RESEARCH ARTICLE

Investigation of Biofilm Formation Efficiency in ESβLs of Pathogenic *Escherichia coli* Isolates

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ABSTRACT

Up to 70 clinical *Escherichia coli* samples were examined, with 30 identified as extended-spectrum β-lactamase (ESβL) isolates. These isolates were gathered from inpatients and outpatients at some Baghdad hospitals. Urine, pus, feces, and blood samples were obtained from government hospital patients of both genders and diverse age groups. Production of ESβL and the quick antibacterial diagnosis was checked using the VITEK2 for susceptibility experimentation and identification of the bacterial sample. The results showed that 30 isolates only in the current study were ESβL producers. Furthermore, all showed biofilm formation after 24 hours of incubation some of these isolates were the ability of biofilm formation decreased after 48 hours compared with negative control. The formation of biofilm has founded a vary from strong to weak. The variation in the bacterial study may depend on their antibiotic resistance and the sample's source. Therefore, this study considers the need to focus in subsequent studies on the effect of biofilm formation and resistance of isolates, which produce beta-lactamase enzymes for many antibiotics, thus avoiding the occurrence of double health crises for infected patients.

Keywords: Biofilm formation, *Escherichia coli*, ESβL, Multiple drug resistance.

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INTRODUCTION

Enterobacteriaceae is the most common group of pathogenic and non-pathogenic, gram-negative bacilli groups.[1] It is a symbiotic bacterium that colonizes the intestines naturally after birth. *Escherichia coli* is the most common pathogen in Enterobacteriaceae. It can potentially infect the human body (Opportunistic pathogen) opportunistically,[2] causing infection from diseases such as urinary tract infections (UTI), cystitis, and other pathogens such as meningitis, septicemia, gastritis, intestines among children, wound infection, and pneumonia.[3] These bacteria possess many virulence factors.[4] The most important of which are resistant to antibiotics and their ability to form biofilm. Characteristic of high and multiple antibiotic resistance.[5] ESβLs are a group of enzymes that lead to resistance increase in aztreonam, ceftazidime, cefotaxime related oxyimino-β-lactams, cephalosporins, and penicillins, but clavulanic acid inhibits them.[6] Until now, more than 400 ESβL variants have been identified.[7]

Biofilms are a complex collection of bacteria with unique properties that facilitate their host avoidance, immune response, and penetration by Antagonists.[8]

The resistance to antibiotics by the bacteria is higher when isolates can form biofilms.[9] The increase in levels of resistance leads to the fact that treatment options will be more difficult. The emergence of multiple resistance changes the infection from something simple to complex. Biofilm formation and extended spectrum beta-lactamase (ESBL) production synergistically contribute to the widespread dissemination of gram-negative bacilli antibiotic resistance (MDR) strains.

These facts may be responsible for chronic and persistent injuries and relapses of infection, which subsequently lead to high rates of infection and death and thus the formation of a health crisis.[10] The present study aims to isolate and identify the ESBLs producing *E. coli* bacteria and to reveal the ability of these isolates to cause biofilm formation.

MATERIALS AND METHODS

Bacterial isolates

Up to 70 clinical *E. coli* samples were examined, with 30 identified as ESβL isolates. These isolates were gathered from inpatients and outpatients at a few Baghdad hospitals. Urine, pus, faces, and blood samples were obtained from patients at government hospitals who came from both genders and diverse age groups between (9/10/2019 to 20/12/2019).

Standard microbiological techniques were used for separation, and 11 and standard microbiological procedures

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were used to identify the isolates. They were re-identified using the VITEK 2 compact system (BioMerieux, France),[11] is intended for bacterial species identification and antimicrobial susceptibility test (AST) detection of quick clinically relevant human bacterial infections.

Identification of *E. coli* Isolates

The VITEK 2 compact system was used to evaluate antibiotic susceptibility profiles, and MIC values on *E. coli* isolate, which normally employs different Antimicrobial Susceptibility using test cards (AST- GN71cards) according to the expected pathogens. The cards were inoculated and incubated in the machine according to the manufacturer's instructions. MIC levels were classed as sensitive, moderate, or resistant according to the National Committee for Clinical Laboratory Standards' (NCCLS) breakpoint.[12] The final results were evaluated using the Advanced Expert System in accordance with (AES).[13]

In the AST-GN71 (bioMérieux) card, 17 different antibiotics were employed and tested, including ampicillin (AMP), ampicillin/sulbactam (SAM), aztreonam (ATM), cefazolin (CFZ), cefepime (FEP), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), ceftriaxone (CRO), tigecycline (TGC), imipenem (IPM), meropenem (MEM), tobramycin (TOB), amikacin (AMK), ertapenem (ETP), gentamicin (GEN), moxifloxacin (MXF), nitrofurantoin (NIT).[14]

Detection of ESβLs

Antimicrobial susceptibility testing was performed on the isolates using the VITEK 2 system and the AST-GN71 card. This method was created to detect ESβLs phenotypically in both screening and confirmatory tests on the same plate.[15] The isolates of *E. coli* were validated as ESβLs generating using a double-disc synergy (DDST) test with 30 mg of aztreonam, ceftazidime, cefotaxime, ceftriaxone, and (20 mg amoxicillin + 10M clavulanic acid) When the zone of inhibition around any antibiotic disc was increased towards the amoxicillin/clavulanic acid disc,[16] ESβL isolates generation was considered positive.

Detection of the Ability of *E. coli* Bacteria to (Biofilm Formation)

The ability of the 30 isolates producing ESβLs was tested to form biofilm by using adopting sterile microtiter 96 plates. It is a quantitative method for determining the production of biofilms by using a microplate reader,[17] as follows: Muller-Hinton proth medium (MHB) containing (1%) glucose was prepared after being incubated for 24 hours at a temperature of 37°C. Using two titration plates, one for 24 hours incubation and the other for 48 hours incubation, added (150) micro-liters of crystal violate dye at a concentration of 1% and for 15 minutes to dye the biofilms that formed on the walls of the fine plates. The dye was emptied from the fine plate pits and washed with a phosphate buffer saline solution twice in succession. The plate was left to dry for 10 minutes at a temperature of (60°C), then added 150 microliters of ethanol alcohol at a concentration of 99%. The readings of the plate were taken at the 560 nm wavelength by a Glomax reader from Promega company. The same steps were made for the second plate, which was incubated for 48 hours, then the efficiency of the isolates was determined by the formation of Biofilm and by comparing the readings that were obtained according to the following equations:

- An isolation is considered to be non-forming of the biofilm when the optical density rate of the control is greater or equal to the optical density of the isolation) ODc ≥ OD).
- An isolation is considered a poorly formed biofilm when the optical density of the isolation is greater than the optical density of the control and is equal to or less than twice the optical density of the control. (ODc < OD≤ 2XODc))
- An Isolation is considered to be a moderate formed biofilm when the optical density rate of the isolation is equal to or less than four times the control (2xODc< OD ≤4xODc)
- An isolation is considered to be highly biofilm-forming when the optical density of the isolation is greater than four times that of the control rate (OD> 4xODc).

RESULTS

A total of 30 positive ESβL isolates were found out of 70 *E. coli* isolates obtained; 70 were identified from various clinical specimens in patients of both genders and ages from various Baghdad hospitals. The VITEK2 automated microbiology equipment was used to investigate isolates up to the species level. Using the double-disc synergy test (DDST), the isolates were confirmed as ESβL generating *E. coli*, as described in (Figure 1). DDST results for 70 isolates revealed that 30 isolates (42.8%) were ESβL producers and 40 isolates (57.2%) were non-producers.

Table 1 shows the positive results from 30 (42,8%) clinical isolates of ESβL produced in bacteria isolated from various

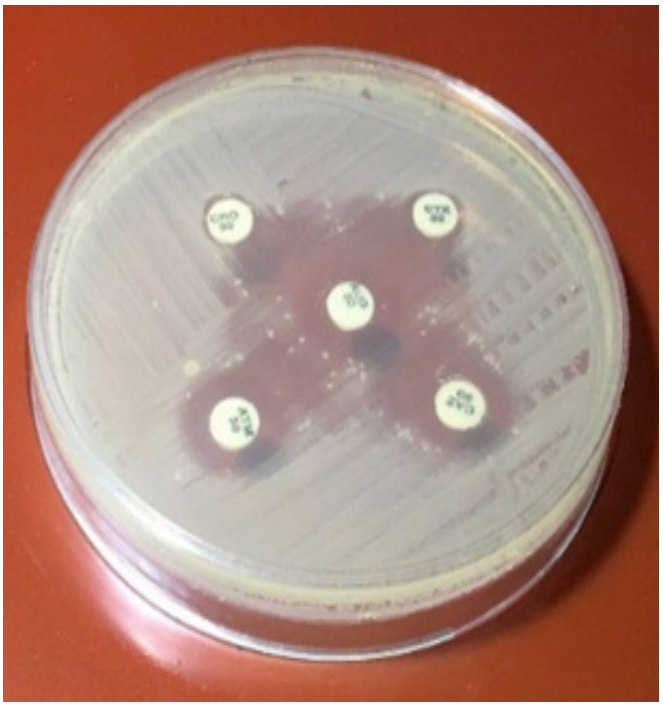

Figure 1: Photograph showing DDST

Table 1: Type and number of positive samples for ESβLs

Samples	*Number of samples*	*Male%*	*Female%*
Urine	18(60%)	7(23.3%)	11(36.6%)
Stool	2(6.6%)	1(3.3%)	1(3.3%)
Pus	4(13.3%)	3(10%)	1(3.3%)
Wound swab	6(20%)	5(16.6%)	1(3.3%)
Total	30	16(53.4%)	14(46.6%)

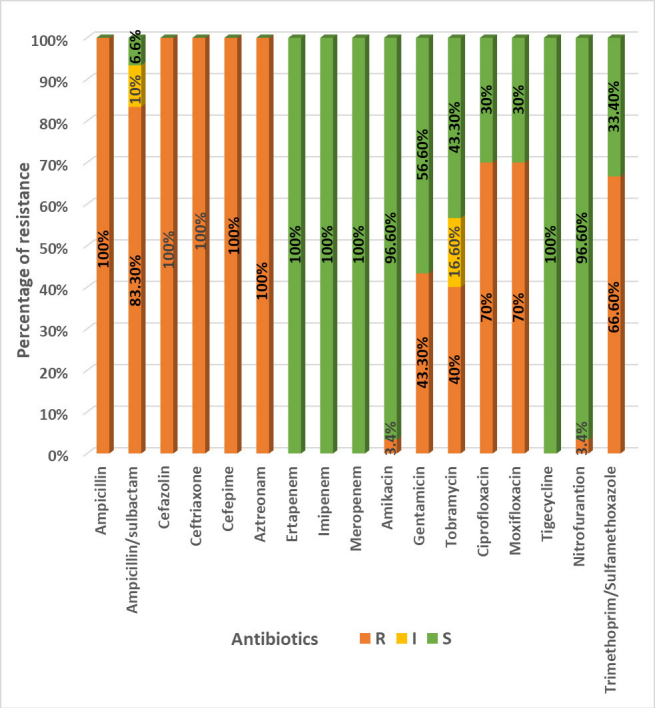

Figure 2: The percentage of antibiotic-resistant *E. coli* isolates producing ESβL

infections. The positive results were distributed as 18 (60%) isolates in urinary tract infections, 2 (6.6%) isolates in stool, 4 (13.3%) isolates in pus samples, and 6 (20%) isolates in wound swab samples.

Sensitivity of Antibiotic

Using the AST-GN71 antimicrobial agent card, the resistance pattern of 30 ESβL producing isolates was discovered using 17 different antibiotics (Figure 2). All of the isolates in this study were susceptible to imipenem, ertapenem, meropenem, tigecycline, and (96.6%) of isolates were susceptible to amikacin and nitrofurantoin and had high resistance (100%) to ampicillin, cefazolin, ceftriaxone, and aztreonam, 96% to cefepime, and 83.3% to ampicillin/sulbactam), whereas resistance to other antibiotic was moderate

Biofilm Formation

The results of the phenotypic detection of *E. coli* isolates producing broad-spectrum beta-lactamase enzymes showed their ability to form biofilm in varying degrees. Using the microtiter plate method under two different incubations, 24 hours and 48 hours periods for each plate.

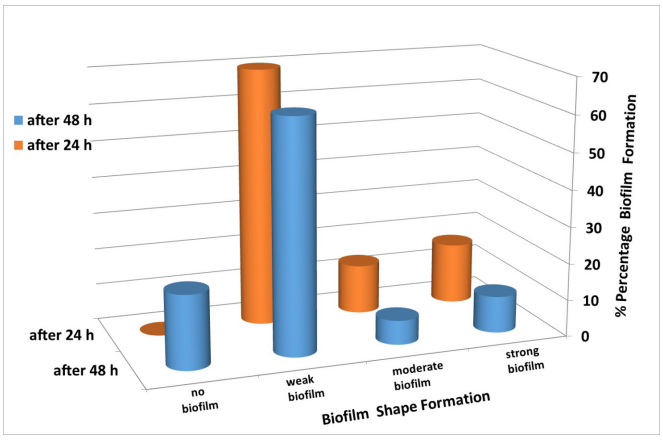

Figure 3: The percentage of biofilm formation in *E. coli* isolates producing ESβLs, after 24 and 48 hours of incubation

Out of the 70 clinical isolates, 30 (42.8%) isolates produced ESβLs, and all showed the ability to form biofilm 100% and be significantly higher after 24 hours of incubation in isolates producing ESβLs that were different in their isolation sources as shown in Table 2. The results showed that 5 (16.6%)bacterial isolates had a strong ability to form a biofilm, 4 (13.3%) bacterial isolates had a moderate ability to form a biofilm, while 21(70%) isolates had a weak ability to form a biofilm.

When incubating the plate for 48 hours, the biofilm formation was reduced 80%. The percentage of isolates has strong biofilm formation was 10% for three isolates, while the average biofilm Formation Moderate ratio for two isolates was 6.6%. The weak Biofilm formation was 19 isolates at a rate of 63.3%, while six isolates had not the ability to form biofilm at a rate of 20%. All these results are shown in Figure 3 and Table 2.

DISCUSSION

In Vitro, to verify the importance of time on the formation of biofilms, the incubation had a significant effect on increasing the growth of biofilms. It was observed that the interaction of the OD values with the incubation days was the most appropriate time for the formation of biofilms after 24 hours' incubation. The formation of biofilms began to slow down after 48 hours. The growth of the formation of biofilms follows a gradual pattern, where the peak of the formation of membranes is achieved according to this study at 24 hours of incubation and then gradually decreases until the second day (48 hours), while the maximum period of formation of E. coli bacteria is within 24 hours of the incubation.

The results of the current study agree on the ability of isolates of E. coli bacteria producing ESβLs to form biofilms with their findings.[18] The isolates ratio producing ESβLs (34%) were varying degrees in biofilms formation, also confirming the presence of multiple resistance among isolates and the isolates producing ESβLs have a greater ability to produce biofilms than isolates that don't produce these enzymes. While the study by.[19] noted that disrupting the resistance of biofilms leads to Enhancing the ability of antibiotics to clear infections caused by bacterial strains forming biofilms membranes resistant.

Table 2: Results of biofilm formation after (48–24) hours for isolates producing ESβLs

Source of isolates	*Isolate No.*	*Biofilm after 24 hours*	*Biofilm after 48 hours*
Pus	6	Strong biofilm	Moderate biofilm
Urine	7	Weak biofilm	No biofilm
Urine	20	Weak biofilm	Weak biofilm
Urine	21	Weak biofilm	No biofilm
Wound	22	Weak biofilm	No biofilm
Urine	24	Moderate biofilm	Weak biofilm
Urine	25	Weak biofilm	Weak biofilm
Urine	28	Weak biofilm	Weak biofilm
Wound	31	Strong biofilm	Strong biofilm
Urine	34	Moderate biofilm	Weak biofilm
Pus	36	Weak biofilm	Weak biofilm
Urine	38	Weak biofilm	Weak biofilm
Pus	40	Moderate biofilm	Weak biofilm
Urine	41	Weak biofilm	Weak biofilm
Stool	45	Moderate biofilm	Weak biofilm
Urine	51	Weak biofilm	Weak biofilm
Stool	54	Strong biofilm	Strong biofilm
Urine	61	Strong biofilm	Strong biofilm
Wound	62	Weak biofilm	Weak biofilm
Urine	64	Weak biofilm	Weak biofilm
Pus	66	Weak biofilm	Weak biofilm
Urine	73	Weak biofilm	No biofilm
Wound	75	Weak biofilm	No biofilm
Urine	81	Weak biofilm	No biofilm
Pus	82	Strong biofilm	Moderate biofilm
Urine	85	Weak biofilm	Weak biofilm
Urine	98	Weak biofilm	Weak biofilm
Urine	104	Weak biofilm	Weak biofilm
Urine	106	Weak biofilm	Weak biofilm
Pus	113	Weak biofilm	Weak biofilm

Same notes in many studies were registered the for different clinical samples of *E. coli*, but the differentiation just in a number of isolates and producing ESβLs and biofilm formation shape in that studies.[20-22]

That broad-spectrum beta-lactamase enzymes (ESβLs) are responsible for the resistance the bacteria possess against third-generation antagonists of cephalosporin and monobactam,[23] and that most of the genes responsible for producing these enzymes are carried on plasmids that are responsible for resistance to other antagonists,[6] and due to the frequent occurrence of multiple resistance and other classes of antibiotics such as (aminoglycosides, fluoroquinolones), treating these bacteria is often a difficult therapeutic challenge.[23]

The growth of organisms that have the ability of biofilm formation and the resistance to many antibiotics increases the resistance of antibiotics to more than 1000 times.[24] This is due to several reasons, including the low spread of the antibiotic between the sticky interlayer of the biofilms, as well as the transmission of genes Resistance within this environment by plasmid or some jumping genes between bacterial cells, or by random mutations that lead to increased resistance of cells to antibiotics or toxins. The production of biofilms in the intestinal bacteria producing ESβLs provides a suitable environment for the exchange of antibiotic resistance genes and can enhance its widespread proliferation,[25] in addition to being able to further express the Efflux pumps in the membranes of living cells, with stabilization of the biofilm although changing the pH and ion concentrations, the biofilm sheet can also inhibit the elimination of enzymes or Nutrients, or even small molecules, that accumulate to leave more suitable microenvironments within biofilms.[26,27] Finally, some bacteria cells it has a significant role as a mechanical protection mechanism in preserving the biofilm.[28]

On the other hand, it also appears that the formation of biofilms by bacterial cells plays a role in the recurrence of some types of infection, making it difficult to eliminate and treat them.[29]

The production of biofilms leads to certain virulence factors that tend to congregate in infected parts and sometimes lead to an increase in virulence and their spread becomes of concern.[25] A study from China,[30] explained that the cancer patients infected by ESβL-producing *E. coli* bacteria which have the ability to biofilms formation had a lower survival rate in the ICU, and that prior exposure to cephalosporins, chemotherapy, and biofilm formation were three indirect risk factors lead to higher levels of pathogenicity and mortality. While the other study emphasizes the importance of forming biofilms in increasing the number of injuries and deaths even in non-cancer patients.[31] The best solution is to use certain antibiotics to prevent the formation of biofilms, such as azithromycin gentamicin and tigecycline, which may inhibit Biofilm formation.

In Vitro, to verify the importance of time on the formation of biofilms, the incubation significantly increased biofilms' growth. It was observed that the interaction of the OD values with the incubation days was the most appropriate time for the formation of biofilms after 24 hours of incubation. The formation of biofilms began to slow down after 48 hours. The growth of the formation of biofilms follows a gradual pattern, where the peak of the formation of membranes is achieved according to this study at 24 hours of incubation and then gradually decreases until the second day (48 hours) while the maximum period of formation of *E. coli* bacteria is within 24 hours of the incubation.

CONCLUSION

The strains of bacteria producing ESβLs and able to form biofilms are Most pathological compared to isolates that do not have biofilms formation and fewer ESβLs production. The effectiveness of many virulence genes, including biofilm formation, respond to the pressure that occurs during the rearrangement of bacterial genes in order to obtain ESβLs plasmids may be the primary mechanism for the

susceptibility of E. coli strains that make up ESβLs to form biofilms.

This study showed that all *E. coli* isolates produced by ESBLs were able to the formation of biofilms after 24 hours of incubation. It also indicates that the combination of biofilm production, production of ESβLs enzymes, and incubation with other factors study can be of benefit for controlling infection and improving clinical outcomes by choosing appropriate antibiotic treatment.

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