, 42(42.8%) were Multi-drug resistant (MDR), 27(27.5%) were XDR while 8(8.2%) exhibited pan-drug resistance (PDR). Of the 74 isolates of <i>Escherichia coli</i> and 24 <i>Klebsiella pneumoniae</i> that were positive for carbapenemase production, 12 (16.2%) and 9 (37.5%) were Metallo beta-lactamase (MBL) producers respectively, Hence, the overall prevalence of carbapenemase-producing <i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i> in this study were 47.3% and 87.5%.</p> <p>Conclusion: Carbapenemase-producing Enterobacteriaceae was indeed recognized in our hospitals. The EDTA-combination disk test was a rapid, cost-effective and suitable method which will be able to identify and distinguish the carbapenem-resistant bacterial isolates within the hospitals especially when molecular detection techniques are not available.</p> <p>Keywords: Carbapenemase, <i>Escherichia coli</i>, EDTA-combination disk test, <i>Klebsiella pneumoniae</i>, Modified Hodge test, Multi-drug resistant.</p>
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Introduction

Klebsiella pneumoniae and *Escherichia coli* are gram-negative bacteria from *Enterobacteriaceae* family that have been increasingly reported to be multidrug-resistant (MDR) and causing life-threatening infections either community-acquired or nosocomial infections [1, 2]. These bacteria have the capability to easily acquire or to transfer genes that are responsible for drug resistance through plasmid or transposons in which when it's activated can result in the production of extended-spectrum beta-lactamase (ESBLs).[3] Harboring of

ESBL by bacteria enables significant resistance to penicillin, narrow and extended-spectrum cephalosporin, and monobactams. They also are sometimes responsible for resistance against aminoglycosides, trimethoprim/sulfamethoxazole, and quinolones [4]. This will leave the carbapenem antibiotic as the last choice for antibiotic therapy, however the worldwide prevalence of carbapenem-resistant *Enterobacteriaceae* has been reported due to high usage of antibiotics and misuse of these antibiotics by the patients, and poor practice of antibiotic policy in hospitals.[5, 6]

Resistance to carbapenems is mediated by loss of the outer membrane proteins, drug efflux or the production of excessive AmpC beta-lactamase with outer membrane porin mutation.[7] Resistance to carbapenems among *Enterobacteriaceae* could be mediated by various families of carbapenemases including NDM, IMP, VIM, KPC, and OXA-48, which are encoded by bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC} and bla_{OXA-48} respectively.[3]

Different mechanisms of detection assays are used to detect the activity of carbapenemase-producing isolates. Detection of carbapenem resistance is crucial for choosing proper antibiotic therapy, infection control measures, and continuous surveillance to prevent the spread of resistant strains in hospital settings. Also, early detection of carbapenemase-producing bacteria in hospital systems can put a stop to further spread of the transmissible genes.[8] Phenotypic detection tests are used to detect the activity of carbapenemases in bacteria isolates while molecular assays are used to identify the genes encoding carbapenemases. Because of the growing risk of infections that are caused by carbapenem-resistant *Enterobacteriaceae* and poor local information, studying the frequency of carbapenem producing bacteria is important to prevent and control the further increase of these resistant strains.[9] Molecular testing methods are considered to be standard for the detection of genes encoding carbapenem resistance, however high cost and unavailability these tests are the major struggles for these methods not to be adapted and practiced in all laboratories, therefore rapid and economic phenotypic tests are being increasingly used.[3, 10]

This study focuses on investigating the Antibiotic susceptibility pattern and determines the frequency of carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from hospitals in Erbil city, using phenotypic assays, we also described the incidence of Multidrug resistance (MDR), extensive drug resistance (XDR) and pan-drug resistance (PDR) among *Escherichia coli* and *Klebsiella pneumoniae* isolates. In this study, we performed two phenotypic tests for predic-

tion of Carbapenemase production: Modified Hodge test and combined disc diffusion. Modified Hodge test can detect almost all carbapenemase-producing isolates, and Combined disc diffusion has been reported to be a simple method to detect metallo beta-lactamase producers, as their activity is inhibited by chelating agents like EDTA.[11, 12]

Material and methods

Sample collection and bacterial isolation

Urine, blood, sputum, and different body site swabs were collected from clinically ill patients during a 9 months period from October 2017 until the start of August 2018, at Rizgary teaching hospital and PAR private hospital in Erbil city. Samples were inoculated on blood agar and MacConkey agar. *Escherichia coli* and *Klebsiella pneumoniae* isolates were identified by using standard Microbiological methods like; differential and selective cultures, Gram stain films and biochemical tests such as; catalase test, oxidase test, sodium citrate test and the Sulfur, Indole, Motility test. Later on, isolates identification were further confirmed by the Vitek system. This study was conducted according to the ethical committing at Hawler Medical University.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out by Kirby-Bauer disc diffusion method on Mueller-Hinton (Oxoid) agar plates. Samples were incubated for 18-24hr at 35°C. Considering guidelines from the Clinical and Laboratory Standards Institute (CLSI), the following discs were used: Imipenem, meropenem, ampicillin, tazobactam, cefazolin, ceftazidime, cefepime, ceftriaxone, ciprofloxacin, levofloxacin, gentamicin, tobramycin, and trimethoprim/sulfamethoxazole. *Escherichia coli* and *Klebsiella pneumoniae* isolates were identified as (sensitive, intermediate, resistant) according to breakpoints defined by the CLSI.[13] Multi-drug resistance (MDR) was defined as sensitivity to at least one antimicrobial agent in three or more antimicrobial categories characterized by the organizations, while XDR is referred as the bacterial isolate being sensitive to at least one antimicrobial agent in

all but two or less antimicrobial categories listed. While PDR, it is described as sensitivity to all antimicrobial agents in all antimicrobial categories for each bacterium listed.[14]

Carbapenemase detection by phenotypic assays

Detection of carbapenemase production was carried out by the following tests:

a) Modified Hodge test (MHT): A dilution of *E.coli* ATCC 25922 prepared in 5ml of normal saline with 0.5 McFarland dilution, the suspension was diluted by adding 0.5ml to 4.5ml of normal saline. The suspension of *E.coli* ATCC 25922 was streaked over Muller Hinton agar plate. A disc of Meropenem was used, placed in the center of the plate. Three to four McFarland suspended tested isolates grown overnight on blood agar plate were inoculated onto Muller Hinton agar plate in a straight line from the edge of the plate to the disc at the center of the plate (minding contamination of the discs by the swab). Interpretation of negative and positive tests was done according to CLSI.[15] Enhanced growth of the indicator strain towards the meropenem disk indicated a positive result, clover leaf-type indentation at the point of intersection of the isolate with the indicator strain. Whereas, the indicator strain showing no enhanced growth of towards a meropenem disk (no clover leaf-type indentation) at the point of intersection of the isolate with the indicator strain is noted to be negative. Indeterminate results were indicated by inhibition of the growth of the indicator strain produced by the test isolate.

b) EDTA-combined disk diffusion test: For the EDTA-disk synergy test, the test strain was cultured on MacConkey agar and incubated overnight was suspended to the turbidity of a 0.5 McFarland tube used to swab inoculate a Mueller–Hinton agar plate. After drying for few minutes, a 10- μ g imipenem disk and a blank filter paper disk were placed 10mm apart from edge to edge, 10 μ L of 0.5M EDTA solution was then applied to the blank disk, which resulted in approximately 1.5mg/disk. After overnight incubation 16 to 18 h at 35°C, inhibi-

tion zones that were presented between the imipenem and imipenem-EDTA disks were compared. the presence of an enlarged zone diameter of 4mm around the IPM-EDTA disk compared to that of the IPM disk alone was interpreted as EDTA-synergy test positive, indicating positive for metallo-beta-lactamase (MBL) production.[11]

Results

Isolates characteristics

After eliminating unwanted isolates from 210 collected samples, 74 non-repetitive clinical isolates identified as *Escherichia coli* and 24 isolates of *Klebsiella pneumoniae*, on the basis of colonial morphology on culture media, some biochemical tests and confirmed by VITEK 2 (bioMérieux). Urine samples (65.3%) were the predominant source of our isolates, the second largest source of the isolates were pus swab samples (15.3%), followed by sputum (10.2%), blood (6.1%), and other samples; like ear discharge and body fluids (3.1%), as expressed in Figure 1.

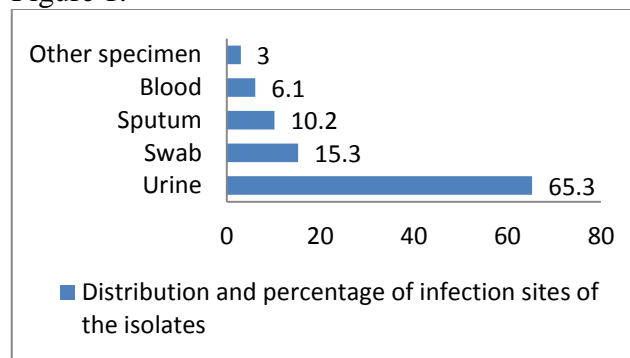


Figure 1: Distributions and percentage of infection sites of the isolates.

Antibiotic susceptibility test (AST)

Clinical and laboratory standards institute (CLSI) breakpoints were used for interpretation of AST profile by standard antibiotic sensitivity test and Vitek II analysis, we found all isolates were resistant at least to two or more tested antibiotic agent. 9 out of 24 (37.5%) isolates of *Klebsiella pneumoniae* were non-susceptible to Meropenem agent and *Escherichia coli* isolates tested 12 out of 74 (16.2%) isolates resistant to Meropenem. (48.5%) and (16.2%) of *Klebsiella pneumoniae* and *Escherichia coli*

showed resistance to Imipenem respectively. The percentage of resistance against antibiotic agents for both *Klebsiella pneumoniae* and *Escherichia coli* are expressed in Figure 2 and Figure 3.

Eight isolates showed resistance to all tested antibiotic meaning they were pan-resistant and thus clinically can be very problematic cases to find a suitable antibiotic choices.

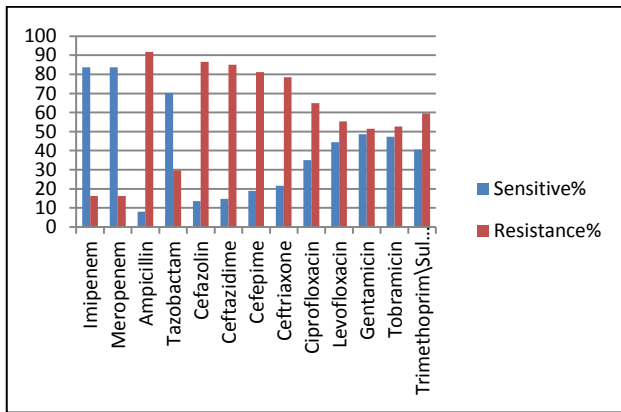


Figure 2: Percentage of antibiotic resistance among *Escherichia coli* isolates

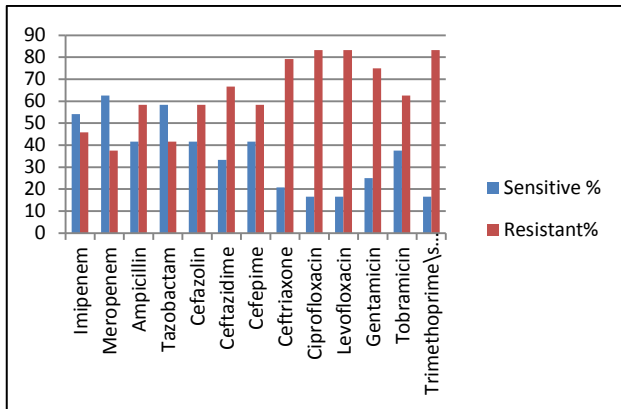


Figure 3: Percentage of antibiotic resistance among *Klebsiella pneumoniae* isolates

Table 1 shows the result of the susceptibility of the bacterial isolates to the carbapenems including imipenem and meropenem. Out of the 74 *E. coli* isolates recovered in this study, a total of 12 isolates (16.2%) were resistant to imipenem and 12 (16.2%) resistant to meropenem, and 11 out of 24 (45.8%) isolates of *Klebsiella pneumoniae* isolates were resistant to imipenem and 9 of 24 (37.5%) were resistant to meropenem.

Table 1: Frequency & percentage of carbapenem-resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates.

Bacteria	Imipenem	Meropenem
<i>Escherichia coli</i> n=74	12 (16.2%)	12 (16.2%)
<i>Klebsiella pneumoniae</i> n=24	11 (45.8%)	9 (37.5%)

The prevalence of MBL-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates is shown in Table 2. Carbapenemase production was phenotypically detected by the modified Hodges test technique and EDTA combined disc test in a total of 74 *Escherichia coli* isolates and 24 *Klebsiella pneumoniae* isolates.

Table 2: Distribution of MBL-producing isolates

Organism	Source	MBL-positives	Percentage
<i>Escherichia coli</i>	Urine	25	33.7
<i>Escherichia coli</i>	swabs	5	8.1
<i>Escherichia coli</i>	Sputum	3	4.1
<i>Escherichia coli</i>	Blood	1	1.3
Total		35	47.3
<i>Klebsiella pneumoniae</i>	Urine	14	58.3
<i>Klebsiella pneumoniae</i>	Sputum	5	20.8
<i>Klebsiella pneumoniae</i>	Blood	2	8.3
Total		21	87.5

Incidence of MDR, XDR, and PDR among Escherichia coli and Klebsiella pneumoniae isolates

Error! Reference source not found. shows the incidence of multidrug resistance, extensive drug resistance and pan-drug resistance among *Enterobacteriaceae* isolates including *Escherichia coli* and *Klebsiella pneumoniae*. The results generally showed 42 of all 98 isolates of *Enterobacteriaceae* were MDR bacteria and 21 of all 98 *Enterobacteriaceae* isolates were non-MDR while 27 of bacterial isolates were XDR. And 8 of all 98 bacterial isolates of this study were PDR.

Prevalence of carbapenemase activity based on phenotypic tests

Modified Hodge test (MHT) and EDTA-combined disc diffusion were performed as a screening test for all *Escherichia coli* and

Klebsiella pneumoniae including both Mero-penem-resistant and sensitive strains. MHT results in *Escherichia coli* isolates tested 12/74 (16.2%) showed positive results and for *Klebsiella pneumoniae* tested 9/24 (37.5%) were positive. EDTA-combined disc diffusion for *Escherichia coli* while for *Klebsiella pneumoniae* tested 23/74 (31.1%) and 11/24 (45.8%) respectively. *Klebsiella pneumoniae* and *Escherichia coli* isolates 2 of each group showed positive for both MHT and CDT presenting (8.3%) and (2.7%) of the isolates respectively. Details of carbapenemase activity among the isolates are shown in Table 4. The negative results did not change even after the isolates were incubated overnight.

Results presented in Figure 4 provide evidence for the effects of IMP-resistance on MBL-production, the study assessed that 19 (82.6%) of the IMP-resistant isolates were MBL-producers while only 4 of the IMP-resistant isolates were non-MBL-producers.

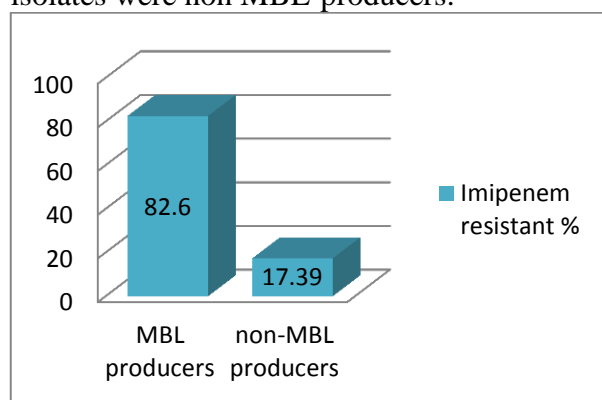


Figure 4: The percentage of MBL and non-MBL producing *Klebsiella pneumoniae* & *Escherichia coli* with IMP-resistance

Table 3: Incidence of MDR, XDR and PDR among *Klebsiella pneumoniae* and *Escherichia coli* isolates

Bacteria	Non-MDR	MDR	XDR	PDR	Total
<i>K.pneumoniae</i>	5 20.8%	6 25.0%	8 33.3%	5 20.8%	24 24.5%
<i>E.coli</i>	16 21.6%	36 48.6%	19 25.6%	3 25.7%	74 75.5%
Total	21 21.4%	42 42.8%	27 27.5%	8 8.2%	98 100%

Table 4: Phenotypic test results for clinical isolates

Positive tests	<i>Escherichia coli</i> n.=74 & %	<i>Klebsiella pneumoniae</i> n.=24 & %
MHT	(12) 16.2%	(9) 37.5%
CDT	(23) 31.1%	(11) 45.8%
Both MHT & CDT	(2) 2.7%	(2) 8.3%

Discussion

Enterobacteriaceae are among the most important causes of nosocomial and community-acquired infections. Unfortunately, extensive and un-prescribed antibiotic agents are resulting in the worldwide crisis of resistance phenomena, and the treatment of such cases is being serious challenges for health care professionals.[16] The prevalence of resistant isolates and antibiotic sensitivity patterns are representing different results due to geographical, population, and environment variations.

For rapid detection and controlling further dissemination and hospital breakouts, phenotypic detection is of great clinical importance. Metallo beta lactamase-carrying organisms can have various phenotypic appearances, depending on the bacterial host. In this study two phenotypic testing methods were carried out to identify MBL-producing organisms; CDT (combined disc test) which is based on the ability of chelating metals by EDTA, and modified Hodge test (MHT). To our knowledge, this is the first report of the presence of MBL among *Klebsiella pneumoniae* and *Escherichia coli* isolates in Erbil city. Number of studies has been carried out in other cities in Iraq among other gram-negative bacteria, including detection of MBL among *Pseudomonas aeruginosa*, in Sulaimaniyah, Erbil and Baghdad city. [17-19]

In the current study, the frequency of *Escherichia coli* isolates were (75.5%) while *Klebsiella pneumoniae* was (24.5%) of the isolates, most of them were isolated from urine samples (66.0%), this result is in conformity with another study conducted by Romero and coworkers[20].

The highest level of antibiotic resistance among *Escherichia coli* isolates was presented against ampicillin (91.8%) which is shown in (Figure 1). This result is in agreement with a previous study that has been done by Akpaka and Swanston, whose clinical result for Ampicillin-resistant *Escherichia coli* isolates was 93.3% [21]. While the highest level of antibiotic resistance levels among *Klebsiella pneumoniae* isolates were among cephalosporins ranging between (58.3%-83.3%), however another study done in Saudi Arabia showed the highest resistance level among *Klebsiella pneumoniae* against ampicillin (100%) [22]. The high resistance level against cephalosporins such as cefazolin, ceftazidime, cefepime probably is a result of the widespread distribution of ESBL producing Enterobacteriaceae isolates in Erbil city hospitals as reported by previous study conducted by Haji and coworkers, in which in their study they stated that 77 (76.2%) and 15 (78.9%) of *Escherichia coli* and *Klebsiella pneumoniae* isolates were ESBL-producers.[23]

All the isolates of the current study were most susceptible to carbapenem agents such as meropenem and imipenem, this indicates the rapid emergence and increase in resistance against carbapenems. Antibiotic sensitivity profile showed (13.5%) imipenem and (14.86%) meropenem resistant *Escherichia coli* isolates, while *Klebsiella pneumoniae* isolates were (33.3%) resistant against both imipenem and meropenem. A study conducted in Urmia university teaching hospital among clinical isolates of *Klebsiella pneumoniae* showed (23.6%) of the total isolates to be resistant against imipenem.[24] Also a study by Ghanbari and coworkers showed (9.5%) and (11%) of *Escherichia coli* and *Klebsiella pneumoniae* isolates resistant to imipenem, respectively.[25] Antibiotics are extensively used and this situation remains uncontrolled at both the community and hospital level. The ease in access to antibiotics without physician's prescription, prescribing last line resort antibiotics without demanding antibiotic susceptibility testing, not finishing antibiotic courses and many other factors are contributing to the continuous rapid emergence and increase in the antibiotic re-

sistance levels, resulting in different patterns of antibiotic resistance among isolates as we mentioned in (Table 1). Approximately (42.9%) of all 98 isolates were found to be MDR bacteria while (27.5%) were XDR and (8.2%) were PDR. Folgiori *et al.* reported similar results of the prevalence of multi drug resistance among Gram-negative bloodstream infections in a tertiary hospital and reported that the most frequently isolated bacteria were *Escherichia Coli* (67.6%), in which 39% of these isolates were MDR.[26] another study from India reported (31.6%) MDR *Escherichia coli* isolates and (30%) MDR *Klebsiella pneumoniae*, while XDR was higher among *Klebsiella pneumoniae* isolates (27.8%)[27] while our study recorded highest percentage among MDR *Escherichia coli* isolates (48.6%) and (33.3%) XDR *Klebsiella* isolates. The presence of PDR bacteria among our isolates indicates advance level of resistance in Erbil city.

All of the isolates were further screened for carbapenemase production, as with a high capacity for horizontal genetic dissemination the resistant strains could result in epidemics. Indeed, all Enterobacterial isolates should be screened for carbapenemase production even with low-level resistance to carbapenems, especially as we don't know the level of carbapenemase produced at sites of infection.

No single carbapenem-screening criterion can be used to identify all isolates. Therefore, it's essential for confirmatory testing to be carried out for the detection of carbapenemase-producing *Enterobacteriaceae*, as usually mobile genetic elements carry these genes[28]. In recent years studies have been reporting the emergence of carbapenem susceptible MBL-carrying organisms, Yan and colleagues reported on the laboratory detection of carbapenem-susceptible MBL-carrying organisms and compared three methods. This is a very challenging issue because selecting all isolates for phenotypic screening creates unnecessary work but to avoid false negative results in this study we selected all isolates for phenotypic screening of MBL production.[29]

The phenotypic testing results showed (16.2%) of *Escherichia coli* isolates tested positive for MBL and (37.5%) of *Klebsiella pneumoniae*

showed positive results by modified Hodge test, while EDTA-combined disc testing results showed positive in (31.1%), (45.8%) of *Escherichia coli* and *Klebsiella pneumoniae*, respectively. The difference between various regions, strains, materials used in the tests, and the difference of sample size of the two strains can contribute in this dissimilarity between the two phenotypic testing methods.

In a total of 98 isolated strains, 23 (23.4%) were resistant to imipenem, and 19 (82.6%) were MBL producers and 4 (17.4%) were non MBL-producers as shown in (Figure 4). Our results are similar to another study reported in Pakistan in which (83.3%) of *Escherichia coli* and (75%) of *Klebsiella pneumoniae* were positive for MBL production.[30]

In this study we detected a higher percentage of the potential carbapenemase activity among the isolates in accordance with the results of other studies that have been carried out in different cities of Iraq and the Kurdistan region. Phenotypic results from the current study showed comparatively higher results than other studies from Iraq, one of them from Sulaymaniyah city, in which (1.69%) and (0.56%) was reported in *Klebsiella pneumoniae* and *Escherichia coli* respectively by EDTA-combined disc test, they also reported (10.16%) and (0.56%) positive MHT in *Klebsiella pneumoniae* and *Escherichia coli*, [17] and a study from Iran reported 16% of *Klebsiella* isolates positive for MHT.[9]

There is no clear report data from Kurdistan region showing the prevalence of MBL producing Enterobacteriaceae, especially in recent years many neighboring countries reporting high statics of the emergence of new MBL prevalence.

Conclusion

This study shows antibiotic resistance pattern among *Klebsiella pneumoniae* and *Escherichia coli* which are rapidly growing and are being distributed among Erbil city population due to antibiotic abuse and more prescribed antibiotics without carrying out laboratory tests for AST. Also, we conclude that phenotypic testing is a necessity to prevent further dissemina-

tion and increase of resistance among *Enterobacteriaceae*. The outcome of this study recommends the declaration of annual reports about antibiotic resistance among *Enterobacteriaceae* in the Kurdistan region. Further surveillance, a screening test for detection of carbapenem-resistant are needed.

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