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Detection of ESBL production in clinical isolates of *Escherichia coli*, their antibiotic resistance and biofilm development on varied catheter materials

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Abstract

The focus of the study was to detect the antibiotic resistance of *Escherichia coli*, their extended spectrum beta-lactamase (ESBL) production and development of biofilm on different catheters. 37 *E. coli* clinical strains were procured from K.A.P. Viswanatham Government Medical College, Trichy, Tamil Nadu, India and screened for their antibiotic resistance through Kirby-Bauer disc diffusion method. 30 strains (81.0%) were highly resistant to ampicillin. The tested strains expressed 29 different antibiotic resistance patterns. Interestingly, it was found that 28 strains (75.6%) were multi - drug resistant (MDR). 33 (89.1%) strains were found to be positive for ESBL. *E. coli* producing ESBL were further tested for Double Disk Synergy Test (DDST) and 30 (81.0%) were positive strains. Similarly, in Modified Double Disk Synergy Test (MDDST), 32 (86.4%) isolates were found to be positive. Whereas in Direct Modified Three Dimensional Tests (DMTDT), only 12 (32.4%) isolates were positive, while in Indirect Modified Three Dimensional Tests (IMTDT), 35 (94.5%) isolates were positive. Biofilm formation of *E. coli* on two different catheters was tested and the results revealed that silicone based catheter reduced the bacterial biofilm effectively than the PVC catheters. In addition, it was found from this study that following biofilm growth, *E. coli* developed increased resistance against most of the antibiotics. Also it was found that the presence of glucose at higher level enhanced biofilm development of *E. coli*. The increasing resistance of biofilm-associated *E. coli* to antimicrobial agents and the potential of the organism to cause infections through indwelling medical devices like catheters is a critical public health concern.

Key words: *E. coli*, antibiotic resistance, ESBL, biofilm development, catheter

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A German microbiologist named Theodor Escherich in 1884 began to study about the gut microbes in infants and their characteristics. In that study, he found a fast growing bacterium which he named, *Bacterium coli commune*, now known as *E. coli*[2]. *E. coli* is a complex gram negative bacterium which is found commonly in the environment, gut of humans, animals, and food. The most prevalent bacterial infection in humans is Urinary Tract Infection (UTI), and *E. coli* is the most predominant bacterial pathogen isolated from hospital and community based patients with this infection[16]. After inappropriate usage of antibiotics for more than 70 years to treat various infections, antibiotic resistance has been recognized as a major issue in healthcare settings[26]. *E. coli* is a MDR bacterium and also produces ESBL. In addition, ESBL-producing strains are a major cause of hospital infections, which are characterized by high morbidity and mortality as a result of inadequate treatment options. Another big issue is biofilm formation, in which the biofilm matrix provides extra resistance power to the bacteria by making them resistant to treated antibiotics[13]. According to numerous earlier researches, bacteria in biofilms are more resistant than their planktonic counterparts in suspension to environmental stresses, including heat[17]. Cells are protected against antibiotics by biofilm, which enables them to endure in adverse environments[1,8]. Biofilms lead the bacteria to cause dreadful infections which are hard to eliminate using antibiotics[20].

E. coli produces ESBL enzymes that degrade and destroy the frequently used antibiotics including cephalosporins, rendering the antibiotics inefficient to treat infections. The antibiotics used in this study were the most widely used for the *E. coli* infections. Also, *E. coli* had been reported to be the significant producer of ESBL worldwide[12]. The treatment of *E. coli* infection is becoming more and more concerned due to the rising cephalosporin resistance, particularly the concurrent rise in the prevalence of MDR *E. coli*. The most common bacterial pathogen involved in serious infections is *E. coli*[6,10,24]. Additionally, the threat posed by antibiotic resistant *E. coli* is growing and is becoming a serious concern for human health worldwide[29]. The goal of this study was to assess the antibiotic resistance profiles of *E. coli* and to screen the isolates for ESBL-production, as well as to evaluate the formation of biofilm on two different catheter surfaces (silicone elastomer bonded (SEB) and polyvinylchloride (PVC)) using the Crystal Violet (CV) staining method. Also, the role of glucose in biofilm formation was examined, and the antibiotic resistance pattern of tested isolates after the biofilm development was determined from this study.

Collection of strains :

A total of 37 clinical isolates of *E. coli* were obtained from K.A.P.Viswanatham Government Medical College, Tiruchirappalli, Tamil Nadu, India.

Confirmation of bacterial isolates :

The collected 37 strains were grown overnight at 37° C in Tryptic Soy Agar (TSA) (HiMedia) and then transferred to MacConkey agar for morphological characte-rization and finally reconfirmed through biochemical confirmation analysis.

Determination of antibiotic resistance pattern :

The antibiotic resistant pattern of all confirmed *E. coli* bacterial isolates was carried out using disc diffusion method. The overnight bacterial suspensions were diluted in 0.85 % Nacl, equivalent to 0.5 McFarland standards according to the guidelines of Clinical Laboratory and Standards Institute[5]. Then the bacterial culture was swabbed on to Mueller Hinton Agar (MHA) (HiMedia) plates and antibiotic discs were placed over it and kept at 37° C for 24 h. After incubation, the resistant pattern was noted. Ampicillin (10µg), cefepime (30µg), cefotaxime (30µg), co-trimoxazole (25µg), tetracycline (30µg), levofloxacin (5µg), gentamycin (10µg), imipenem (10µg), ertapenem (10µg), meropenem (10µg), tigecycline (30µg), colistin (10µg), and doripenem (10µg) (HiMedia) were the antibiotics used in this study.

Detection of ESBL *:*
Phenotypic confirmatory disc diffusion test (PCDDT) *:*

An inoculum of the test strain with the McFarland standard of 0.5 was swabbed on MHA plate. Antibiotic discs containing ceftazidime, aztreonam, cefotaxime or ceftriaxone and a disc of amoxyclav were placed on the MHA plate and then incubated at 37° C for 24 h[28].

Double disk synergy test (DDST) *:*

This test was used to detect the ESBL production in *E. coli*. It was performed using cefotaxime (30µg), cefepime (30µg), cefpodoxime (30µg), and aztreonam (30µg) discs encircling amoxyclav (30µg) (20µg amoxicillin +10µg clavulanic acid disc) of 16 to 20 mm away from it. The plates were incubated for 24 h at 37° C. If the zone of inhibition around any of the cephalosporin discs expressed a clear synergy towards the amoxyclav disc, then the organism was said to produce ESBL[15].

Modified double disk synergy test (MDDST)*:*

This test was used to determine ESBL in *E.coli* strains which produce Amp C. A disc of amoxyclav (30µg) was kept at the centre of the plate, whereas aztreonam (30µg), ceftazidime (30µg), cefepime (30µg), imipenem (10µg), cefpodoxime (30µg) and cefoxitin (30µg) were positioned around it at 16 – 20 mm distance and piperacillin – tazobactam (100µg /10µg) was placed at 22 and 25 mm. *E.coli* was considered to produce ESBL, only if the zone of inhibition surrounding cefepime or any of the extended spectrum cephalosporin discs displayed a clear – cut raise towards the piperacillin – tazobactam antibiotic disc or amoxyclav disc[15].

Modified three dimensional test (MTDT) *:*

(i) Direct modified three dimensional test :

The test strains were swabbed on sterile MHA plates with 0.5 McFarland turbidity standards, as conducted in DDST, and a disc of ceftazidime, cefotaxime, or aztreonam were positioned in the centre of the plate. 30µl inoculum of the test strain equivalent to 5.0 McFarland standards was seeded into a well with a diameter of 6 mm. ESBL production was distinguished by a heart-shaped distortion of the zone of inhibition, with test organism

growth emerging behind and approaching the well[23].

(ii) Indirect modified three dimensional test (IMTDT) *:*

E. coli ATCC 25922 was grown overnight in TSA at 37° C and was adjusted equivalent to 0.5 McFarland standards and then swabbed on the sterilized MHA plates. 30µl inoculum of the test strain equivalent to 5.0 McFarland standards was seeded into a well with a diameter of 6 mm. ESBL production was indicated by a heart-shaped distortion of the zone of inhibition around the β – lactam disc[15].

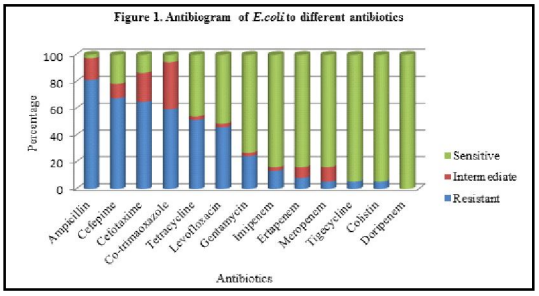
Quantification of biofilm production on catheters:

Biofilm formation on two different surfaces (SEM and PVC) was quantified using crystal violet (CV) assay. Two types of catheters were used (Teleflex Medical Pvt. Ltd., and Royal Surgicare Pvt. Ltd., India). They were cut into 13mm long vertically and then placed inside the corresponding sterile glass tubes containing 10 ml tryptic soy broth (TSB). All the glass tubes containing a piece of catheter were inoculated with 100 µl of bacterial culture. The glass tubes were then incubated at 37°C for 24 h. After incubation, the cells which were not bound were washed with sterile water. The catheter pieces were then transferred carefully to sterile glass tubes. The biofilm attached to the catheter was stained with 1ml of 0.5% CV. The excess stain was rinsed with water and the remaining stain on biofilm cells was decolorized using 1 ml of 99% ethanol. It was then transferred to a cuvette and the Optical Density (OD) of cells adhered in catheter in each glass tube was estimated at 595 nm (Cary - 60 UV-Vis Spectrophotometer, Agilent Technologies, USA)[17].

Assessment of antibiotic resistant pattern following biofilm development on SEB and PVC catheter materials:

Disc diffusion technique was used to obtain the antibiogram profile of *E. coli* following biofilm development on the tested catheter materials. After the development of biofilm, the cells were collected from both the catheter materials using a sterile cotton swab and diluted in sterile 0.85% saline which is equivalent to 0.5 McFarland standards and then swabbed on the MHA plate. The antibiotics that were employed in the antibiotic susceptibility test before the development of biofilm were used in this assessment.

Antibiotic resistant pattern of E. coli :



Antibiogram of *E. coli* isolates tested against different classes of antibiotics are shown in Figure 1. All the isolates were resistant to at least one antibiotic tested. The test isolates showed high resistance against ampicillin (81%), followed by cefepime (67.5%), cefotaxime (64.86%), co-trimoxazole (56.7%), tetracycline (51.3%), levofloxacin (51.3%), gentamicin (24.3%), imipenem (10.8%), ertapenem (8.1%), meropenem (5.4%), and tigecycline (5.4%) and colistin

(5.4%). 75.6% of the isolates were found resistant to more than three classes of antibiotics, and identified as MDR *E. coli*. All the isolates were found to be completely sensitive to doripenem. Totally 29 different types of patterns were found in *E. coli* (Table-1).

Table-1. Antibiotic resistant pattern of *E. coli*

S. No.	Antibiotic Resistance Patterns	No. of Strains
1	AMP	02
2	CPM	02
3	AMP,COT	02
4	AMP,CPM	01
5	AMP,COT,IPM	01
6	AMP,CPM,CTX	02
7	AMP,CTX,TE	01
8	CPM,CTX,LE	01
9	CTX ,IPM,MRP	01
10	AMP,COT,CPM,CTX	01
11	AMP,COT,IPM,LE	01
12	AMP,COT,MRP,TGC	01
13	AMP,CPM,CTX,TE	01
14	AMP,GEN,MRP,TE	01
15	AMP,COT,CPM,MRP,TE	01
16	AMP,COT,CTX,IPM,TE	01
17	AMP,COT,GEN,LE,MRP	01
18	AMP,COT,CPM,CTX,TE	01
19	AMP,CL,COT,CPM,MRP,TE	01
20	AMP,COT,CPM,CTX,GEN,LE	01
21	AMP,COT,CPM,CTX,LE,TE	02
22	AMP,COT,GEN,LE,TE,IPM	01
23	AMP,COT,CPM,CTX,GEN,LE,TE	04
24	AMP,COT,ETP,GEN,IPM,LE,TE	01
25	AMP,CPM,CTX,ETP,IPM,LE,TE	01
26	AMP,CPM,CTX,ETP,GEN,LE,MRP, TE	01
27	AMP,CL,COT,CTX,LE,TE,CPM,GEN,MRP	01
28	AMP,COT,CPM,CTX,ETP,GEN,LE,MRP,TE	01
29	AMP,COT,CTX,ETP,TE,CPM, GEN,LE,TGC	01

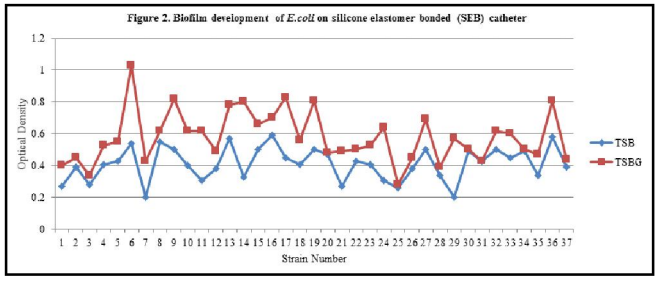
ESBL *detection :*

Table 2. Prevalence of ESBL positive *E.coli*

ESBL detection	Positive
Phenotypic confirmatory disk diffusion test (PCDDT)	32 (86.4%)
Double disk synergy test (DDST)	30 (81.0%)
Modified double disk synergy test (MDDST)	33 (89.1%)
Direct modified three dimensional test (DMTDT)	12 (32.4%)
Indirect modified three dimensional test (IMTDT)	31 (83.7%)

The prevalence of ESBL-producing *E. coli* was determined and the results are given in Table-2. Out of 37 isolates, ESBL positive bacteria were found in 32 (86.4%) isolates in the PCDD test followed by 30 isolates (81.0%) in the DDST method, 33 (89.1%) isolates in MDDST, 12 (32.4%) isolates in DMTDT, and 31 (83.7%) isolates in the IMTDT method. In comparison, it was found from the study that the MDDST approach was the most sensitive than others and the DMTDT method exhibited least sensitive.

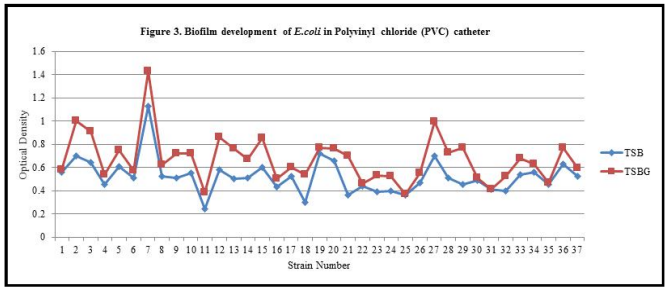
Biofilm development of E. coli on (SEB) *catheter in* (TSB) *and* TSB *supplemented with glucose* (TSBG) *:*



Biofilm formation of *E. coli* in SEB catheter in TSB and tryptic soy broth supplemented with glucose (TSBG) is illustrated in Figure 2. The growth of *E. coli* on SEB catheter was found to be higher in TSBG than in TSB.

In TSB and TSBG, all 37 isolates expressed consistent biofilms. In TSB, strain 16 and in TSBG, strain 6 exhibited the maximum growth. Strain 14 also had shown a notable increase in growth trend in TSBG than TSB. Strains 7 and 29 showed the least growth in TSB whereas strain 25 expressed the least growth in TSBG. In the case of TSBG, strains 9, 11, 17, 19, 24 and 29 showed a substantial rise in growth trend. Similarly, strains 7, 10, 13, 21, and 36 showed a considerable rise in TSBG and strains 1, 4, 5, 12, 15, 16 18, 23, 27, 32, 33, 35. Strains 2, 3, 8, 20, 22, 25, 26, 28, 30, 34, and 37 showed minimal rise in growth, while strain 31 had no growth difference.

Biofilm development of E. coli on PVC *catheter in* TSB *and* TSBG *:*



Biofilm formation of *E. coli* in PVC catheter in TSB and TSBG are illustrated in Figure 3. The growth of *E. coli* on SEB catheter was found to be higher in TSBG than in TSB. Both in TSB and TSBG, strain 7 exhibited the highest growth and strain 11 exhibited the least growth, whereas strain 25 also marked a lesser growth in TSBG. The growth peak of strains 21 and 29 in TSBG were higher than in TSB, whereas those of strains 2, 3, 7, 9, 12, 13, 15, 18, 27 and 28 were also considerably higher. Similarly, in TSBG, strains 5, 10, 11, 14, 23, 24, 32, 33, and 36 exhibited a notable growth. Strains 1, 4, 6, 8, 16, 17, 19, 20, 22, 25, 26, 30, 34, 35 and 37 showed a slower rate of growth. No difference in growth was observed in strain 31.

Comparison of biofilm development of E. coli on SEB *and* PVC *catheters in* TSB *and* TSBG *:*

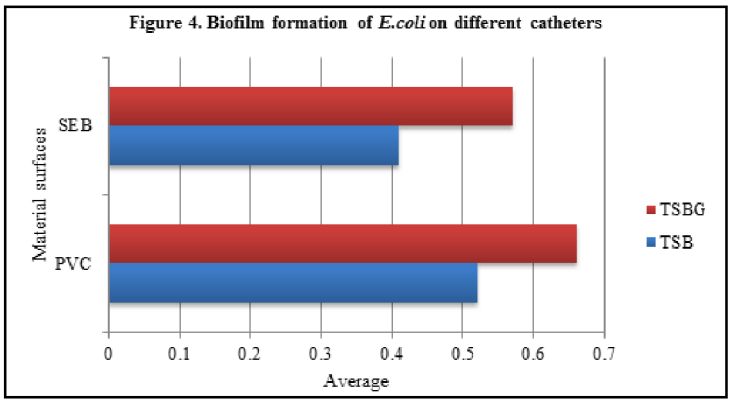
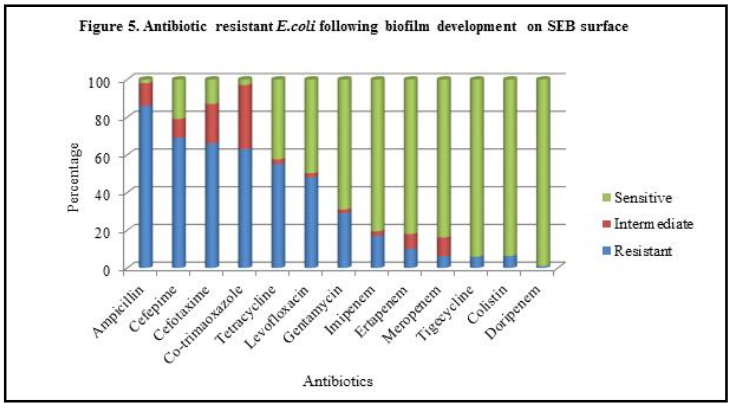
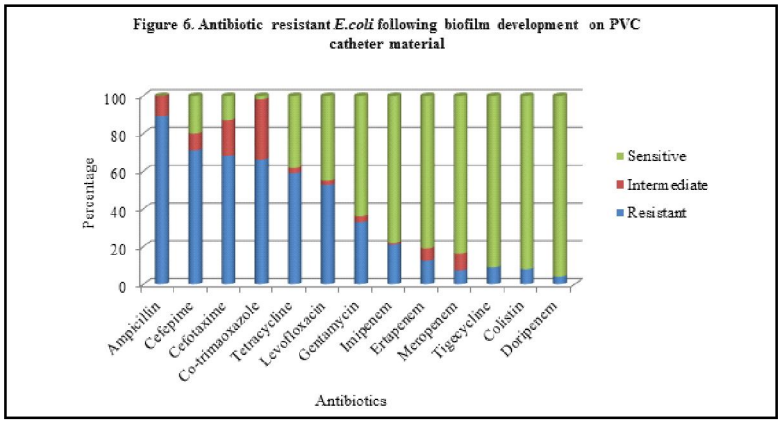


Figure 4 demonstrates the comparison of biofilm development of *E. coli* on PVC and SEB catheters in TSB and TSBG. It is evident from the figure that the PVC catheter supported the highest biofilm formation in TSB and TSBG. *E. coli* tested with TSBG showed good growth than in TSB both on SEB and PVC surface catheters. PVC catheter had good biofilm development in both TSB and TSBG with an average range of 0.5 to 0.7. And, SEB catheter had comparatively less growth between 0.4 and 0.6.





Antibiotic resistant pattern of E. coli on SEB *catheter :*

Antibiotic resistance profile of all *E. coli* isolates was assessed after the development of biofilm on SEB catheter material and the results are shown in Figure 5. It is observed that the resistance of the isolates had increased considerably after the development of biofilm. Ampicillin resistance following the establishment of the biofilm increased up to 4.9%, while gentamycin resistance increased up to 4.6%. For tetracycline and co-trimoxazole, the resistance rates were increased to 3.7% and 3.6%, respectively. Imipenem resistance was found to be raised up to 3.5%, levofloxacin resistance increased to 2.1%, ertapenem increased to 1.9%, resistance for cefepime increased to 1.5% compared to before biofilm formation, while resistance for cefotaxime increased to 1.2%; colistin to 1.0%, meropenem to 0.8% and tigecycline, the least to 0.6%. On the SEB catheter, 1.0% increase in doripenem resistance was seen after biofilm growth.

Antibiotic resistant pattern of E. coli on PVC *catheter :*

Antibiotic resistance profile of all *E. coli* isolates after biofilm formation on PVC catheter material is depicted in Figure 6. It was found that the isolates resistance increased substantially after the development of biofilm. After the biofilm growth, the tested isolates demonstrated the highest resistance of 89.0% to ampicillin, which was 7.9% increase. All the other antibiotics tested also had increased resistance against cefepime which exhibited 3.5% increase while cefotaxime had 3.2% high. Resistance to co-trimoxazole increased by 6.6% and tetracycline resistance was 59.0% with an increase of 7.7%. Resistance to levofloxacin increased by 6.6%, gentamycin resistance increased by 8.6%, and imipenem resistance increased by 7.5%. Ertapenem had 4.5% more resistance, meropenem 2.0%, tigecycline 3.6% and colistin 2.6% raise in resistance on PVC. After biofilm development, a 4.0% increase in doripenem resistance was observed on PVC catheter. While comparing the antibiotic profiles of *E. coli* on both SEB catheter and PVC catheter, resistance was found to be higher on PVC catheter material.

After the discovery of penicillin during Second World War by Alexander Fleming, it saved many lives and still the antibiotics are

being used to treat for various illnesses in humans. This apart, it is also being used indiscriminately in livestock, poultry farm, farming activities and aquaculture practices. The indiscriminate use of antibiotics by a human being leads to the development of resistant microbes against numerous antibiotics. Considering the importance of this, in the present study, a total of 37 clinical *E. coli* isolates were subjected against different antibiotics before and after biofilm formation in medical device with two different materials. Results revealed that high resistance was observed in ampicillin (81%), and low resistance was noticed against meropenem (5.4%), tigecycline (5.4%) and colistin (5.4%). All the strains were completely sensitive (100%) to doripenem. Cepas *et al*.[4] concluded from their study that 30.0% of the *E.coli* strains tested were positive for MDR[4]. However, in the present study, 75.6% of *E.coli* was found to be MDR. Similarly, Martinez-Vazquez *et al*.[22] revealed that 72.7% of the tested *E. coli* strains were MDR and also found that there were 82.0% of resistant *E. coli* strains from their investigation[22]. In another study by Wu *et al*.[29] found that only 26.0% of the *E. coli* isolates were MDR[29]. Interestingly, the resistance rate against cefepime (67.5%), cefotaxime (64.8%), and gentamicin (24.3%), also, ESBL producing *E. coli* was (89.1%) from the present study positively correlated with the report of Wu *et al.,*[29]. The *E. coli* strains that produce ESBL makes the strains resistant to antibiotics especially to cephalosporin class which worsens the therapeutic approaches. Dhara *et al*.[7] had reported that ESBL producing clinical strains were so high in prevalence leading to higher mortality rates[7]. Khan *et al.*[15] had concluded from their study that DDST was positive in 62.5% isolates; MDDST had 100% positive isolates[15]. The present study correlates with their results; DDST had 81.0% isolates, whereas MDDST had 89.1% isolates. ESBL producing enterobacterial isolates causes major infections and treating them is much challenging because of their resistance level to the extended spectrum cephalosporins. The ESBL producing *E. coli* isolates were largely found or isolated in hospital environments, in food, aquatic environments, and in animals[9] and they are the main source of childhood infections and pose serious problems leads to treatment failure due to MDR, and high morbidity and mortality[14].

Li *et al*.,[18] had reported that high incidence of urinary tract infection (UTI) is caused mainly due to *E. coli*[18] and 80.0% of the UTI cases are caused by catheter associated urinary tract infections (CAUTI)[19]. The indwelling catheter device enhances the growth of disease causing organisms like *E. coli* in the urinary tract. A patient develops 5.0% risk of CAUTI within a day of indwelling catheter and then by day 30 they are all infected[21]. An earlier study claimed that biofilm contributes to 80.0% of human infections and that 31.12% of the examined isolates from patients who were catheterized, was *E. coli*[25]. The impact of biofilm growth on different catheter materials has not received much attention. The present study quantified the growth of biofilms over two different catheter materials and examined the effect of glucose supplementation on development of biofilms. The findings revealed that the highest biofilm development of *E. coli* was exhibited on PVC rather than on SEB with and without glucose supplementation. Glucose is the most prevalent monosaccharide and it is the main source of

energy for cells in several types of bacteria, including *E. coli*[27]. Furthermore, in comparison with other carbohydrates, glucose promotes growth of *E. coli* rapidly and acts as a primary carbon source[3].

According to the study of Huang *et al*.,[11] PVC catheters lack in retaining physical properties and flexibility and also, they support growth of *E. coli* biofilm[11]. Hence silicone catheters can be preferred as it had less effect on biofilm development and also it was found to have less sepsis, longer life and fewer insertions in patients. In addition, silicone had been reported to minimize injuries and allergies of the urinary mucosa. The quantitative associations between biofilm-forming ability and the antibiotic resistance before and after the biofilm development have not been adequately studied. Therefore this study had been carried out to investigate the relationship between them.

In India, ESBL – producing *E. coli* is becoming increasingly widespread. In this study, tested *E. coli* strains indicated notable levels of resistance to the various antibiotic classes. The impact of antibiotic resistance can be established by disclosing its resistance patterns for successful treatments. Development of biofilm is more frequent in *E. coli* that produces ESBLs. And this study also shows that silicone – based catheters are less likely to produce biofilm, which has an impact on the emergence of antibiotic resistance even with the sugar supplements, making them less susceptible to CAUTIs, which are another consequence of prolonged catheter use. Additionally, silicone – based catheter is frequently more flexible and poses a lower risk of infections.

Declarations :

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Authors contribution :

The work was designed by KS. BAM performed the experiments, collected the data, and prepared the manuscript. LSB helped in generating data and preparing the manuscript. KPK supervised and designed part of the work. KS read and approved the manuscript.

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Conflicts of interest – The authors declare no conflict of interest.

Ethical statement – This article does not contain any human participants or animals performed by any of the authors.

Data availability – This manuscript contains all of the data collected during the study.

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