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Original Article

Evaluation of antibiotic resistance and biofilm production in Escherichia coli isolated from clinical samples in Kirkuk city

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Abstract

Escherichia coli (E. coli) is a prominent etiological cause of bacterial infections. Biofilms form when cells are embedded within a matrix of extracellular polymeric substances that bond to each other or to either inert or living surfaces. It increases antibiotic resistance by maintaining cells. The study aims to determine the biofilm formation and antibiotic susceptibility patterns of *E. coli* isolates. A total of 200 clinical samples were collected from Kirkuk city hospitals, including urine, sputum, wound, and burn swabs. Forty isolates were classified as *E. coli*. An antibiotic sensitivity test was conducted using the disc diffusion method with fourteen antibiotic discs. A microtiter plate method was used to detect biofilm. The study revealed antibiotic resistance rates against Ceftriaxone 33 (82.5%), Ceftazidime and Trimethoprim-sulfamethoxazole 32 (80%), Tetracycline 28 (70%), Cefepime 27 (67.5%), Ciprofloxacin and Aztreonam 32 (57.5%), Amoxicillin-clavulanate 12 (30%), and Piperacillin-tazobactam 11 (27.5%). The most effective antibiotics were nitrofurantoin, meropenem, imipenem, chloramphenicol, and gentamycin, with susceptibilities (100%, 90%, 85%, 85%, 75%), respectively. The biofilm production of the *E. coli* isolates ranged from weak 30 (75%) to moderate 9 (22.5%), and strong 1 (2.5%). The study concluded that all antibiotic-resistant isolates were biofilm producers.

Keywords: *Escherichia coli*, Biofilm, Antibiotic resistance, Kirkuk city

Introduction

Escherichia coli is a Gram-negative straight rod-shaped non-spore-forming bacterium, motile by peritrichous flagella, belonging to the Enterobacteriaceae family [1]. Several different *E.coli* strains cause various intestinal and extraintestinal diseases; enteropathogenic strains (EPEC) are responsible for diarrheal infections worldwide, extraintestinal pathogenic strains (ExPEC) includes uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), avian pathogenic *E. coli* (APEC), and neonatal meningitis *E. coli* (NMEC), were linked to cystitis, pyelonephritis, respiratory tract infection, sepsis, cervicovaginal infection (CVEC), otitis media, meningitis (NMEC), cholecystitis, and wound infection. UPEC strains constitute more than 80% of community-acquired and 30% of nosocomial urinary tract infections. [2,3,4]. Pathogenicity islands (PAIs) encode a plethora of virulence factors associated with pathogenic *E. coli* (EXPEC)

that exhibit a diverse array of functions, including adhesins, iron acquisition factors, toxins, lipopolysaccharides, polysaccharide capsules, and invasins [5]. Antimicrobial resistance in the last few decades has surfaced as a significant threat to global public health systems. Various resistance mechanisms are identified in microbes, including natural resistance in specific microbes to certain antimicrobials, genetic mutations, and acquired resistance from other species. The concept of multidrug resistance (MDR) is used when an organism becomes resistant to more than one antibiotic [6]. In the last twenty years, a significant increase has been observed in the prevalence of *E. coli* strains exhibiting resistance to commonly utilized antibiotics, including β-lactams, quinolones, aminoglycosides, sulfonamides, and Fosfomycin. Due to its implications for human health, it has been categorized, alongside other members of the Enterobacteriaceae family according to the World Health Organization's (WHO) list of the 12 bacterial families that pose the greatest risk to human health [6,7]. In order to protect themselves from environmental stresses such as changes in PH and temperature, UV radiation, dryness, oxidation, metal ions, or biocides, bacteria form organized 3D communities called biofilms on either living or nonliving surfaces [8,9]. The National Institutes of Health reports that biofilms are responsible for 80% of all infections in humans. Biofilms significantly promote the development and spread of antibiotic resistance, enhancing treatment resistance by factors ranging from 100 to 1000 times compared to their planktonic counterparts. Furthermore, biofilms evade both innate and adaptive immune defences, rendering their treatment and eradication particularly difficult [10,11].

Materials and Method

Ethical approval

Certain official agreements were obtained from the Kirkuk Health Directorate before establishing the study. The sample was collected with the patient's informed and analytical permission.

Patients and sample collection

The study was carried out in Azadi Teaching Hospital, Kirkuk General Hospital, and the Public Health Laboratoryin Kirkuk City from December 2023 to March 2024. Different clinical samples (urine, sputum, wound, and burn swabs) were obtained from 200 clinical specimens. The specimens were cultivated on blood agar, MacConkey agar, eosin methylene blue agar, and brain heart infusion broth (BHI). After growth, the colonies formed were evaluated for lactose fermentation, and biochemical responses; the vitek2 system was used to confirm the identification.

Bacteriological isolation and identification

Blood agar, MacConkey agar, eosin methylene blue agar, and BHI broth were used to culture all of the samples, which were subsequently incubated at 37°C for 24 hours. The isolated colony was recognized by lactose fermentation on MacConkey agar, colony shape, gram stain, the green metallic sheen colony on EMB agar (figure 1), and hemolytic activity on blood agar.

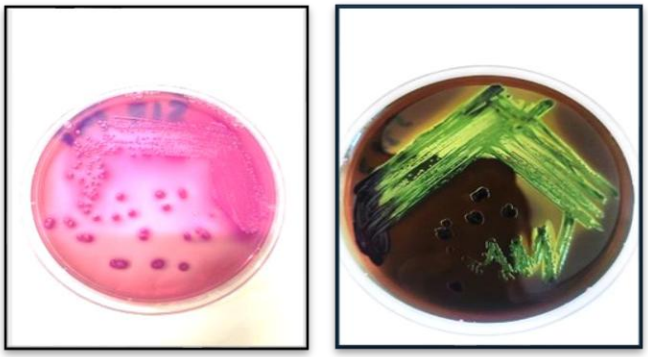

Figure 1. *E. coli* on Macconkey agar (Right), on EMB agar (Left)

Antibacterial sensitivity test

Fourteen antibiotic discs were employed to assess the susceptibility of all *E. coli* isolates in the investigation (figure 2). Following overnight bacterial proliferation at 37°C, a sterile inoculating loop was employed to suspend the organism in 2 ml of sterile saline, after which the saline tubes were vortexed to achieve a homogenous suspension. To calibrate the turbidity of the suspension, a standard of 0.5 McFarland was used. Using a cotton swab, an aliquot of 2µL of bacterial suspension was spread out on the Muller Hinton agar in

three different orientations, rotating the plate by 60° each time. The plate was inverted at room temperature for several minutes. Following the application of antibiotics, the plates were incubated for 24 hours at 37°C. Using a zone inhibition ruler, the zone of inhibition was measured in millimeters. The data were analyzed according to the National Committee for Clinical Laboratory Standards. [12].

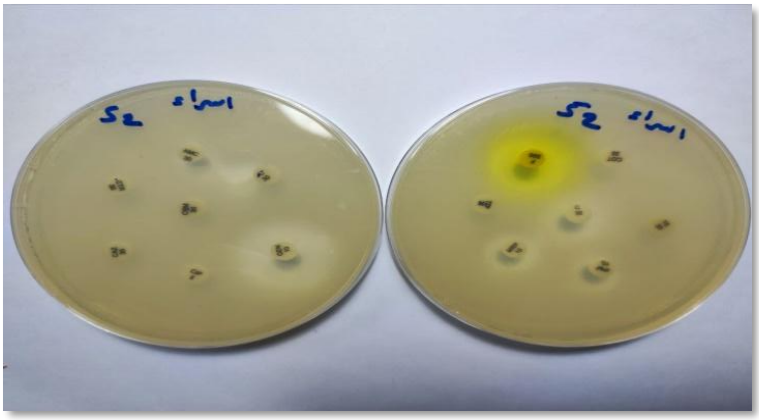

Figure 2. Antibacterial susceptibility test for *Escherichia coli*

Evaluation of biofilm formation by Microtiter plate method

The study isolates were incubated in 5 ml of tryptone soy broth (TSB) for 24 hours at 37°C. The solution was subsequently diluted with tryptone soy broth containing 1% glucose (TSB glu) at a ratio of 1:100. Each well of the 96-well microtiter plate were filled with 200 µL of the diluted culture, with broth utilized as the control. The culture was incubated for 48 hours at 37°C and the plate was carefully cleaned three times with phosphate-buffered saline (PBS) after incubation to remove nonadherent cells. Subsequently, 200 µL of 0.1% crystal violet was introduced to each well and incubated for 10 to 15 minutes at ambient temperature. After the removal of crystal violet via three washes with distilled water, the microtiter plate was inverted for several hours to promote drying. To solubilize the dye, 200 µL of a 95% ethanol solution was introduced to each well, thereafter covering the microtiter plate with a lid and allowing it to equilibrate at room temperature for 30 minutes. The absorbance of the destaining solution was measured at 630 nm utilizing an ELISA reader (figure 3) [13]. The Biofilm is described as:

ODi ≤ ODc = Non-biofilm former

ODc < OD <= 2 ODc = Weak biofilm former

2 ODc < OD <= 4 ODc = Moderate biofilm former

4 ODc < OD = Potent biofilm former

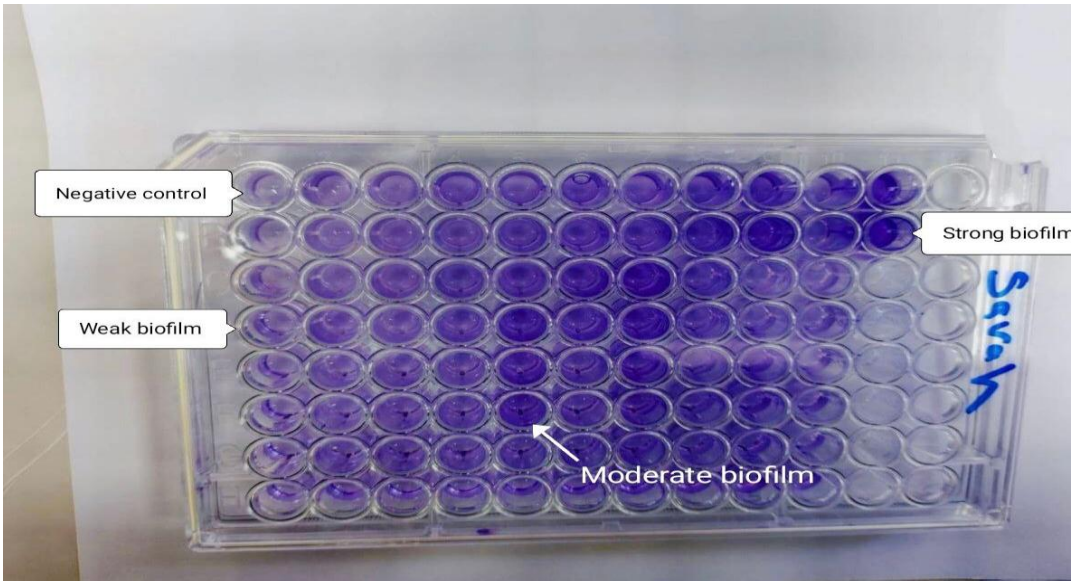

Figure 3. Biofilm determination in *E.coli* by microtiter plate method

Results and Discussion

Isolates of *Escherichia coli* in various clinical samples

Two hundred samples were obtained from patients at Azadi Teaching Hospital and Kirkuk General Hospital in Kirkuk City. Samples were dispersed as follows: urine sample (102), wound swab (38), burn swab (23), and sputum sample (37) as shown in (table 1). Forty isolates (20%) were isolated as *E.coli* andurine samples had the highest incidence of *E. coli* isolates, followed by wound swabs, sputum, and burn swabs, with percentages (70%, 20%, 5 %, and 5%) respectively. These findings are in agreement with those [14] in Kirkuk, who mentioned that *E.coli* represented 42(27.10%) of clinical isolates with a high percentage among urine samples. According to the current findings, urine samples had a high incidence (70%) of *E. coli*. Uropathogenic *Escherichia coli* (UPECs) were the predominant etiological agent of UTIs, accounting for upwards of 75% of all cases [15] . Also are compatible with [16]. However, it contrasted with a study [17] who showed that 400(66.6%) of isolates were *E.coli*. The disparity in the findings can be attributed to variations in the sample size, isolation source, number of hospitals investigated and sample collection season.

Table 1. Isolation source and percentage of *E. coli*

Isolation source	*Total Number of samples*	*Number of E.coli isolates*	*Percentage of E.coli isolates*
Urine	102	28	70%
Wound swab	38	8	20%
Burn swab	23	2	5%
Sputum	37	2	5%
Total	200	40	100%

Identification and isolation of *E. coli*

MacConkey agar, Blood agar, and Eosin Methylene Blue (EMB) have all been employed in morphological investigations to identify *E. coli*. The colonies under observation have a convex, round morphology. The colonies' surfaces seemed smooth when they were first isolated. Due to lactose fermentation, the colonies displayed a pink colouration on MacConkey agar. A green metallic sheen is a characteristic of *E. coli* proliferation that could be associated with dyes' metachromatic properties in EMB agar making the identification easier. Gram stain showed gram-negative non-spore-forming bacilli[18]. Biochemical tests were performed to characterize suspected *E. coli* isolates. All the isolates showed positive results for indol test, and Motility test, but were negative for the citrate utilization test, and urease test, and triple sugar iron test (TSI) was acid/acid (figure 4) [19]. Vitek 2 system was utilized to validate the results (figure 5).

Figure 4. Biochemical tests for *E. coli*

Bionumber: 0405610450006611
Organism Quantity: **Selected Organism: Escherichia coli**

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2412496103	**Expires:**	Sep 26, 2024 13:00 CDT
	Status:	Final	**Analysis Time:**	2.60 hours	**Completed:**	Feb 12, 2024 19:15 CST
Organism Origin	VITEK 2					
Selected Organism	99% Probability **Escherichia coli** Bionumber: 0405610450006611 Confidence: Excellent identification					
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	+	64	lLATa	-			

Figure 5. Vitek 2 compact system results for *E. coli*

Antimicrobial susceptibility test

The overall susceptibility patterns of *E. coli* isolates from various clinical sources are displayed in figure (6). The isolates exhibited varying degrees of resistance towards each antibiotic used in the study. Ceftriaxone exhibits the highest resistance rate 33(82.50%), followed by trimethoprim-sulfamethaxole and ceftazidime 32(80%), tetracycline 28(70%), cefepime 27(67.50%), azetronam and ciprofloxacin 23(57.5%), Amoxicillin- clavulanate 12(30%), pipracillin-tazobactam 11(27.50%), while the most effective antibiotics were nitrofurantoin (100%), meropenem(90%), imipenem and chloramphenicol(85%), and gentamycin(75%). Results in this study showed an elevation in the rate of resistance to third- and fourth-generation cephalosporin classes represented by ceftriaxone, ceftazidime, and cefepime. The (40) isolates of *E. coli* demonstrate the highest levels of antibiotic resistance to ceftriaxone reaching (82.50%). The resistance rate agreed with the study results [20] which reported the resistance rate towards ceftriaxone (80%). Resistance rate against trimethoprim-sulfamethaxole was 80% which is compatible to (54.1%) reported in a study from Nepal [21] This signifies daily increase in *E.coli* resistance to Co-trimoxazole. However, it disagreed with a study [22] showing that resistance to Co-trimoxazole was 48.5%. Ceftazidime and cefepime are broad-spectrum cephalosporins commonly employed in the treatment of gram-negative nosocomial infections.. The resistance rates towards these two antibiotics was high 80% and 67.50% respectively, contrary [23] Turkey, who reported that resistance rate for ceftazidime and cefepime were 22.2% and 20%, respectively. In contrast, it was compatible with some studies [24,25] who demonstrated that the resistance rate for ceftazidime and cefepime were 70%, and67.5%, respectively. *E.coli* isolates show high resistance to tetracycline (70%) which is consistent with the (57.5%) resistance rate reported by[20]. Also, it is incompatible with the (25%) resistance obtained [26] in Al-Najaf Province. Resistance to aztreonam and ciprofloxacin was (57.5%) which is in accordance with[27] who reported resistance to ciprofloxacin as 59.3% and [28] reported resistance to aztreonam (56%), but were inaccordance with [23] with aztreonam resistance (18.9%). Amoxicillin-clavulanate revealed a resistance rate at 30% at (27.8%) reported by some studyies [23] but disagreed with another [29] in Erbil City, which reported a high resistance at (91.4%). Additionally, the current study's piperacillin-tazobactam resistance (27.5%) contracted a study[30] who found the resistance to this antibiotic was (8%). The rise in resistance to this antibiotic could result from self-medication and the excessive use of antibiotics, which is usual in this region. The lowest antibiotic resistance was observed in carbapenems exemplified by imipenem, and meropenem, the ratio of resistance of the isolates against these two antibiotics was (10%), which was concordant witha scholar‘s [31] resistance rate for the same antibiotics (4.4%). As for chloramphenicol from the antibiotic group phenicol had a low resistance rate (12.50%), compatible results have been shown [29] but high resistance (88%) was detected[32]. Gentamycin is an aminoglycoside antibiotic that is broad-spectrum and an inhibitor for protein synthesis. *E.coli* is resistant to this antibiotic and had a resistance rate (20%). A similar resistance rate (22.2%) was detected [26], however high resistance was reported [33] at 75%. A high-level sensitivity of *E. coli* to Nitrofurantoin was observed at (100%); this could be a result of the antibiotic's limited indication, narrow spectrum of action, restricted tissue distribution, and limited interaction with bacteria outside of the urinary tract. An equivalent sensitivity rate of 96.4% was noted [28], whereas a significant resistance level of 50% against this antibiotic was observed in Samarra [33]. Infections resulting from multidrug-

resistant *E. coli* are increasingly concerning, with resistance caused by the production of extended-spectrum β-lactamases (ESBLs). In the current study, 21 (52.5%) of the isolates were identified as ESBL producers. This finding aligns with the results [34], which reported 59.7% of the isolates as ESBL producers, whereas a study [26] observed that 23.7% of the isolates were ESBL producers. Third generation cephalosporins and most other β-lactam antibiotics can be hydrolyzed by ESBLs. Furthermore, coresistance to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole may occur, but carbapenems are considered the most effective treatment for ESBL *E. coli* infections since they are not affected by the ESBL enzyme. [3]. This may explain the findings of the current study regarding the previously mentioned classes of antibiotics.

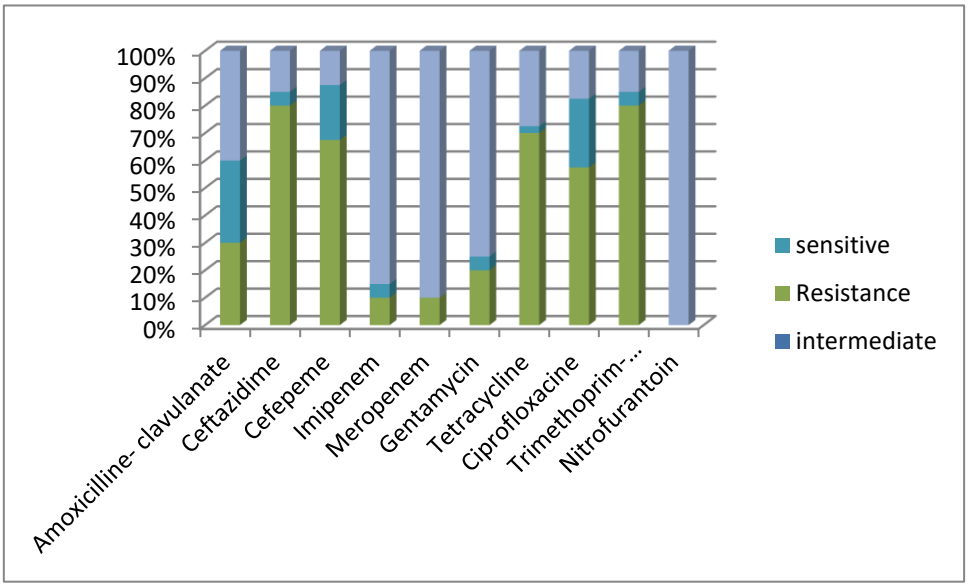

Figure 6. Antibiotic susceptibility test pattern in *E. coli*

Biofilm production

The microtiter plate method was used to quantify 40 *E. coli* isolates. Of these, 40 (100%) produced biofilm, 30 (75%) produced weak biofilm, 9 (22.5%) produced moderate biofilm, and 1 (2.5%) produced strong biofilm (figure 7). According to a scholarly work [35], 92% of the isolates were able to create biofilm and prior studies utilizing MTP to examine biofilm generation showed a similar outcome. Another investigation was done in Mexico also demonstrated that all identified *E. coli* isolates exhibited the capability to form biofilm.

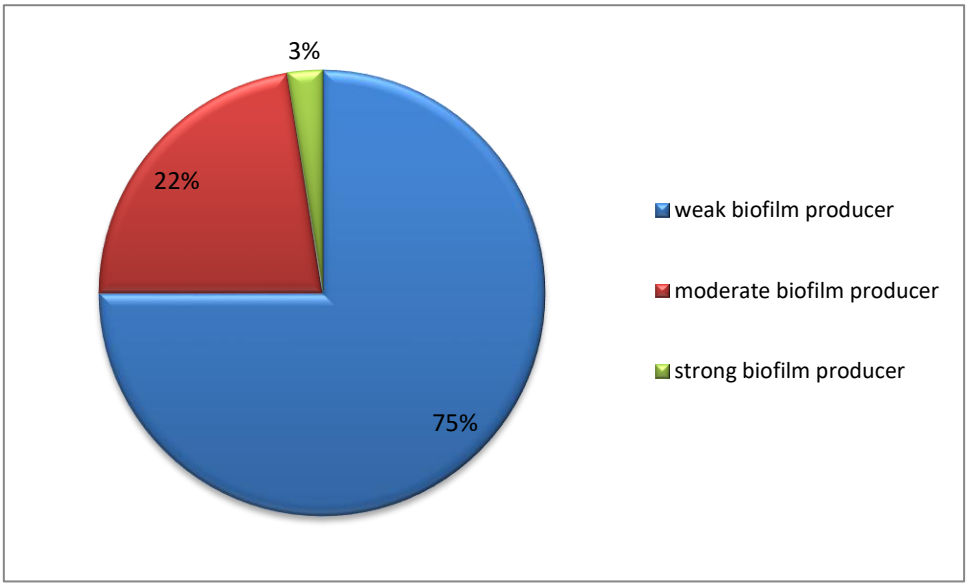

Figure 7. Biofilm production in *E.coli*

Conclusion

This study has come up with the following conclusions:

1. *Escherichia coli* is a famous gram-negative bacillus implicated in several intestinal and extraintestinal infections, mainly UTIs.
2. *E. coli* exhibits significant resistance to numerous common antibiotics.
3. All clinical isolates of *E. coli* demonstrate the capacity for biofilm formation.

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