



## Microbiology Research Journal International

30(1): 26-32, 2020; Article no.MRJI.53999

ISSN: 2456-7043

(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

# Assessment of Biofilm Formation among the Clinical Isolates of *Escherichia coli* in a Tertiary Care Hospital

Bedobroto Biswas<sup>1</sup>, Naik Shalini Ashok<sup>1\*</sup>, Deepesh Nagarajan<sup>2</sup>  
and Md Zaffar Iqbal<sup>1</sup>

<sup>1</sup>Department of Microbiology, M. S. Ramaiah Medical College, Rajiv Gandhi University of Health Sciences, India.

<sup>2</sup>Department of Biochemistry, Indian Institute of Science Bengaluru, India.

### Authors' contributions

This work was carried out in collaboration among all authors. Author BB designed the study, conducted the experiments, wrote the protocol and wrote the first draft of the manuscript. Author NSA managed the analyses and proof reading of the manuscript. Authors DN and MZI managed the literature searches. All authors read and approved the final manuscript

### Article Information

DOI: 10.9734/MRJI/2020/v30i130187

#### Editor(s):

(1) Prof. Essam Hussein Abdel-Shakour, Department of Botany and Microbiology, Faculty of Science, Al- Azhar University, Cairo, Egypt.

(2) Dr. Zahid Anwar, Department of Biochemistry, University of Gujrat, Pakistan.

(3) Dr. Lachhman Das Singla, Professor-cum-Head, Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, India.

#### Reviewers:

(1) Ruhul H Kuddus, Utah Valley University, Utah, USA.

(2) Teresa Fasciana, University of Palermo, Italy.

(3) Byron Baron, University of Malta, Malta.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/53999>

Original Research Article

Received 25 November 2019

Accepted 27 January 2020

Published 07 February 2020

## ABSTRACT

**Aims:** Identification and grading of the *Escherichia coli* according to their biofilm production capability.

**Study Design:** Cross-sectional study.

**Place and Duration of Study:** This was conducted in Department Microbiology at M.S. Ramaiah Medical college and Hospital, Bengaluru from March 2017 to August 2017.

**Methodology:** A total of 55 non repetitive *Escherichia coli* isolates were identified from various clinical samples like urine, pus, tissue and peritoneal fluids. All the organisms were isolated in pure

\*Corresponding author: E-mail: [nshaliniamicro@yahoo.in](mailto:nshaliniamicro@yahoo.in);

culture and biofilm formation was detected *in vitro* by Gold standard TCP (Tissue culture plate) method. Organisms were incubated for an extended period of 48 hours and the biofilms were detected by acetone alcohol elution method. Organisms were categorized as strong, moderate, weak and no biofilm producers based on the obtained OD value of the elute.

**Results:** Majority of the isolates of *Escherichia coli* were obtained from catheterized urine culture (67.03%) followed by pus (25.50%). Most of the isolates were capable of forming biofilm *in vitro* by tissue culture plate method except a few (9.1%). 40% of the isolates were strong biofilm formers which had >4 ODC. 25.5% showed medium biofilm-forming capability and rest 25.5% showed weak biofilm formations *in vitro*.

**Conclusion:** The ability to form biofilm from a species can give us a better understanding of the biofilm-related infections pertaining to the particular group. Detection of biofilms remains a most important determinant to approximate the incidence of such infections. Categorization of organisms according to their biofilm formation may help us understand the frequency of biofilm-associated infections, and thus take necessary precautions to avoid the problem. Further studies involving the detection of biofilm may be conducted and the tests can be implemented in routine diagnostic microbiology to assess the usefulness of the methods in detection of biofilm-related infections.

**Keywords:** *Escherichia coli*; biofilm; Foley's catheter; tissue culture plate.

## ABBREVIATIONS

TCP : Tissue culture plate;

ODC : Optical density of control;

## 1. INTRODUCTION

Microorganisms usually exhibit two stages of life namely planktonic and biofilm stage. In the biofilm stage, the microorganisms are usually adhered to an external surface as they are embedded in the extracellular polysaccharide or protein or protein-polysaccharide matrix which is produced by them. Due to impermeability to most of the antimicrobial agents, biofilms render decreased susceptibility to most of the available antibiotics [1]. According to Ceri *et al*, *Escherichia coli* in the biofilm stage requires >500 times minimum inhibitory concentration (MIC) of Ampicillin to achieve a 99.9% reduction in population [2]. The patients with indwelling medical devices namely catheters and implants are the most affected population due to biofilms [1]. Therefore the treatment options for biofilm related infections are mostly limited and often removal of the affected indwelling device is required for radical cure.

Both Gram negative and Gram positive can form biofilms. *Escherichia coli* being one of the most predominant colonizers of the human intestinal tract can be a potential pathogen if it acquires enterotoxins or invasive factors from plasmid DNA or bacteriophages. Apart from causing diarrhoeal disease, it also causes neonatal meningitis and sepsis and urinary tract infections

[3]. *Escherichia coli* is an appropriate model for colonization study due to availability of wide array of genetic tools. *Escherichia coli* ST 131 is the emerging drug resistant organism which is referred as "superbug" recently in England. The organism being mostly resistant to cephalosporin and fluoroquinolones is responsible for increasing number of community acquired urinary tract infections [4]. In the biofilm stage, *E. coli* produces an extracellular polymeric matrix which prevents diffusion and acts as a sink for antimicrobials and superoxide which makes biofilm related infections most difficult to treat [5]. More studies on biofilm formation should be conducted which will give insight to the pathogenicity of the organisms and study the dynamics of biofilm formation. Detection of biofilm formation, although is not a part of routine microbiology laboratory, can help us determine the rates of biofilm formation by clinically relevant strains isolated from patients suffering from hospital acquired or community acquired infections.

The study aims to identify *Escherichia coli* from various clinical samples and grade the organism according to its biofilm forming category.

## 2. MATERIALS AND METHODS

This cross sectional study was carried out in Department of Microbiology, M.S. Ramaiah Medical College, Bengaluru. Study duration was from March 2017 to August 2017. A total of 55 non repetitive isolates of *Escherichia coli* was taken from various clinical samples.

The study proposal was presented before by the Institutional review board and was accepted. The sample collection and processing was conducted as per standard guidelines.

## 2.1 Sample Size Calculation

A study carried out by MA Ansari et al. [6] has revealed that moderate to high-intensity biofilm production in the *Escherichia coli* is 55%. Based on the above findings of the study with a relative precision of 25% and the desired confidence level of 95% it is estimated that 50 samples need to be included for the study. Categorical variables such as biofilm production in various grades- weak, moderate, high were expressed in percentage with 95% confidence interval.

## 2.2 Subjects and Selection Method

The study was carried out on *Escherichia coli* isolates, recovered from different samples like urine, pus, wound swab, peritoneal fluid and tissue. All the isolates were representative strains and were clinically correlated with the infections.

## 2.3 Inclusion Criteria

All isolates of *Escherichia coli* from samples like urine, pus, wound swab, peritoneal fluid and tissue were included in the study.

## 2.4 Exclusion Criteria

Isolates not showing biofilm formation was not be further processed in the study. Urine samples other than catheterized urine were not taken into the study.

## 2.5 Objective

Identification and grading of the *Escherichia coli* according to their biofilm formation.

## 2.6 Sample Collection

Urine samples were collected from catheterized patients after proper decontamination of the Foley's catheter, maintaining sterility during collection. Samples were collected in sterile urine containers and then transported to the lab for processing within 1 hour of collection. Aspirated pus samples were collected aseptically from the infected site and were transported in intact disposable syringes to the laboratory

immediately for processing. Swab samples were collected maintaining strict asepsis only from open wounds after proper decontamination. Transport of the swabs was done immediately for processing in the laboratory. Tissue samples were collected intraoperatively and were immediately transported in a sterile container with normal saline for culture. Peritoneal fluid samples were also collected aseptically in a sterile container during abdominal paracentesis and were immediately transferred in laboratory for culture.

## 2.6.1 Isolation of the organism

Cysteine–lactose–electrolyte-deficient (CLED) medium and 5% sheep blood agar were used exclusively for culture of urinary samples. MacConkey's agar medium was used for isolation of organisms from swab, pus, tissue, peritoneal fluid and urine samples. Additionally, 5% sheep blood agar and MacConkey's agar was also used for isolation of organisms from peritoneal fluids. All organisms were isolated and identified using routine biochemical testing media according to the standard guidelines. Colony characteristics were noted from the culture plates. Upon confirmation of a Gram-negative rod by Grams staining, organisms were identified by standard methods. Biochemical reactions included indole reaction, mannitol motility test (MM), triple sugar iron agar test (TSI), citrate utilization test, urea hydrolysis test, Methyl red test (MR) , Voges proskauer tests (VP), oxidative fermentative test with dextrose (OF), nitrate reduction test, sucrose and inositol fermentation tests.

## 2.7 Demonstration of Biofilm Formation

Tissue culture plate (TCP) method was deployed to demonstrate the biofilm formation and categorize the isolates according to various biofilm-forming capability. The TCP method by Christensen *et al* is most widely accepted and is a standard test for testing for biofilm formation [7]. Christensen's method of TCP culture for biofilms was used with a modification of the incubation period which was extended to 48 hours for each TCP. Isolates from respective agar plates were sub-cultured in Tryptic soy broth (TSB) with 1% glucose and incubated for 18 hours at 37°C in stationary condition and diluted 1:100 with fresh TSB (Individual wells of a 96 well flat bottomed TCP was filled with 0.2 aliquots of diluted cultures ). It was then vortexed for 30 seconds.

Sterility control was served by broth only in the control well. TCPs were incubated at 37°C for 48 hours. Post incubation, content of each well was removed by gentle tapping. Wells were washed four times with 0.2 mL of phosphate buffered saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. The wells of the plate were fixed by incubation at 60°C for 60 min and stained with 200 microliter of crystal violet (1%). Excess stain was rinsed by thorough washing with deionized water. 200 µl Acetone alcohol (20:80) -was then transferred into each well to elute the biofilms and then it was transferred to a fresh assay plate. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at a wavelength of 600nm. These OD values was considered as an index of bacteria adhering to surface and forming biofilms. Biofilm producing strain of *Pseudomonas aeruginosa* (ATCC 27853) and non-biofilm producing strain of *Staphylococcus aureus* (ATCC 25923) were used as controls for the assay [8]. The categories of biofilm formation for the test isolates were defined as follows –

**Table 1. Chart of Biofilm category**

Biofilm category	Mean OD
Strong	> 4 ODC (>0.464)
Moderate	2ODC to <4ODC (0.232 to <0.464)
Weak	ODC to <2ODC (0.116 to <0.232)
Non biofilm	<ODC = <0.116

ODC = OD Control, ODC = 0.116 (Average OD of Negative control + 3 Standard deviation of Negative control)

## 2.8 Statistical Analysis

Data was analyzed using SPSS version 20 (SPSS Inc., Chicago, IL). The level P < 0.05 was considered as the cutoff value.

## 3. RESULTS AND DISCUSSION

### 3.1 Results

The organisms showing the following biochemical reactions were identified to be *Escherichia coli* – indole – produced, mannitol – fermented, motile, triple sugar iron (TSI) – acid slant /acid butt with no gas and no H<sub>2</sub>S, citrate – not utilized, urea –not hydrolyzed, methyl red test – positive, Voges proskauer test – negative, oxidative fermentative glucose test – fermentative reaction, nitrate – reduced, sucrose – not fermented, inositol – not fermented.

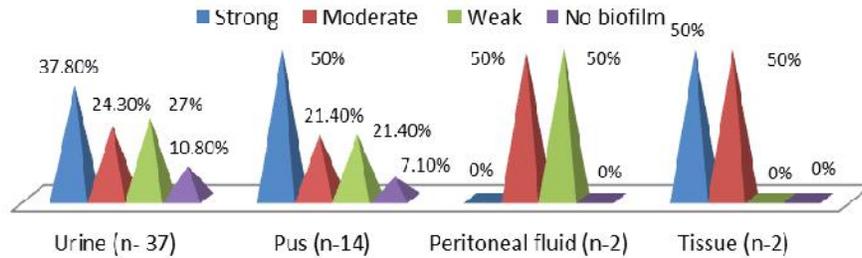
Majority of the isolates of *Escherichia coli* were obtained from catheterized urine culture (n-37) followed by pus (n-14). Few of them were isolated from tissue culture (n-2) and peritoneal fluid culture (n-2). Most of the isolates were capable of forming biofilm in vitro by tissue culture plate method except a few (9.1%). Categorization of biofilm formation was determined by the mean OD value as shown in Table 1. 40% of the isolates were strong biofilm formers which had >4 ODC. 25.5% showed medium biofilm forming capability and rest 25.5% showed weak biofilm formations in vitro. The following chart shows the category of biofilm formation according to the specimens received (Fig. 1).

It was observed that strong biofilm producers were mostly isolated from urine (37.8%), pus (50%) and tissue samples (50%) only. Moderate biofilm formers were isolated from all samples – urine (24.3%), pus (21.4%), peritoneal fluid (50%), tissue samples (50%). Both the isolates from tissue samples and peritoneal fluid were biofilm formers. Fig. 2 shows the categorization of biofilms in the Tissue culture plate post elution by acetone alcohol method.

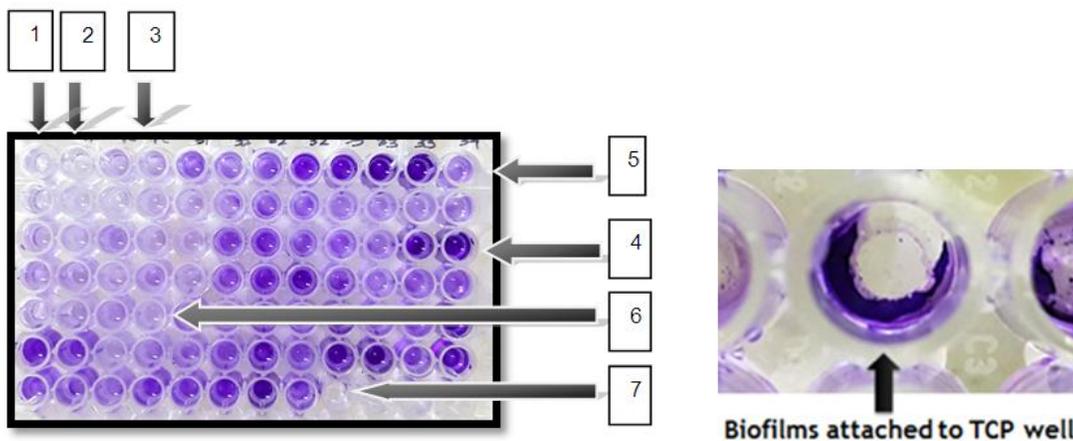
Well marked 1 shows sterility control. Well marked 2 shows the growth of non biofilm-forming strain as negative control - *Staphylococcus aureus* (ATCC 25923). Well marked 3 shows biofilm formed by positive control strain - *Pseudomonas aeruginosa* (ATCC 27853). Well marked 4,5,6,7 shows strong, moderate, weak biofilms formed by *Escherichia coli*. Similarly well-marked 7 shows a non biofilm forming strain of the organism.

### 3.2 Discussion

Indwelling medical devices including the Foley's catheters are frequently used for more than 25% of the patients admitted in a hospital. Prolonged duration of placements of such devices in the body gives rise to an increased risk of acquiring infections by organisms forming biofilms. Thus resulting in increased morbidity, mortality, length of hospital stay and finally incur a huge cost for the treatment which often requires removal of the implant /device [9]. In our study we had 37 catheterized urine samples from which *Escherichia coli* was isolated (n-37). Catheter-associated urinary tract infection (CAUTI) is the most common amongst the nosocomial infections. *Escherichia coli* and *Proteus mirabilis* are the most common organism isolated in cases



**Fig. 1.** The above figure shows different category of biofilm formation by *Escherichia coli* isolated from various samples



**Fig. 2.** The figure shows the categorization of biofilms formed by *Escherichia coli* in a 96 well tissue culture plate; after elution by acetone alcohol

of CAUTI [10]. The organisms can thrive well in the hospital environment in spite of having adverse growth conditions and initiation of antibiotic treatment. It is due to their characteristics and virulence, they are successful colonizers of medical devices [11]. Similarly in pus and wounds, multiple factors are responsible for biofilm formation. One of the most important determinants is the failure of the planktonic organism to get eliminated from the surface of a wound by application of antimicrobial drugs or any other physical agents. Thus biofilms are easily formed over wounds [12]. In our study extended static incubation period of 48 hours in the 96 well microtiter plate for detection of biofilms was found to be optimum.

We demonstrated only 50 strains out of 55 isolated to be biofilm formers (90.9%), which correlates with other studies around the globe. In a similar study done by FM Patel et al. on 50 isolates, 84% of organisms produced biofilm in which *Escherichia coli* constituted 12% of the

isolates [13]. In another similar study carried out by Summaiya Mulla et al. 88% of the isolates identified from patients having medical devices has shown biofilm formation [14].

Although *Escherichia coli* have been responsible for most CAUTI worldwide, it is the *Enterobacter cloacae* which exhibit the highest biofilm production in a similar setting [15]. In a study involving clinical isolates of *Pseudomonas spp* and *Acinetobacter baumannii* by R. Papa et al., it was demonstrated that 52.27% and 34.1% of the strains were strong and moderate biofilm producer respectively. In our study, only 40% and 25.5% of *Escherichia coli* formed strong and moderate biofilm respectively [16]. In a similar study involving clinical isolates of *Acinetobacter spp.*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus spp.*, and *E. coli*, it was demonstrated that 57.14% of the isolates formed strong biofilm and 35.7% isolates were weak biofilm producers [13]. In most of the microbiology laboratories, routine diagnostic

work does not include the detection of biofilm formation from the clinical samples. In cases of prosthetic joint infections, routine culture from the implant may render the culture sterile if the pathogenic organism has formed biofilm over the surface of the implant. Hence more advanced methods like short term vortexing and sonication should be deployed to detect biofilm formed *in vivo* from surfaces of devices and catheters [17].

#### 4. CONCLUSION

Our study shows *Escherichia coli* isolated from clinical samples of catheterized urine, pus, peritoneal fluid and tissue samples were able to form biofilms *in vitro*. Amongst all the isolates, 40 % was capable of forming strong biofilm *in vitro*. The ability to form biofilm by a species can give us a better understanding of the biofilm-related infections pertaining to the particular group of organism. Biofilm is responsible for a vast number of device related infections in humans worldwide; hence detection of biofilm remains the most important determinant to approximate the incidence of such infections. Proper methods of detection of biofilm from tissue /catheter / indwelling medical devices must be followed which includes sonication and vortexing of the samples to elude the biofilms. Till date very few studies have categorized biofilm of *Escherichia coli* according to severity of formation. Our study has standardized the method of detection of biofilm formation by the TCP method and categorization of isolates into severe, moderate and weak biofilm formers. Further studies involving the detection of biofilm may be conducted and the tests can be implemented in routine diagnostic microbiology to assess the usefulness of the methods in detection of biofilm related infections.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

#### REFERENCES

1. Donlan Rodney M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*. 2001;33(8):1387-1392. DOI:10.1086/322972
2. Ceri H. et al. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*. 1999;37(6):1771–1776. DOI:10.1128/jcm.37.6.1771-1776
3. Makvana S, Krilov LR. *Escherichia Coli* Infections. *Pediatrics in Review*. 2015; 36(4):167–171. DOI:10.1542/pir.36-4-167
4. Fasciana et al. Virulence factors and antimicrobial resistance of *Escherichia coli* ST131 in community-onset healthcare-associated infections in Sicily, Italy. *Pharmacologyonline (Special Issue)*. 2017; 12-21.
5. Beloin C, et al. *Escherichia Coli* Biofilms. *Current Topics in Microbiology and Immunology Bacterial Biofilms*. 2008;249–289. DOI: 10.1007/978-3-540-75418-3\_12
6. Ansari Ma, et al. Anti-Biofilm efficacy of silver nanoparticles against MRSA and MRSE Isolated from Wounds in a Tertiary Care Hospital. *Indian Journal of Medical Microbiology*. 2015;33(1)101. DOI:10.4103/0255-0857.148402
7. Christensen GD, et al. Adherence of coagulase-negative *Staphylococci* to Plastic Tissue Culture Plates: A Quantitative Model for the Adherence of *Staphylococci* to Medical Devices. *Journal of Clinical Microbiology*. 1985;22(6):996–1006. DOI:10.1128/jcm.22.6.996-1006.1985
8. Prashanth K, et al. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. *Indian Journal of Medical Microbiology*. 2008; 26(4):333. DOI:10.4103/0255-0857.43566
9. Dror Naama et al. Advances in microbial biofilm prevention on indwelling medical devices with emphasis on usage of acoustic energy. *Sensors*. 2009;9(4):2538–2554. DOI:10.3390/s90402538
10. Jacobsen SM, et al. Complicated catheter-associated urinary tract infections due to *Escherichia Coli* and *Proteus Mirabilis*. *Clinical Microbiology Reviews*. 2008;21(1): 26–59. DOI:10.1128/cmr.00019-07
11. Gelosia A, et al. Phenotypic and genotypic markers of *Staphylococcus Epidermidis* Virulence. *Clinical Microbiology and Infection*. 2001;7(4):193–199. DOI:10.1046/j.1469-0691.2001.00214.x

12. Neopane Puja, et al. *In Vitro* Biofilm formation by *Staphylococcus aureus* Isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. International Journal of General Medicine. 2018;11:25–32.  
DOI:10.2147/ijgm.s153268
13. Patel Fm, et al. Detection of biofilm formation in device associated clinical bacterial isolates in cancer patients. Sri Lankan Journal of Infectious Diseases. 2016;6(1):43.  
DOI:10.4038/sljid.v6i1.8086
14. Stickler DJ. Bacterial biofilms and the encrustation of urethral catheters. Biofouling. 1996;9(4):293–305.  
DOI:10.1080/08927019609378311
15. Majumder Md. Mahabubul Islam, et al. Microbiology of catheter associated urinary tract infection. Microbiology of Urinary Tract Infections - Microbial Agents and Predisposing Factors; 2019.  
DOI:10.5772/intechopen.80080
16. Papa R, et al. Biofilm formation in carbapenemase-producing *Pseudomonas Spp.* and *Acinetobacter baumannii* Clinical Isolates. International Journal of Infectious Diseases. 2018;73:119–120.  
DOI:10.1016/j.ijid.2018.04.3688
17. Mandakhalikar Kedar Diwakar et al. Extraction and quantification of biofilm bacteria: Method Optimized for Urinary Catheters. Scientific Reports. 2018;8(1).  
DOI:10.1038/s41598-018-26342-3

© 2020 Biswas et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Peer-review history:*

*The peer review history for this paper can be accessed here:*  
<http://www.sdiarticle4.com/review-history/53999>