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Molecular Detection of Plasmid-Mediated AmpC in Gram Negative Bacteria Isolated from Intensive Care Unit Patients in Wasit Province, Iraq

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ABSTRACT

Background: The intensive care unit (ICU) plays a crucial role in managing and treating some of the most complex and serious disorders that affect the human body.

Patients and Methods: A total of 100 clinical specimens (urine, sputum and pus) were collected from patients admitted to the ICU. in this study admitted to the intensive care units (ICUs) of Alzahraa and Alkarama hospitals for more than 48 hours between 3rd October 2021 to 20th February 2022. Each patient's name, age, gender, underlying clinical condition, ICU admission date, indoor admission date, previous antibiotic intake history, current ICU therapy, and clinical outcome were recorded.

Results: Thirty (36.5%) female and 52 (63.5%) male patients were admitted to the ICU. They were distributed 69 (84.1%) gram-negative bacteria. Gram positive 13 (15.9%). Nitrofurantion was most active against *E. coli*. Piperacillin-tazobactam was most active against *Proteus mirabilis. AmpC* ß-lactamase was detected by phenotypic and genotypic procedures, phenotypically *AmpC* producers for *klebsiella pneumoniae* 13 (68%). Genotypically isolates bla_{EBC} 4 (30.7%), bla_{CIT} 13 (53.8%). However, phenotypically survey of *AmpC pseudomonas aeruginosa* was 9 (45%). Genotypically isolates bla_{EBC} was 6 (50%). Phenotypically survey of AmpC producers for *E. coli* was 9 (52%) and genotypically isolates bla_{EBC} 3 (30%), bla_{CIT} 1 (10%). While, *DHA*, *MOX* ACC, *FOX* genes were absent among all isolates. **Conclusion:** *klebsiella pneumoniae* showed AmpC ß-lactamase comprised (**bla_{EBC}** and **bla_{CIT}**. while no isolate have *DHA, MOX ACC, FOX* genes). As for *E.Coli* AmpC ß- lactamase comprised (**bla_{EBC}** and **bla_{CIT}**, while no isolates have AmpC *DHA, MOX ACC* and *FOX*.

Keywords: Intensive care unit, Bacteria characterization, Antibiotic susceptibility, Phenotypes, genotype, Plasmic AmpC, Gram-negative bacteria.

INTRODUCTION

The intensive care unit (ICU) plays a crucial role in managing and treating some of the most complex and serious disorders that affect the human body. Despite the ICU's are crucial and well-established role in patient care, ICU-acquired infections raise expenditures significantly, while also increasing morbidity and death for patients are found there. Large multicenter research conducted in the United States and Europe have shown that hospital infection rates in ICUs are the worst of all hospital-acquired infections **Iwashyna and Viglianti** [(1)]. Intensive care unit (ICU) patients frequently have consequences from healthcare-associated infections (HAIs), which include bacteremia, pneumonia, urinary tract, skin, or soft tissue infections **Salonia and Sotelo** [(2)].

The ICU personnel and doctors may act as conduits for the transfer of germs from other inpatient units to ICUs **Amaan *et al*** [(3)]. Some Gram-negative bacteria include chromosomal genes that encode ampC β-lactamases, which are significant resistance mechanisms. With the exception of carbapenem and cefepime, these enzymes are resistant to all β-lactam antibiotics, and atypical extended spectrum β-lactamases (ESBLs), they act as cephamycins hydrolyzer so the commercially available β-lactamase inhibitors does not suppressed them. Substrate profile such as monobactam, penicillins, cephalosporins were belong these enzymes **Castanheira *et al*** [(4)]. When exposed to certain β-lactam antibiotics, AmpC β-lactamase synthesis increases and wide spectrum cephalosporin resistance is induced **Meini *et al*** [(5)]. However, certain genetic mutations result in overexpression and production of AmpC β-lactamase.

AIM OF THE STUDY

The aim was presented to detect spread of bacterial isolates from patients in ICU in Kut City, Wasit Province, Iraq and to characterize it at the level of molecular analyses of its phylogenic and antimicrobial resistance genes.

PATIENTS AND METHODS

A total of 100 clinical samples including: urine, sputum and pus culture media such as mannitol salt agar, MacConkey agar, blood agar, and chocolate agar were collected in this study admitted to the intensive care units (ICUs) of Alzahraa and Alkarama hospitals for more than 48 hours between 3rd October 2021 to 20th February 2022. The growth showed different bacterial colonies whose morphological and biochemical characteristics were tested. Then DNA was extracted; purity and concentration were confirmed with Nanodrop. The purity of gram-negative bacteria was (1.8-2), and the concentration was between 50-360 ng/µl. Primers were mentioned as **Dahwash *et al.*** [(7)] procedure.

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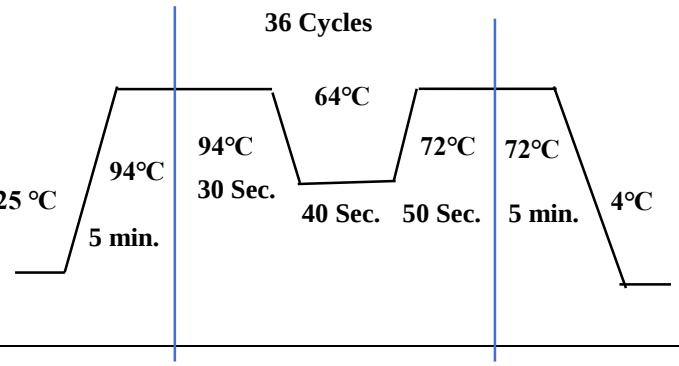
Table (1): Primers' sequence of gram-negative bacteria (Ugwu ***et al***.[29].

MultiplexPCR pool	Primers	Sequences (5'-3')	Size(bp)	References
Multiplex III:AmPC group-1 EBCM, MOXM and DHAM	Multi-CaseEBC-F	TCGGTAAAGCCGATGTTGCGG	302	(Ugwu *et al.*, 2020)
	Multi-CaseEBC-R	CTT CCA CTG CGG CTG CCA GTT		
	Multi-CaseMOX-F	GCTGCTCAAGGAGCACAGGAT	520	
	Multi-CaseMOX-R	CACATTGACATAGGTGTGGTGC		
	MultiCaseDHA-F	AACTTTCACAGGTGTGCTGGGT	405	
	MultiCaseDHA-R	CCGTACGCATACTGGCTTTGC		
Multiplex-IV: AmpC group 2 CIT, FOX and ACC	MultiCaseCIT-F	TGGCCAGAACTGACAGGCAAA	462	
	MultiCaseCIT-R	TTTCTCCTGAACGTGGCTGGC		
	MultiCaseFOX-F	ACATGGGGTATCAGGGAGATG	190	
	MultiCaseFOX-R	CAAAGCGCGTAACCGGATTGG		
	MultiCaseACC-F	AACAGCCTCAGCAGCCGGTTA	346	
	MultiCaseACC-R	AACAGCCTCAGCAGCCGGTTA		
	MultiCaseSIM-R	TAATGGCCTGTTCCCATGTG		

AmpC β-lactamase in isolates resistant to cefoxitin. In addition, Phenotypic Screening of AmpC β-Lactamase Production .The AmpC disc test has been used for confirming (phenotypically) the existence of AmpC β-lactamase in isolates resistant to cefoxitin using the technique indicated via **Gautam *et al***.[30]. In addition, the plates have been studied for resistant ≥ IZD 18 mm after an overnight incubation at a temperature of 37 C **Ugwu *et al***.[29].

Table (2): Thermal cycling program for multiplex pool.4AmPC group

Genes	PCR cycling profile	Products size
bla*$_{MOX}$, *CIT,DHA, ACC,EBC, FOX		520bp 462bp 405bp 346bp 302bp 190bp

This protocol as a reliable and efficient tool for the identification via multiplex PCR assays of the most common encoding of the beta-lactamase genes for gram negative bacteria. Thereafter Gel Electrophoresis and Documentation ,the PCR products and DNA ladder were placed into the wells of a 1.5-2 percent agarose gel containing 1 µl of Ethidium Bromide, and the gel was processed through an electrophoresis apparatus at either 70 or 100 volts. Agarose was removed from tank visualized under U.V light to measure the DNA bands with DNA ladder according to **Sambrook**[31]

Ethical Consideration:

The study was approved by the Ethics Board of Wasit University and an informed written consent was taken from each participant in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. WMA [32].

Statistical analysis

Finally, the data's statistical analysis has been carried out with the use of SAS (Statistical Analysis System - version 9.1).

RESULTS AND DISCUSSION

The current study was conducted on a total one hundred clinical specimens (urine, sputum and pus). The specimens were collected from patients admitted to the ICU. Eighty-two were positive growth culture distributed according to the patient's age, the highest incidence was among 20-29 age groups with (25.6%). While, the lowest incidence was among (50-59) and (70-79) age groups (3.6%), (3.6%) respectively, the mean ±SD of age was 15.556 ranging from 1 years to 90 years. Eighty-two were pure positive culture, 30 (36.5%) of patients were females and 52 (63.5%) were males. They admitted to the intensive care unit and distributed as 69 (84.1%) gram-negative bacteria, while gram positive were 13 (15.8%) patients.

Bacterial isolates that were obtained from the clinical specimens have been initially characterized based on the cultural morphology as well as the biochemical tests. Results of the culture showed colonies on MacConkey agar pink color with precipitation of bile salt around colonies, the refers to *Escherichia coli* isolates while results of biochemical tests for *Escherichia coli* were had given positive test for catalase, indole, methyl red, but negative for oxidase, voges-proskauer, simmon citrate, and TSI test showed A/A with gas, without H_2S. These results were diagnostic for *Escherichia coli*. Similar results were recorded by **Abdul-hussein *et al.*** [6] and **Nasser *et al.*** [8].

Results of identification of *klebsiella pneumonia* showed pink lactose fermenter mucoid colonies on MacConkey agar. Positive test for urease, voges-proskauer, simmon citrate, but negative for oxidase, methyl red, motility test, indole and TSI test showed A/A with gas, without H2S. Further confirmation done using Vitek2. Similar results were recorded by **Raheema *et al.*** [9] and **Jassim *et al.*** [10].

Results of *Acinetobacter baumannii* showed nonmotile and give negative result to oxidase, indole, methel red, voges proskauer and variable to urea. All isolates were positive to catalase, Simmons citrate and triple-sugur-iron test was alkaline/no change, these results are identical with those obtained by **Raheema *et al.*** [9] and **Jassim *et al.*** [10]. The results of *Pseudomonas aeruginosa* showed that all isolates had given negative result for indole, methyl red, voges proskauer and urease test also, citrate assimilation was positive, motility was positive and TSI alkaline/alkaline or Alkaline/no change with no production of H2S and gas and isolated from cetrimide agar colonies appeared mucoid, smooth in shape, fruity odour, fluorescent green, and creamy pigments these results agreed with what mentioned by **Dahwash *et al.*** [7], **Raheema & Alsaidi** [12] and **Al-Saeedi & Raheema** [13].

The results of *Proteus mirabilis* appear as bacilli, rapidly motile by flagella. Swarming phenomena on blood agar plate. It is non-lactose fermenter so it gives a pale colony on macConky agar. Negative test for indole and voges-proskauer but positive test for urease, simmon's citrate, H_2S is produced ,nitrate reduction motility and methyl red. Similar finding was recorded by **Raheema *et al.*** [9], **Albadri** [11] and **ALjeelizy *et al.*** [26].

The results of *Serratia marcescens*, showed that isolate had given result for indole, urease test, citrate assimilation, motility and catalase positive. Methyl red, voges proskauer and oxidase negative and TSI alkaline/acidic or acidic/acidic with no production of H_2S and gas. The results of *Burkholderia* cepacia complex showed that isolate had given a result at negative for indole. But positive for methyl red, voges proskauer, urease test, citrate assimilation, motility, catalase and TSI oxidase, alkaline/acidic with no production of H_2S and gas.

The results of *Pantoea spp* showed that isolate had given result for indole methyl red, urease test, motility, and oxidase negative. Voges-proskauer citrate assimilation, catalase positive, TSI alkaline/acidic with no production of H2S and gas. The results *Enerobacter cloacae* indicated that isolate had given result for indole, methyl red, urease test, motility and oxidase negative. Voges-proskauer, citrate assimilation and catalase was positive. TSI alkaline/acidic with no production of H_2S and gas. The results of *staphylococcus aureus* appeard that isolates had given result for indole was negative, methyl red was positive, voges-proskauer was positive, urease test was positive, citrate assimilation was positive, motility was negative and TSI acidic/acidic with no production of H_2S and gas, catalase was positive, coagulase was positive and oxidase was positive this results are consistent with findings from other Iraqi studies **Al-Saeedi & Raheema** [13] and **Raheema** [14]. Also similar result was recorded by **Raheema** [15] and **Raheema *et al.*** [17]. On blood agar *staphylococcus haemolyticus* fermenters as yellow colonies. Further biochemical tests were necessary for identification of *staphylococcus haemolyticus* from other species, all isolates were positive to catalase test, but negative for coagulase and oxidase **Raheema** [18]. The results of *Enterococcus faeciu* showed that isolate had given negative results for indole, urease test, citrate assimilation, motility, catalase and oxidase. But positive result for voges-proskauer.

Antibiotic susceptibility test of *E. coli*

The maximum resistance level of study for Ampicillin, Ceftriaxone, Cefotaxime and Azitreonam were 100% for each of them; followed by Ceftazidime and Piperacillin (94%) for each of them; Cefixime and Cefepime (88%) for each of them; Ciprofloxacin (82%); Meropenem, Nalidixic acid and Levofloxacin (75%) for each of them; Trimethoprime (69%); Cefoxitin (56%); Piperacillin-tazobactam (50%); Amoxicillin-clavulanic acid and Imipenem (44%) for each of them. As shown in figure (**1**).

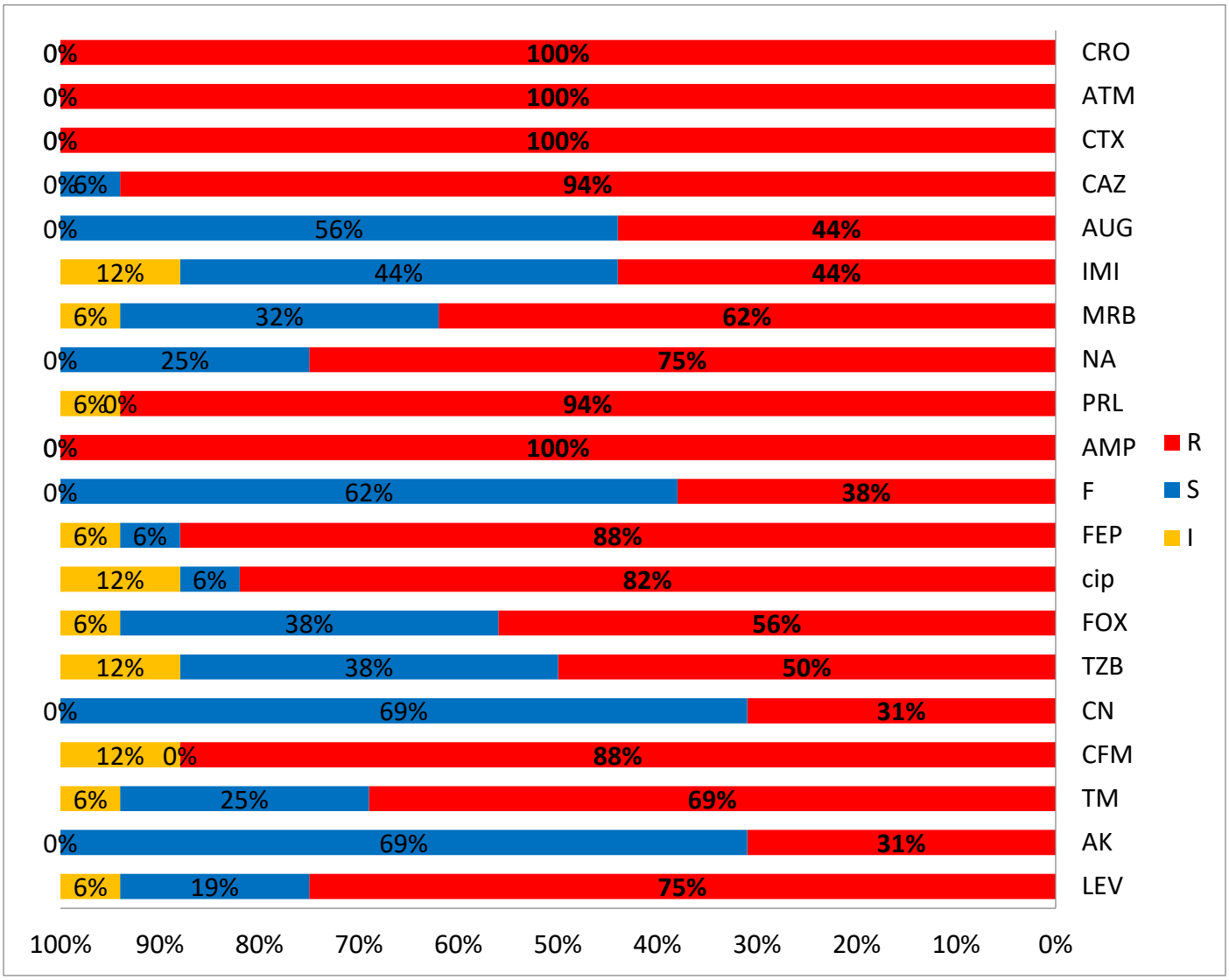


Figure (1): The percentage of antibiotics susceptibility profiles of *E.Coli*

CRO=Ceftriaxone, AZT=Aztreonam, CTX=Cefotaxime, CAZ=Ceftazidime, AUG= Amoxicillin-clavulanic acid, IMI=Imipenem, MRB=Meropenem, NA=nalidixic acid, PRL=piperacillin, AMP=Ampicillin, F=Nitrofurantoin, FEP=Cefepime, CIP=Ciprofloxacin, FOX=Cefoxitin, TZP=piperacillin-tazobactam, CN=Gentamicin, CFM=Cefixime, TM=Trimethoprime, AK=Amikacin, LEV=levofloxacin

In this study, the maximum resistance level for ceftriaxone, azitreonam, and cefotaxime were (100%) for each of them and ceftazidime was (89%). It is observed that it produces ESBLs in the study performed by **Nasser *et al.*** [8] found that the maximum resistance to the Ampicillin was (100%). Sensitivity of *E. coli* appears for gentamicin was (69%), amikacin was (69%).

Antibiotic susceptibility test of *Proteus mirabilis*

The maximum resistance level of this study for Ampicillin, Trimethoprime and Cefotaxime were (100%) for each of them, followed by Ceftriaxone, Gentamicin and Nitrofurantion were (80%) for each of them, followed by Piperacillin was 60%. The intermediate resistance level of study Cefepime was 60%, Cefixime was 40%. As shown in figure (**2**).

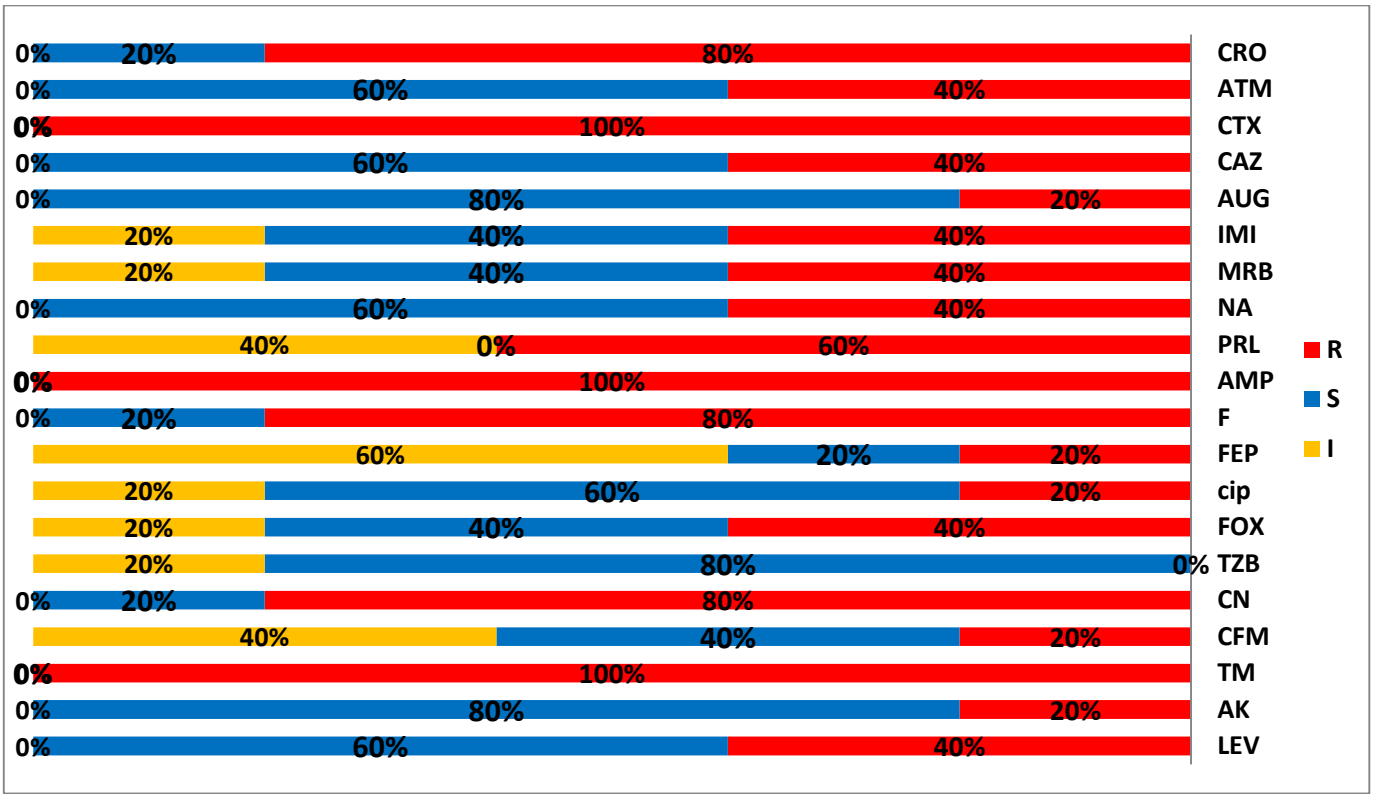


Figure (2): The percentage of antibiotics susceptibility profiles of *P. mirabilis*
CRO=Ceftriaxone, AZT=Aztreonam, CTX=Cefotaxime, CAZ=Ceftazidime, AUG=Amoxicillin-clavulanic acid, IMI=Imipenem, MRB=Meropenem, NA=nalidixic acid, PRL=piperacillin, AMP=Ampicillin, F=Nitrofurantoin, FEP=Cefepime, CIP=Ciprofloxacin, FOX=Cefoxitin, TZP=piperacillin-tazobactam, CN=Gentamicin, CFM=Cefixime, TM=Trimethoprime, AK=Amikacin, LEV=levofloxacin.

The maximum resistance level of this study for Ampicillin was 100%. A study performed by **Gorems *et al.*** [19] was100% in Jimma Town, Southwest, Ethiopia. Also with **ALjeelizy *et al.*** [26].

Phenotypic prevalence of AmpC ß- lactamase

In this screening test the result was cefoxitin (69%) of *klebsiella pneumoniae*, clinical isolates were AmpC β-lactamases producers. Similar results (75%) and (68%). These results have been reported in many other studies around the world. In our study, this result was cefoxitin (100%) of *klebsiella oxytoca*, clinical isolates were AmpC β-lactamases producers, while the result was cefoxitin (20%) of *pseudomonas aeruginosa*. Result AmpC β-lactamases in the current study was cefoxitin (56%) of *E. Coli* are in agreement with the study performed by **Rameshkumar *et al.*** [20] which was the result (57.1%). However, the result was cefoxitin (40%) of *Proteus mirabilis* similar results (40%). It was reported in another study by **Milton *et al.*** [21]. Result AmpC β-lactamases in the current study was cefoxitin (100%) of *Acinetobacter baumannii* are similar with the study performed by **Shali *et al.*** [22] which was the result (100%).

Molecular detecting Antibiotic Resistance Genes in gram negative bacteria

The three types of AmpC resistance include chromosomal resistance that is not capable of inhibiting owing to mutations in the promoter and/or attenuator, plasmid-mediated resistance, and inducible resistance caused by the encoding of chromosomal ampC genes. The pAmpC family of AmpC β-lactamases (*MIR/ACT*, *ACC*, *DHA*, *FOX*, *CIT*, and *MOX*) includes these enzymes.

EBC, DHA, MOX

In this study, no PCR-amplification products with bla_{EBC}, bla_{DHA}, and *bla* $_{MOX.}$genes in all isolates of *Burkholderia cepacia* and *Klebsiella oxytoca*). In the present study the results demonstrated that bla_{EBC} (50%) for 6 *Pseudomonas aeruginosa* from (12) isolates and this result is compatible with in Zahedan city, Iran by **Muthukumar *et al.*** [27] and **Adabi *et al.*** [28] consist of this gene bla_{EBC} (38%) and (50%) respectively. But as for bla_{EBC} (30.7%) for 4 *Klebsiella pneumoniae* from (13) the results of the gene. It does not correspond to **Jassim *et al.*** [10] in Wasit since he does not have this gene *EBC*. While the results of our study were of bla_{EBC} (30%) for 3 *E. Coli* from (10). It is inconsistent with this study in Wasit by **Nasser *et al.*** [8] because it does not have this gene.

The result was bla_{EBC} (50%) for1*Acinetobacter baumannii* from two. However, the result was bla_{EBC} (50%) for 1 *Proteus mirabilis* from two. No PCR-amplification products with *DHA* and *MOX* genes in any isolates of gram negative bacteria in this study. As for the *DHA*, *MOX* any positive results in all isolated bacteria in this study did not find. The agarose gel of PCR products was shown in Figure (**3**).

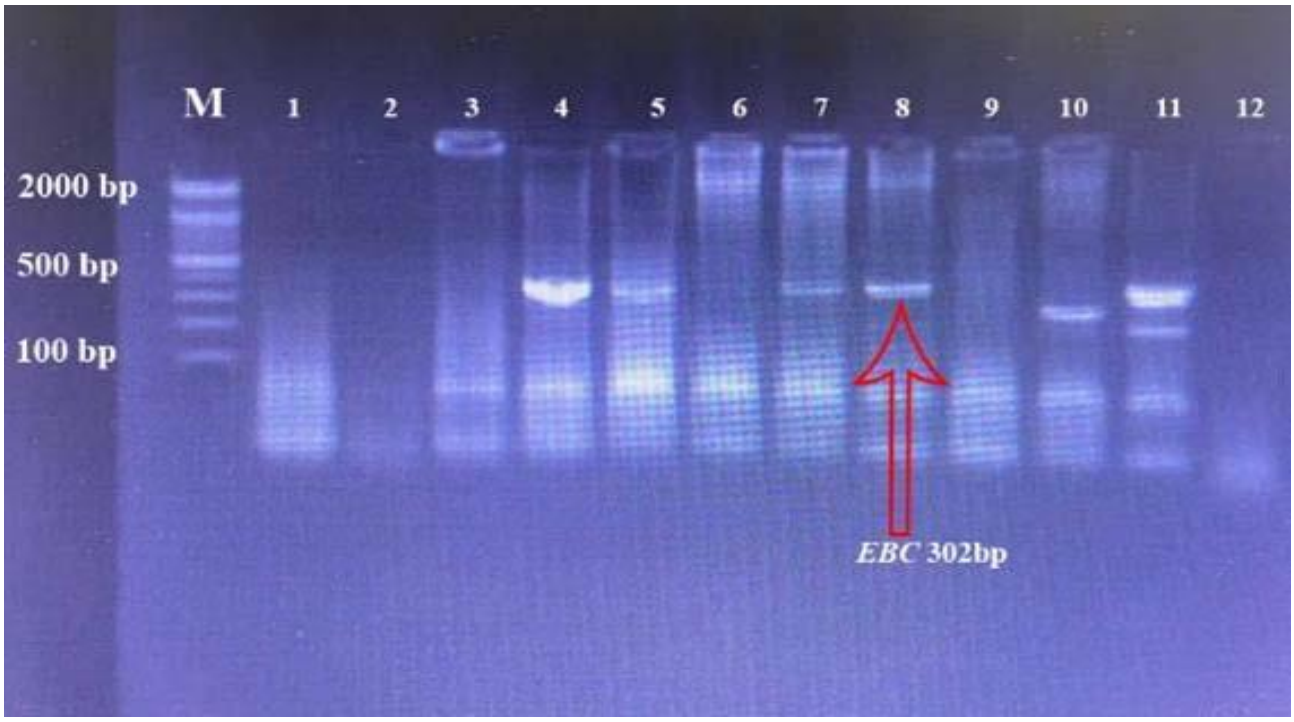


Figure (3): Gel electrophoresis of amplified products for detection of gram negative bacteria Ampc genes. Agarose 2 %, 100 V/cm for 40 min, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane (m): DNA Ladder (2000bp). Some *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, E. Coli , Proteus mirabilis* isolates at *EBC* (302 bp.

ACC, FOX, CIT

In this study, no PCR-amplification products with *bla* $_{CIT}$, *bla* $_{FOX}$, and *bla* $_{ACC}$ .Genes in all isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Burkholderia cepacia* and *Klebsiella oxytoca*

In the present study the results demonstrated bla_{CIT} were (53.8%) among seven *Klebsiella pneumoniae* isolates from (13). It also came identical to a study in Wasit **Jassim *et al.*** [10] showed that the prevalence of gene bla_{CIT} was (23%) among isolates. And that results of bla_{CIT} (50%) among 1 *Proteus mirabilis* from (2) this results are similar to our study in Wasit **P**rovince, Iraq **Shallal *et al.*** [23]. that it has this gene bla_{CIT} (60%), While the results of bla_{CIT} (10%) among 1 *E. Coli* from (10).

It also corresponds to a study in Wasit **Nasser *et al.*** [8] showed that the prevalence of gene bla_{CIT} was (24.40%) among isolates. As for bla_{FOX}, we have one isolate from a bacteria *Proteus mirabilis* that carry this gene, and the result was (50%) from 2 *Proteus mirabilis* the results are compatible with the result at **Mol *et al.*** [25]. In this study, we found the presence of this gene bla_{FOX} was (30.4%). The agarose gel of PCR products was shown in Figure (**4**).

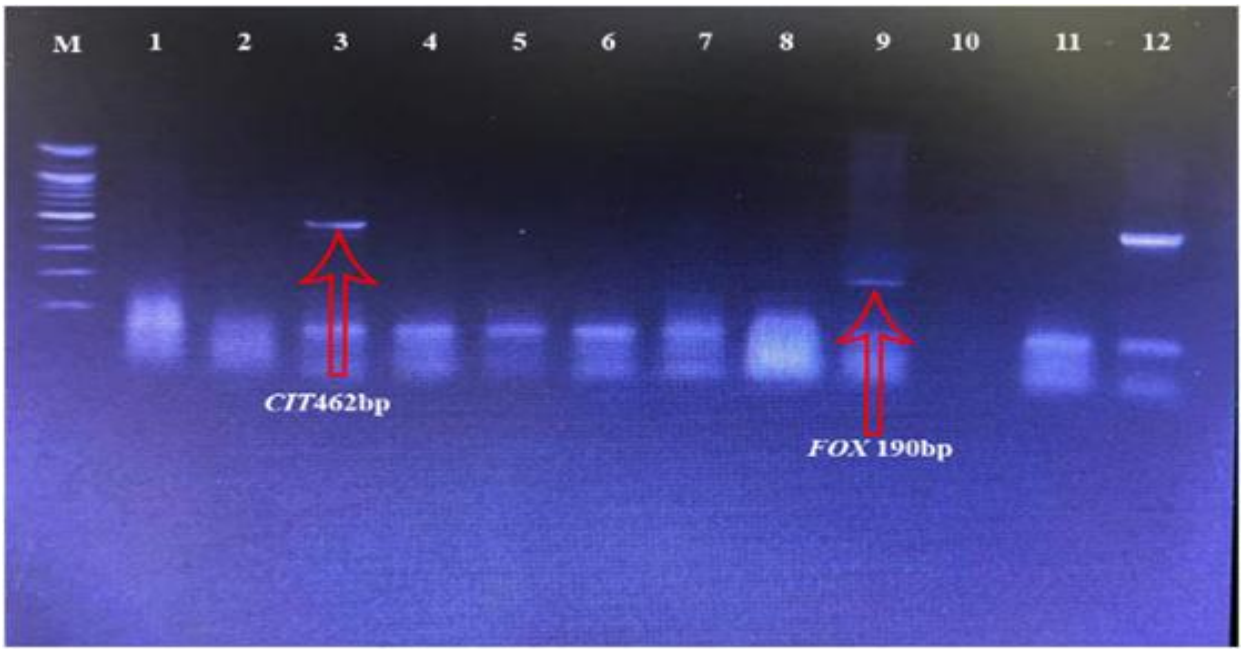


Figure (4): Agarose gel electrophoresis of image that showed PCR product analysis for *ACC, FOX, CIT* gene from gram negative bacteria. M (Marker ladder 100-2000bp). Lane (1-12): some *FOX*, *CIT*gene *Proteus mirabilis, E.Coli* isolates at (190 bp), (462 bp) gene product size.

In this study, no PCR-amplification products with ACC gene in all isolates. Resistance due to plasmid-mediated AmpC enzymes is less common than extended-spectrum beta-lactamase production in most parts of the world but may be both harder to detect and broader in spectrum. AmpC enzymes encoded by both chromosomal and plasmid genes are also evolving to hydrolyze broad-spectrum cephalosporins more efficiently.

CONCLUSIONS

According to the findings of current study in ICU, males are more frequently admitted and admitted more frequently than females. Results of antibiotic susceptibility test revealed that the most active compound against *E. Coli* was Nitrofurantion. Piperacillin-tazobactam, levofloxacin and Ceftazidime were most active against *Proteus mirabilis*. Phenotypically and genotypically for *klebsiella pneumoniae* showed AmpCß-lactamase comprised (***bla***$_{EBC}$ and ***bla***$_{CIT}$, while, no isolate have *DHA, MOX ACC, FOX* genes). AmpC ß-lactamase comprised (***bla***$_{EBC}$). Whereas, no isolate have AmpC *DHA, MOX ACC* and *FOX* genes for *pseudomonas aeruginosa*. As for *E.Coli* AmpCß- lactamase comprised (***bla***$_{EBC}$ and ***bla***$_{CIT}$, while no isolates have AmpC *DHA, MOX ACC* and *FOX*.

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