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Detection of resistance genes (gyrA,qepA,drf1,drf17) for *E.coli* in Iraqi aquatic environment

Zinah Mohammed Mahdi [1], ***Shahad Husham Mahmood*** [2], ***Noor Nihad Baqer**** [1]

[1]Ministry of Science and Technology, Environment and Water Directorate, Baghdad, Iraq.
[2]Department of Biotechnology, College of Applied Science, University of Fallujah, Al Anbar, Iraq.
*Corresponding Author.

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Abstract

The control of water represents the safe key for fair and optimal use to protect water resources due to human activities, including untreated wastewater, which is considered a carrier of a large number of antibiotic-resistant bacterial species. This study aimed to investigate the prevalence of antibiotic-resistance to *E. coli* in Tigris River by the presence of resistance genes for aminoglycoside(*qepA*) ,quinolone (*gyrA*), and sulfa drugs(*dfr1* ,*dfr17*) due to the frequent use of antibiotics and their release into wastewater of hospitals. Samples were collected from three sites on Tigris River: S1(station wastewater in Adhamiya), S2 (station wastewater in Baghdad Medical city hospital), S3 (station wastewater in Abu Nuwas) from February-July 2021. Out of 67 isolates of bacteria, only 40 isolates of *E. coli* were detected by Vitek2. The antibiotic-resistance was estimated by the disk diffusion method. All *E.coli* isolates were tested against 6 antibiotics. The results showed the high resistance antibiotic of *E. coli* against Ceftazidime 70%, with intermediate resistance to Cefotaxime 47.5%, and low resistance to the sulfa drugs as Trimethoprim 27.5% and quinolones antibiotics as ciprofloxacin 17.5%, aminoglycosides as Amikacin and Gentamycin 5% and 7.5%. Moreover, the results revealed that *gyrA* gene was detected in 4 isolates (10%) while *drf1* and *drf17* genes were in 2 isolates of each gene (5%).Whereas *qepA* gene has not appeared in isolates. In conclusion, the isolates of *E.coli* from the Tigris River showed low resistance to sulfa drugs and quinolones , aminoglycosides. The resistance genes (*gyrA,drf1,drf17*) were detected in a few isolates which may be explained by the horizontal transfer of plasmids that carried genes and their distribution among the family Enterobacteriaceae.

Keywords: aquatic environment**,** *drf1* gene ,*drf17* gene, *E.coli*, *gyrA* gene.

Introduction

Aquatic environment pollution remains a foremost public health hazards, and symbolizes an important reservoir of releasing antibiotic-resistance bacteria due to human activities, including untreated wastewater, which is considered a carrier of a large number of antibiotic-resistance bacterial species [1]. The excessive use of quinolones and sulfa drugs, in different proportions, according to the health institutions that use antibiotics, and the geographic region, has led to the emergence of new types of resistance to antibiotics. Therefore, studying the genes responsible for resistance to antibiotics against

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bacteria is important, including the genes responsible for resistance to quinolone and sulfa drugs. *E. coli* is one of the intestinal microbiomes for more than 90% of people [2], *E. coli* represents commensal bacteria in the intestine as microflora of animals and humans, some *E. coli* strains can cause disease in humans, mammals, and birds[3].*E. coli* is one of the bacteria that have recently become resistant to different classes of antibiotics through various mechanisms including efflux pump, and decreased cell permeability[4]. They are highly adaptable bacteria that can survive and grow in outdoor environments, involving the river [5]. Quinolones are a class of synthetic and broad-spectrum antibacterial agents that interfere with bacterial DNA gyrase (bacterial topoisomerase II) and topoisomerase IV, preventing the supercoiling of DNA, and ultimately promoting DNA strand breakage [6,7].Resistance to fluoroquinolone (ciprofloxacin), which is of the hydrophilic type, appears by plasmid efflux controlled by the *QepA* pump, Trimethoprim resistance in gram-negative bacteria occurs through the horizontal transfer of *dfr* gene that is responsible for coding for folic acid reductase [8, 9]. Poirel *et al* [10] showed in their study the *dfr* , *dfrA7*, *dfrA12* and *dfrA17* genes were found in 15 isolates of non-pathogenic and resistant to *E. coli* that were found in food and animals and of human origin. Also, Alwash and Al-Rafyai. [11] appeared in their study that the spread of antibiotic resistance of *E. coli* isolates was associated with the proximity of Marjan Hospital for Internal Medicine and Cardiology to the river , where there was a direct discharge of wastewater in Hilla. The Hilla River showed a high prevalence of antibiotic-resistance to *E. coli* with MDR rates of 80.3%.

The study aimed to investigate the prevalence of antibiotic-resistance to *E. coli* in Tigris River by the presence of resistant genes for aminoglycoside (*qepA*) quinolone (*gyrA*) and sulfa drug(*dfr1* ,*dfr17*) , due to the frequent use of antibiotics and their release into wastewater of hospitals.

Materials and Methods

Samples collection

The samples were collected from three sites of the station including S1 (station wastewater in Adhamiya), S2 (station wastewater in Baghdad Medical city hospital) which was thrown into the river and S3 (station wastewater in Abu Nuwas). A volume of (1000ml) was collected samples to estimate the rate of river pollution by medical wastewater. The period collection was from February -July 2021 with three times for each month so the total samples were 21 samples.

Identification 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. Plates were incubated for 48 hours at 37°C. *E.coli* colonies were initially identified by macroscopic and microscopic methods (gram stain).Further biochemical tests (oxidase, catalase, methyl red, indole test, Voges-Proskauer, and Citrate utilization) were used followed by VITEK2 Compact system. The isolates were subcultured and were stored at 4˚C for further analysis[12].

Antibiotic sensitivity screening

The antibiotic sensitivity of *E. coli* was examined according to Assefa *et al*. [13] applying the Kirby-Bauer method with Muller Hinton agar. Transported(3-5) colonies from MacConkey agar after incubation 18-22 hour at 37 ˚ C into a tube containing 5 ml of normal saline (9%), and turbidity was estimated with 0.5 McFarland solution is equivalent to 1.5×10^8. In the sensitivity test a total of 6 antibiotics were utilized including Ceftazidime (CAZ,30µg), Cefotaxime (CTX,30µg) as β-lactam; Amikacin (AK,30µg), Gentamycin (GN,10µg) aminoglycosides ,Ciprofloxacin (CIP,5µg) as fluoroquinolones .Finally, Trimethoprim (TM,5µg) belonged to the Sulfa drug (Fig.1). The results were compared with standard tables for standard *E.coli* according to CLSI [14].

Genetic analysis for genes from *E. coli* isolates

The whole genome DNA of bacteria isolates was extracted using Bacterial DNA MiniPrep™ Zymo Inc. Catalog No. D6005. Primers for the genes from IDT (Integrated DNA Technologies company, Canada).Moreover, the concentration and purity of DNA estimated by nanodrop spectrophotometer

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(Nabi /Korea), concentration was 77-295 ng/µL and purity was 1.8-2. Primers for genes were chosen according to [15,16] as shown in Table 1. Polymerase chain reaction (PCR) was conducted on 40 isolates of *E. coli*. The PCR reaction contents involved Taq PCR PreMix (2X) 5µl (iNtRON, Korea), forward and reverse primers 10 picomoles/µl (1 µl), DNA sample was 1.5µl, free nuclease water was 16.5µl with final volume 25 µl. PCR condition for each gene is shown in Table 2. Electrophoresis was conducted by applying agarose gel (1.5%) to observe the amplicons of PCR staining by Red safe stain.

Table 1.The primers for genes

Genes	Primer Sequence 5` -3`	Tm (°C)	GC (%)	Product size
gyrA	F-5` AAATCTGCTCGTGTCGTTGG-3`	68	60	349bp
	R- 5`GCCATACCTACAGCAATACC-3`	68	60	
qepA	F- 5`AACTGCTTGAGCCCGTAGAT- 3`	68	60	596bp
	R - 5`GTCTACGCCATGGACCTCAC -3`	72	64	
dfr1	F- 5`TGGTAGCTATATCGAAGAATGGAGT-3`	70	70	425bp
	R- 5`TATGTTAGAGGCGAAGTCTTGGGTA-3`	72	72	
dfr17	F- 5`GAAAATATCATTGATTTCTGCAGTG - 3`	67	66	465bp
	R-5`TTTTTCCAAATCTGGTATGTATAATTT-3`	65	66	

Table 2. PCR condition for genes

Genes	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
gyrA	94°C/5min 1cycle	94°C/45sec 35cycle	52°C/45sec 35cycle	72°C/45sec 35 cycle	72°C/7min 1 cycle
qepA	94°C/5 min 1 cycle	94°C/45sec 35cycle	54°C/45sec 35cycle	72°C/45min 35cycle	72°C/7min 1cycle
drf1	94°C/5 min 1 cycle	94°C/45sec 35cycle	52°C/45sec 35cycle	72°C/45min 35cycle	72°C/7min 1cycle
drf17	94°C/5 min 1 cycle	94°C/45sec 35cycle	55°C/45sec 35cycle	72°C/45min 35cycle	72°C/7min 1cycle

Statistical analysis:

MedCalc Software Ltd 2023 online was used for statistical analysis by applying chi-square when a p-value of less than 0.05 there are significant differences while p-value of more than 0.05 there are no significant differences. It was used three replicas in this study.

Results and Discussion

Identification of *E. coli*

It was diagnosed 40 isolates of *E.coli* out of 67 bacteria species by biochemical tests that showed positive for Methyl red, Catalase, Indole and negative for Citrate utilization, Voges-Proskauer,and Oxidase. The Vitek 2Compact outcomes appeared with a probability of 95% for isolates of *E.coli*. The results of antibiotics resistance showed different resistance for the forty isolates. The results of antibiotics sensitivity for *E .coli* appeared its resistant to one or ≥3 antibiotics categories,it was represented MDR (Multidrug-resistant) which was represented in 7 isolates that were resistant to four antibiotics .The highest resistance to all antibiotics appeared in two isolates representing XDR (Extensive drug-resistant) and the rest of *E. coli* isolates were less resistant to antibiotics (Fig.1).

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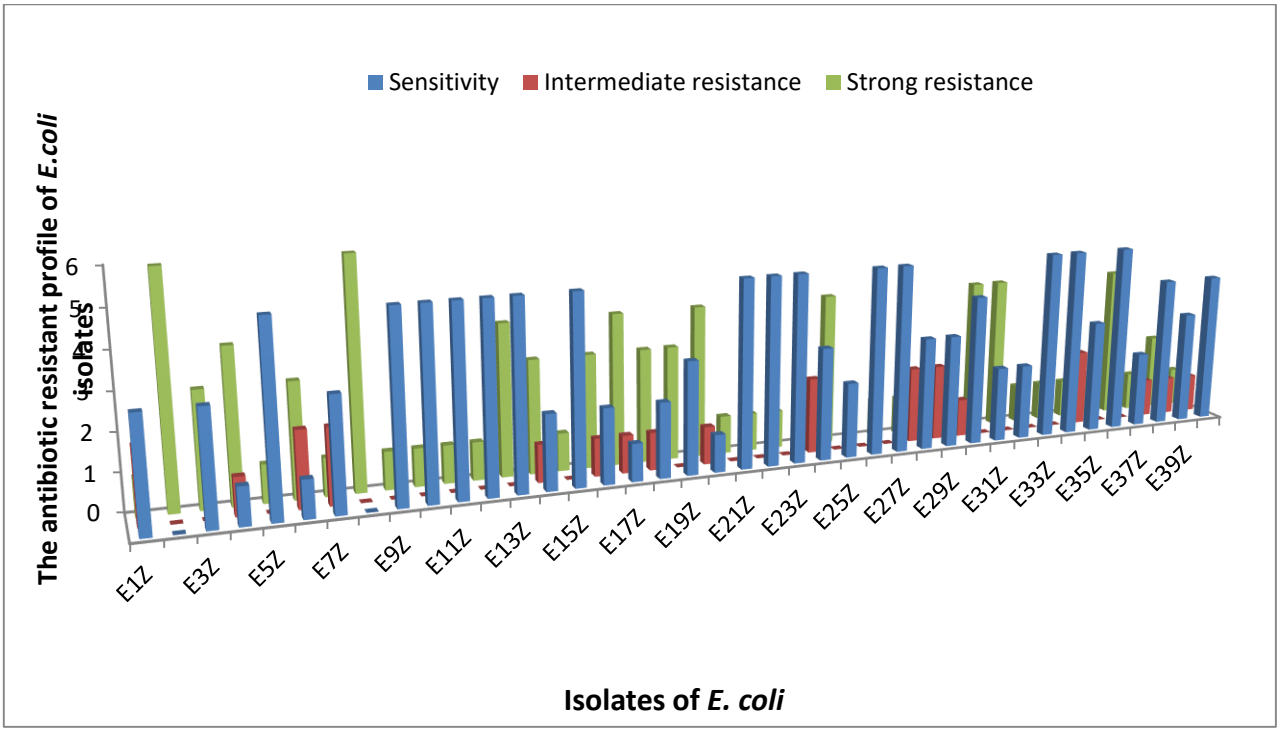

Figure 1. Antibiotics resistant profile of *E. coli* isolates (Blue color represent sensitivity of antibiotics ,Red color represent intermediate resistance for 3 antibiotics and Green color represent strong resistance for more than 3 antibiotics

The number of isolates in each station was shown in Table 3, in which the high percentage was in station wastewater of Baghdad Medical City Hospital (S2) at a percentage of 50% with significant differences among the stations of wastewater P<0.05.

Table 3. The distribution of *E. coli* isolates in 3 stations

Stations	No. of isolates	Percentage(%)
Adhamiya (S1)	9	22.5
Baghdad Medical City Hospital(S2)	20	50
Abu Nuwas (S3)	11	27.5
Total	40	100
P value	<0.05	

Antibiotic sensitivity screening

The outcomes showed the high resistance antibiotic against Ceftazidime 70%(28/40 isolates) , moderate resistance against Cefotaxime 47.5%(19/40 isolates) ,and low resistance against sulfa drug (Trimethoprim) 27.5% (11/40 isolates) and quinolones antibiotics (ciprofloxacin)17.5%(7/40 isolates) , aminoglycosides (Amikacin and Gentamycin) 5% and 7.5% respectively (2/40 and 3/40 isolates respectively) as shown in Fig. 2 .

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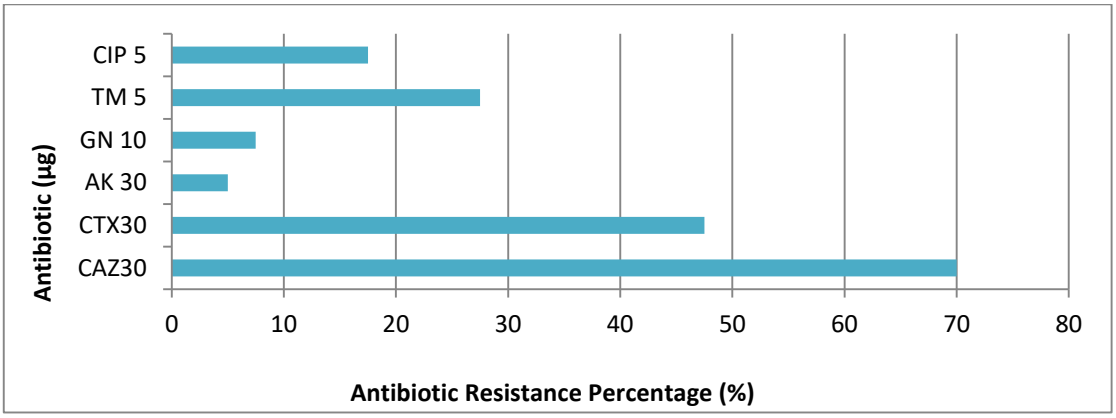

Figure 2. The percentage of antibiotic resistance *E.coli* isolates (Ciprofloxacin: CIP 5µg, Trimethoprim: TM 5µg; Gentamycin: GN 10 µg, Amikacin: AK30 µg; Cefotaxime: CTX 30 µg; Ceftazidime: CAZ 30 µg)

Genetic analysis for genes from *E. coli* isolates

Concerning the outcomes of resistance genes, four isolates have *gyrA* genes and two isolates for each of *drf1* and *drf17* genes (Fig. 3 ,4,5). The distribution percentage for *gyrA* gene was 10% (4/40 isolates), and 5% (2/40 isolates) for each of *drf1* and *drf17*.While *qepA* gene has not appeared in isolates.

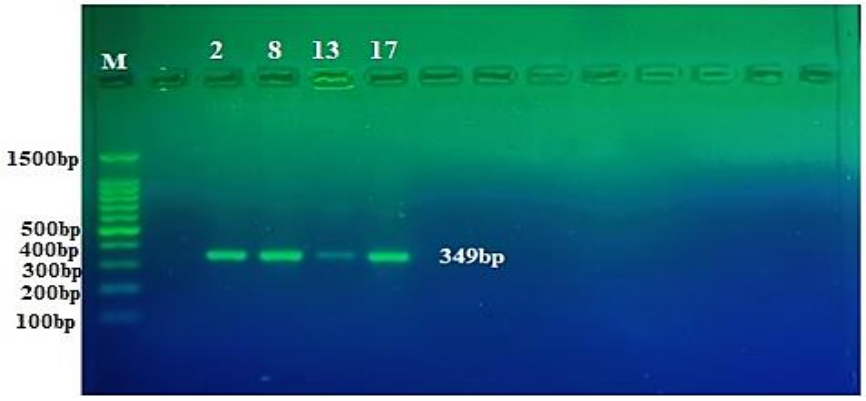

Figure 3. PCR product (349bp) for *gryA* gene of *E.coli* isolates number (2,8,13,17) (electrophoresis in 1.5% agarose in TBE 1x at 75 volt/cm^2) for one hour(DNA ladder 1500bp).

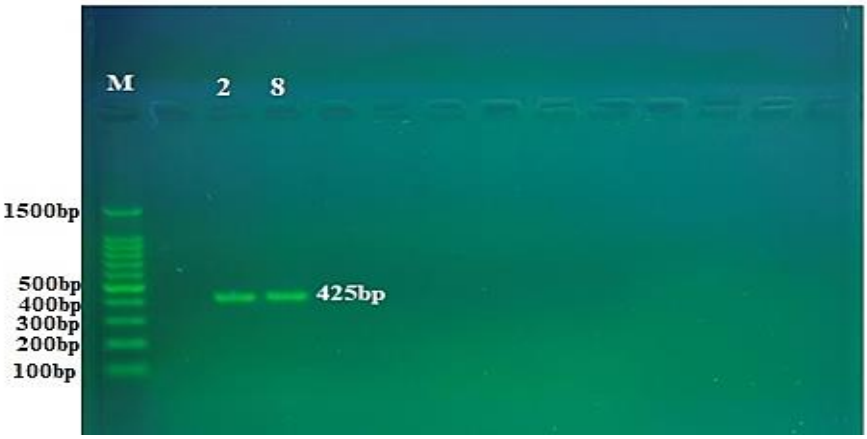

Figure 4. PCR product (425bp) for *drf1* gene of *E.coli* isolates number (2,8) (electrophoresis in 1.5% agarose in TBE 1x at 75 volt/cm^2) for one hour(DNA ladder 1500bp).

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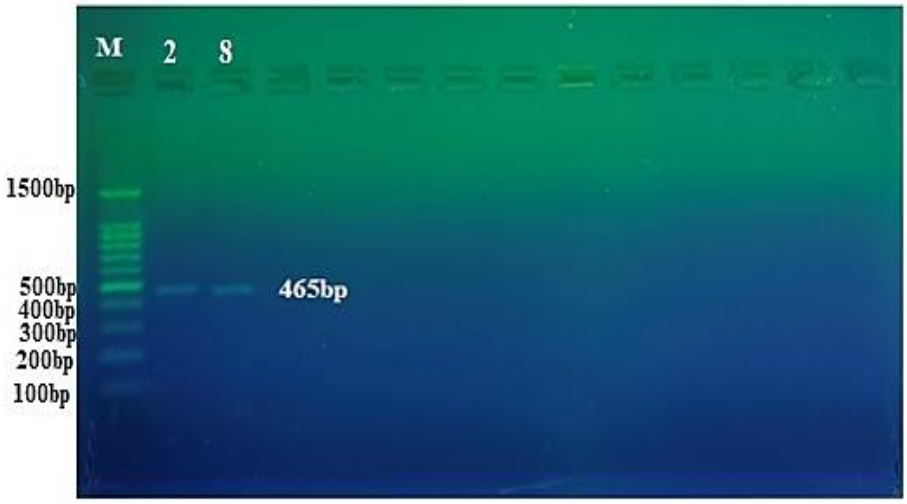

Figure 5. PCR product (465bp) for *drf17* gene of *E.coli* isolates number (2,8) (electrophoresis in 1.5% agarose in TBE 1x at 75 volt/cm^2) for one hour(DNA ladder 1500bp).

Discussion

Wastewater considers the reservoir and environmental source of antibiotic-resistance. It has been suggested to be a point for horizontal transmission of genes, enabling the diffusion of antibiotic-resistant genes among different bacterial spp[17]. In the current study, one or more than 3 of *E .coli* isolates were resistant to antibiotics , it was shown that seven isolates resistant to four antibiotics represented MDR. While two isolates were highly resistant to all antibiotics representing XDR.

So this study agreed with other study found that most *E.coli* isolates were MDR and XDR respectively [18].In one study conducted to know the resistance of *E. coli* isolated from urine, it appeared high resistance with a percentage 80.56 % of multidrug *E. coli* isolates also showed high resistance against β-lactamase [19].

Besides, in the current study appeared *E .coli* isolates in all stations due to the waste excreted from the Baghdad hospitals in the Tigris river. Where another study conducted in Iraq, *E.coli* was shown to be the majority as MDR at 78.5% and XDR at 22.5% [11] . The results of the current study were similar to another study which observed that *E.coli* isolates a high resistance against Ceftazidime and a little resistance to Amikacin and Gentamycin [20].

Meanwhile, one study in Iraq reported spreading resistance of *E. coli* to antibiotics involving: cephalosporins, penicillins, imipenem, fosfomycin, and aminoglycosides[11]. Also, other studies showed the main antibiotic resistance types of E. coli noticed in the current study were prevalent in aquatic environments[21,22]. This resistance paradigm is real because, due to a lack of awareness and education, antibiotics can be purchased without a prescription in Iraq. Furthermore, these antibiotics are used to promote growth in animal farming and agricultural applications. Thus, wastewater discharges and compost effluents may contain antibiotics that pollute the aquatic environment [11].

Antibiotic resistance is activated by several mechanisms, either through genetic resistance, including the efflux mechanism or through acquired mechanisms and mutations in the genetic material and transferring by plasmids[23]. *E. coli* isolates appear resistant to quinolone (Ciprofloxacin).This can occur by either chromosomal mutations in DNA gyrase genes or the acquisition of transferable plasmid-mediated quinolone resistance (PMQR) genes [24]. Generally, plasmid-mediated resistance is a rising concern and can be transferred among various bacterial species and stimulated for transferring into other pathogenic species through horizontal gene transfer (HGT) [25].

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Bacterial resistance to trimethoprim occurs as a result of increasing production of dihydrofolate reductase (DHFR) in the antibiotic's targeted promoter via promoter mutation, and *E. coli* resistance to sulphonamide antibiotics occurs as a result of massive production of the enzyme p-aminobenzoic acid (PABA), where sulphonamides mimic PABA. So antibiotics have difficulty linking to the target. Meanwhile, trimethoprim resistance in *E. coli* is caused by chromosomal mutations in the *dhfr* or *dhps* genes, which cause antibiotic resistance[26].

In the current study had been observed four isolates of *E.coli* had resistance gene *gyrA* and two isolates had *drf1 and drf17* genes. There is no previous Iraqi study about the prevalence of *gyrA* and *dfr* genes in the aquatic environments. Whereas, one Iraqi study observed the presence of *gyrA* gene in clinical isolates of *E.coli*[27].

Also, another study in Iran observed resistance of quinolone that related to the mutation in *gyrA* in uropathogenic *E.coli* isolates[28].Moreover, it was observed the distribution of *dfrA1* gene in uropathogenic *E.coli* that was resistant to Trimethoprim[29].

It was considered that quinolone resistance was only chromosomally regulated by nucleotide substitution in the quinolone resistance-determination location of DNA gyrase (*gyrA* and *B*) and also topoisomerase IV, which are the major target molecules for quinolones. Besides, the PMQR gene represents *qnrA*, which encodes a protein that conserves topoisomerase type II from quinolone [30]. The reason for the resistance due to a mutation in their target proteins; DNA gyrase and topoisomerase IV ,and changes in the permeability of the cell membrane [31,32]

A sulfa drug such as trimethoprim appeared to have a low resistance to *E. coli*, caused by modifications in the target enzyme dihydrofolate reductase (*dfr*) encoded by *dfr*-genes[33]and can bacteria use folic acid that they get from the environment to undergo changes in their metabolism, allowing them to acquire resistance to trimethoprim and sulfonamides [34] Moreover, the *E. coli* resistance to aminoglycosides, which include Amikacin and Gentamycin, was low. The reason to penetrate the inner cytoplasmic membrane of *E. coli* and bind the 30S subunit of the bacterial ribosome inhibiting [35] Also, one mechanism of PMQR was the covalent modulation of particular quinolones such as ciprofloxacin by a plasmid-encoding various of aminoglycoside acetyltransferase. another mechanism of PMQR is the quinolones efflux via the efflux pump-encoded genes such as *qepA* and *oqxAB* [30]. Furthermore, the presence of determinants PMQR on mobile genetic factors may result in their distribution within the family Enterobacteriaceae [15].

Numerous factors might have been involved in the release of resistance in the environment. Insufficient sanitation facilities [36] Indiscriminate use of antibiotics, and lack of well-managed sewerage systems are important contributing factors [37,38].

Conclusion

It was found that wastewater from Baghdad Medical City Hospital had more *E. coli* number, where seven isolates of *E .coli* were MDR and two isolates were XDR. The *gyrA* genes were found in four isolates. While two isolates had *drf1*, and *drf17* genes.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.

- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in Ministry of Science and Technology.

Authors' Contribution Statement

All authors contributed to study conceptualization; Data curation; Investigation; Methodology; Project administration; Resources; Software. Z. M. M. and S. H. M.: Wrote- original draft and editing. N. N. B. Wrote- original draft,Writing - review and editing.

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الكشف عن جينات المقاومة (*gyrA*، *qepA*، *drf1*، *drf17*) لبكتريا الاشريشيا القولونية في البيئة المائية العراقية

زينة محمد مهدي[1]، شهدهشام محمود[2]، نور نهاد باقر[1]

[1]وزارة العلوم والتكنولوجيا، دائرة البيئة والمياه، بغداد، العراق.
[2]قسم التقانة الاحيائية، كلية العلوم التطبيقية،جامعة الفلوجة، الانبار، العراق.

الخلاصة

يمثل التحكم في المياه المفتاح الآمن للاستخدام العادل والأمثل لحماية موارد المياه نتيجة الأنشطة البشرية ، بما في ذلك مياه الصرف الصحي غير المعالجة ، والتي تعتبر حاملة لعدد كبير من الأنواع البكتيرية المقاومة للمضادات الحيوية. هدفت هذه الدراسة إلى التحقق من انتشار بكتريا *E. coli* المقاومة للمضادات الحيوية في نهر دجلة من خلال وجود جينات مقاومة أمينوغليكوزيد وكينولون وسلفا بسبب الاستخدام المتكرر للمضادات الحيوية وإطلاقها في مياه الصرف الصحي للمستشفيات. تم التعرف على 40 عزلة فقط من أصل 67 عزلة من البكتريا وتم الكشف عنها بواسطة Vitek2. تم تقدير مقاومة المضادات الحيوية بواسطة طريقة الانتشار القرصي. اختبرت جميع عزلات الإشريشيا القولونية ضد 6 مضادات حيوية. كانت العزلات شديدة المقاومة لـ السفتازديم 70 ٪ ، مع مقاومة معتدلة لـ السيفوتكسام 47.5٪ ، ومقاومة منخفضة لـترايمثبريم 27.5٪ ،السايبروفلوكسسين 17.5٪ ، 17.5٪ ، أمينوغليكوسيدات مثل امكايسين و الجنتاميسين5 ٪ و 7.5٪. أوضحت النتائج أن جين *gyrA* تم اكتشافه في 4 عزلات (10٪) بينما تم الكشف عن الجين drf1 و drf17 في 2 عزلة (5٪) من كل جين. في الختام ، أظهرت عزلات الإشريشيا القولونية من نهر دجلة مقاومة منخفضة لعقار السلفا والكوينولينات والأمينوغليكوسيدات. تم الكشف عن الجينات المقاومة (*gyrA* و *drf1* و *drf17*) في هذه العزلات والتي يمكن تفسيرها بالنقل الأفقي للبلازميدات بين العائلة المعوية.

الكلمات المفتاحية: البيئة المائية ،وجين *drf1* ، و جين *drf17* ،وبكتريا القولون ،و جين *gyrA*.