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Phenotypic and Genotypic Detection Ampc β-Lactmase Producing *E.coli* Isolated from UTI in Anbar Governate

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Abstract :

The plasmid facilitates the transfer of the AmpC enzyme gene to other bacteria through conjugation and transformation . Urine samples of 130 patients were collected from both genders and different ages of patients with suspected urinary tract infections according to the clinical manifestations and symptoms diagnosed by the examining physician in Alramadi teaching hospital and pediatric hospital , during the period from October 2019 to February 2020 . The antibiotic susceptibility results for 9 antibiotics showed that the a highest resistance isolates to Ceftazidime (100%), Ampicilin(92%), Ceftriaxone(86%) , Trimethoprim/ Sulfamethoxazole (86%) , Cefepime(70%) ciprofloxacin (62%), Gentamicin(46%) , Cefoxitin (14%) and Nitrofurantoin (8%).

Phenotypic detection for AmpC productin show that in CC-DDS test appeared 17 positive isolates , in(mCIM) method all isolate gave positive result ,EDTA use to differentiate Metallo-β-lactamase from serine cephalosporinase ,EDTA use as inhibitor of Metallo-β-lactamase ,while Modified Hodge test showed 14 isolate were positive result. The polymerase chain reaction results of twenty-three isolates showed the highest prevalence for *blaFOX* gene was detected in 13 isolate (56.5%), *blaACC* gene was detected in 12 isolate (52.1%) , *bla DAH* gen was detected in 12 isolate (52.1%),8 isolate (34.7%) had *blaCIT* gene , bla EBC Where was identified in 6 isolate (26.08), while the lowest percentage for blaMOX gene was identified in 5 isolate (21.7%) .

Key words : AmpC , E. coli , FOX , MOX

Introduction

Escherichia coli is the most common type of pathogen that causes disease. Infection in the urinary tract (UTI), caused by E. Coli is the most serious infectious disease with substantially higher morbidity , mortality and health-care costs(Raeispour & Ranjbar,

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2018). β-lactamase synthesis by gram-negative bacteria is considered to be the most important and crucial mechanism of resistance to β lactam drugs (Ahmed & Al Meani, 2019) .

The β lactamases are classified in four groups; classes A, C and D are serine-β-lactamases, while class B is β-lactamases metallo enzyme (Sah & Hemalatha, 2015) . β-lactamases were categorized by Ambler and Bush-Jacoby-Medeiros based on their function and molecular structure. (Al Meani et al., 2020). According to Ambler and Bush-Jacoby, Ampβ-lases belong to Class C and Group 1. These enzyme inhibitors do not function on oxyimino-cephalos and cefobactam, for example. Cloxacillin and bororonic acid were used in phenotypical studies to validate the presence (Bush & Bradford, 2020) . the global production of AmpC and the possibility of becoming a 'plasmid gene dispersion to other plasmids' is no longer a real one (M. O. Ibrahim & Al Meani, 2020). This enzymes are typically coded by chromosomal genes in several gram negative bacterium. While the encoding genes of these enzymes were described in plasmids at the end of the 1980s, they could be passed between various species (Hoseini et al., 2017).Commonly reported genotypes of AmpC genes are blaMOX, blaCIT, blaDHA, blaFOX , blaACC and bla EBC.

This work aimed to determine the prevalence of (AmpC) β-lactamases genes in local clinical isolates of *Escherichia coli* , evaluate phenotypic methods for detection of this β-lactamase.

Materiales and Methods :

Sample Collection:

The specimens were collected from patients in Alramadi city from two hospital, during the period from October 2019 to February 2020, that is by taking midstream urine samples from the patients. And then it was transferred directly to laboratory for the purpose of its development and diagnosis, as it was cultured using the Streaking method on petri dishes containing MacoCongy agar media and EMB agar, and incubated at 37 ° C for 18-24 hours (MacFaddin, 2000).Identification of bacteria using conventional and automated vitek -2 system .

Antimicrobial susceptibility testing :

Antimicrobial susceptibility testing performed using conventional disk diffusion method. According to the guidelines recommended by the CLSI(2019) ; antibiotic resistance was determined by using antibiotic discs listed **:** Ampicilin , Gentamicin

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, Ceftriaxone Ceftazidimim ,Ciprofloxacin , trimethoprime-sulfamethoxazole , Nitrofurantoin , Cefepime , and Cefoxitin**.**

Phenotypic detection of AmpC in *E. coli* :

Estimation of ESBls was carried out by modified Hodge test, synergy test, mCIM and **CC-DDS** under the CLSI guidelines (Clinical And Laboratory Standars Institute, 2018)and as described elsewhere (Poirel et al., 2011).

Genotypic detection of AmpC in *E. coli* :

10ml of nutrient broth was inoculated with over night bacterial culture and incubated at 37C°. Genomic DNA was extracted from the culture of bacterial isolates by using HiPurA® Bacterial Genomic DNA Purification Kit (HIMEDA) depending on the instruction of a manufacturing company. The DNA concentration of samples was estimated by using Nano drop Spectrophotometer, by putting 1 µl of the extracted DNA in the instrument to detect concentration in ng/µl, and purity detected by noticing the ratio of absorbance at wavelength 260/280 nm. All primers were supplied by company Bioneer (Korea) as lyophilized product Primer were dissolved with deionized water (ddH2O). Final concentration of (100 pM/µl) (as stock solution) was prepared table 1. To prepare 10 pM concentration as work primer, re-suspended 10 µl of stock solution in 90 µl of ddH2O to reach a final concentration 10 pM, mixed well and kept in -20 C. They were mixed by vortex to homogenize before use at table 2.

Table :(1) Sequences of PCR primers

Gene	Primers'Sequences (5'→3')	Product size (bp)
DHA	F:AACTTTCACAGGTGTGCTGGGT R:CCGTACGCTTACTGGCTTTGC	405 bp
ACC	R:AACAGCCTCAGCCGGTTA R:TTCGCCGCAATCCCTAGC	346bp
MOX	F:GCTGCTCAAGGAGCACAGGAT R: CACATTGACATAGGTGTGC	520 bp
FOX	F:AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	190bp
CIT	F:TGGCCAGAACTGACAGGCAAA R:TTTCTCCTGAACGTGGCTGGC	462 bp
EBC	F:TCGGTAAAGCCGATGTTGCGG R: CTTCCACTGCGGCTGCCAGTT	302 bp

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*** F: Forward sequences, R: Reverse sequences.**

Table (2): Program of PCR reaction:

Cycles	**1**	**35**			**1**
Temp. **Gen**	Initial denaturation	denaturation	annealing temp	extension	final extension
DHA	94 for 2 min	94°C for 1min	61 C for 30 sec	72C for 35sec.	72 C for 7min
ACC	94 for 2 min	94°C for 30 sec	65 C for 30 sec	72 C for 30sec	72 C for 7min
MOX	94 for 2 min	94°C for 30 sec	60 C for 30 sec	72 C for 45sec	72 C for 7min
FOX	94 for 2 min	94°C for 30 sec	64 C for 30 sec	72 C for 20 sec.	72 C for 7min
CIT	94 for 2 min	94°C for 30 sec	60 C for 30 sec	72 C for 35sec.	72 C for 7min
EBC	94 for 2 min	94°C for 30 sec	63 C for 30 sec	72 C for 30sec.	72 C for 7min

Result and disscusion :

Isolation and identification of E. coli

All methods such as morphological , microscopic , biochemical test and automated test gave positive result for E. coli . table3

Table 3 : Phenotypic characteristics and biochemical tests results of the *E.coli*. ' + ' indicated positive result, ' - ' indicates negative result.

Characteristic	***E coli***
Gram Staining	**-**
Shape	**bacilli**
Catalase test	+
Oxidase test	**-**
Indol Test	+
Methyl Red Test	+
MR-VP Test	**-**

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Citrate Utilization Test	-
Fermentation of Glucose	+
Fermentation of Lactose	+
H2S	-
Urease	-
Kilgler	**A/A**
gas	+

Antimicrobial susceptibility testing :

Fifty isolates of *Ecoli* from UTI patients were tested for antibiotic sensitivity by disk diffusion method according to the recommendation(Institute, 2019) as figure 1 . Antimicrobials including: Ampicilin, Gentamicin, Ceftriaxone , Ceftazidime , ciprofloxacin, Trimethoprim/ Sulfamethoxazole , Nitrofurantoin , Cefepime and Cefoxin. The present study shows a highest resistance to Ceftazidime (100%), Ampicilin(92%), Ceftriaxone(86%) , Trimethoprim/ Sulfamethoxazole (86%) , Cefepime(70%) ciprofloxacin (62%), Gentamicin(46%) , Cefoxitin (14%) and Nitrofurantoin (8%) figure (4-12) . In our study resistance to third generation it was as follows; ceftazidime was (100%) this agree with results of study conducted by Odongo (Odongo et al., 2020) on Patients with UTI at Mulago Hospital, Kampala, Uganda. Another study was conducted in Kashan, Iran It did not agree with the results of our study , where was the percentage of resistance 49.3% (Neamati et al., 2015) . While Ceftriaxone resistance was (86%) this result was approach to result from Patient in Zakho City/Iraq made by the researcher on Escherichia coli was the resistance is 87% (M. S. Ibrahim et al., 2020) .

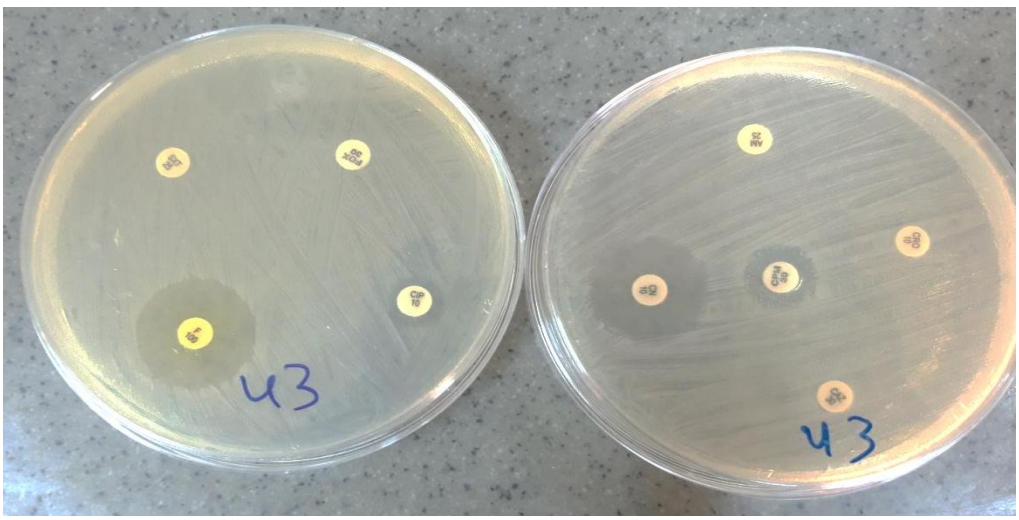

Figure (1): Antimicrobial susceptibility test of *Escherichia coli*

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Phenotypic detection of AmpC β-lactamases

InCC-DDS test appeared 17 positive isolates . In(mCIM) all isolate gave positive result. EDTA use to differentiate Metallo-β-lactamase from serine cephalosporinase ,EDTA use as inhibitor of Metallo-β-lactamase . The Modified Hodge test showed 14 isolate were positive result . Figure (2) , table 3

Table 3 : Phenotypic detection of AmpC β-lactamases

Result	CC-DDS	mCIM n=23	Modified Hodge test n=23
Positive	**17**	**23**	**14**
Negative	**6**	**0**	**9**

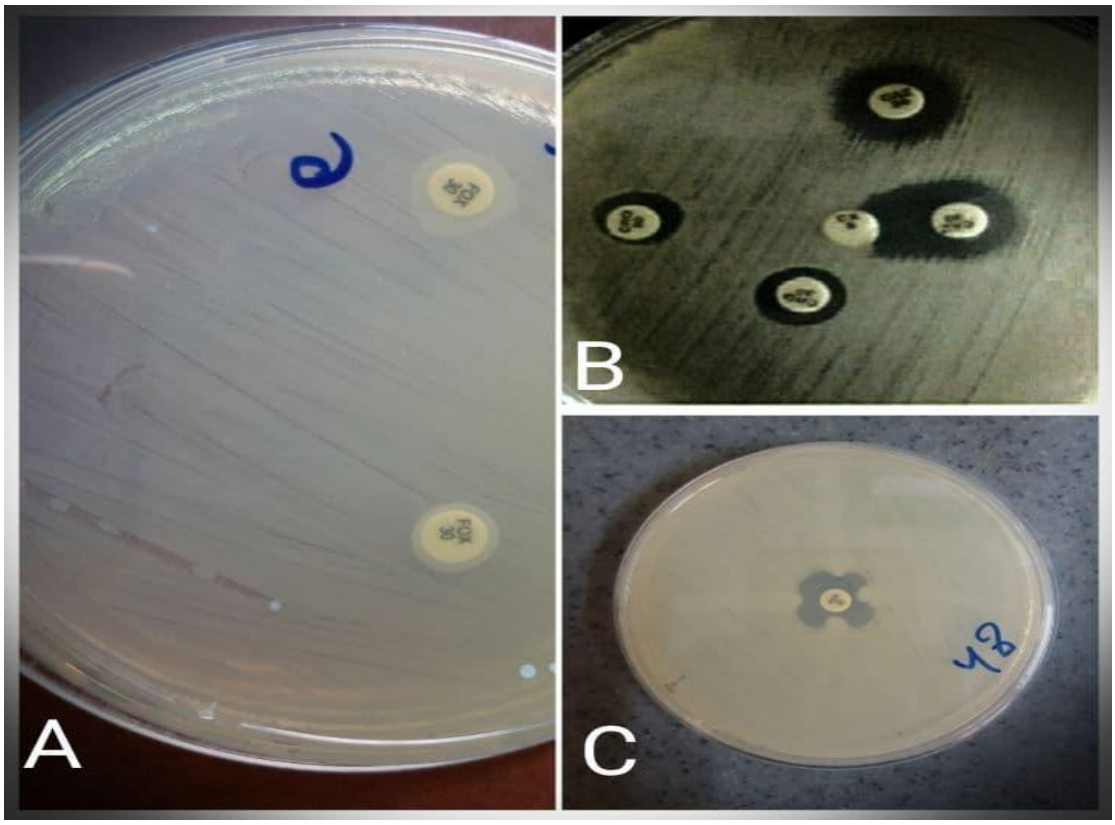

Figure (2): A) positive modified cephalosporinase inactivation methods no inhibition zone around disk,

B) Positive Ceftazidime -cloxacillin is a double-disk synergy test

C) Modified hodge test for AmpC detection in E.coli positive as clover leaf shape

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However, this is not certain and resistance can be due to disorder in the permeability of bacteria external membrane and may have no enzymatic sources (Nikaido & Pagès, 2012) .

AmpC b-lactamases are a new threat because they confer resistance to 7-methoxy-cephalosporins like cefoxitin or cefotetan, are unaffected by commercially available b-lactamase inhibitors, and can confer carbapenem resistance in strains lacking outer membrane porins., (Philippon et al., 2002). The widespread use of cephamycins and β-lactamase inhibitor combinations (e.g. clavulanic acid/amoxicillin and tazobactam/piperacillin) has contributed to selection of AmpC β-lactamase producing strains, worldwide (Najjuka et al., 2019) .

Genotypic Detection :

The polymerase chain reaction results of twenty-three isolates showed the highest prevalence for *blaFOX* gene was detected in 13 isolate (56.5%), *blaACC* gene was detected in 12 isolate (52.1%) , *bla DAH* gen was detected in 12 isolate (52.1%),8 isolated (34.7%) had *blaCIT* gene , bla EBC Where was identified in 6 isolate (26.08), while the lowest percentage for blaMOX gene was identified in 5 isolate (21.7%) figure 3 , 4 , 5 , 6 , 7 , 8,9.Study In Najaf Hospitals detected blaFOX (44.4%), blaCIT (38.9%), blaDHA (27.8%), and blaEBC (50%) (Al-jubouri & Alasadiy, 2014). In study conducted in India hospitals the prevalence ,*of BlaCIT* gen and *blaDHA* was equal percent, it was found in 40% ,followed by bla MOX gen in (18.3%)while gene of the blaACC type was present in only one isolate. No genes belonging to the bla FOX or bla EBC were detected in this study mentioned (Mohamudha et al., 2012) . Study in Turkey the distributions of the genes for *E coli* isolate were: 10.1% CIT, 8.08% MOX, 3.03% EBC, and 1.01 FOX genes. Meanwhile DHA and ACC group genes were not detected (Yilmaz et al., 2013). Other study show that blaCIT 78.9% followed by blaMOX 15.7% bla DAH 15.7, blaFOX 10.5% , bla EBC 5.2 %,and there is no gen belong to bla ACC (Helmy & Wasfi, 2014) .

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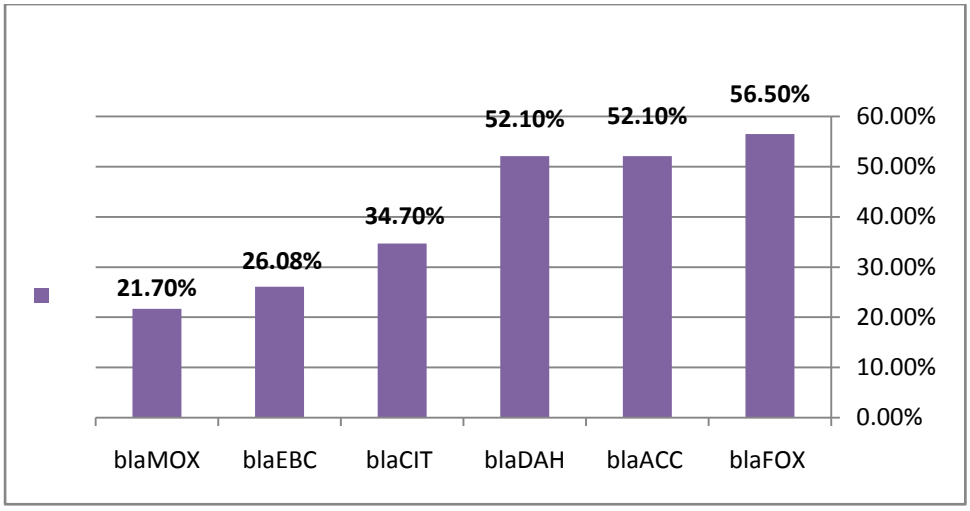
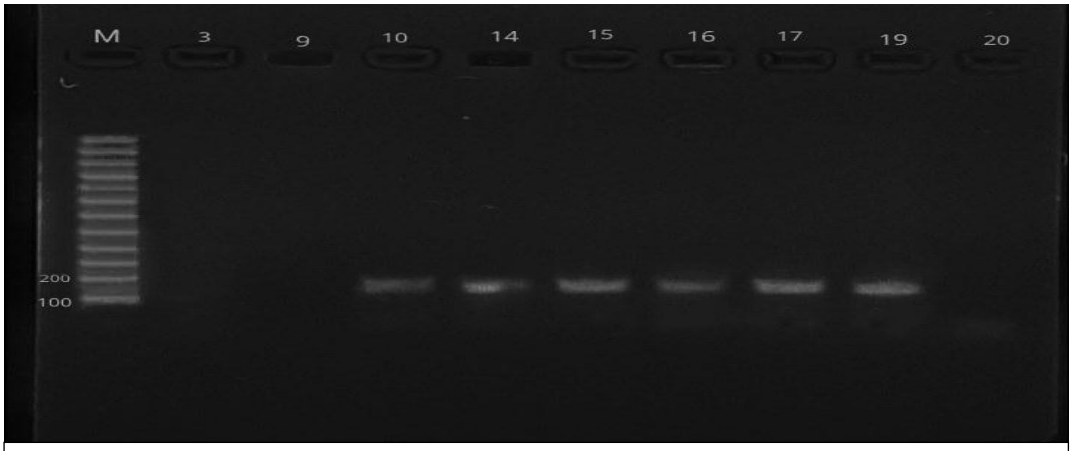
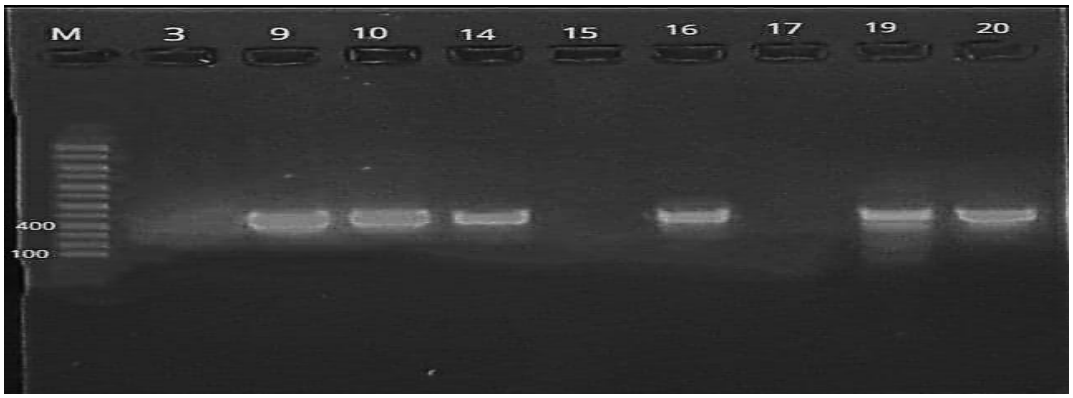


Figure(3) Distribution of the AmpC genes of all clinical isolates of *E.coli*



Figure(4):Detection of bla FOX gen .



Figure(5) Detection of *bla DAH* gen.

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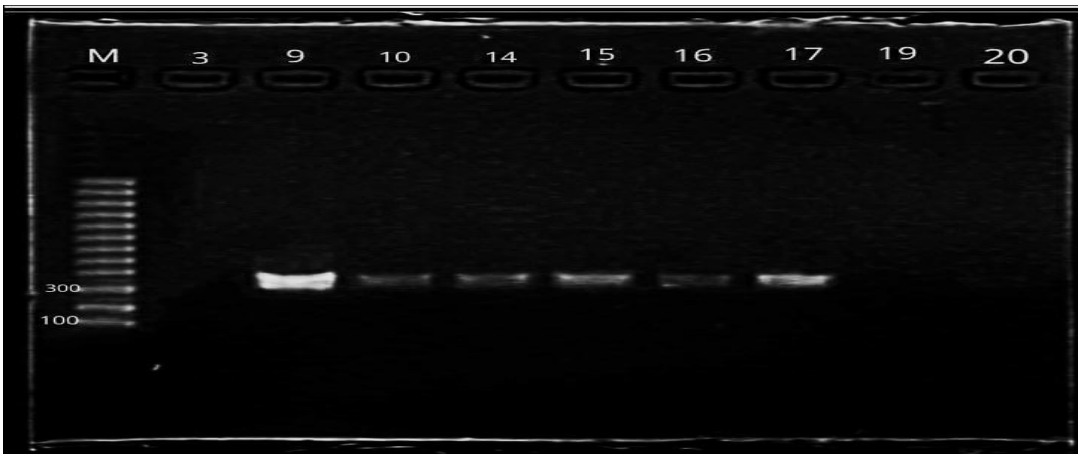
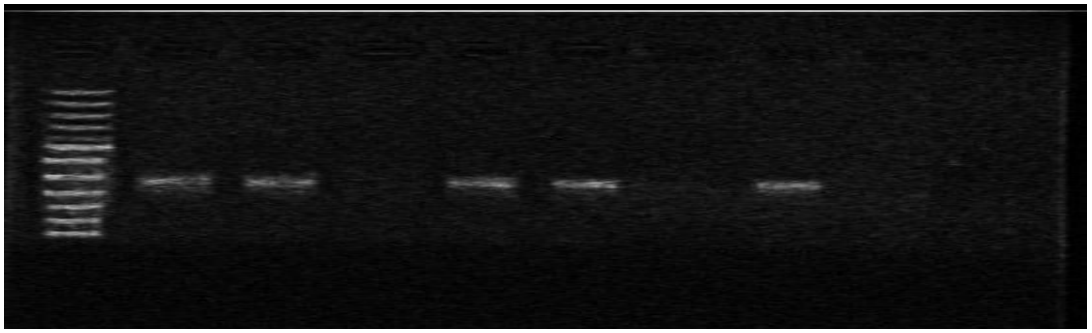
Figure (6): Detection of bla ACC .



Figure(7): Detection of *bla EBC* gen



Figure(8): Detection of *bla CIT gene*

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Figure(9): Detection of BlaMOX

Conclusion

FOX gene was prevalence in most isolates of E.Coli . E.coli isolates which have AmpC genes appeared resistance 100% to Ceftazidime, %), Ampicilin(92%), Ceftriaxone(86%) , Trimethoprim/ Sulfamethoxazole (86%) , Cefepime(70%) ciprofloxacin (62%), Gentamicin(46%) , Cefoxitin (14%) and Nitrofurantoin (8%).prevalence of resistance to third and fourth generation of cephalosporins among isolates in al Anbar hospitals .

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