ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 4, Issue 1, January 2019

Study of Biofilm formation among Clinical Escherichia Coli isolates

[1] Jigna P. Naik, [2] Prutha N. Patel
[12] Department of Microbiology& MLT, Arts, Science and Commerce Collage, Kholwad, Surat, Gujarat, India

Abstract: -- **Introduction: Biofilm forming microorganisms are related to chronic and recurrent human infections as well as are highly resistant to antimicrobial agents. Various methods have been used in laboratory for the detection of biofilm production which includes visual assessment by electron microscopy and polymerase chain reaction, whereas quantitative method like Tissue Culture Plate (TCP) method and two qualitative methods such as Tube Method (TM) and Congo Red Agar (CRA) method are also used in routine laboratories.**
Objectives: To detect the prevalence of biofilm formation in Escherichia coli (E.coli), to evaluate different qualitative methods for the detection of biofilms and to see its relation with antimicrobial resistance.
Materials and Method: The study was conducted at Department of Microbiology and MLT, Arts, Science and Commerce College, Kholwad, Surat during the period January 2017 to December 2017. Standard microbiological procedure was done to identify the isolates. E.coliclinical isolates were subjected to qualitative biofilm detection methods. Biofilm detection was tested by TM and CRA. Kirby-Bauer disc diffusion technique was performed to do antibiotic susceptibility test of biofilm producing bacteria according to CLSI guidelines.
Results and Conclusion: Biofilm productions among E.coli were more than 73%. We have also observed higher antibiotic resistance in biofilm producing E.coli than non-biofilm producers. We can conclude from our study that the CRA method can be recommended as a general screening method in laboratories for detecting biofilm forming bacteria. Biofilm production is associated with persistent infections and antibiotic therapy failure thereby posing a major challenge for the physicians along with economic relevance as well. Hence, such problems can be prevented by detection of biofilm producers and appropriate antibiotic doses modification.

Keywords: -- **Escherichia coli (E.coli), Antimicrobial resistance, Congo red agar method(CRA), Test tube method(TM).**

I. INTRODUCTION

Biofilms are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype[1].It's a distinct phenotype helps in a) altering the gene transcription and growth rate, b) increased resistance to chemical and physical therapy, c) survival of bacteria in hostile conditions, d) responsible for chronic, persistent, relapsing and the recurrence of infections, e) protecting the pathogens from host immune cells, f) increased resistance to antimicrobial agents[2].According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms [3].

Escherichia coli (E.coli)are such a commensal microbe which is the major part of normal aerobic microbial population of the intestine of humans and warm blooded animals [4].However,E.coli can cause variety of infectious diseases. Diseases associated with E.coli includediarrhoea, dysentery, haemolytic uremic syndrome,bladder and kidney infections, septicaemia, pneumonia and meningitis. The ability of E.coli to cause infections depends largely on several virulence factors, which help in the survival of E.coli under adverse conditions in host[5].

Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000fold [6]. Biofilms increases resistance to antibiotics relative to freely growing bacterial cells, because some antibiotics bind to exopolysaccharides on the surfaces of cells, bacterial populations produce a subset of cells known as "persisters," which are hibernating cells that neither grow nor die in the presence of antibiotics [7]. The phenotypic changes caused by growth on biofilm surfaces also protect cells from the effects of antibiotics [8].

Antibiotic resistance in E.coli is the major concern among Gram negative bacteria.Decreasing activity of antibiotics, especially the beta-lactam groups and cephalosporins because of the production β-lactamase and extended spectrum β-lactamase (ESBL) by E.coli and others factors mainly due to the production of biofilm leading to multi drug resistance in E.coli[9].

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 4, Issue 1, January 2019

There are various methods to detect biofilmproduction. These include the Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination [10].

In this study clinical isolates of E.coliwere subjected to qualitative biofilm detection methods. Biofilm detection was tested by TM (Tube method) and CRA (Congo Red Agar) method. Biofilm production of E.coliand antimicrobial drug resistance among them is studiedas it is the main source of multidrug resistance. Antimicrobial drug resistance is rising worldwide with regional difference and the frequency of occurrence [11].Among the practices that contribute to emergence of MDR strains are the prolonged and unjustified use of antibiotics as well as non-investigation based prescriptions. Such practices promote the spread of antibiotic resistance.

II. MATERIALS AND METHOD

The study was conducted in the department of MLT and Microbiology, Arts, Science and Commerce College, Kholwad, Surat,Gujarat for a period of one year. A retrospective review was done on results of cultures of clinical samples i.e. human feces, urine, ear discharge, pus swab from wounds, and eye discharge that had been performed previously in another study [12]. The demographic data of patients as well as E.coli isolates sources data were collected from the registration records using a standard data collection form.

E.coliisolates:

Ninety isolates ofE.coli were used in this study which were previously isolated and identified as E. coli were reconfirmed and characterized by its colony morphology on MacConkey agar and Eosin Methylene Blue (EMB) agar plate and biochemical- IMViC reactions [13]. All confirmed E.coli isolates were subjected to qualitative biofilm detection.Biofilm detection was tested by TM (Tube method) and CRA (Congo Red Agar) method.

Detection of biofilm production:

CRA method:

Biofilm production by the CRA method determined as per protocol of the Nachammai SM et al[2].According to that, the suspensions of the E.coli strains were inoculated into plate which contained Brain Heart Infusion broth, which was supplemented with 5% sucrose and Congo Red. Congo red was prepared as a concentrated aqueous solution and then plates were incubated aerobically for 24-48 hours at 37°C.Positive result was indicated by black colonies with dry crystalline consistency, non-biofilm producing strains developed red colonies as shown in Figure 1.

Figure 1.CRA method

EC-1: Black colonies of biofilm producer E.coli
EC-2: Red-pink colonies of non-biofilm producer E.coli

TM method:

Biofilm production by the TM method determined as per protocol of thePandaet al[14]. BHI broth with 2% sucrose (10 ml) was inoculated with loop full of microorganism from overnight culture plates and incubated for 24 h at 37°C. The tubes were then decanted and washed with PBS (pH 7.3) and dried. Dried tubes were then stained with crystal violet (0.1%) for half an hour. Excess stain was removed; tubes were dried and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined on the wall and bottom of the tube as shown in Figure2.

Figure 2. TM method

Presence of layer of stained material adhered to bottom of tubes which is appear in violet color indicate biofilm formation

Antibiotic Susceptibility Testing:

The antibiotic susceptibility testing was done using modified Kirby-Bauer disk diffusion method. The antibiotics had been selected as per the CLSI guidelines [15]. The Commercially available (HiMedia Laboratories Pvt. Limited, India) antibiotics disk and their concentrations (µg) used in this study were as shown in Table 2. After 18 hours of incubation at 37 °C, the diameter of the zone of

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 4, Issue 1, January 2019

inhibition was measured using a millimeter scale around each antimicrobial disk on the undersurface of the plate.The zone size around each antimicrobial disk was interpreted as sensitive, intermediate or resistant using standard interpretative chart provided commercially with the disk in accordance to Performance Standards Antimicrobial Disk Susceptibility Tests, CLSI. Reference organism E.coli (ATCC 25922) stock culture was maintained and tested by the above procedure using antibiotics under study.

III. RESULTS

There are various methods for biofilm detection. In this study we evaluated 90 clinical isolates by two screening methods CRA and TM for their ability to form biofilms. 66 no of isolates were detected biofilm producer by the CRA method and 74 no of isolates were detected by TM method. Among that 73% isolates showed biofilm positive by CRA method and 82% isolatesshowed positive results by TM method as shown in Chart 1 given below.

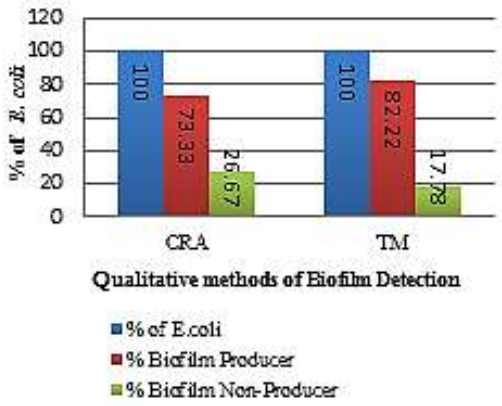

Chart1. Screening of the isolates for biofilm formation by Congo Red Agar (CRA) and Tube method(TM) 73% isolates showed biofilm positive by the CRA and TM methods. This resultis illustrated in Figure 3 given below.

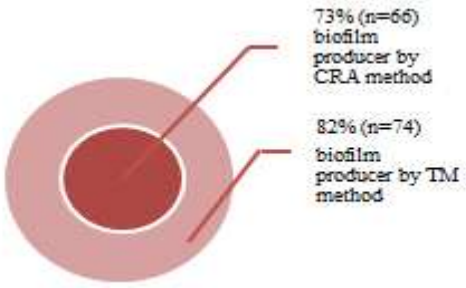

Figure 3. Biofilm positive E.coliisolates detected by CRA and TM method

Among 90 isolates 50 were from males and 40 were from females patients. Total 66 isolates were confirmed as biofilm producing isolates by both method CRA as well as TM methods were further studied. In clinically isolatedE.colibiofilm forming isolateswere found higher in female(90%) than male (60%) as shown in Chart 2.

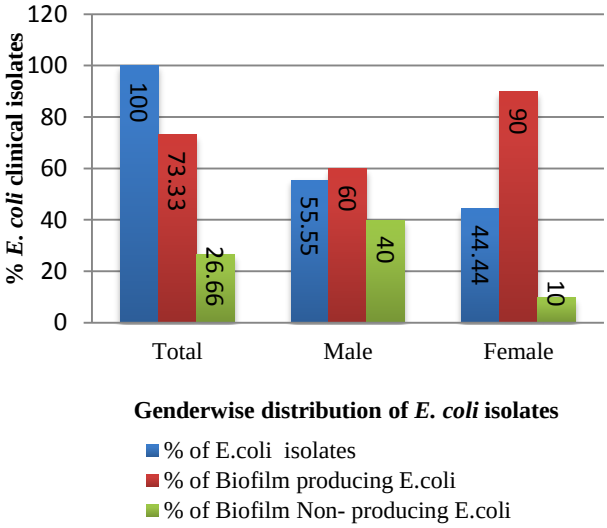

Chart 2. Gender-Wise distribution of biofilm producing and biofilmnon-producing E.coliclinical isolates.

Specimen-wise and ward-wise distribution of biofilm producer and non-biofilm producer E.coli isolates as illustrated inTable1. E.coli clinical isolates from various specimens sources high number of biofilm producers 42 out of 90 isolates wereobtained from stool and 22 were from urine. From ward wise distribution of E.coliclinical isolatesPaediatrics wardand ICU shows high number of biofilm producer.Considering the differences found between the isolates from the different sources sample type wise and ward type, OPD or ICUs detailed study is needed.

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 4, Issue 1, January 2019

Table 1: Specimen-wise and Ward-wise distribution of Biofilm producer and Non-producer E.coli clinical isolates

Specimen source	Out Door Patients (OPD)		Paediatrics ward (PED)		Intensive Care Units (ICU)		Surgical Ward (SW)		Other Ward (OTR)		Total No.of *E.coli*Isolates	
	BFP	BFNP	BFP	BFNP	BFP	BFNP	BFP	BFNP	BFP	BFNP	BFP	BFNP
Urine	12	6	0	0	0	0	0	0	0	0	12	6
Stool	4	0	22	12	14	2	0	0	2	0	42	14
Swab	0	0	0	0	0	0	2	2	2	0	4	2
Pus	0	0	0	0	0	0	2	0	0	0	2	0
Body Fluid	0	0	0	0	4	0	2	0	0	0	6	0
Blood	0	0	0	0	0	0	0	0	0	2	0	2
Total No. of *E.coli* Isolates	**16**	**6**	**22**	**12**	**18**	**2**	**6**	**2**	**4**	**2**	**66**	**24**

BFP: Biofilm producing number of *E.coli* clinical isolates
BFNP: Biofilm Non-producing number of*E.coli* clinical isolates

The 90E.coliisolates studied forantibiotic susceptibility testing were found highly drug resistant.Antibiotic susceptibility pattern ofE.coli clinical isolates, were as shown in Table 2. Among the 90E.coliclinicalisolates high susceptibility rates werefound among the Imipenem,Meropenem,Gentamicin,and Chloramphenicol. The commonly used antibiotics cephalosporins, aminoglycosides and quinoloneswerefound to be resistant among both biofilm producer as well as non-biofilm producer groups. Among the 90 isolates > 60 isolates were resistant to all tested antimicrobialsor resistant to 3 or more classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides and quinolones) and exhibited multidrug resistance.

Table 2: Antibiotics susceptibilitypatternof E.coli clinical isolates

Antibiotics	Sensitive			Intermediate			Resistance		
	Total	BFP	BFNP	Total	BFP	BFNP	Total	BFP	BFNP
Cephoxitin (CN)	40	32	8	22	14	8	28	20	8
Ceftriaxone (CI)	16	10	6	0	0	0	74	56	18

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 4, Issue 1, January 2019

Ceftazidime (CA)	4	2	2	4	4	0	82	60	22
Cefotaxime (CE)	4	2	2	6	6	0	80	58	22
Cefuroxime (CU)	14	8	6	0	0	0	76	58	18
Cefpodoxime (CEP)	0	0	0	2	2	2	88	64	22
Cefepime (CPM)	14	8	6	2	2	0	74	56	18
Cefpirome (CFP)	0	0	0	2	0	2	88	66	22
Aztreonam (AO)	10	6	4	12	10	2	68	50	18
Imipenem (I)	88	64	24	0	0	0	2	2	0
Meropenem (MR)	54	38	16	8	8	0	28	20	8
Ampicillin (A)	0	0	0	4	2	2	86	64	22
Co-trimoxazole (CO)	12	10	2	12	6	6	66	50	16
Gentamicin (G)	52	42	10	22	12	10	16	12	4
Chloramphenicol (C)	46	30	16	10	6	4	34	30	4
Tetracyclin (T)	14	12	2	12	6	6	64	48	16
Ciprofloxacin (CF)	2	2	0	12	6	6	76	58	18

BFP: Biofilm producing number of E.coli clinical isolates isolates
BFNP: Biofilm Non-producing number of E.coli clinical

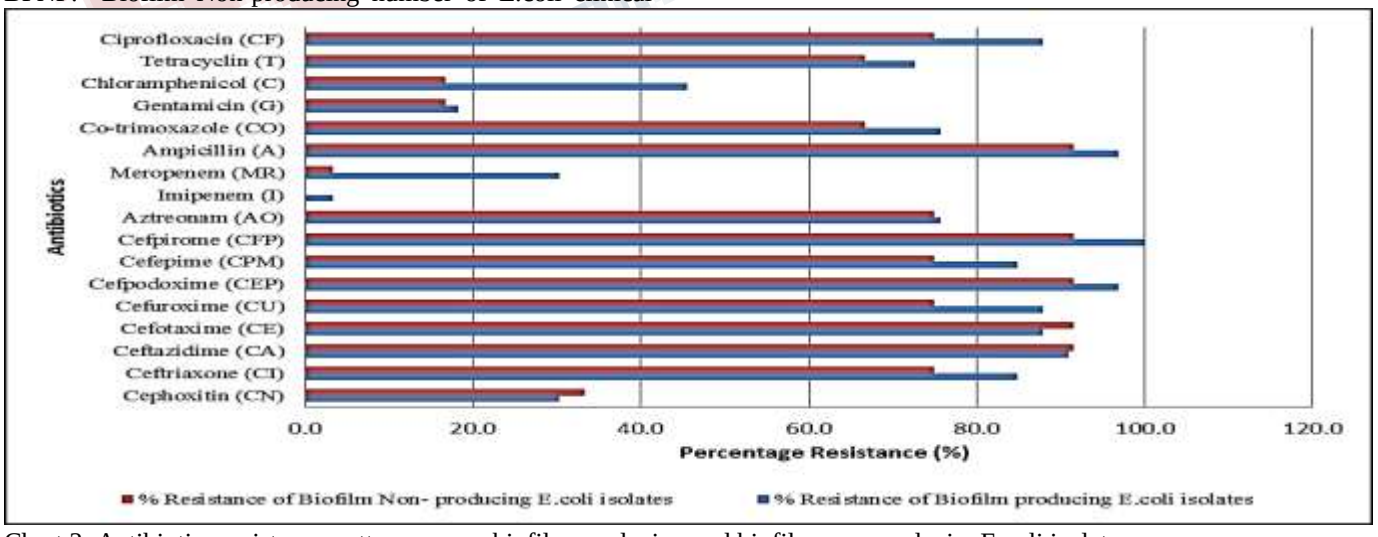

Chart 3. Antibiotics resistance pattern among biofilm producing and biofilm non-producingE.coli isolates

The resistance pattern of E.coli against antibiotics is shown in Chart 3.High resistance was observed in biofilm producing isolates compared to biofilmnon-producing isolates.In comparing antimicrobial resistance to the ability of biofilm formation in the individual strains, we observed that strains capable of forming biofilms were more frequently observed to be an MDR phenotype (Chart 3).In our study isolates shows susceptible to penems antibiotics. Compare to biofilm producing, biofilm non-producing isolates shows 3.3% resistance to meropenem and 0.0% resistance to imipenem. Most of the penems antibiotic resistance exhibited by biofilm producing isolates.However, despite the high levels of antibiotic resistance, there was a significant association between biofilm production and resistance

IV. DISCUSSION

The formation of biofilms increase bacterial resistance to environmental stresses such as, nutritional and oxidative stresses, desiccation, UV light exposure, sanitizing agents and antimicrobials [8]. Due to this increased resistance to stresses biofilms pose a threat in clinical setting.Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. There are various methods for biofilm detection. In this study we evaluated 90 E.coli clinical isolates by two screening methods for their ability to form biofilms. The Congo red method, presents significant relation in context to result interpretation convince when compared with TM of analysis to biofilm producers. In current study antibiotic resistance was higher among biofilm producers to commonly used antibiotics as compared to non-biofilm producers. This may be because bacterial biofilms are often associated with long term persistence of organism in various environments, decreased bacterial growth rate in a biofilm, expression of resistance genes, and restricted penetration of antibiotics into biofilm, similar results were obtained by Sarvaet al, 2016[16]. Furthermore, proximity of cells within a biofilm can facilitate a plasmid exchange and hence enhance the spread of antimicrobial resistance.

V. CONCLUSION

We found a high prevalence of biofilm-forming phenotypes among a large number of E.coli clinical isolates. In addition, biofilm formation was prevalent among isolates with a MDR phenotype. CRA method is reproducible, simple, cost effective method for screening biofilm formation and does not require technical expertise. From this study we have concluded that biofilm formation is the major virulence determinant of E.coli and pose significant risk to therapy as well as infection control purpose so it is necessary to screen all clinical E.coli isolates for biofilm production.

REFERENCES

1. Donlan RM, Costerton W. Biofilms: Survival mechanisms of clinically relevant Microorganisms.ClinMicrobiol Rev.2002; 15(2):167-93.

2. Nachammai SM, Jayakumar K, Aravazhi AN. The effectiveness of antibiotics against a major uropathogen-Escherichia coli and its biofilm assay by phenotypic methods. Int J Res Med Sci.2016; 4:4820-8.

3. Research on microbial biofilms (PA-03-047). NIH, National Heart, Lung, and Blood Institute. 2002-12-20.

4. Parul, Bist B, Sharma B and Jain U. Virulence associated factors and antibiotic sensitivity pattern of Escherichia coli isolated fromcattle and soil, VeterinaryWorld. 2014;7(5): 369-372.

5. Sharma S, Bhat G K, Shenoy S.Virulence factors and drug resistance in Escherichia coli isolated from extraintestinal infections. Indian J Med Microbiol.2007;25:369-73

6. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lanc.2001;358(9276):135-8.

7. Keren I, Kaldalu N, A. Spoering, Y. Wang, and L. Kim. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett.2004;230:13-18.

8. CostertonJW Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science.1999;284(5418):1318-22.

9. Pfaller MA, Jones RN. MYSTIC (Meropenem Yearly Susceptibility Test Information Collection) results from the Americas: resistance implications in the treatment of serious infections. J AntimicroChemothe. 2000;46:25-37.

10. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15:305-11.

11. Goossens H. MYSTIC (Meropenem Yearly Susceptibility Test Information Collection) results from Europe: comparison of antibiotic susceptibilities between countries and centre types. J AntimicroChemothe. 2000;46:39-52.

12. Naik JP, Desai PB.Prevalence of E.coli in case of intestinal and extra intestinal infection. Int. J. Biosci. ISSN. 2012;2231-0304.

13. Collee, JG, Miles RS Watt, B. Tests for identification of bacteria In: Mackie and McCartney Practical Medical Microbiology 14thedColleeJG.Fraser AG. MarnionBP.SunmonA editors (Churchill Livingston: New York).1996;p.131–49.

14. Panda PS, Chaudhary U, Dube SK. Comparison of four different methods for detection of biofilm formation by uropathogens. Indian J PatholMicrobiol.2016;59:177-9.

15. CLSI 2012. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 22nd informational supplement. Wayne PA. CLSI document M100-S22.

16. Sarva K, Chitra G, Mandal J, Manikandan R. Phenotypic Characterization of Escherichia Coli Isolated From Urine and Stool from Short Term and Long Term Catheterized Patients. Eastern J Med Sci.2016; 1(1): 17-23.