Iraqi Journal of Science, 2023, Vol. 64, No. 12, pp: 6193- 6203
DOI: 10.24996/ijs.2023.64.12.9

ISSN: 0067-2904

Detection of Resistance Genes (*blaNDM* and *blaVIM*) of *Escherichia Coli* in the Aquatic Environment

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Received: 14/10/2022 Accepted: 25/1/2023 Published: 30/ 12 /2023

Abstract
The study aims to detect the presence of carbapenems genes and the prevalence of antibiotic-resistant *E. coli* in the Tigris River. Samples were collected from three sites of the Tigris River: S1Adhamiya, S2 Medical city hospital, and S3 Abu Nuwas. It diagnosed 40 isolates of *E. coli* out of 67 isolates of bacteria by Vitek2. The antibiotic sensitivity was determined by the disk diffusion method. *E.coli* isolates were tested against 7 antibiotics these belonged to β-lactam, Carbapenem. Also, the resistance genes (*blaVIM* and *blaNDM*) detected for these isolates of *E. coli*. The results appeared resistance of *E.coli* against AMC 82.5%, PRL 62.5%, AM 55%, and moderate resistance against CRO 45% whereas little resistance against ATM 27.5% and carbapenem including IPM 5%and MRP 5%. Also, the results of resistance genes have appeared in two isolates that have the resistance gene *blaVIM* (5%) and one isolate has *blaNDM* genes (2.5%). These isolates appeared resistant to β-lactam group antibiotics and little resistance to *E. coli* to carpamenes antibiotics (IMP, MER).

Keywords: *blaNDM*, *blaVIM*, Resistance antibiotics, *E coli*, Tigris River.

الكشف عن جينات المقاومة (*blaNDM* و *blaVIM*) للإشريكية القولونية في البيئة المائية

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الخلاصة
تهدف الدراسة إلى الكشف عن وجود جينات carbapenems وانتشار الإشريكية القولونية المقاومة للمضادات الحيوية في نهر دجلة. تم جمع العينات من ثلاثة مواقع من نهر دجلة S1 :الأعظمية ، مستشفى المدينة الطبيةS2 ، S3 أبو نواس. وتم تشخيص 40 عزلة من أصل 67 عزلة من الإشريكية القولونية بواسطة .Vitek2 تم تحديد حساسية المضاد الحيوي بواسطة طريقة انتشار القرص. تم اختبار عزلات الإشريكية القولونية ضد 7 مضادات حيوية تنتمي إلى بيتا لاكتام ، كاربابينيم. كما تم الكشف عن جينات المقاومة *blaVIM* و*blaNDM* لهذه العزلات من الإشريكية القولونية. أظهرت النتائج مقاومة الإشريكية القولونية لـ AMCبنسبة 82.5 %، PRL 62.5% ، AM 55% ومقاومة متوسطة لـ CRO 45% بنسبة بينما مقاومة قليلة لـ ATM بنسبة 27.5٪ والكاربابينيم بما في ذلك IPM بنسبة 5٪و MRP بنسبة 5% كما ظهرت نتائج جينات المقاومة في عزلتين لهما جين المقاومة *blaVIM* (5%) وعزلة واحدة بها جين *blaNDM* (

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2.5%) بدت هذه العزلات مقاومة للمضادات الحيوية من مجموعة بيتا لاكتام وقلة مقاومة الإشريكية القولونية للمضادات الحيوية للكاربامينيم (IMP ، MER) .

1.Introduction

Antibiotics are medicines that use to fight bacterial infections in people and animals. They work by many methods to kill the bacteria or by making it hard for the bacteria to grow and multiply. *E. coli* is one of the most important bacterial types because it is related to human health and also because its original origin is the intestines of humans and animals Therefore, it has been emphasized in this research. There are many sources that can supply the rivers with various materials and antibiotics such as waste from hospitals, water for irrigation of crops that wash away soil containing pesticides, as well as veterinary waste that may cause resistance to many bacterial species as a result of the horizontal transfer of genes and thus the spread of resistance to them [1]. The greatest danger is the formed antibiotic multiple resistances in species, which is currently the problem of the century since it is difficult to treat because more dangerous for the patient and secondly, the transfer of resistance and its spread via plasmids to other bacterial species[2]. Microorganisms, including bacteria, have many mechanisms for achieving drug resistance, including enzymatic inactivation of the antibiotic, decreased cell wall permeability of the antibiotic, Alteration target site, extraction mechanisms for antibiotics clearance, and increased mutation rates in response to severity therefore, antibiotics enter to the aquatic environment can be caused pollution and effect on the community structure and influences on functions of water ecosystem[3]. The bacteria resistance to Carbapenem increased ~~in~~ worldwide, which leads to infections that cannot treat [4] New Delhi Metallo-beta-lactamases (NDM) were discovered prevalent in India by patients traveling to numerous countries[5]. Verona imipenemase (VIM) metallo-beta-lactamases were first detected in Japan [6]. When the prevalence of the pandemic COVID-19, ~~the~~ carbapenem resistance has been recorded in hospitals. The rapid identification of bacteria that are resistant to carbapenem would permit hospitals to get protective measures and restrict the prevalence of infections [7]. Due to the current conditions that lead to an increase in the resistant bacteria species and also the resistance pattern of these species, this study aimed to detect the presence of carbapenems genes and the spread of antibiotic-resistant *E. coli* in the Tigris River.

2 . Methods

2.1 Samples collection

Samples 67 were collected from three sites S1 Adhamiya before to wastewater of Baghdad Medical City's Hospital and thrown into the river S2 and S3 Abu Nuwas to find out the pollution caused by medical wastewater dumped into the river and its impact on the rest of the sites through the spread of resistance genes. Samples were collected on duplicate by sterile glass container (1000 ml). All samples were transported to a laboratory for analysis.

2.2 . Isolation and identification of E. coli

2.2.1 Preparation of culture media

Blood agar, MacConkey agar, Simon citrate agar, Eosin Methylene Blue(EMB) agar and Mueller-Hinton agar were prepared according to the company manufacturer (Himedia/ India) then adjusting pH and sterilizing the culture media by autoclave at 121ºC/inch2 for 15 min .The media were cooled until 45ºC to use them for the isolation and identification of *E.coli*. Regarding the preparation of Blood base agar after autoclaving sterilization, it is cooled, and then added 5% of human blood to use in the isolation.

2.2.2 Isolation of E. coli

E.coli were isolated by serial dilution method in normal saline, 1 ml from each dilution was dispensed into Petri-dishes; then poured Nutrient agar, MacConkey agar, and EMB agar were. Plates were incubated for 48 h at 37°C.

2.2.3 Microscopic examination

Gram stain (BD medical/UK) is ready to use for staining bacteria isolates to examine by a microscope, it included crystal violet; iodine gram; ethanol 70 %; safranin stain.

2.2.4 Biochemical tests preparation

Biochemical tests were prepared according to Collee *et al*. [8]. The method of catalase test was to put the colonies of bacteria on glass slides after then drops of hydrogen peroxide (3%) were added, and the presence of bubbles indicated a positive reaction.

The Oxidase test method involved taking the colonies of bacteria by sterile stick and smearing them on filter paper saturated with drops of tetramethyl-p-phenylene diamine dihydrochloride. The positive results are shown by changing the color of a violet or purple in10 seconds.

Methyl red test method was the broth tubes of MR-VP that were cultured with bacteria and incubated at 37°C for 48 hours. After then it was added 5 drops of the reagent of methyl red and mixed. Positive results appeared shining red and negative results appeared yellow.

Voges-Proskauer test method was the broth tubes of MR-VP were cultured with bacteria and incubated at 37°C for 24 hr. After then it was added 1 ml reagent A (40 g KOH was dissolved into 100 ml distilled water) and 3 ml reagent B (5 g α-naphthol dissolved in 100 ml of absolute ethanol) to 5 ml of culturing broth and shaken for 30 seconds. The color of pink to red formation indicated positive results. The citrate utilization method was the preparation of the slant of Simmons citrate agar after then it was cultured with bacteria and incubated at 37°C for 24 hr. The color royal blue indicated positive results whereas the color green indicated negative results.

2.2.5 Identification of E. coli by VITEK2 Compact

VITEK2 Compact has been used for the identification of the isolates of bacteria (Identification card: GN ID card Reference number 21341). The method was performed according to the manufacturer company (BioMe'rieux/ France).

2.3. Antibiotic resistance test for *E.coli*

E. coli sensitivity against antibiotics was tested according to Vandepitte *et al*.[9] using the Kirby-Bauer method with Muller Hinton agar. Transferred 3-5 colonies from MacConkey agar after incubation 18-22 hr. at 37 ° C into a tube containing 5 ml of normal saline, and turbidity was determined with 0.5 McFarland solution is equivalent to 1.5×10^8. The sensitivity test was performed by using an antibiotic disk for Ampicillin (AM,10µg), Amoxicillin /Clavulanic acid (AMC,30µg), Piperacillin (PRL,100µg), Aztreonam (ATM, 30µg), Imipenem (IPM10µg), Meropenem (MRP10µg) and Ceftriaxone (CRO,30µg). The results of the diameter inhibition zone were compared with standard tables for standard *E.coli* according to CLSI[10].

2.4. DNA Isolation and PCR Amplification of genes from *E. coli* Isolates

Genomic DNA was extracted for all isolates by Bacterial DNA MiniPrep™ Zymo Inc. Catalog No. D6005. Primers of genes from IDT (Integrated DNA Technologies company, Canada) are the sequences of the primers for genes as in Table (1). Polymerase chain reaction (PCR) was performed on 40 isolates of *E. coli*.The mixture contents as in Table (2) with a final volume of 25 µl and DNA concentration ranging (from 85-295 ng/µL).Amplification for each gene was done according to Table (3). Electrophoresis was carried out by using agarose gel (1.5%) to visualize the amplification of PCR products which staining by Red safe stain.

Table 1: The primers for *blaVIM* and *blaNDM* genes[11]

Genes	Primer Sequence	Tm (°C)	GC (%)	Product size
blaVIM	F:5'-GGTGTTTGGTCGCATATCGCAA- 3' R:5'-ATTCAGCCAGATCGGCATCGGC-3'	66 65	60 60	520bp
blaNDM	F: 5'-GGTTTGGCGATCTGGTTTTC - 3' R: 5'- CGGAATGGCTCATCACGATC- 3'	68 70	60 62	624bp

Table 2: The mixture of PCR reaction

Contents	Volume
Taq PCR PreMix (2X) (iNtRON, Korea),	5µl
Forward primer10 picomoles/µl	(1 µl)
Reverse primer10 picomoles /µl	(1 µl)
DNA sample	1.5µl
Free nuclease water	16.5µl
Final volume	25 µl

Table 3: Conditions of PCR amplification

Stages	Tm (°C)/Time	cycles
Initial denaturation	94°C/5 min.	1 cycle
Denaturation 2	94°C/45sec	35 cycles
Annealing	60°C/45sec	
Extension	72°C/45sec	
Final extension	72°C/7 min.	1 cycle

3. Results

The forty tested isolates clarified different antibiogram resistance. *E. coli* appeared gram-negative coccobacilli or bacilli shaped in microscopic examination The biochemical tests appeared positive results for Methyl red, Indole, Catalase, and EMB agar. Citrate utilization, Voges-Proskauer, and Oxidase were negative results ~~for~~ as shown in Figures (1-4). The Vitek 2 Compact results showed ~~for~~ isolates of *E.coli* with a probability of 95% (Figure 5).

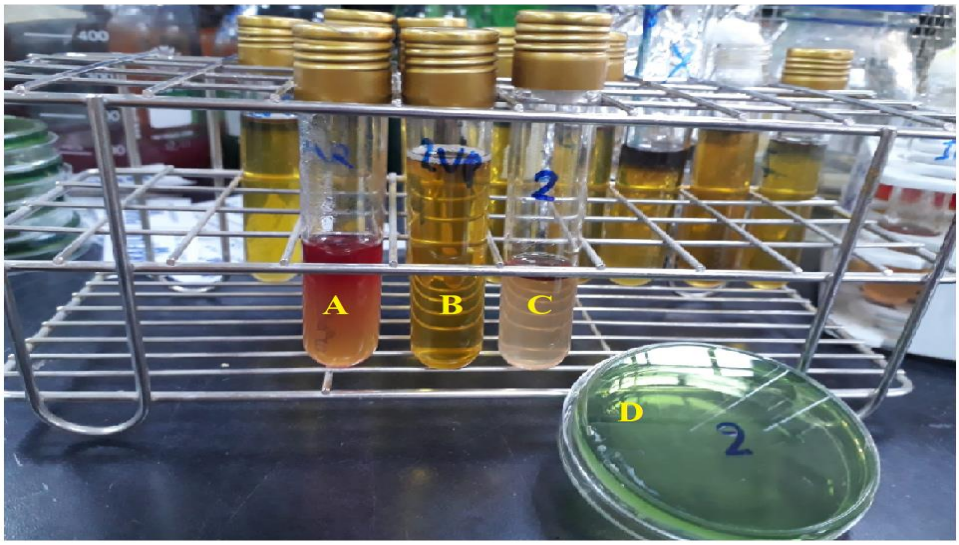

Figure 1: Biochemical test of *E.coli* :A-Methyl red; B-Voges-Proskauer;C-Indole; D-Citrate utilization

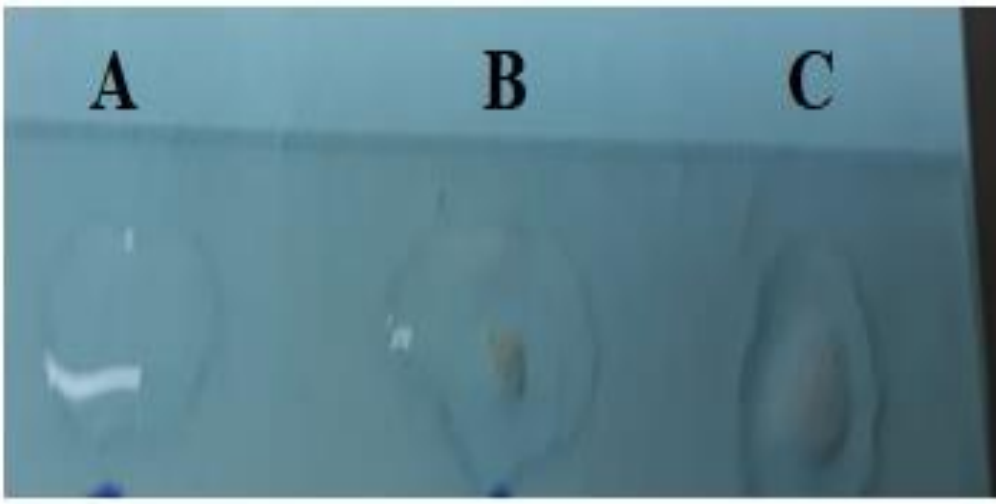

Figure2: Catalse test of *E.coli* :A-Control;B-Neagative result;C-Positive result

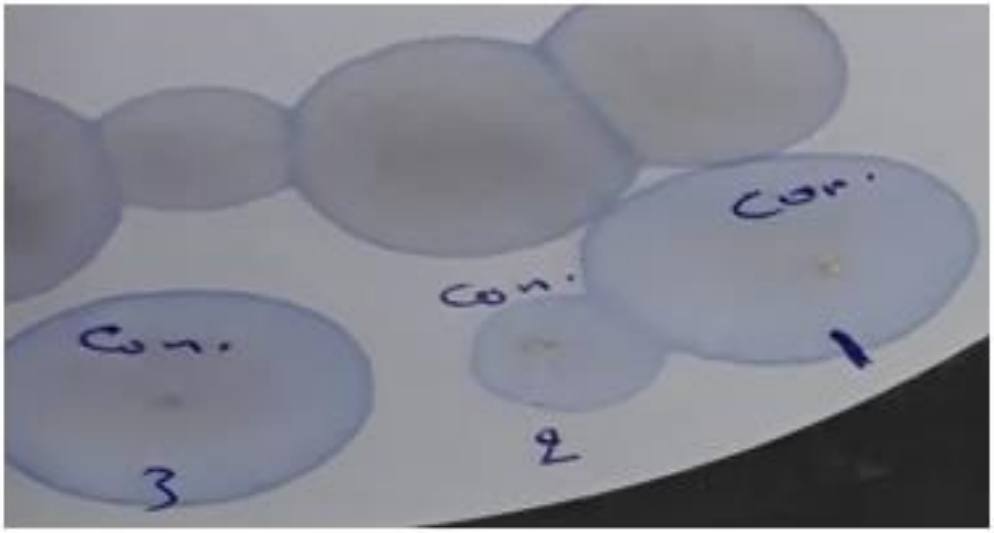

Figure 3: Oxidase test of *E.coli*

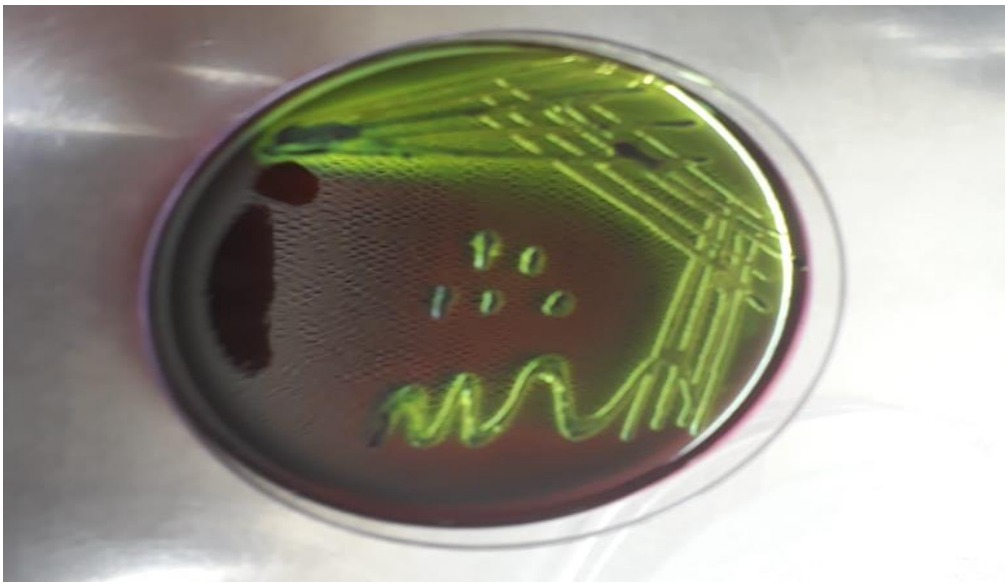

Figure 4: EMB agar of *E.coli*

bioMérieux Customdr: Microbiology Chart Report Printed May 10, 2021 21:34 CDT

Patient Name:
Location:
Lab ID: 3
Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:
Collected:

Comments:

Identification Information	Analysis Time:	3.90 hours	Status:	Final
Selected Organism	95% Probability	Escherichia coli		
	Bionumber:	0405610754526610		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 5: VITEK2Compact identification of *E.coli*

Depending on the results of the disk diffusion method as shown in Figure(6). Most *E .coli* isolates were detected to be resistant to one or more antibiotics tested. The results of the current study for antimicrobial sensitivity of *E .coli* have shown susceptibility to at least one or ≥3 antimicrobial categories, it was considered MDR(Multidrug-resistant). The strongest resistance to all antibiotics appeared in isolates E2Z, E8Z, E13Z E17Z, E31Z, and E37Z Also, E18Z, E20Z, and E25Z were resistant to six antibiotics and the other *E. coli* isolates were less resistant to six antibiotics as shown in Figure (6) which represent number antibiotics resistance of *E. coli* isolates.

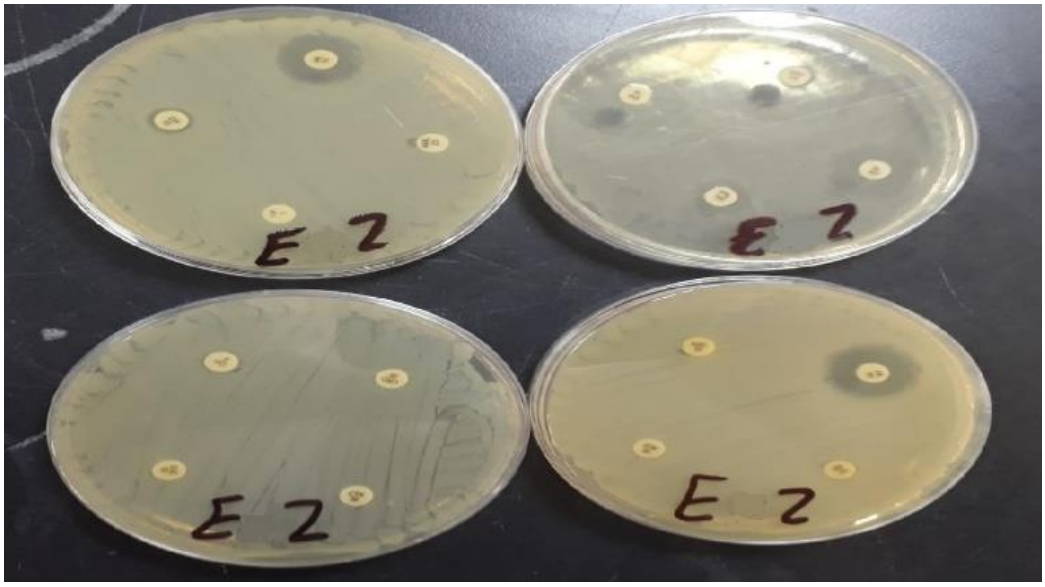

Figure 6: Sensitivity test of *E.coli* isolates

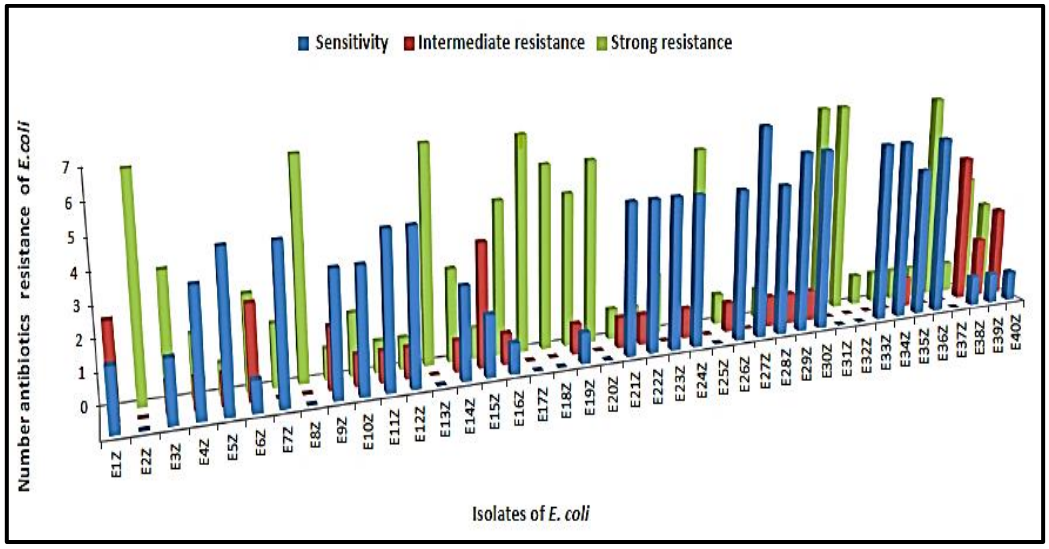

Figure7: Resistant-antibiotics of *E. coli* isolates (Blue column represents the sensitivity of antibiotics, the Red column represents intermediate resistance for 3 antibiotics and the Green column represent strong resistance for < 3 antibiotics

The results observed high resistance towards many groups of antibiotics, these results revealed high resistance to β-lactam groups like aminopenicillins and extended spectrum were AMC 82.5% (33/40), PRL 62.5% (25/40), AM 55% (22/40) and moderate resistance to CRO 45% (18/40) whereas little resistance to ATM 27.5% (11/40) and carbapenems including IPM 5% (2/40) and MRP 5% (2/40) (Figure 7). Concerning the results of resistance genes, it has appeared two isolates that have resistance gene *blaVIM* and one isolate has *blaNDM* gene (Figures 8 and 9). The distribution percentage for each gene *blaVIM* was 5% (2/40) and *bla NDM* was 2.5% (1/40).

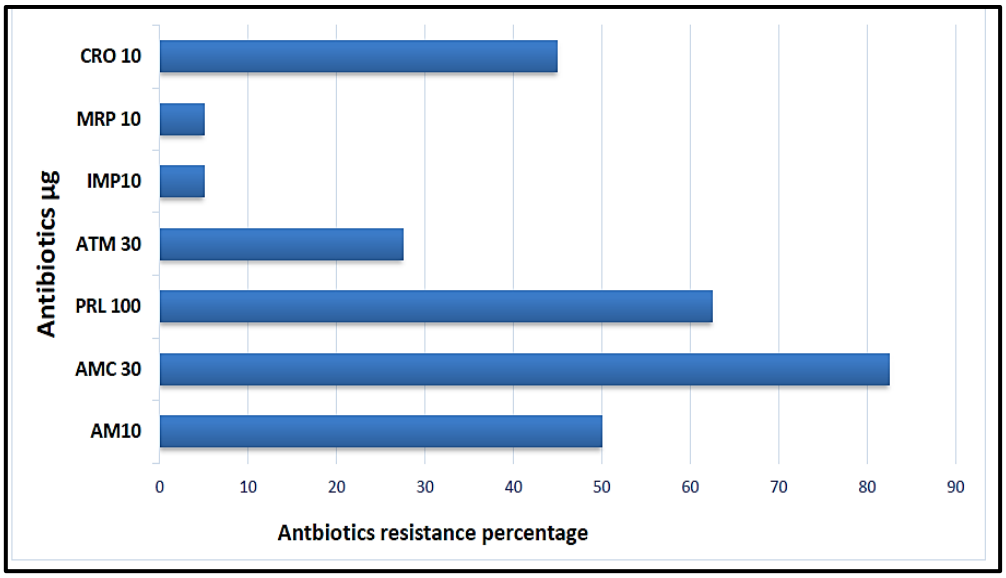

Figure 8: The percentage of antibiotic resistance for all *E.coli* isolates (Ampicillin: AM, Amoxicillin /Clavulanic acid: AMC; Piperacillin: PRL, Aztreonam: ATM, Imipenem: IPM, Meropenem :MRP and Ceftriaxone: CRO)

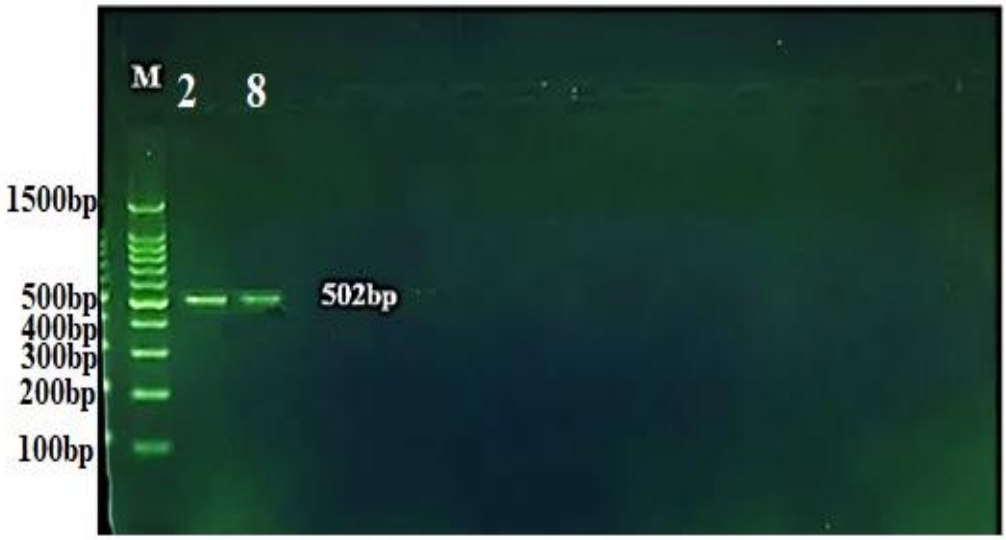

Figure 9: Electrophoresis of PCR amplicon (502 bp) for *blaVIM* gene for multiple isolates of *E.coli* but the gene appear in isolate number 2 and 8(2% agarose in TBE 1x at 75 volt/cm^2) for 1:15 hr. DNA ladder (1500bp).

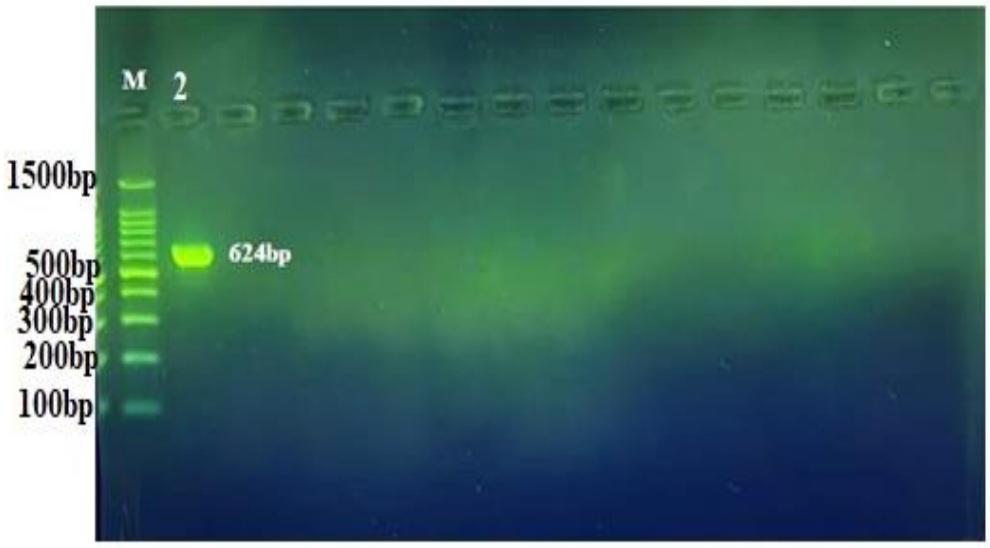

Figure 10: Electrophoresis of PCR amplicon (624bp) for *blaNDM* gene for multiple isolates of *E.coli* but the gene appears in isolate number2 (2% agarose in TBE 1x at 75 volt/cm^2) for 1:15 hr. DNA ladder (1500bp).

4. Discussion

Most *E .coli* isolates were detected by disk diffusion method in the current study to ~~be~~ phenotypes resistant against one or more antibiotics tested that in agreement with [12] who define MDR (Multidrug-resistant) and XDR (Extensively drug-resistant) in *E .coli* isolates and the present results agreed with another study [13,14]. Although in the present study, it was detected the resistance gene in a few bacterial isolates that have phenotypes resistant to most antibiotics studied. Where the current study agreed with another study that detects resistance genes(*blaNDM* and *blaVIM*) the resistance mechanism was not diagnosed by any of the resistance genes primers applied in the study for *E.oli* isolates [11].

According to these findings, it has appeared this resistance of antibiotics in isolates for all stations. The reason for the resistance of this group with *E. coli* is that the secretion of the enzyme beta-lactamase inactivates this antibiotic. Where there may be resistance genes other than those that have been studied that can confer resistance to the studied bacterial isolates. Bacteria may also develop mechanisms to reduce drug penetration into their cells, therefore *E.coli* has many mechanisms of resistance to β- lactam antibiotics like loss of porin and efflux pumps [15-17]. The findings ~~were~~ agreed with other previous research which illustrated high resistance of *E. coli* to penicillin and most cephalosporins antibiotics [18] and agreements with other studies in low resistance to some antibiotics [19]. Further, the current study agreed with one of study that appeared to the resistance of *E.coli* from an aquatic environment to amoxicillin and some cephalosporins [20].

The findings in the current study agreed with Hoelle *et al*. [21] showed ~~in~~ their study there was found *blaVIM* in *E. coli* isolates with 55% positive and the IMP gene in isolates was 1% positive whereas the presence of *blaNDM* gene in isolates was negative. These differences in the finding may be caused by the variations in the study period and sample size [11]. Moreover, the recent study targeted a limited number of resistance genes also there are else mechanisms that cause resistance like mutations and the absence of porin [22]. Besides, the transmission of antibiotic-resistant genetic factors from commensal strain to pathogenic strain may happen in reservoirs so those groups mix together, involving wastewater treatment plants[23]. Natural

environment (water, wastewater treatment plants, soils) was deemed to be genetic reactors of bacteria, where efficient genetic interchanges happen among various bacteria [24]. Genes that encode antibiotic-resistant are related to mobile genetic factors like plasmids and transposons, which may be interchanged among bacteria pertinence to various phylogenetic descent [25] . Several studies have appeared to multidrug- resistance of *E. coli* strains exists in the environment, this shows the potential risks to public health that can obtain from population activities [26,27].

5. Conclusion

It was diagnosed that 40 out of 67 isolates of *E. coli* from three sites in Tigris River, these isolates appeared resistant to β-lactam group antibiotics whereas ~~it~~ was shown ~~the~~ little resistance to *E. coli* to carpamenes antibiotics (IMP, MER). The presence carpamenes genes were *blaVIM* (5%)and *blaNDM* (2.5%) .

Ethics approval and consent to participate

The study protocol was approved by Environment and Water Directorate /Ministry of Science and Technology.

Competing interests

The authors declare that no conflict of interest.

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