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Research article

Impact of Biofilm Formation on Antibiotic Resistance in *Escherichia coli*

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ABSTRACT

Escherichia coli is a main nonpathogenic bacterium naturally present in the human gut. However, certain strains have adapted to cause infections, leading to illnesses in the gastrointestinal tract, urinary system, or central nervous system, even in otherwise healthy individuals. The study focused on the role of biofilm formation on resistant isolate of *E. coli.* Biofilms can be defined as groups of microorganisms attached to a surface. In this study 25 strains of *E. coli* have been used, the isolates have been detected by using Gram-stained, biochemical test and VITEC 2 system, As for antibiotic resistance, six antibiotics have been used, nitrofurantoin, piperacillin, amikacin, ceftriaxone, chloramphenicol, and Vancomycin. The concentrations have been used according to the clinical and laboratory standard Institute (CLSI). Biofilm Assay Protocol was used. All isolates exhibited sensitivity to nitrofurantoin and chloramphenicol, but they were resistant to piperacillin (100%), vancomycin (100%), and ceftriaxone (50%), followed by amikacin (50%). Biofilm production of 25 *E. coli* isolates showed that 25 isolates were able to form biofilm, 11 isolates indicated strong reaction, 6 were medium and 8 of them were weak biofilm production. Among isolations creating biofilm more than 44 % of bacteria exhibit strong biofilm production, 24% moderate, and less than 32 % weak biofilm production.

Keywords: Antibiotic resistance, Biofilms, *Escherichia coli*, Urinary tract infection, Virulence Factors

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1. INTRODUCTION

Urinary tract infections (UTIs) are currently the most frequently diagnosed bacterial infections. The principal cause of these infections is *Escherichia coli*, which is responsible for 65 to 75 percent of all cases, according to research. Other bacteria, such as *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, also contribute to UTIs. Globally, approximately 150 million people experience UTIs every year, with about 10.5 million cases occurring in the United States [1,2].

Women get urinary tract infections up to 30 times more often than men do, with up to 60 percent of women getting a UTI at least once in their lives. *E. coli* is normally found in human intestine, but when it spreads to another body location, it can cause different diseases (e.g. diarrhea, meninges, UTI, and infect soft tissues), and in this cases it is called opportunistic bacteria [3]. Biofilm is one of the virulence factors of bacteria, and it is one of the mechanisms of resistance [4]. Biofilm refers to a group of microorganisms that are permanently attached to

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a surface and cannot be easily washed away. These microbes are surrounded by a protective matrix primarily made of polysaccharides [5,6].

The formation of biofilms on medical devices is a leading cause of hospital-acquired infections. These infections are particularly stimulating to treat because biofilms can resist significantly higher concentrations of antibiotics compared to free-floating bacteria [7,8]. The study focused on the role of biofilm formation on the resistance of *E. coli*

2. MATERIAL AND METHODS

2.1. Isolates

Fifty specimens were collected from patients and other urinary tract infections. All isolates were collected from some laboratories in the Baghdad province of Iraq from September 2023 to February 2024. The samples were collected with sterile cups properly labeled for each patient including patient name, age, and sex the samples were taken to the Medical Bacteriology Laboratory. The specimens were streaked on Nutrient agar, blood agar, MacConkey agar, and EMB agar. Bacterial isolates were finally identified through Gram staining, analysis of colony morphology, and various biochemical tests. Antimicrobial susceptibility was evaluated using the modified Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The VITEK 2 System was used according to the manufacturer's instructions. Normal saline (3 ml) was placed in a Kahn tube and inoculated with a loop full of isolated colonies and mixed by the vortex. The Kahn tube was checked for standardization to McFarland standard (1.5 X 10^8 CFU/ml) by using the Dens Check machine. The density must be (0.50 – 0.63). Then was transferred 145 µL by red pipette for G-ve bacteria to another Kahn tube. In the Kahn tube, an ID card was inserted into the cassette, and the identification number of a sample was entered via barcode. In the filler module, the cassette was then placed, and when the cards were filled, the cassette to the reader and incubator module. The incubation temperature, the optical reading of the cards, and monitors were controlled by the instruments, and data was transferred to the computer for analysis. Then the system automatically ejected the cards into a waste container when the test cycle was completed.

2.2. Antibiotic Sensitivity Test

The anti-biogram patterns of *E. coli* isolates were analyzed using the disc diffusion method, as outlined by Bauer et al. (1966) and by the Clinical Laboratory Standards Institute (CLSI) guidelines (2019). The isolates were tested against six commonly used antibiotics, as detailed in (Table 1). The standard inoculum was prepared by diluting the overnight bacterial growth with sterile Mueller-Hinton broth; until the turbidity was equal to the 0.5 McFarland standards (containing approximately 1.5 x 10^8 CFU / ml). The standard inoculum was smeared on Muller-Hinton agar (MHA) plates using a sterile cotton swab.

Plates were allowed to dry for a few minutes and antibiotic discs were placed on the agar surface and plates were incubated for 12-24 hours at 37°C.

The sensitivity or resistance of isolates for a particular antibiotic was determined by measuring the diameter of the zone of inhibition of growth with an antibiotic zone scale. The results were taken as sensitive or resistant based on CLSI 2019 [9].

Table 1. Antibiotic discs that used in the study

No.	Antibiotic disc	Code	Concentration Mcg/disc	Origin
1	Nitrofurantoin	NI	300	USA
2	Piperacillin	PRL	100	USA
3	Amikacin	AK	30	USA
4	Ceftriaxone	CL	30	USA
5	Chloramphenicol	C	30	USA
6	Vancomycin	VA	30	USA

2.3. Microtiter Plate Biofilm Assay Protocol

Preparation of inoculating culture by overnight cultivation of the selected isolate to obtain the mid-log phase (OD600 ~0.5). Then dilute to OD600 = 0.2 the growth, in 96-well microtiter plates, and distribute 95 µl of selected medium per well. Then add to each well 5 µl of culture prepared in step 1. Negative controls include media-only wells and avoiding using wells on the plate's periphery (fill with media alone to avoid drying). Seal at the desired temperature with parafilm and incubate, gently transfer the media remaining in the wells containing unattached cells after the required incubation time (24 hours) and gently submerge the plate in deionized water. Then remove the plate, gently transfer out the contents again, and wash again. Additional washing steps may also be required, depending on the desired stringency, 200 µl 0.1% Crystal violet (w / v) has been applied to each well and stained at room temperature for 10 minutes. Three times wash the plate gently. Allow air-dry on the plate, measuring dye absorbed by adherent cells and matrix. 200 µl of 95%ethanol (or another solubilizing agent) were added, to each well, and incubated for 10 min at RT. Pipette this solution containing re-solubilized dye up and down twice to mix, transfer to a clean 96-well plate, and read absorbance was checked, the strength of biofilm formation was calculated as per the given formula below [10].

Table 2. The categories of biofilm levels.

Average OD value	Adherence	Biofilm production
OD ≤ ODc	None	None
ODc<OD ≤ 2 × ODc	Weak	Weak
2 × ODc<OD ≤ 4 × ODc	Moderate	Moderate
4 × ODc< OD	Strong	Strong

3. RESULTS

3.1. Identification of *E. coli*

50 urine samples cultured on selective media for Gram-negative bacteria, 25 isolates appeared as pink, with green metallic sheen on MacConkey agar and EMB agar, respectively, indicating its ability to ferment lactose. They were confirmed as Gram-negative isolates based on microscopic examination of Gram-stained 25 isolates were subjected to confirmatory identification by using VITEC 2 system and the results of the VITEC 2 system proved that all 25 isolates were *Escherichia coli*. The *E. coli* pink on MacConkey agar and green metallic sheen on EMB agar positive to indole and methyl red tests and were negative to Voges – Proskauer and Citrate utilization tests when tested for traditional biochemical tests (IMViC test), as shown in (Fig. 1).

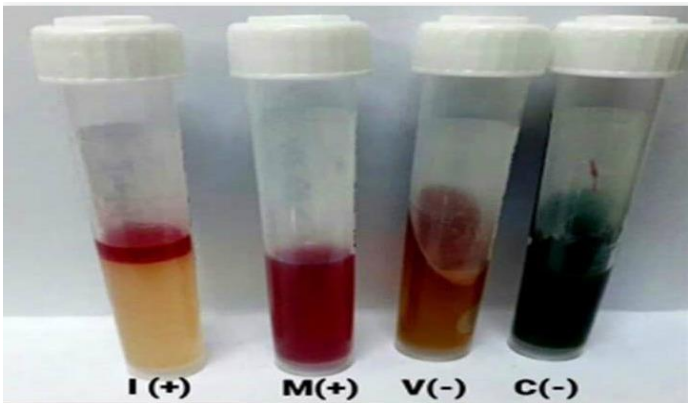

Fig. 1. IMViC test results for E. coli (after 24h at 37°C).

3.2. Antibiotic Sensitivity Test

As shown in Fig. 2, all isolates exhibited sensitivity to nitrofurantoin and chloramphenicol, but they were resistant to Piperacillin (100%), Vancomycin (100%), and Ceftriaxone (50%), followed by Amikacin (50%). Resistance to Piperacillin, *Escherichia coli* has mostly been associated with mechanisms that discuss resistance to third-generation cephalosporins [11,12].

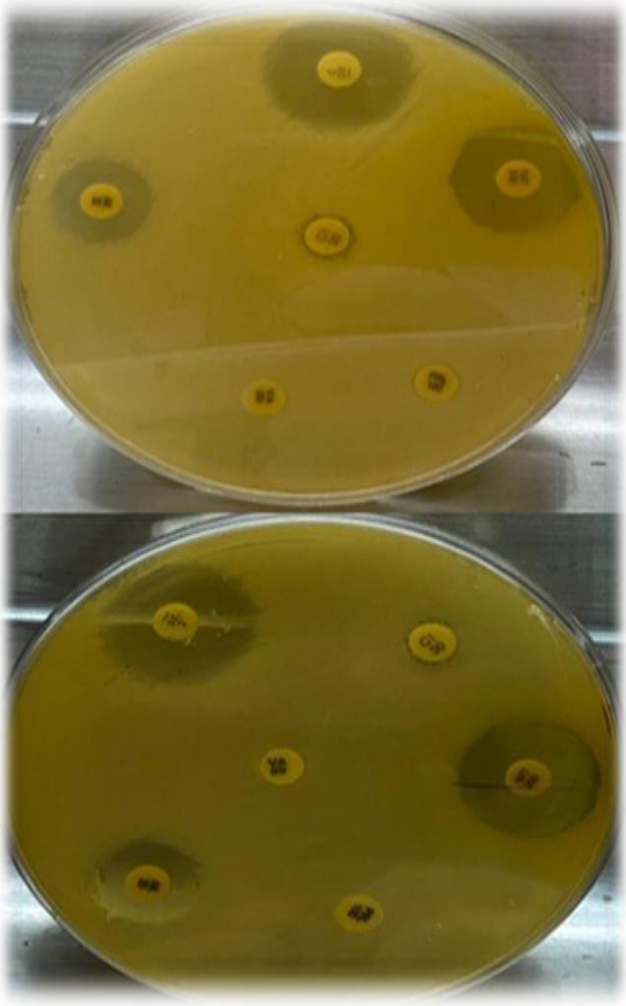

Fig. 2. Antibiotic Sensitivity Test.

3.3. Biofilm production

Among the 25 *E. coli* isolates, all 25 were able to produce biofilm: 11 (44%) exhibited strong biofilm production, 6 (24%) exhibited medium,8 (32%) exhibited weak biofilm production. Among isolations creating biofilm as shown in Table 2 and Figure 3.

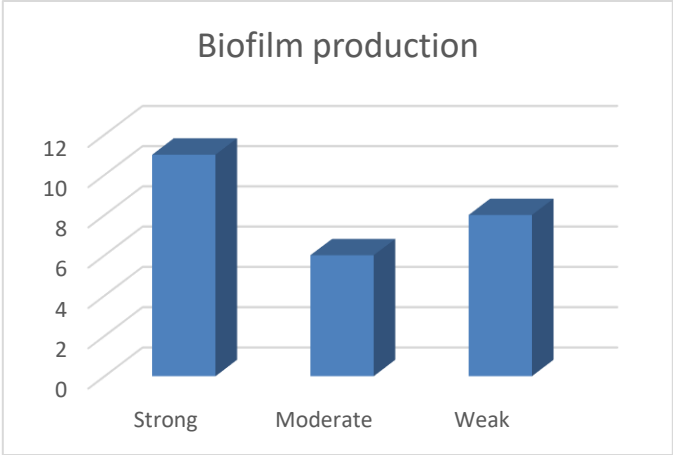

Fig 3. Percentage of biofilm production of E. coli isolated from UTI, The figure shows that 44% of isolates exhibited strong biofilm production, 24% showed moderate production, and 32% displayed weak biofilm production.

4. DISCUSSION

Most *E. coli* isolates resistant to piperacillin-tazobactam (TZP) also exhibit resistance to third-generation cephalosporins (3GC). However, a new phenotype of *E. coli* that is resistant to TZP but remains susceptible to 3GC has recently been identified. The underlying mechanisms of resistance and this emerging phenotype's population structure are still poorly understood. In this study [13], we agree with this source, as all isolates were resistant to piperacillin.

In this research, all *E. coli* isolates were found to be sensitive to nitrofurantoin, a drug commonly used to treat uncomplicated urinary tract infections, with rising resistance to other antibiotics, there is growing interest in nitrofurantoin antibiotics. Despite being in use for over 60 years, resistance to these antibiotics remains minimal, as supported by the current findings showing complete sensitivity among the isolates studied [14,15].

All isolates exhibited sensitivity to Chloramphenicol (CHL) is a broad-spectrum antibiotic that was widely used in veterinary medicine until concerns about its potential toxicity raised issues regarding its safety [16]. Ceftriaxone is a third-generation cephalosporin antibiotic commonly used to treat invasive infections caused by Enterobacteriaceae, including *E. coli* [17,18]. The results showed that half of the isolates were sensitive to ceftriaxone. This rising resistance, the World Health Organization has recently classified ceftriaxone-resistant Enterobacteriaceae as significant pathogens of critical importance. The most common mechanisms of ceftriaxone resistance in Enterobacteriaceae are the production of extended-spectrum ß-lactamases (ESBLs) and AmpC ß-lactamases [19]. On the other hand, the results showed that the bacteria resistant to the antibiotic have the biofilm property, which is one of the virulence factors of the bacteria, and it is possible that the gaining of resistance was due to the biofilm formation, The production of an exopolysaccharide matrix, or

glycocalyx, is a key characteristic of biofilms. It is believed that this matrix assistances prevent antibiotics from reaching the bacterial cells within the biofilm. In a previous study, *Pseudomonas aeruginosa* biofilms inhibited the diffusion of antibiotics. In contrast, biofilms of *S. epidermidis* formed by a similar method allowed antibiotics to diffuse through the membrane, suggesting that these antibiotics can effectively penetrate the biofilm. These findings indicate that inhibition of diffusion may not always reason for resistance to antimicrobial agents [20].

5. Conclusion

The study covers most of the bacteria associated with UTI, which are the most common type of bacterial infection diagnosed today. The most common bacteria to cause these infections are *E. coli.* All isolates exhibited sensitivity to nitrofurantoin and chloramphenicol, but they were resistant to Piperacillin (100%), Vancomycin (100%), and Ceftriaxone (50%), followed by Amikacin (50%). In this study, more than 44% of bacteria exhibit strong biofilm production, 24% moderate, and less than 32% weak biofilm production, The results showed that the bacteria resistant to the antibiotic have the biofilm property, which is one of the virulence factors of the bacteria, and it is likely that the acquisition of resistance was due to the biofilm. Recent genomic studies, such as DNA microarray analysis, have identified several new *E.coli* genes associated with biofilm formation. However, many other genes involved in this process may have been overlooked due to factors such as low expression levels, regulatory mechanisms, or variations in genetic backgrounds.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The study on bacteria was approved by the Research Ethics Committee of AlMustansiriyah University College of Pharmacy/ Iraq under protocol number (136x, 2024). All procedures adhered to ethical guidelines for microbiological research and ensured compliance with international standards.

Author contributions

Mohammed NS: Investigation; Methodology; Project administration; Resources; Supervision; Validation; Roles/Writing - original draft; and Writing - review & editing.

Obaid MM: Conceptualization, Data curation, and Formal analysis, Roles/Writing - original draft; Visualization and Writing - review & editing.
Jasem MA: Investigation; Methodology; Project administration.
Noaman TM: Roles/Writing - original draft; Visualization and Writing - review & editing.

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