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Identification of 16S rRNA Gene and Detection of Some Virulence Factors in Multi-Antibiotic Resistant *E. coli* Isolated from Clinical and Water Samples

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ABSTRACT

The study included collecting 140 clinical samples (urine, urine of patients with renal failure, burn swabs, wound swabs) from Baquba Teaching Hospital and Baladrooz General Hospital. 71 water samples were also collected from the Public Health Laboratory in Diyala Governorate to investigate the presence of *E. coli* bacteria. The bacteria were diagnosed morphologically, microscopically, by biochemical tests, and by Vitek device. 44 isolates of *E. coli* bacteria were obtained and their sensitivity to antibiotics was examined. The highest resistance was recorded to vancomycin (100 %), ampicillin and amoxicillin (86.4 %), and cefotaxime (84.2 %). The highest sensitivity was to ambienem and meropenem (100 %). The virulence factors were examined and the ability of bacteria to produce strong biofilms (16 %) from water samples, metallo-beta-lactamase (4 %), clinical (8 %) water, broad-spectrum beta-lactamase (20 %), clinical (4 %) water, and for the purpose of accurate diagnosis by molecular methods, bacteria were diagnosed using the 16S ribosomal RNA (16S rRNA) gene, as the isolates appeared at a rate of (100 %).

Keywords: Antibiotic resistance, Clinical sample, *E. coli*, Water Sample.

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تشخيص جين 16SrRNA والكشف عن بعض عوامل الضراوة في البكتيريا الاشريشيا القولونية المقاومة للمضادات الحيوية المتعددة المعزولة من العينات السريرية وعينات المياه

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الملخص

تضمنت الدراسة جمع 140عينة سريرية (بول ، بول مرضى الفشل الكلوي ، مسحات الحروق ، مسحات الجروح) من مستشفى بعقوبة التعليمي و مستشفى بلدروز العام، كما و تم جمع 71 عينة ماء من مختبر الصحة العامة في محافظة ديالى للتحقيق من وجود بكتريا اي كولاي،تم تشخيص بكتريا مظهريا ،مجهريا، اختبارات كيموحيوية ، جهاز Vitek ، تم الحصول على 44 عزلة من بكتريا *E.coli* ، فحصت حساسيتها تجاه المضادات الحيوية اذ سجلت اعلى مقاومة لمضاد Vancomycin بنسبة (100 %) Ampicillin و Amoxicillin بنسبة (86.4 %) و Cefotaxime بنسبة (84.2 %) كما و كانت اعلى حساسية اتجاه مضادين Meropenem و Imipenem بنسبة (100 %). تم فحص عوامل الضراوة وكانت قدرة البكتريا لإنتاج الاغشية الحيوية (16 %) القوية من عينات ماء، البيتا–لاكتاميز المعدنية (4 %) سريري (8 %) ماء، بيتا–لاكتاميز واسعة الطيف (20 %) سريري (4 %) ماء، ولغرض التشخيص الدقيق بالطرائق الجزيئية تم تشخيص بكتريا بواسطة جين 16SrRNA اذ ظهرت العزلات بنسبة (100 %).

INTRODUCTION

Escherichia coli (E. coli) is classified as a member of the gut microbiota because it is commonly found in the typical bacterial population of the large intestine [(1)]. It is a commensal bacterium found in the intestines of humans and animals. However, it can also act as an opportunistic pathogen, causing various diseases, including meningitis, bacteremia, sepsis, and diarrhea. In addition, it is one of the most common bacterial causes of urinary tract infections. The pathogenicity of this bacterium is due to the presence of several virulence factors it carries [(2)]. *E. coli* bacteria are bio indicators, such as being used as a source of fecal contamination in water samples. *E. coli* strains vary depending on the host in which they are found. The presence of these strains (which are native to the intestines of humans and animals) in water indicates fecal contamination from humans or animals and thus causes environmental pollution. Water contamination by bacteria is one of the problems facing water consumers. The only way to confirm the presence or absence of bacteria in water is to examine water samples and identify the type of bacteria present [(3)]. *E. coli* bacteria use different means to survive and persist in the environment. One of these means is the formation of biofilms, and the formation of biofilms can promote antibiotic resistance, making it difficult to eradicate and control these organisms [(4)]. Antibiotic resistance is a major issue that leads to many deaths worldwide [(5)]. Pathogenic strains of *E. coli* are increasingly evolving due to their resistance to several types of antibiotics, such as beta-lactams, tetracyclines, fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides [(6)]. Given the wide variation in resistance patterns across different regions, it is essential to determine the antimicrobial resistance (AMR) profile of bacteria in the local area. This is vital to avoid treatment failure and reduce the likelihood of consequences, especially in cases where infections are caused by multidrug-resistant

strains [7, 8]. This study aims to determine the extent of *E. coli* resistance to the antibiotics used, as well as its ability to produce virulence factors that help it cause disease.

MATERIAL AND METHODS

Bacterial specimens' collection

During the period from (1 December 2023 to 30 March 2024), 140 clinical samples were collected from various sources (urine, burn swabs, urine of patients with renal failure, wound swabs) and from both sexes. The number of isolated samples from females was (103) samples at a rate of (73.5 %) and the number of isolated samples from males was (37) at a rate of (26.4 %) and their ages ranged between (3-81 years) from (Baqubah Teaching Hospital and Baladruz General Hospital), in addition to collecting 71 water samples (water) from the Public Health Laboratory, Food Department in Diyala Governorate.

Bacterial isolation and diagnosis

Bacterial isolates were isolated and identified based on microscopic examination, culture on (MacConkey agar, eosin methylene blue (EMB) and HiChrome medium, MacConkey Broth (for water samples)) shown in Figure (1), biochemical tests (catalase test, oxidase test), as well as diagnosis using Vitek device, and confirmation using 16S rRNA gene [9, 10]. Bacterial isolates were incubated for (24 h) at (37 °C).

Antibiotic sensitivity test

Antibiotic susceptibility testing of *E. coli* isolates was performed using Mueller-Hinton-Agar medium using Kirby-Bauer method based on (CLSI, 2023),[11]. The antibiotics in this study were (Norfloxacin (10 µg), Amikacin (30µg), Gentamicin (10µg), Cefotaxime (30µg), Cefoxitin (30µg), Ciprofloxacin (5µg), Ampicillin (10µg), Amoxicillin (10 µg), Imipenem (10µg), Meropenem (10 µg), Vancomycin (30 µg).

Detection of virulence factors

Detection of biofilm using the micro titer plate (MTP) method

Biofilm production was detected by micro titer plates. Isolates were cultured on nutrient broth medium and incubated for (24 h) at (37 °C). Fixation was performed with methanol and (0.5 %) crystal violet. Absorbance was read using an enzyme linked immunosorbent assay (ELISA) reader at (630 nm) and the cultures were placed in three wells of medium with bacteria and the last three wells of medium without bacteria [12].

Production of Metallo-beta-lactamase enzymes (MβLS)

The synergistic method of antibiotic disk (Imipenem) and ethylene diamine tetra acetic acid (EDTA) was used to determine metallo-beta-lactamase enzymes. Isolates were cultured using Mueller Hinton agar medium. An imipenem disk was placed alone and a disk with EDTA was placed (2 cm) apart and incubated for (24 h) at (37 °C) [13].

Production of extended-spectrum beta-lactamase enzymes by E. coli bacteria (ESBL)

CD synergy method was used to determine extended spectrum beta-lactamases (ESBLs). A combination of antibiotics (Amoxicillin/Clavulanic acid, Pipercillin, Cefotaxime, Cefixime) was used for this test and cultured on Mueller-Hinton agar medium and incubated for (24 h) at (37 °C) [14].

Identification of 16SrRNA gene

Genomic deoxyribonucleic acid (DNA) was extracted from the overnight culture using (ABIOpure TM Total DNA, USA). The concentration of the DNA extract was determined by measuring the absorbance using a Quantus Fluorometer which was used to detect the concentration of the extracted DNA in order to assess the quality of the samples. For (1µL) of DNA, (200 µL) of diluted Quantifluor dye was mixed. After (5 min) of incubation at room temperature, the DNA concentration values were detected. The polymerase chain reaction (PCR) products were amplified using Go Tag Green master mix (promega, USA). To identify the *E. coli* species, the multiplex PCR method was used with the primer

sequences listed in Table (1) [5]. The amplification reaction mixture (25 µl) consisted of (12.5 µl) of (2x) Go-Tag master mix, (1 µl) of primer (10 pmol), (8.5 µl) of nuclease-free water and (2 µl) of DNA. The amplification reaction was performed using a thermal cycler (Thermal Fisher Scientific, USA). Under the following conditions: Amplification steps: initial denaturation at (95 °C) for (5 min) followed by (30 cycles) of denaturation at (95 °C) for (30 s), annealing at (60 °C) for (30 s), extension at (72 °C) for (30 s), final extension at (72 °C) for (7 s), and holding at (10 °C) for (10 s).

Table 1: Primer sequence and annealing temperature used.

Primer	Sequence 5-3	Annealing Temp. (°C)	Product size (bp)	Reference
27F	AGAGTTTGATCCTGGCTCAG	60	1500	15
1492R	TACGGTTACCTTGTTACGACTT			

RESULTS AND DISCUSSION

Isolation and Identification of *Escherichia coli*

The study included the collection of 211 samples (140) clinical samples, at a rate of (66.35 %), and (71) water samples, at a rate of (33.64 %). There were (44) positive cases, with a rate of (20.85 %). The isolation results for these cases (urine, Kidney failure, burns, wounds) showed that the highest numbers of isolated bacteria were obtained from urine samples, which included (93) samples from patients with urinary tract infections, at a rate of (66.42 %), followed by samples collected from Kidney failure patients included (21) samples, at a rate of (15 %), then wound samples, which included (15) samples, at a rate of (10.71 %). The lowest clinical percentage obtained was from burn samples, which included (11) A sample rate of (7.85 %) As for water samples, (71) samples were collected, a rate of (33.64 %). The percentage of all isolates that gave a positive test was (20.85 %).

A study conducted in Dohuk Governorate[15]. showed that E. coli bacteria were isolated from different clinical sources, and the highest percentage was in urine samples, where (92.2 %) was obtained, followed by wound samples (3.9 %). A study [16] showed that *E. coli* bacteria were isolated from water sources and 97 isolates of *E. coli* bacteria were obtained, representing (18.7 %).

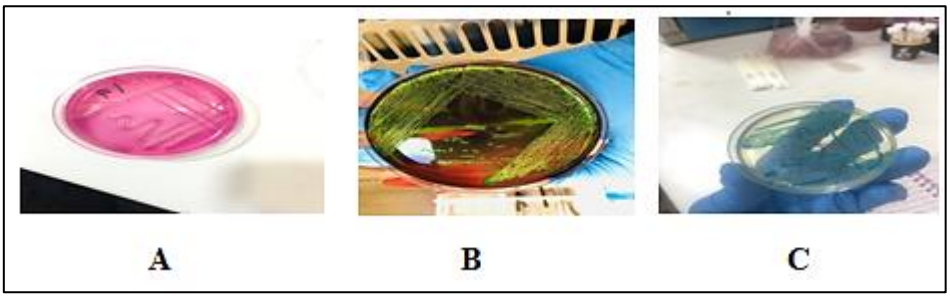

Fig. 1: A: *E. coli* bacteria on MacConkey culture medium. B: *E. coli* bacteria on Eosin Methylene Blue culture medium. C: *E. coli* bacteria on the culture medium, HiChrome *E. coli* Agar.

E. coli antibiotic resistance test

The susceptibility of 44 *E. coli* isolates to 11 antibiotics was tested, the results of the isolations showed a clear difference between the resistance of clinical and environmental isolates to the antibiotics used. shown in Table (2). A study [17] in Baghdad, which isolated *E. coli* from clinical sources, showed that the resistance to vancomycin was (100 %), and a study [17] where the highest resistance was to the antibiotics ampicillin (84 %) and amoxicillin (86 %), and a study in Babylon [18],The highest sensitivity was reported to be imipenem, with a sensitivity rate of (95 %). The study [19] also showed that the highest sensitivity was to the antibiotic

meropenem, as its sensitivity reached (100 %). And in Baghdad, where bacteria were isolated from some hospitals, the percentage of resistance to cefotaxime was (84 %), and these are the results of studies that agree with the results of the current study [20].

Sensitivity testing is important in identifying antibiotics for treatment as well as reducing the randomness of antibiotics, as *E. coli* bacteria show multiple resistance to different types of antibiotics as a result of their misuse in the treatment of various diseases. It has become well known that multi-resistant bacterial strains are widespread, especially in hospitals, especially beta-lactam antibiotics among members of the Enterobacteriaceae family [21].

Table 2: Antibiotics used in the study for isolates (clinical and Water).

	Antibiotic groups	Group antibiotics	Number of resistant isolates and percentage %(clinical)	Number of resistant isolates and percentage % (Water)
1	Cephems (Parenteral) Including Cephalosporins I, Ii Iii, Iv	Cefotaxime	32 (84.21 %)	4 (66.66 %)
		Cefoxitin	13(34.21 %)	0
2	Glycopeptides	Vancomycin	38 (100 %)	(100 %)
3	Penicillins	Ampicillin	33 (86.84 %)	6 (100 %)
		Amoxicillin	33 (86.84 %)	5 (83.33 %)
4	Carbapeneams	Imipenem	0	0
		Meropenem	0	0
6	Aminoglycoside	Gentamicin	8 (21.05 %)	1 (16.66 %)
		Amikacin	3 (7.89 %)	0
7	Fluoroquinolones	Ciprofloxacin	17 (44.73 %)	2 (33.33 %)
		Norfloxacin	19 (50 %)	2 (33.33 %)

Virulence factors

Several virulence factors were detected for *E. coli* bacteria, as biofilm formation was detected by the micro titer plate (MTP) method and the production of beta-lactamase enzymes.

Detection of biofilm using the micro titer plate (MTP) method

The current study showed 25 isolates (62.5 %) were biofilm producers, and 4 environmental isolates (water) at a rate of (16 %) were strong biofilm producers, while 6 clinical isolates at a rate of (24 %) were moderate biofilm producers and 2 environmental isolates (water) at a rate of (8 %) were moderate biofilm producers, and 13 clinical isolates at a rate of (52 %) were weak biofilm producers shown in Figure (2).

A study [22] showed that the *E. coli* bacteria produced strong biofilms (35 %), medium (60 %) and weak (5 %). These results do not agree with the current study, it agreed with the study [23] where the average biofilm production rate reached (24 %), while it differed in the production of strong biofilms where the production rate reached (73 %).

Biofilms act as a defense mechanism that enhances the survival rate of microorganisms. It plays an important role in the development and persistence of microbial diseases as well as being the basis for genetic exchanges. It acts as a shield that protects the microbial community from the action of many antimicrobial agents such as antibiotics, preservatives and chemical disinfectants [24].

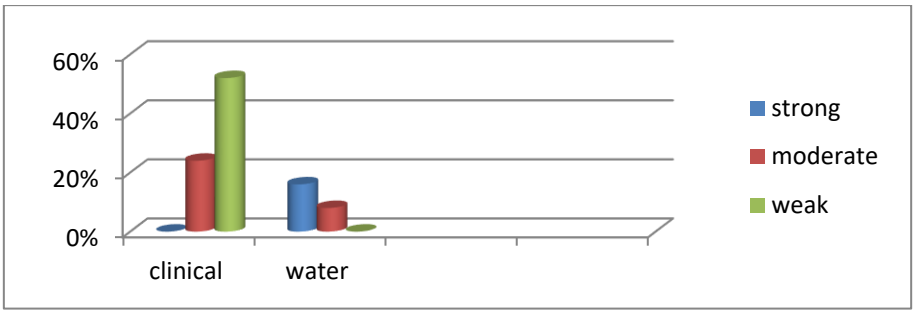

Fig. 2: Percentage of *E. coli* bacteria's ability to produce biofilm.

Production of metallo-beta-lactamase enzymes (MβLS)

25 *E.coli* isolates were tested for the production of metallo-beta-lactamase enzymes (MBLs). The results showed that out of 19 clinical isolates, only one isolate produced (MBLs) at a rate of (4 %). As for the water isolates, the results showed that out of 6 isolates from water, only two isolates produced (MBLs) at a rate of (8 %). Shown in Figure (3).

The results of the study [25] appeared in Diyala Governorate, where the MBL production rate reached (20 %), and this is a result that contradicts the current study. The results of the current study were close to the results of the study [23] where the percentage of *E.coli* bacteria production of metallo-beta-lactamase enzymes was (9.4 %) and non-productive (16.5 %). As for the water isolates.

The production of MBL enzymes in Enterobacteriaceae isolates has an increasing prevalence pattern and prevalence may vary significantly across geographic locations. The increasing prevalence of pathogens that produce MBL enzymes may be a driving factor behind the increased production of carbapenemase-producing organisms [24].

Production of extended-spectrum beta-lactamase enzymes by *E. coli* bacteria (ESBL)

25 isolated *E. coli* samples were tested for the production of extended-spectrum beta-lactamase enzymes (EBLS). the results showed that out of 19 clinical isolates, only 5 clinical isolates of *E. coli* bacteria were producers of extended spectrum beta-lactamase enzymes at a rate of (20 %), as for the water isolates, the results showed that out of 6 water isolates, only one water isolate was producers of extended spectrum beta-lactamase enzymes at a rate of (4 %) shown in Figure (3).

The results of the current study were inconsistent with the results of the study [25] where the production rate of broad-spectrum beta-lactamase enzymes was (47.8 %), In a study conducted in Diyala Governorate[22], bacteria were isolated from different infections if the ESBL production rate was (10 %), and this rate is close to our current study.

The difference in ESBL production between this study and other studies may be due to the difference in isolates, the difference in local antibiotic use in each country, the overuse of broad-spectrum antibiotics, and the extent of drug resistance suffered by pathogens in the local study area. Beta-lactams are one of the most important virulence factors that help in destroying the beta-lactam ring in some antibiotics, thus increasing antibiotic resistance and virulence of *E. coli* [26].

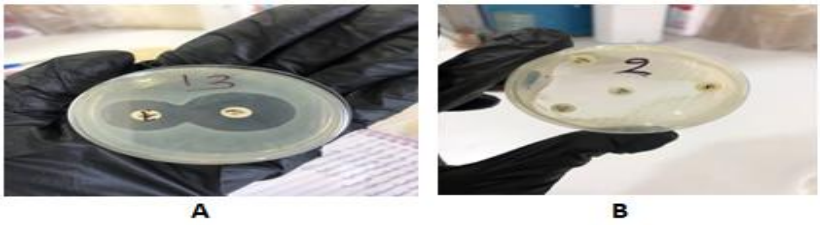

Fig. 3: A: Metallo-beta-lactamase enzymes, B: Broad-spectrum beta-lactamase enzymes.

Molecular diagnosis of *E. coli* using 16SrRNA gene

E. coli bacteria were identified by molecular methods using the 16S rRNA gene for clinical and water isolates, where the optimal PCR conditions were used, as explained in the previous paragraph. Polymerase chain reaction was performed for 11 bacterial isolates, numbered (3, 4, 5, 8, and 11) clinical and (20-25) water, where the gene appearance rate was (100 %). where the gene appearance rate was (100 %) Shown in Figure (4). Then, the PCR results were sent to the National Center for Biotechnology Information (NCBI) for the purpose of sequencing the isolates. Three new isolates were detected with a rate of (99 %), two clinical isolates (3, 4) and one water isolate (20). They were registered in NCBI, and each isolate has its own Accession number, as the Accession number is specific to isolate No. 3 (PP977164), No. 4 (PP977166), and No. 20 (PP977172).

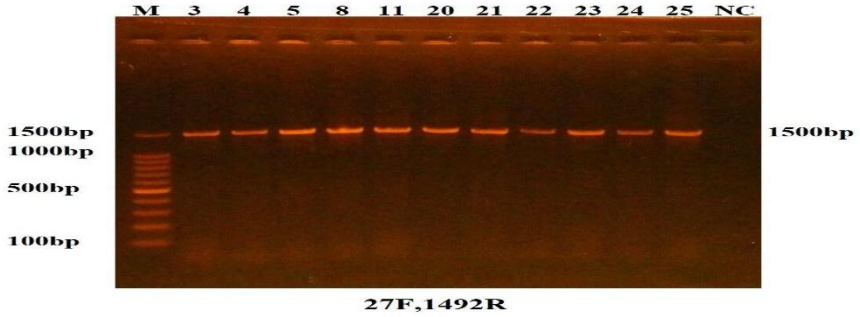

Fig. 4: Results of the amplification of 16SrRNA gene of *Escherichia coli* were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (3-4-5-8-11) clinical isolate and 20-25 water isolate resemble 1500bp PCR products.

CONCLUSIONS

The excessive use of antibiotics has made *E. coli* bacteria resistant to groups of antibiotics. This is primarily due to the lack of cultural and health awareness when taking antibiotics. The presence of *E. coli* isolates with multiple resistance in water samples is considered a factor threatening public health as a result of the acquisition of dangerous factors from environmental sources.

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Author contribution: Authors contributed equally in the study.

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