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PREVALENCE OF ANTIBIOTIC-RESISTANT *ESCHERICHIA COLI* IN SOME DRINKING WATER TREATMENT PLANTS IN BAGHDAD CITY

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ABSTRACT

This study was aimed to investigate antibiotic-resistant genes in *Escherichia coli* found in drinking water is a global public health concern. The isolation and identification of *Escherichia coli* in three drinking water treatment plants in Baghdad city was carried out using a mix of biochemical and molecular analysis on selective medium. LacZ gene amplification by Polymerase Chain Reaction was carried out for molecular identification, and the study showed that lacZ was present in each isolate. The molecular method was used to confirm the culture method's findings. Both the molecular diagnostic and the culture method produced the same results. Applying the McFarland standard and the Kirby-Bauer disk diffusion method, the antimicrobial susceptibility test was done against 14 distinct antibiotics. The most commonly found antibiotics to which the isolates were resistant (80%–100%) were tetracycline, erythromycin, and ampicillin, with a significant difference (p = 0.0001). Multidrug resistance was present in all of the isolates, as they exhibited resistance to a minimum of three agents across the nine antibiotic classes tested. Escherichia coli is defined as showing resistance to three or more classes of antibiotics. The blaM gene was detected in *E. coli* isolates 12/36 (33.3%) that produce extended-spectrum beta-lactamase (ESBL).

Keywords: **diagnostic gene , resistant gene, pollution indicator bacteria ,**
***Part of M.Sc. thesis for the [1st]author**

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INTRODUCTION

The dissemination of antibiotic-resistant bacteria (ARBs) and antibiotic-resistant genes (ARGs) in The environment is affected by the improper use of antibiotics in veterinary and human clinics, the improper disposal of antibiotics that have expired (Mahdi *et al*,2023), the increased discharge of pharmaceutical industrial wastewaters, and the decreased effectiveness of drinking water and wastewater treatment facilities (Bortolotti *et al* ,2019). Reducing antibiotic use alone won't stop ARBs and ARGs from finding their way into the environment and drinking water (Collignon & McEwen, 2019). Antibiotic resistance in bacterial species can manifest itself in an intrinsic way, which is connected to innate structural or functional traits that are shared by the species regardless of prior antibiotic exposure (Al-Kareem,2023), modified antibiotic targets (by genetic mutation or post-translational modification of the target) (Hano *et al*,2018), and acquired mechanisms (acquire antibiotic resistance mechanisms); (Hala,2024), Including decreased cell permeability, increased expression of efflux pumps .According to (AL-Lami *et al* ,2022), and antibiotic enzymatic inhibition or degradation (Hammadi *et al*,2015). Bacteria may decrease cell permeability by changing the expression and/or activity of porin proteins, which either block antibiotics from reaching their bacterial targets or cause drug inactivation. The physicochemical properties of the water are taken into consideration while choosing the

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techniques that are employed in drinking water treatment facilities. The most often utilized procedures globally are coagulation/flocculation, sedimentation, filtration, and disinfection (Abbood & Awda, 2020, Sanganyado & Gwenzi, 2019). Nevertheless, it is typically ignored to track the prevalence of ARB and ARG following treatment (Tan *et al*,2015), Furthermore, newer water remediation technologies like membrane filtration, biological and granular activated carbon filtration, and advanced oxidation .either fail to remove ARGs entirely or even help them spread throughout the water distribution systems (Xu *et al*,2016). Thus, creating easily available and effective methods for water cleanup to get rid of antibiotics, ARBs, and particularly ARGs is still difficult (Jara *et al*,2020). Investigate the spread of resistance genes in drinking water with the least human interference. Research has discovered clinically significant antibiotic resistance genes at sampling locations near field research stations, supporting human-to-water wastewater plant transmission routes (Reygaert,2018). This study was aimed to investigate how common antibiotic resistance is in *Escherichia coli* isolates that come from a range of sources, such as surface waters and wastewater treatment plants (WWTPs) and the waterways that are directly impacted by the discharge of these facilities.

MATERIALS AND METHODS

This study focused on drinking water treatment plants in Baghdad city, firstly for the isolation and identification of *E. coli* as an indicator of pollution, and secondly, for the detection of antibiotic-resistant *Escherichia coli*, which depends on phenotyping and genotyping methods. Twelve water samples were taken from different places throughout of Baghdad city. for a period of three months from October to December 2023in Baghdad city .at different points within the three drinking water treatment plants (Al Wathba, Al Wahda, and Al Karama), including influent, effluent, and various stages of the treatment process, each site using sterile bottles, preserved on ice, and transported to the laboratory (within 2 hours) for further processing. All bacteriological activity was carried out in the laboratories of the Environment, Water, and Renewable Energy Directorate in the Ministry of Science and Technology. Sample collection Among the 12 samples, there were 4 samples of each Al Wathba, Al Wahda, and Al Karama.(Table 1)

Table 1. code and location of the *E.coli* isolotion strains

Strain code	strain	location
A1 , A2 , A3 , A4 , A5 , A6 , A7 A8 , A9 , A10 , A11 , A12 , A13 A14	***E.coli***	**Al-Karama**
B1 , B2 , B3 , B4 , B5 , B6 , B7 B8 , B9 , B10 , B11 , B12 , B13 B14 , B15	***E.coli***	**Al-Wathba**
C1 , C2 , C3 , C4 , C5 , C6	***E.coli***	**Al-Wahda**
	a	

E. coli isolation and identification

Using the accepted techniques for examining water and wastewater (multiple-tube fermentation technique) (Abu-Sini *et al*,2023), water sample analysis for bacterial analysis was performed to isolate and identify *Escherichia coli* (Young *et al*,2005), culture on MacConkey agar and the Eosin Methylene Blue Agar from nutrient broth and after that, incubated for 24 hours at 37°C. the suspected *E. coli* isolates were confirmed using bioch-emical tests (Tambi *et al*,2023)

Vitek-2 Compact : All the *Escherichia coli* isolates were tested for phenotype identification

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and confirmation using The Vitek-2 compact appears to be a digital microorganism identification system that displays the tested isolates' phenotypic and worked according to the protocol (BioMerieux, Marcy L'Eyoily, France) (Hordijk *et al*,2013), .The results automatically came out in the form of a pint out. Before being used, *E. coli* isolates were stored at -80ºC in Nutrition broth with 15% glycerol.

Antibiotic sensitivity testing

E. coli isolates were then placed in tubes of sterile saline, and the turbidity was adjusted to match a 0.5 McFarland standard. plates of Mueller Hinton agar were inoculated on the surface using cell suspensions and sterile swabs. Using impregnated disks, the Kirby-Bauer method was used to test antibiotic resistance (Humphries *et al*,2018). with the following antibiotics: Amoxicillin-Clavulanic acid (30µg), Ampicillin (10µg), Aztreonam (30µg), Ciprofloxacin (15µg), Gentamicin (10µg), Imipenem (10µg), Meropenem (10µg), Ceftrazidime (30ug), Tetracycline (10µg), Cefotaxime (30 µg), Ceftriaxone (30µg), Erythromycin (15µg), Chloramphenicol (30µg), and Azithromycin (15ug). To evaluate resistance, intermediate, and susceptibility, inoculated plates were incubated at 37°C for twenty-four hours. The inhibitory zone sizes were then measured in millimeters in compliance with the manufacturer's guidelines.

DNA isolation and template production

Specific *E. coli* isolates were inoculated into 1 mL Trypticase Soy Broth and cultured for 18–24 hours at 37 °C in order to obtain molecular confirmation, and then *E. coli* isolates was extracted according to the instructions of the Wizard Kit (Promega, USA).using the nano-drop technique, the DNA concentration was measured and then stored at -20°C for additional analysis (García-Alegría *et al*,2020).

***lacZ* gene *Bla*M gene amplification**

Using lacZ3 primers particular to *E. coli*, In order to confirm the reliability of the isolates, the β-galactosidase gene was identified using polymerase chain reaction (table 1), as previously described ,and the detection of the antibiotic-resistant gene using the *E. coli*-specific labM gene (Table 2), as previously described (Dallenne *et al*,2010).

Table 2. the product size, primer sequence, and targeted gene

Primer name	Primer Sequence	Gene	Amplicon size
lacZ3	F: 5- TTGAAAATGGTCTGCTGCTG -3 R: 5- TATTGGCTTCATCCACCACA -3	β-galactosidase	243bp
*bal*M	F: CAGCGGTAAGATCCTTGAGA R: ACTCCCCGTCGTGTAGATAA	beta-lactamase	643 bp

The Polymerase chain reaction test amplified the sequence of the LabM and LacZ (Table 1). The entire volume of the PCR reaction is 25 µL. In the end, the target sequence amplicons were identified with the application of gel electrophoresis, a ladder DNA marker measuring 100 bp, and a 2% agarose gel. (Peqlab, Erlangen, Germany) (Korajkic *et al*,2018). (Table 3) shows the optimal conditions for detecting the (balM, lacZ3) genes under study using PCR technology.

Table3. optimal conditions for detecting the (balM, lacZ3) genes under study using PCR technology

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60,58,50,55	00:30	30
Extension	72	00:30	30
Final extension	72	07:00	1
Hold	10	10:00	1

Statistical Analysis: Statistical Analysis: To determine the impact of different elements in research parameters, the Statistical Analysis System- SAS (SAS,2017) program was utilized. In this study, the chi-square test was utilized to compare percentages (0.05 and 0.01 likelihood) statistically significant.

RESULTS AND DISCUSSION

In this study, of sixty isolates of coliform bacteria isolated from drinking water, thirty-six isolates were identified as *E. coli* (60%). On the EMB agar, the positive *E. coli* isolates showed a metallic green sheen, according to the morphology of colonies. There is a red ring on the peptone water buffer media when added with the Kovach reagent in the peptone water buffer media. In addition, Each of the *E. coli* isolate tested positive for the

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distinctive biochemical assays that were utilized to identify the isolates. (Table 4).

Table 4. Biochemical Tests Results

Tests	Results
Indole test	+
Methyl Red (MR)	+
Voges-Proskauer (VP)	-
Citrate utilization test	-
Triple Sugar Iron Agar	**Alk/Acid, Gas**
Motility	+

The percentage of *E. coli* in three drinking water stations was (Al-Karama and Al-Wathba stations) was 41.6% (15/36), and Al-Wahda station was 16.6% (6/36) (Table 5).

Table 5. The quantity and percentage of *E. coli* positive isolates from the drinking water station

Station name	No. of isolates	Positive No. (%)
Al- Karama	**15**	**41.6 % (15/36)**
Al Wathba	**15**	**41.6 % (15/36)**
Al-Wahda	**6**	**16.6% (6/36)**

To provide molecular validation, polymerase chain reaction was used to validate the presence of the lacZ gene in isolates. *E. coli* has a gene called lacZ that codes for the beta-galactosidase protein, which breaks down lactose. The results of the PCR analysis showed that every isolate had lacz (Dinakaran et al,2022), By comparing the band size to the DNA marker, this was determined on a 1.5% agarose gel on the basis of 243 bp of lacZ3 (Figure 1). The PCR method was used to validate the culture method's results. Conclusions of the PCR test and the culture procedure yielded the same results. Methods for *E. coli* genotyping are an effective diagnostic tool for *E. coli* infections. (Rasheed *et al*,2024) . Genotyping methods are accurate, fast, and cost-effective. However, genotyping methods can be complex to perform and interpret, and may not be available in all settings. (Favate *et al*,2023).

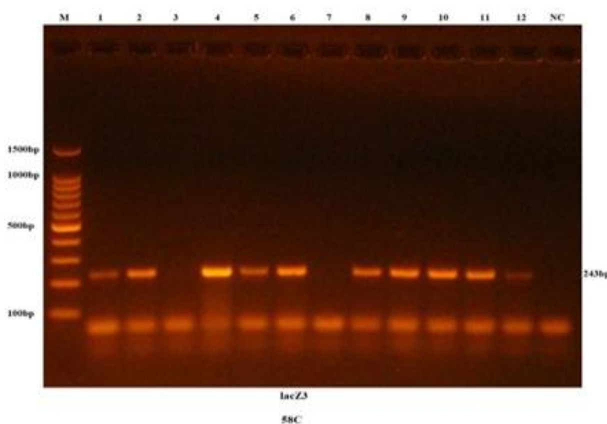

Figure 1 . lacZ 3 gene product in *E. coli* isolates (243 bp).

The presence of coliform is a measure of the bacteriological quality of water since coliforms, such as *E. coli*, are frequently used as indicators of the microbiological condition of ground and surface waters (Sugiah *et al*,2023). As these organisms are typically agents of gastroenteritis in humans, the isolation of coliform, particularly *E. coli*, from water sources is attributable to contamination by human and animal origin. This is of public health significance (Agustina,2021). The source of the drinking water contamination may have been leaking water pipes since the tap water was left running for five minutes before the sample being taken (Pratama *et al*,2020).

Antibiotic Susceptibility of E. coli Isolates

All 36 *E. coli* isolates were tested for their susceptibility using a panel of 14 antibiotics (from different classes). The most commonly found antibiotics to which the isolates were resistant (80%–100%) were Tetracycline, Erythromycin, and Ampicillin. (7%–27%) of the isolates showed resistance to Aztreonam, Meropenem, Imepenem, Azithromycin, Amoxicillin-Clavulanic Acid, Except for the Al-Wahda station, all isolates were sensitive (resistant zero) to Meropenem,and then isolates were resistant (0%–100%) to Ceftrazidime, Ciprofloxacin, Cefotaxime, and Ceftriaxone, to which the isolates were resistant. Finally, 7%–33% of isolates were resistant to gentamicin, chloramphenicol,Chloramphenicol, Resistance was more common among the isolates from three drinking water treatment plants for the following three out of fourteen antibiotics (Tetracycline, Erythromycin, and Ampicillin), with a significant difference (p = 0.0001)

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(Tables 6,7, and 8). Antimicrobial Resistance (AMR) in normal flora of Escherichia coli, or *E. coli*, may serve as an alternative indicator of resistance in other gut-dwelling pathogenic bacteria. (Escudeiro *et al*,2019). It has also been demonstrated that commensal *E. coli* carrying AMR poses a risk for developing resistant *E. coli* infections.

Table 6. Percentage of antibiotics resistant *Escherichia coli* isolated from (Al-Karama) drinking water plant

Number of *E.coli* isolates (15)/in the (Al-Karama) drinking water plant					
NO.	Antibiotics Disks	Code	Resistant%	Sensitive%	P-value
1	Aztreona	ATM	13	87	0.0001 **
MEM	7	93	0.0001 **	Meropenem	2
3	Gentamicin	CN	13	87	0.0001 **
4	Ceftrazidime	CAZ	20	80	0.0001 **
5	Ciprofloxacine	CIP	13	87	0.0001 **
6	Cefotaxime	CTX	53	47	0.548 NS
7	Ceftriaxone	CRO	0	100	0.0001 **
8	Tetracycline	TE	80	20	0.0001 **
9	Erythromycin	E	93	7	0.0001 **
10	Chloramphinicol	C	13	87	0.0001 **
11	Imepenem	IPM	13	87	0.0001 **
12	Azithromycin	AZM	27	73	0.0001 **
13	Ampicillin	AM	93	7	0.0001 **
14	Amoxicillin-Clavulanic	AUG	27	73	0.0001 **
	P-value		0.0001 **	0.0001 **	---

** (P≤0.01)

Table 7. Number and percentage of Multi-drug resistant *Escherichia coli* in the drinking water plant / (Al Wathba) project in Al Kilani

Number of E.coli isolates (15)/in the / (Al Wathba) drinking water plant					
NO.	Antibiotics Disks	Code	Resistant%	Sensitive%	P-value
1	Aztreona	ATM	7	93	0.0001 **
MEM	20	80	0.0001 **	Meropenem	2
3	Gentamicin	CN	7	93	0.0001 **
4	Ceftrazidime	CAZ	80	20	0.0001 **
5	Ciprofloxacine	CIP	67	33	0.0007 **
6	Cefotaxime	CTX	73	27	0.0001 **
7	Ceftriaxone	CRO	73	27	0.0001 **
8	Tetracycline	TE	80	20	0.0001 **
9	Erythromycin	E	87	13	0.0001 **
10	Chloramphinicol	C	7	93	0.0001 **
11	Imepenem	IPM	7	93	0.0001 **
12	Azithromycin	AZM	7	93	0.0001 **
13	Ampicillin	AM	93	7	0.0001 **
14	Amoxicillin-Clavulanic	AUG	13	87	0.0001 **
	P-value		0.0001 **	0.0001 **	---

** (P≤0.01)

Table 8. Number and percentage of Multi-drug resistant *Escherichia coli* in the drinking water plant / (Al Wahda) plant in Al Zafaranyia

Number of E.coli isolates (6)/in the / (Al Wahda) plant in Al Zafaranyia drinking water plant					
NO.	Antibiotics Disks	Code	Resistant%	Sensitive%	P-value

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1	Aztreona	ATM	17	83	0.0001 **
MEM	0	100	0.0001 **	Meropenem	2
3	Gentamicin	CN	17	83	0.0001 **
4	Ceftrazidime	CAZ	50	50	1.00 NS
5	Ciprofloxacine	CIP	0	100	0.0007 **
6	Cefotaxime	CTX	100	0	0.0001 **
7	Ceftriaxone	CRO	0	100	0.0001 **
8	Tetracycline	TE	100	0	0.0001 **
9	Erythromycin	E	100	0	0.0001 **
10	Chloramphinicol	C	33	67	0.0001 **
11	Imepenem	IPM	17	83	0.0001 **
12	Azithromycin	AZM	17	83	0.0001 **
13	Ampicillin	AM	100	0	0.0001 **
14	Amoxicillin-Clavulanic	AUG	17	83	0.0001 **
P-value			0.0001 **	0.0001 **	---

** (P≤0.01)

All of the isolates were resistant to at least three agents in the six classes of antibiotics assayed, which represents Multidrug resistance *Escherichia coli*, defined as showing resistance to three or more classes of antibiotics(Fig 2,3,4), has become a serious problem in the mismanagement of infectious diseases (Catalano *et al*,2022). Pharmaceutical extrusion by efflux drug pumps is one of the many pathways that lead to antibiotic resistance, and it plays a significant role in multidrug resistance. Clinically significant drug resistance has been linked to a decrease in intracellular drug accumulation through increased drug efflux and overexpression of drug transporters (Thomas & Tampé, 2020), This is the main process by which resistance to erythromycin and tetracyclines develops. One of the main challenges to effectively treating bacterial infections is the emergence of continuous resistance to several antibiotics with different chemical structures and targets (Imran *et al*,2022). Alternative antimicrobial agents and strategies to combat bacterial infections are urgently required, as the improper and overuse of antibiotics has led to the rapid development of Multidrug-resistant (MDR) bacteria and their ineffectiveness against hard-to-treat biofilm-related infections (BRIs). (Garcia *et al*,2021). Multiple pathways can lead to the generation of MDR in bacteria. First, on resistance R-plasmids, bacteria usually accumulate many genes, each of which codes for resistance to a distinct medication, within a single cell. Furthermore, higher levels of the genes encoding multidrug efflux pumps may potentially contribute to multidrug resistance (Alenazy,2022), extruding a wide range of drugs. Furthermore, the development of MDR can be achieved by adding a chemical group to the drug or by breaking it down enzymatically. Hydrolyzing certain medications (tetracycline, penicillin, etc.) might render them inactive. Acetyl, phosphoryl, and adenyl groups are frequently transferred in order to inactivate drugs through chemical group transfer .

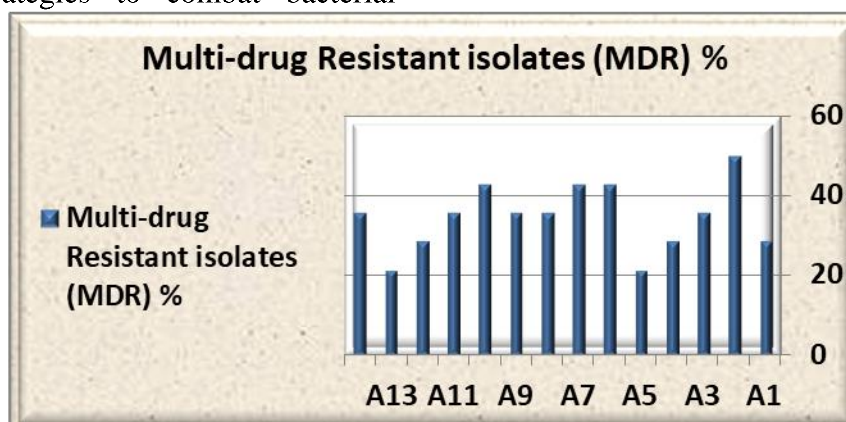

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Figure 2. Number and percentage of Multi-drug resistant *Escherichia coli* in the drinking water plant / (Al-Karama) plant in Al-Atifiyah

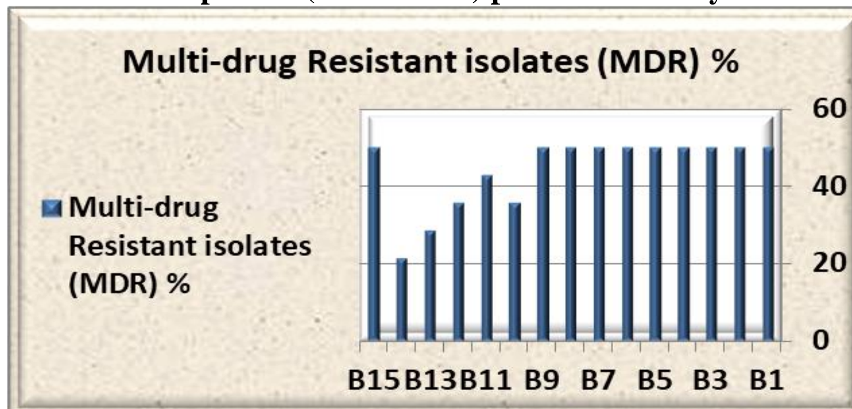

Figure 3. Number and percentage of Multi-drug resistant *Escherichia coli* in the drinking water plant / (Al Wathba) project in Al Kilani

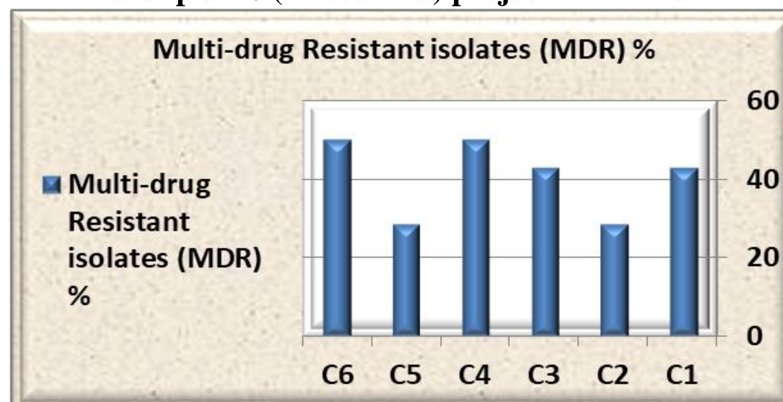

Figure 4. Number and percentage of Multi-drug resistant *Escherichia coli* in the drinking water plant / (Al-Wahda) plant in Al Zafaranyia

In 12 out of 36 (33.3%) *E. coli* isolates, the blaM gene was found (Figure 5). The production of extended-spectrum beta-lactamases (ESBLs) by *E. coli* has been identified as a significant multidrug-resistant bacterium linked to severe hospital and community-acquired infections globally, particularly in areas with high rates of unsanitary conditions and inadequate hygiene practices (Verma *et al*,2022), Compared to traditional phenotypic approaches, (PCR)-based molecular technologies are more sensitive, quick, and accurate at identifying ESBL-resistant genes. They support medical professionals in treating patients with a focused approach, controlling outbreaks, and putting infection control procedures into practice . Various ecological niches within the population and environment have been found to harbor multidrug-resistant *Escherichia coli* (Odonkor & Addo, 2018), For instance beta-lactamase -producing *Escherichia coli* were detected in drinking water, It has played an essential role in the field of water microbiology as a fecal contamination indicator.In the instance that these *E. coli* samples prove to be multidrug-resistant bacteria, there is increased cause for concern for public health (Tornberg-Belanger *et al*,2022), additionally, it's critical to understand the possibility of gastrointestinal illnesses and antibiotic resistance (AMR) spreading. According to (Mahmud *et al*. 2020), aquatic habitats offer the perfect conditions for the horizontal transfer of AMR genes encoded on different types of mobile genetic components (Mahmud *et al*,2020). The results of this research indicate that the drinking water samples examined here may be a significant conduit for the exposure and spread of pathogenic, ESBL-producing, and MDR *E. coli* variants, which also represent a health danger to the city's human population (Ibrahim *et al*,2023), Based on the findings of

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this study, we advise policymakers to exert significant effort in putting into practice effective infection control measures by emphasizing the provision of high-quality water and guaranteeing the city's water quality monitoring programs.

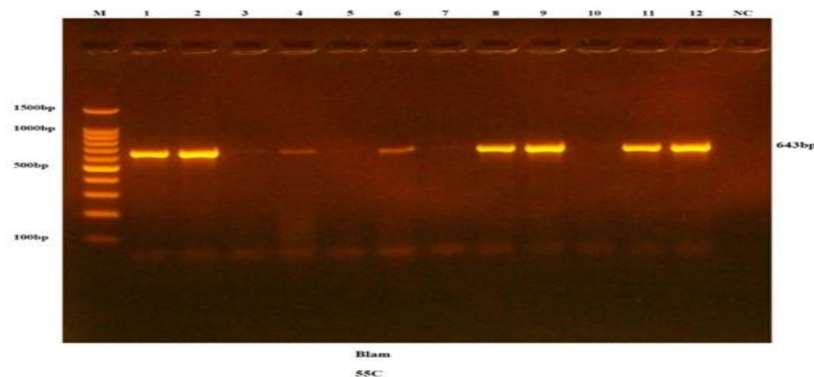

Figure 5 . *bla*M encoded to extended-spectrum beta-lactamase (ESBL) in *Escherichia coli* isolates(643bp

blaM gene, which encodes the TEM-1 beta-lactamase enzyme in *E. coli*, As previously reported, the risk of isolating ESBL-producing *E. coli* increased by more than twice after receiving an antibiotic recently. This suggests that regular use of antibiotics in hospitals creates significant drug pressure that drives AMR. (Doan et al, 2020), Because of its epidemiological relevance, the growing abundance of extended-spectrum β-lactamase (ESBL) generating bacteria in water poses a serious risk to human health (Sivakumar *et al*,2021), Due to their connection to antibiotic resistance, *Escherichia coli* have emerged as significant and deadly waterborne pathogens worldwide (Hartantyo *et al*,2020).

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انتشار بكتيريا الإشريشيا القولونية المقاومة للمضادات الحيوية في بعض محطات معالجة مياه الشرب في مدينة بغداد

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المستخلص

يعد وجود جينات مقاومة للمضادات الحيوية في الإشريشيا القولونية الموجودة في مياه الشرب مصدر قلق عالمي للصحة العامة. تم إجراء عزل وتشخيص بكتيريا *Escherichia coli* في ثلاث محطات لمعالجة مياه الشرب في مدينة بغداد باستخدام مزيج من التحليلات الكيموحيوية والجزيئية على وسط انتقائي. تم إجراء تضخيم جين LacZ بواسطة تفاعل البلمرة المتسلسل للتعرف الجزيئي، وأظهرت الدراسة وجود lacZ في كل عزلة. تم استخدام الطريقة الجزيئية لتأكيد نتائج الطريقة الزراعية، أنتج كل من التشخيص الجزيئي والطريقة الزراعية نفس النتائج. وبتطبيق معيار ماكفارلاند وطريقة انتشار القرص كيربي – باور، تم إجراء اختبار الحساسية المضادة للميكروبات ضد 14 مضادًا حيويًا مختلفًا. كانت المضادات الحيوية الأكثر شيوعًا والتي كانت العزلات مقاومة لها (80٪ – 100٪) هي التتراسيكلين والإريثروميسين والأمبيسيلين، مع وجود فروقات كبيرة (P=0.0001) . كانت المقاومة للأدوية المتعددة موجودة في جميع العزلات، حيث أظهرت مقاومة لما لا يقل عن ثلاثة عوامل عبر فئات المضادات الحيوية التسعة التي تم اختبارها. يتم تعريف الإشريشيا القولونية على أنها تظهر مقاومة لثلاث فئات أو أكثر من المضادات الحيوية. تم اكتشاف جين blaM في عزلات الإشريشيا القولونية 36/12 (33.3%) التي تنتج بيتا لاكتاماز ممتد الطيف (ESBL).

الكلمات المفتاحية: البكتريا مؤشر للتلوث، الجين التشخيصي، الجين المقاوم

جزء من رسالة الماجستير للباحث الأول