Phenotypic and Molecular Correlation between Biofilm Production and Antibiotic Resistance *Escherichia coli* that Isolated from Heart Catheterization Patients

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ABSTRACT

Several types of bacteria enhance their survival by attaching to non-living surfaces or tissues, presenting them as multicellular communities covered by a protective extracellular matrix called biofilm. There has been a clear interest in assessing the relationship between antibiotic resistance phenotype and biofilm production. The aim of this paper was to present additional experimental results on this topic and to test the biofilm formation ability of *Escherichia coli* isolates using *in vitro* methods in the context of their antibiotic resistance. In our case study, (24) isolates of *E. coli* were included, and the colony morphology of the isolates was recorded after 24 hours, while the antimicrobial susceptibility test was performed by adhesion using the Kirby Power disc diffusion method. The biofilm formation of the isolates was evaluated using the method. All 24 clinical specimens 100% showed strong biofilm formation and all clinical specimens showed the presence of *pml* gene by clear bands in gel electrophoresis. The results of the antibiotic sensitivity test of *E. coli* isolates by disk diffusion method against 18 types of commonly used antibiotics showed a large variation in their rates of antibiotic resistance, as the highest percentage of resistance to the antibiotic to *E. coli* resistance to the most common antibiotics used in treatment. The highest rate of resistance was seen with cephalothin and Clindamycin 24/24 (100%) followed by ceftazidime, cefotaxime Levofloxacin, Ciprofloxacin and Erythromycin 18/24 (75%), Netilmicin, Chloramphenicol, Amoxicillin, and Tetracycline 16/24 (66%), Gentamicin 14/24 (58%), Tobramycin and Trimethoprim 11/24 (45%), Ampicillin and Impenem 6/24 (25%), Colistin sulfate and Azithromycin 4/24 (16%) as all isolates were sensitive. The study found a positive correlation between interesting study genes and biofilm formation and antibiotics in *E. coli*. Strains that appeared multidrug-resistant (MDR) were given a high mean of biofilm. It has been demonstrated that some correlations exist between antibiotic resistance and the biofilm-forming ability of *E. coli* isolates. There is an association between biofilm production with persistent infection and antibiotic failure.

INTRODUCTION

A significant number of deaths result from antimicrobial resistance, which is a global health issue. Antibiotic-resistant bacterial strains can cause serious infections with a number of negative effects, such as increased mortality and morbidity, prolonged hospital stays, and financial losses. Pathogenic bacteria may re-become resistant to a wide range of chemically unrelated antigens through several resistance mechanisms (either chromosomal or plasmid-encoded) (*Ali et al.,* 2018). Multidrug resistance (MDR) is defined as insensitivity to at least one antimicrobial compound in three or more antimicrobial classes Based on projections of the "Cooperative Group's Burden of Antimicrobial Resistance" while according to the O'Neill report (isolated by the National Health Service in the United Kingdom), MDR infections may lead to 10 million excess deaths by 2050 (Cassini *et al.,* 2015; O'Neill, 2014).

Enterobacteriaceae are of greatest interest, given their overall disease burden, mortality rates, and ever-increasing rates of resistance. In experiments in vitro (that is when bacteria are grown in different culture media), bacteria are often found in their planktonic (or free-living) states (Domonkos *et al.,* 2016*)*. However, in extreme environmental conditions or in vivo in an infected host, bacteria maintain their viability by attaching to non-living surfaces or tissues, forming complexes consisting of more than one cell encapsulated in a cell-enveloping matrix, known as a biofilm *(*Lebeaux *et al*., 2014*)*. In fact, according to a recent estimate by the National Institutes of Health (NIH), more than 60% of in vivo infection is caused by microorganisms present in biofilms. Biofilms composed of exopolysaccharides (EPS), nucleic acids (environmental DNA), proteins, lipids, various ions and water, secreted by multiple bacterial communities, will create good conditions for the survival of all bacteria (Chatterjee *et al.,* 2014*)*. In addition to the "classical" resistance factors seen in bacteria, biofilm formation is another risk factor, and may be the main cause of recalcitrant chronic infections (such as catheter-associated infections, skin and soft tissue infections, and dental caries) (Stájer *et al*., 2020). Biofilms provide bacteria with protection from penetration of harmful substances and antibiotics with effective concentrations leading to lower inhibitory concentrations. Strong biofilm production is an important feature of all disease-causing microbes (Santajit *et al*., 2016). Thus, the main objective of this study was to present results that show the association between the ability of intestinal bacteria to produce biofilms and the antibiotic resistance phenotype using in vitro experiments.

MATERIALS AND METHODS

The study was conducted at Bacteriology and Molecular Laboratories in Biology Department, Sciences Faculty, Kufa University, Iraq.

Clinical Specimens:

The study included a collection of 126 clinical specimens obtained from patients who were attending in Al- Najaf (Al-Sadder Medical City, suffering from cardiac disease during the period from July 2022 to December 2022.

Isolation and Identification of *Escherichia coli*:

E. coli was isolated and identified according to traditional biochemical diagnostic, by using routine methods e.g. according to MacFadden, (2000). These isolates were cultured on brain heart infusion broth at 37°C and on MacConkey agar plate (Himedia), blood, mannitol and eosin methylene blue media.

Vitek–2 for Confirm Identification:

GN identification card was used for the identification of Enterobacteriaceae (Guido *et al*., 2005).

Antibiotic Susceptibility Test:

In this study used 18 types of commonly used antibiotics include: Amoxicillin 30 µg, Ciprofloxacin 10 µg, Chloramphenicol 30 µg, Piperacillin 100 µg, Azithromycin 15 µg, Doxycycline 10 µg, Meropenem 10 µg, Gentamicin 30 µg, Ampicillin 25 µg, Cefoxitin 30 µg, Levofloxacin 5 µg, Cefotaxime 10µg, Imipenem 10 µg, Tobramycin 10µg, Clindamycin µg and Tetracycline 30µg. The antibiotic sensitivity report was performed according to Kirby-Bauer disc diffusion fashion on Mueller-Hinton agar (Morello *et al*., 2006). Briefly, the investigated isolates were allowed to multiplication overnight at 37ºC in BHI broth referred to 0.5 McFarland turbidity standard equal to $1.5X10^8$ CFU/ml (Mcfarland, 2000), the MH agar plates were fully spreading with 0.1ml of growth suspension and then fixed antibiotics disks on the surface. The applied plats were left for 10-15 minutes and then incubated for 24 h at 37ºC as a standard cultural condition. The fixed antibiotics were classified as sensitive (S), Intermediate (I), or resistant (R) according to the diameters of halo zone in millimeters (mm) around the individual disk, the results

were compared with the clarifying list of (CLSI, 2021).

Detection of Biofilm Formation Activity:

Using the TCP approach, the ability of each isolate to produce biofilm was evaluated. Before being diluted 1 in 100 times with the new brain heart infusion broth medium containing 3% glucose, the isolate was injected into the brain heart infusion broth medium and cultivated for approximately 18 hours at 37°C in a static environment. Adding 0.2 ml aliquots of the diluted culture were placed in several wells of a sterile polystyrene 96-well flat bottom culture plate. Broth was used as a sterility and non-specific medium binding control. The tissue culture plate was kept at 37°C for 18 and 24 hours of incubation. After incubation, the contents of each well were carefully taken out by hitting the plate. The wells were cleaned four times with 0.2ml of phosphate buffer saline to get rid of floating "planktonic" microorganisms (pH 7.2). Crystal violet (0.1% w/v) was used to stain the biofilm formed in the plate by adherent "sessile" bacteria. Sodium acetate (2%) was used to fix the biofilm. The remaining stain was removed from the plates using deionized water, and they were then left to dry. Both sides of the wells were covered in biofilm from adhesion bacteria, which were consistently stained with crystal violet. After adding 200 µl of absolute ethanol to each well, the optical density (OD) of stained adhesion bacteria at 570 nm wavelengths was calculated using a micro ELISA auto reader (OD 570). The experiment was conducted three repeated and three times, with the data averaged and the outcomes described in accordance with Stepanovic et al., (2004).

Molecular Study:

Extraction of DNA and PCR Conditions:

Total DNA was extracted using the boiling method according to Shah et al., (2017). Thermo cycle PCR was used to use the molecular method to confirm the formation of biofilms once more. This method requires specific primers for the *pml* gene, which have the sequence F 5' GGATCATCTATAATGAAACTG 3' and R 5' CTGATAATCAACTTGGAAGTT 3', and are 563 bp in size (Abbas et al., 2015). Then, 12 µl of master mix, 5 µl of template DNA, 2 µl of each set of primers, and complete to 25 µl of sterile nuclease-free water were placed in an appropriate PCR tube. The mixture was vortexed well. The PCR designed to amplify the *pml* gene included a primary denaturation step for 2 minutes at 94°C, denaturation, 52°C/30 sec for annealing, and flowed by extension for 72°C for 60 seconds. The reaction mixture was held at 4°C until use while the final extension step took place at 72°C for about 10 minutes (Abbas et al., 2015). Each and every PCR amplification was performed using a Verity Thermal Cycler (Agilent, UK). Then, 1% agarose gel electrophoresis was used to analyze all of the PCR products, and they were all stained with red ethidium bromide dye. Finally, the gel documentation system was used to identify the electrophoresis results.

Statistical Analysis:

Experimental data were presented in terms of observed number and percentage frequencies; SPSS (Statistical Package for Social Science) program version was used. Regarding other data were analyzed Correlation.

RESULTS AND DISCUSSION

Patient Population Statistics:

A total of 126 patients attended the Open Heart Center at Al-Sadr Hospital in Al-Najaf Governorate. They were distributed according to the type of catheter: the first group 60 (47.61%) patients with therapeutic catheters, 30 (23.8%) patients with diagnostic catheters, 16 (12.6%) patients with diagnostic and therapeutic catheters together, 10 (7.9%) patients with electric catheters and 10 (7.9%) patients with peripheral catheters (Table 1). Following that, the specimens underwent identification and culturing on media (Morphological, Biochemical test, and VITEK-2 Compact System). The results demonstrate that only 24 isolates from different catheter types of cardiac disease

were confirmed as *E. coli* with ID message confidence levels ranging was very good (Probability percentage from 88 to 98).

Table 1: Percentage of Cardiac Catheter Types patients in the Open Heart Center at the Chest Hospital.

Heart disease patients	Number of Samples	Percentage
Therapeutic catheters	60	47.61
Diagnostic catheters	30	23.80
Diagnostic and therapeutic catheters together	16	12.69
Electric catheters	10	7.93
Peripheral catheters	10	7.93
Total	126	100%

Bacterial infection may be a factor in the development of rheumatic heart disease, coronary artery disease, and atherosclerosis (Zavareh *et al*., 2016). This method was considered as a factor and proof of diagnosis of infections related to catheterization and confirmed by Bouza *et al*. (2003) who explained that cardiac catheterization using a catheter tip is one of the risk factors for bacteremia by pathogens. Stewart and O'Grady, (2011) show that veins peripheral catheterization indicator of the risk of bacteremia which measures by catheter tip methods. Fätkenheur *et al*., (2003) recommended that the semi-quantitative catheter tip culture method be regarded as the industry standard for bacteria adhering to the catheter's outer surface. The semi-quantitative approach is effective at identifying catheter-associated bacteremia (Ripa *et al*.,2018). Because of its ease of use and affordability, this test is a popular method for diagnosing infections linked to catheterization. The risk of bacterial colonization of cardiac catheters used in humans is increased by the longer indwelling times of IV catheters (Parra-Flores *et al.*, 2016). Catheter colonization with series pathogens and catheterization duration are statistically significantly correlated (Ripa *et al*., 2018).

E. coli is straight, cylindrical, Gram-negative bacilli, a rod-shaped cell with rounded ends that are 0.5 μm in diameter and 2.0 μm in length, they occur singly or in pairs (Gupte, 2010). It has distinct metallic green sheen colonies on EMB agar and pink to red colored colonies on MacConkey agar plate that can grow at 37°C (AI-Tememy, 2014).

Antibiotic Resistance Profile:

Selective antibiotics are used to demonstrate their effect on *E. coli* isolates, which are 18 commonly, used antibacterial agents (Table 2). The results were interpreted according to the diameter of the inhibition zone and compared with standard zones of inhibition determined by CLSI, (2021). All the identified *E. coli* isolates were subjected to *in vitro* susceptibility testing by the modified Kirby-Bauer disc diffusion method in this study, which revealed that 24 isolates, from, *E. coli* were resistant to the most common antibiotics used in treatment. The highest rate of resistance was seen with cephalothin and Clindamycin 24/24 (100%) followed by ceftazidime, Levofloxacin and Ceftazidime and Erythromycin 18/24 (75%), Netilmicin, Chloramphenicol, Amoxicillin and Tetracycline 16/24 (66%), Gentamicin 14/24 (58%), Tobramycin and Trimethoprim 11/24 (45%), Ampicillin and Impenem 6/24 (25%), Colistin sulfate and Azithromycin 4/24 (16%). As shown in Table (2).

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Table 2: Antibiotic susceptibility test for 24 *E. coli* isolates**.**

Type of antibiotic class (subclass)	Antibiotic disk	Resistance	Intermediate	Susceptible
Quinolones	Levofloxacin	18 (75%)	0	6 (25%)
	Ciprofloxacin	18 (75%)	0	6 (25%)
β-lactams Carbapenemase	Impenem	6 (25%)	0	18 (75%)
Aminoglycosides	Gentamicin	14 (58%)	0	%)41.6(10
	Tobramycin	11 (45%)	0	13 (54%)
	Netilmicin	16 (66%)	0	%)33 (8
Sulfonamides (antifolate)	Trimethoprim	11(45%)	0	13 (54%)
Macrolides	Erythromycin	18 (75%)	2 (8%)	4 (16%)
Tetracycline	Tetracycline	16 (66%)	0	%)33 (8
Phenicol	Chloramphenicol	16 (66%)	0	%)33 (8
β-lactams cephalosporines	Azithromycin	4 (16%)	0	20 (83.3%)
	Ceftazidime	18 (75%)	0	%)25(6
	Cephalothin	24 (100%)	0	0
	Cefotaxime	18 (75%)	4 (16%)	2 (8%)
Lincomycin	Clindamycin	24 (100%)	0	0
Polymyxin	Colistin sulfate	4 (16%)	4 (16%)	16 (66%)
Penicillins	Ampicillin	6 (25%)	0	18 (75%)
	Amoxicillin	16 (66%)	0	%)33 (8

Antibiotic resistance is a significant clinical issue in the treatment of infections. One of the most significant multidrug-resistant (MDR) opportunistic Gram-negative bacteria, *E. coli* causes a variety of illnesses with high mortality and morbidity due to hospital-acquired infections and nonhospital-acquired infections. Acquired infections and antibiotic susceptibility patterns vary by geographic area, and *E. coli* has been identified by the WHO, (2004) as one of the key bacteria representing significant increases in antibiotic resistance rates, which is a major concern. Antibiotic resistance among *E. coli* isolates has also been reported with an increased frequency worldwide due to aberrant use of antibiotics (Maraki *et al*., 2013; Soltani *et al*., 2018). Numerous factors, including overuse of antibiotics, the availability of antibiotics without a prescription, and poor surveillance, have contributed to the high rates of antibiotic resistance in hospitals and the general population. Quinolones are a crucial class of antibiotics for the empirical treatment of *E. coli* in various infections because of their high efficacy, low cost, and safety profile. They are also used as a broad-spectrum antibiotic (Remy *et al*., 2012). However, the widespread use of quinolones resistant strains, particularly in developing nations like Iraq, was a result of the overuse of these antibiotics (Dalhoff *et al*., 2012),

The current study's findings regarding (75%) Levofloxacin-resistant *E. coli* isolates were corroborated by AL-Azawi's, (2015) findings of (62.5%) Levofloxacin-resistant isolates. Similar studies that were published by Tajbakhsh *et al*., (2016) found that resistance to this antibiotic was (56.25%), and (Rameriz-Castillo *et al*., 2018) exposed the percentage of bacterial resistance to this antibiotic was 47.3%. The results of the current study regarding the antibiotic ciprofloxacin of the quinolones group, and it was shown that the high resistance to ciprofloxacin is due to the fact that this group of antibiotics was the first choice for the treatment of cardiac diseases.

Third-generation cephalosporines were used as empirical antibiotics to treat a variety of clinical cases, and the rate of antibiotic resistance to these drugs has been startlingly high globally (Rajivgandhia *et al*., 2018; Majeed and Aljanaby, 2019). It has been suggested that the main cause of *E.*

coli's high resistance is their possession of efflux pumps, which facilitate the efficient flow of antibiotics outside of the cell and remove their harmful effects. It has also been suggested that one cause of *E. coli*'s antibiotic resistance to β-lactams is their production of the enzymes β-lactamases, which include the enzymes cephalosporinase and penicillinase, as these enzymes break down the β-lactam (Platansing, 2015). According to the results, 75% percent of *E. coli* was found to be resistant to ceftazidime and cefotaxime however, Nalini *et al*., (2014) found that the resistance rate for these antibiotics was 73.31%.

The findings demonstrated the prevalence of gentamycin resistance (58%) According to Zaman *et al*., (2017), possessing Aminoglycoside Modifying Enzymes (AMES), N-acetyl transferase and phosphotransferase, as well as possessing efflux systems and change in membrane permeability on the outer surface of the bacterial cell are two causes of *E. coli* resistance to antibiotics in the aminoglycosides group. Comparable studies were conducted when (Sabir *et al*., 2014) discovered that. The findings revealed macrolide-group antibiotic erythromycin resistance (75%), whereas other research by (Kibret and Abera, 2011) revealed that bacterial resistance to this medication was extremely high (89.4%). The percentage of Trimethoprim-Sulfamethoxazole (45%) was also revealed by the results, which are nearly identical to those reported by (Karaaslan *et al*., 2013), who discovered that the resistance rate for *E. coli* isolates is 50.6%. In addition, 25% of *E. coli* isolates are resistant to imipenem, according to results reported by (Al-Mamouri, 2015), who recorded a 6.8% resistance rate, while (Moş *et al*., 2010) stated that imipenem resistance was 50%.

This study found that tetracycline and chloramphenicol resistance was each at 46.6%, whereas (Mohammed, 2018) in Iraq found that the same antibiotics were each resistant to 88%, and (Abdel Hamid and Abozahra, 2017) in Egypt found that tetracycline resistance was 64.3% by *E. coli*. This might be because the bacteria altered the permeability of its outer membrane and had efflux pumps. Chloramphenicol acetyltransferase (CAT) is an enzyme that adds new acetyl groups to chloramphenicol antibiotics. When this enzyme is inhibited, the antibiotic is modified, which increases resistance to it.

Phenotypic Biofilm Detection

The ability of *E. coli* bacteria to form a biofilm was detected using the microliter plate method with 96 a hole, and the results showed that a high percentage of the *E. coli* isolates were biofilm-producing, so 24 bacterial isolates belonged to *E. coli* bacteria that have the ability to produce a biofilm (Fig. 1 and Table 3). The results agree with Tajbakhsh, (2016). The current study on the ability of bacteria to form a biofilm (100%) with the results reached by the researcher Poursina *et al*., (2018) showed that the percentage of bacterial isolates that have the ability to produce a biofilm (80%)

The ability of bacteria to produce a biofilm is one of the most important factors in the virulence of bacteria that are endowed by bacteria that are resistant to most antibiotics (Soto,2013). When bacteria are present in the liquid environment and when the biofilm is available, they live on solid surfaces, as the bacteria make several modifications and changes to suit the new environment. Including changes in the expression of surface molecules such as receptors on the surface of flagella and metabolic waste such as acids, carbohydrates, toxins, etc., opening up for bacteria a greater opportunity to survive in unfavorable conditions **(**Hayta *et al*., 2021**)**. Biofilm-producing bacteria are 1000 times more resistant to antibiotics than non-bacteria producing, Transmission of resistance genes within the biofilm environment between bacterial cells, whether by plasmids or jump genes or by the occurrence of random mutations that lead to an increase in cell resistance to

toxins and antibiotics. Expression of efflux pumps more in biofilm-forming cells. Stabilization of the biofilm by a change in concentrations of Ions, pH, and finally the presence of metabolically ineffective

resident cells that have a role in maintaining bacterial cells under the biofilm as a mechanical self-defense mechanism (Soto, 2014).
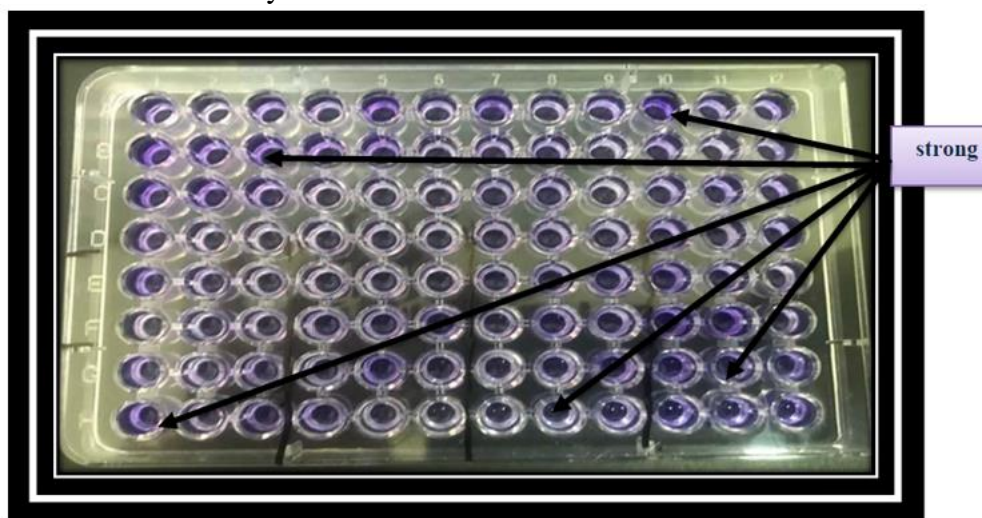

Fig. 1: Biofilm formation of *E. coli*.

Table 3: Distribution of *E. coli* Isolates According to the Types of Biofilms.

Stander rang of OD	Biofilm	*E. coli* No. (%)
<0.120	Non	0(0)
0.120-0.240	Moderately	0(0%)
>0.24	Strong	24(100)

Molecular Identification of Biofilm Production:

As shown in Figure (2), the molecular detection of the *pm1* gene using a specific primer for *E. coli* isolates resulted in positive amplification for every isolate (100%) tested. According to the study's findings, all of the isolates are members of the PM1 bacteria strain, which is distinguished by its unique phenotypic traits and by its possession of all the virulence factors necessary to spread infection. The study's findings revealed that 100% of isolates carried the *mrpA* and *pm1* genes, indicating the prevalence of the PM1 *P. mirabilis* strain in UTI and vaginal infections. One vaginal swab isolate's *mrpA* and pm1 gene content suggests that the

bacteria moved from the urinary tract to the cervical region through biofilm formation and swarming. According to Al-Dahhan, (2017) and Fusco *et al*., (2017), the PM1 isolate has a high capacity for adhesion in epithelial cells and is distinguished by the homogeneity of its colonies on solid media and the absence of clumping growth in liquid culture media (Brooks *et al*., 2007). And the development of the biofilm on biological surfaces after two hours of incubation, up to the full phenotypic characteristics of the biofilm after six hours of incubation due to its high ability to swarm, and using this strain to develop medications aimed at particular virulence factor pathways.
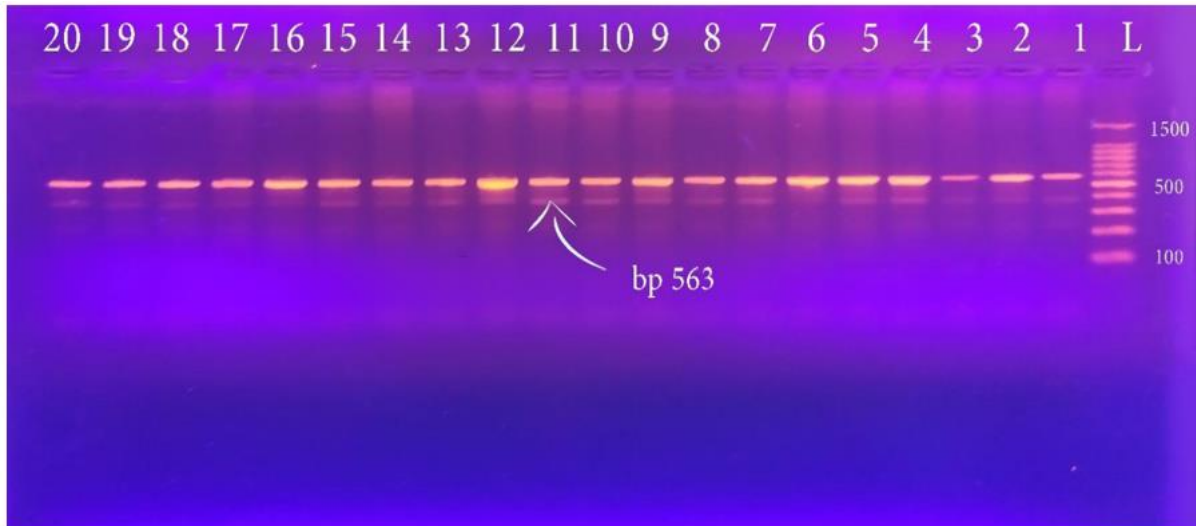
Fig. 2: Electrophoresis on agarose gel of *pm1* gene amplification products (563 bp) in isolates of *E. coli* (1% agarose, 70 V for 120 minutes). Column L: DNA size guide -100 base pairs. Column 1-20: isolates carrying the *pm1* gene.

Correlation of Antibiotic Resistance *E. coli* with Biofilm Formation:

Figure (3) revealed a positive significant moderate correlation (0.569**) in *E. coli* isolates between the number of antibiotic resistance and the mean of biofilm formation.
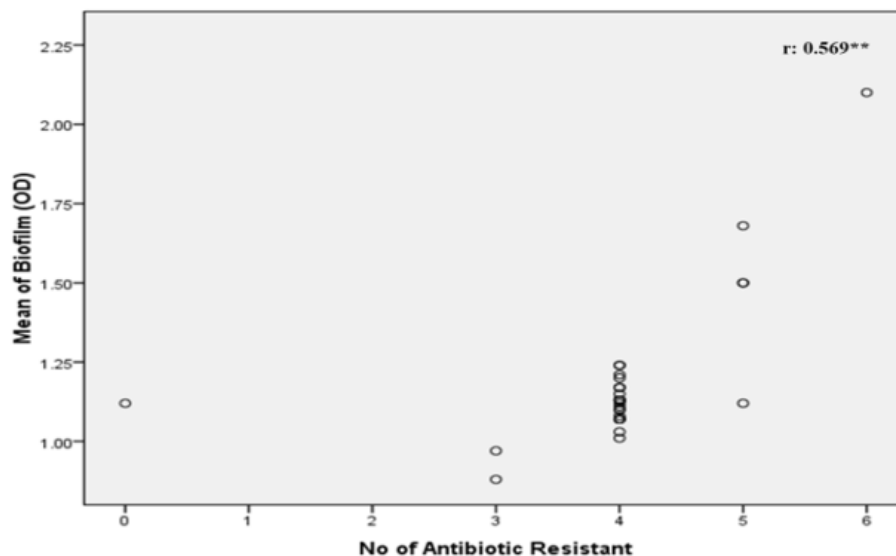

Fig. 3: Correlation between the number of antibiotic resistance (No.) and the mean of biofilm formation (OD) in 24 *E. coli* isolates **. Correlation is significant at the 0.01 level (2-tailed).

To date, it has been demonstrated that some correlations exist between antibiotic resistance and the biofilm-forming ability of *S. typhi* isolates. There is an association between biofilm production with persistent infection and antibiotic failure. Gilbert *et al*., (2002) reported that biofilm producers were to be 10-1000 times less susceptible to antibiotics than the equivalent cells growing planktonically. Curtin *et al*., (2003) revealed that biofilm hampered the penetration of antimicrobials and the concentrations required to eradicate biofilm-producing bacteria are higher than those required to eradicate strains that did not produce biofilm. Also, Keren *et al*., (2004) explained this issue as bacterial populations produce persister cells that

neither grow nor die in the presence of antibiotics and that persisters are largely responsible for high levels of biofilm tolerance to antimicrobials.

So, the process of biofilm formation is particularly relevant for the clinician because biofilm-associated microorganisms exhibit dramatically decreased susceptibility to antimicrobial agents (de-Silva *et al*., 2002). There are many virulence factors in *Enterobacteriacae* strains associated with its pathogenicity. Among these factors is the capacity of *Enterobacteriacae* strains to form a biofilm that protects it from the host immune response as well as from antibiotics (Bandeira *et al*., 2014; Chung and Rimal, 2016).

Globally, there has been an increase in the incidence of disease caused by biofilm-related microbes in the last year. Biofilm-producing microbes have drastically increased resistance to both antimicrobial drugs and the host immune response, posing a serious threat to public health. Notably, the rise in MDR bacterial and fungal strains has many people concerned about crises (Ghaly *et al*., 2020). Biofilm development and medication resistance make bacterial eradication more challenging in clinical settings. Therefore, the relationship between biofilm formation and drug resistance should be investigated. As previously said (Algburi *et al*., 2018), our findings revealed that biofilms play a significant role in building antibiotic resistance and that there is a link between biofilm production and antimicrobial resistance. Optimizing the use of vancomycin and imipenem is a viable technique for reducing biofilm-associated infection treatment failures (Broussou *et al*., 2018).

Conclusions:

Pathogens that form biofilms are a significant clinical concern because they frequently affect co-morbid patients who are hospitalized and who have recalcitrant, difficult-to-treat infections. Catheters and other implanted devices removal may be a crucial part of treating infections caused by biofilms.

This study demonstrated that *E. coli*, has a high resistance to many common antibiotics, especially ß-lactam. 100% of bacterial isolates have the capacity to produce a biofilm that is capable of holding them together. high resistance to many common antibiotics. 100% of bacterial isolates have the capacity to produce a biofilm that is capable of holding their formation, and antibiotics.

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