

Research Paper

Extraction and Screening of Biofilm producing Bacterial isolates in Short-and Long-term Catheters

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Abstract

Biofilm is defined as microbial-derived sessile communities enclosed with extra polymeric substances and characterized by cells that are irreversibly attached to a surface. Biofilm bacteria are a serious threat to public health as biofilm formation of bacteria in indwelling medical devices especially in urinary catheters causes persistent infections resistant to treatment with antimicrobial agents. The present study focused to screen the biofilm-producing bacteria from the indwelling urinary catheters. Both short-term and long-term urinary catheters were collected from the catheterized patients admitted in the national and a private hospital. Bacterial population removed from the catheters were tested for biofilm production by widely used three methods such as Tube method, Congo Red Agar method, and Tissue Culture Plate method. In this preliminary screening, the major biofilm producer identified in the present study is *E.Coli* in all types of catheters. Further, biofilm-producing bacteria were predominantly detected in long-term catheters than short-term catheters. In addition, the identification of more than one bacterial strain in long-term catheters revealed that bacterial diversity increases with the duration of catheterization. The results of the present study revealed that long-term urinary catheters have the potential for survival and diverse biofilm-producing bacteria.

Keywords: Biofilm production, biofilm-producing bacteria, indwelling urinary catheters

Introduction

Biofilm is a community of microbial organisms that are irreversibly attached to a surface and generally enclosed in a polysaccharide matrix. The bacteria become more resistant after adhering to a surface and subsequently develop more resistivity over the following days of biofilm production [1]. Mature biofilms are highly resistant to the action of the human innate and adaptive immune defense system, as well as to the

action of antimicrobial agents and disinfectants. Gram-positive bacteria commonly involved in biofilm production are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* [2].

Biofilms have become a major source of device-related infections in hospitals. The urinary catheter is the second most internally placed human object which is used most often in hospital settings. The number of catheter-associated urinary tract infections (CAUTI) increases every year as the pathogens get through this catheter simply and easily attack the urinary tract and the bladder [3]. Nearly 97 % of urinary tract infections are related to urinary catheters. About 60 % of the infections developed due to biofilm production [4] as urinary catheters more tend to get biofilm-producing bacteria [2, 5].

Generally, urinary catheters are inserted into around 12 to 16% of adult patients in the initial stage of their admissions at hospitals [6]. The tendency of biofilm production of the organism increases with the duration of catheterization [1]. Therefore, the risk of infections may be related to the length of time the catheter in place. Most patients catheterized for less than one week possibly would not get the infection, however, elderly and disabled patients who are catheterized for more than a month have a 100 % possibility of getting infections [1, 4]. If the infection is not treated, it may end up with the diseases such as bacteremia, bacterial vaginosis, chronic renal infection, acute pyelonephritis, bladder cancer, and in certain cases death [4].

Nosocomial infections are a major problem in any healthcare setting and several folds higher in developing countries including Sri Lanka. Since CAUTI develops mostly due to biofilm production, the study of microbial biofilm has received significant attention over the past few decades among researchers. Due to inadequate surveillance and reporting system, catheter-associated biofilm infection has been underestimated and developed into a common problem in developing countries. Therefore, studying the causative bacteria responsible for biofilm production is necessary to develop effective strategies for controlling biofilm and improvement of patient care. In Sri Lanka, a study carried out in Colombo North teaching hospital indicates that urinary catheterization is one of the major sources of acquiring nosocomial infection in the country [7]. Another study reported by Atukorala [8], revealed that, though the healthcare workers had been trained in infection control education program, there was no significant reduction in catheter-associated urinary tract infection (CAUTI) compared to other device-related

infections [8]. However, there is a scarcity of reports with regard to biofilm formation in urinary catheters and responsible microorganisms in Sri Lankan setup.

As catheter-associated biofilm infections significantly contribute to patient morbidity rate. Therefore, novel strategies and other measures are urgently required to treat biofilm infections. There are several challenges to be met in the development of novel anti-biofilm therapies. Further, lack of knowledge about biofilm-producing bacteria leads to slow progression of detecting biofilms in urinary catheters and consequently leads to therapeutic failure. Hence, the identification of bacteria responsible for biofilm production is most important to establish treatment strategies and preventive measures against catheter-associated infections. The present study investigated biofilm bacteria and their ability of biofilm production with respect to the time duration of catheterization.

Materials and Methods

Sample Collection

This study was approved by the Ethics Committee, National Hospital of Sri Lanka. The indwelling urinary catheters were randomly collected from patients with or without nosocomial infections admitted in the National Hospital of Sri Lanka as well as from the New Delmon Hospital during the period from May 2019 to March 2020. The factors such as sex, age, race of patients, disease conditions, and treatments were excluded in this study. Both short-term and long-term urinary catheters were collected in sterilized HiDispo™ bag in aseptic conditions. The collected catheters were immediately brought to the laboratory for screening of bacterial isolates for biofilm production.

The catheters collected were grouped as follows;

1. Urinary catheters collected as short term - Catheterized for ≤ 7 Days
2. Urinary catheters collected as midterm - Catheterized for 7 to ≤ 28 Days
3. Urinary catheters collected as long term - Catheterized for > 28 Days

Extraction and Biochemical Identification of Bacteria

Each urinary catheter was sectioned into small parts and each part was suspended into 10 ml of ringer's solution separately. Biofilm was removed from the catheter parts by continuous shaking by keeping in the shaker at 150 rpm [1]. Finally, the extracted inoculum of each part of the catheter was mixed together to prepare a composite sample. The inoculum taken from the prepared composite sample was cultured in a nutrient agar medium using the serial dilution technique. Ringer's solution was used as

the diluents and the cultured plates were incubated at 37 °C for 24 hr.

All together 44 bacterial colonies were randomly selected from the bacterial population extracted from each of the short-term, mid-term and long-term catheters. Every four colonies isolated from each catheter were named according to the duration of catheter usage. For example, Catheter coded B which was inserted into the patient's bladder for 2 days grouped as short-term (S). Therefore, 4 selected colonies (1, 2, 3, and 4) isolated from this catheter were named BS21, BS22, BS23, and BS24. Similarly, all the colonies were named as mentioned in following Table 1.

Table 1. Coding numbers given for the colonies

Code	Days	Duration	Code no.	Tested Bacterial colonies
AA	2	S		No significant growth
AB	2	S		No significant growth
B	2	S	BS2	BS21; BS22; BS23; BS24
C	5	S	CS5	CS51; CS52; CS53; CS54
D	5	S	DS5	DS51; DS52; DS53; DS54
E	6	S	ES6	ES61; ES62; ES63; ES64
F	7	S	FS7	FS71; FS72; FS73; FS74
G	7	S	GS7	GS71; GS72; GS73; GS74
H	8	M	HM8	HM81; HM82; HM83; HM84
M	30 (X)	L	MLX	MLX1; MLX2; MLX3; MLX4
N	30 (X)	L	NLX	NLX1; NLX2; NLX3; NLX4
O	30 (X)	L	OLX	OLX1; OLX2; OLX3; OLX4
P	30 (X)	L	PLX	PLX1; PLX2; PLX3; PLX4

Detection of Biofilm Production

The selected isolated colonies were tested for biofilm production by using Tube Method, Congo Red Agar Method, and Tissue Culture Plate Method. The selected bacterial colonies were subjected to the above-mentioned three experimental methods separately and the triplicates were used for each experiment. Reference strain of positive biofilm producer *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* ATCC 35556, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, and *Staphylococcus epidermidis* ATCC 12228 (non-slime producer) were used as controls.

Tube Method (TM)

The tube method was performed to determine qualitatively the adherence and biofilm-forming ability of the isolates as described by Hassan *et al.* 2011 [9]. The bacterial

isolates along with the controls inoculated in 10 ml of trypticase soy broth and kept overnight at 37 °C. The amount of biofilm formed was scored as 1-weak/none, 2-moderate, and 3 -high/strong comparing with the biofilm-producing and non-biofilm bacterial reference strains. The absence of a film is represented as a negative result.

Congo Red Agar Method (CRA)

Congo Red Agar (CRA) method was also followed to detect biofilm production using the simple qualitative method as described by Hassan *et al.* 2011 [9], CRA medium was prepared by supplementing 37 g/L BHI (Brain Heart Infusion) with sucrose 50 g/L, 10 g/L agar, and 8 g/L Congo red indicator. CRA plates were inoculated with test organisms along with the controls and incubated at 37 °C for 24 hr aerobically. Crusty black colonies with a dry filamentous appearance were recorded as biofilm producers and smooth pink colonies as non-producers.

Tissue Culture Plate (TCP)

The biofilm production was determined quantitatively in the present study using the Tissue Culture Plate method (TCP). 10 ml of Trypticase soy broth with 1 % glucose was inoculated with a loopful of test organism from the fresh overnight culture isolated on nutrient agar. The broth was incubated at 37 °C for 24 hr. The overnight culture was further diluted with a fresh medium. The control organisms were also processed in the same way. Ninety-six-wells flat-bottom tissue culture plates were filled with diluted cultures individually. The only sterile broth was used as a negative control. The culture plates were incubated at 37 °C for 24 hr. After incubation, contents of each well were removed, and wells were washed with phosphate buffer saline. Biofilms remained adherent to the walls and the bottoms of the wells were fixed with sodium acetate and stained with crystal violet. Optical densities (OD) of stained adherent biofilm were measured using ELISA microplate reader at wavelength 490 nm and 630 nm [2, 3, 6, 9].

The OD values were considered as an index of bacteria adhering to the surface and producing biofilm. The interpretation of the results of biofilm production was done according to the criteria given by Hassan *et al.* 2011 [9]. Optical Density Cut-off (OD_c) value was calculated. The OD of the sample higher than the OD_c value was considered positive for biofilm production.

Table 2. Interpretation of results of Tissue Culture Plate Method

*Average OD Value	Biofilm Production
$\leq \text{ODc} / \text{ODc} < \sim \leq 2x \text{ODc}$	Non/weak biofilm producer
$2x \text{ODc} < \sim \leq 4x \text{ODc}$	Moderate biofilm producer
$> 4x \text{ODc}$	Strong biofilm producer

*Optical Density cut-off value (ODc) = Average OD of negative control + 3x Standard Deviation (SD) of the negative control.

Identification of Biofilm Isolates

All the biofilm-producing colonies selected randomly were investigated by using morphological appearance, Gram's staining, and other biochemical tests such as Catalase test, Oxidase test, Coagulase test, and Indole test to identify the bacterial isolates. The bacterial isolates were identified based on the combination of methods used for the preliminary screening.

Results and Discussion

Biofilm production in urinary catheters greatly promotes urinary tract infection which leads to prolonged hospitalization and high mortality rates [4]. However, this problem is still remained unsolved in Sri Lankan hospitals due to a lack of research in this area. Therefore, as a preliminary attempt, the present study focused to evaluate the biofilm production of the catheter-associated bacteria in indwelling urinary catheters collected from both the National Hospital of Sri Lanka and New Delmon Hospital.

Biofilm Producing Bacterial Isolates

Altogether, 44 randomly selected bacterial colonies were tested for biofilm production by three different standard methods such as tube method, Congo red agar method, and tissue culture plate method. The tested methods showed a good correlation with each other and revealed the same results (Table 3). Further, the results were found to be consistent when each experiment was repeated three times. Therefore, high false-positive could be very minimum in the present study.

In the tube method, biofilm production was confirmed by a visible film in the walls of

the tube and those isolates were then interpreted as biofilm producers. The colonies NLX1 & NLX2 isolated from the catheter inserted for 30 days were found to be positive for biofilm production and showed strong biofilm production compared with the positive control confirming that the long-term catheters may have the potential risk for producing biofilm from the catheter-associated bacteria. However, the colonies NLX3 and NLX4 obtained from the catheter inserted for 30 days did not produce biofilm. This result clearly indicates that there may be passive members of the biofilm community while some microbial species have greater potential to produce biofilms in Figure 1.



Figure 1. Tube method with different degrees of biofilm production

In CRA method, crusty black colonies with a dry filamentous appearance were recorded as biofilm producers and smooth pink colonies as non-producers. Among the screened catheter-associated bacteria, colony NLX1 from the catheter inserted for 30 days showed strong biofilm production while colony HM81 from the catheter inserted for 8 days showed mild production and there was no biofilm production showed by the colony

BS21 from the catheter inserted for 2 days in Figure 2. These results clearly indicate that the biofilm production increases with the duration of the catheterization as the same results obtained by the tube method.

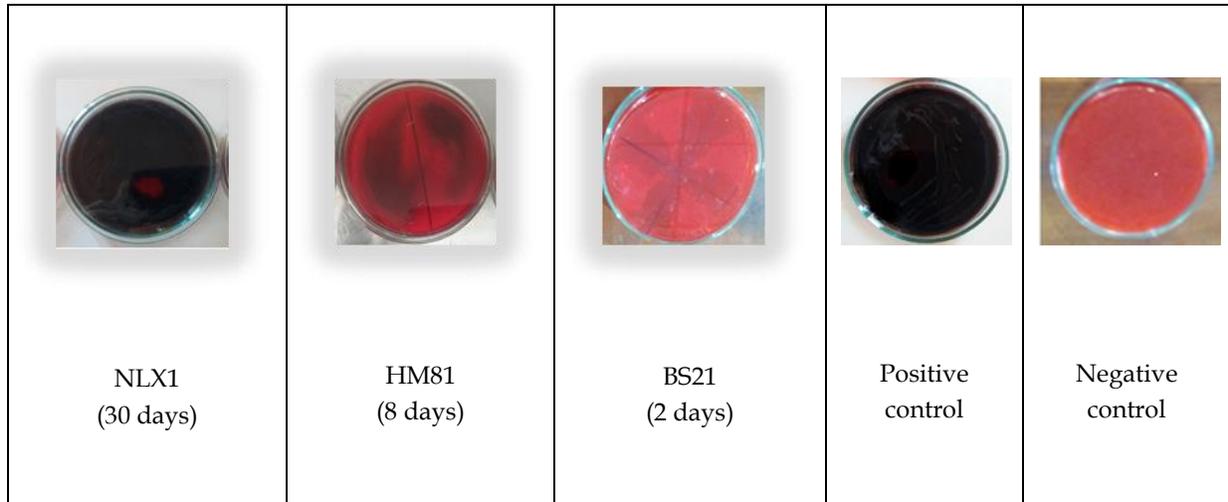


Figure 2. Varying degrees of biofilm production detected in CRA plates

The tissue culture plate (TCP) method is a more quantitative and reliable method with fewer subjective errors for the detection of biofilm-producing microorganisms compared to the other two methods [9]. In this method, the OD values measured by ELISA reader were considered as an index of biofilm-producing bacteria adhering to the surface. The Optical Density Cut of value (ODc value) was calculated for each catheter isolate and then biofilm production was analyzed. The OD value of bacterial isolates MLX1 and MLX2 from the catheter inserted for 30 days was found to be 0.17 and it was 4 times greater than the ODc value of 0.040. Based on the results of the screened bacterial isolates, MLX1 and MLX2 showed as positive for biofilm production, and these bacterial isolates were found to be the strongest biofilm producer compared to the rest of the screened isolates. Whereas the value of bacterial isolates BS21, BS22, BS23, and BS24 extracted from the catheter inserted for 2 days was found to be 0.020 which was lesser than ODc value of 0.037. These isolates are considered as negative for biofilm production in Table 3.

Overall, the present study showed a very good correlation between biofilm production tested by different methods and the duration of catheter usage. The biofilm production was increased with a number of days. The risk of biofilm production of bacterial isolates could be higher in long-term catheters inserted for 30 days. Among the colonies tested

for biofilm production, there was no biofilm production or very weak biofilm production detected in bacterial isolates in the catheters inserted for less than 7 days (Table 3). Especially, there were no significant bacterial growth detected in the bacterial isolates in the agar medium from the two short-term catheters which had been inserted for 2 days into the patient's bladder indicating that catheter usage for lesser number of days could minimize the bacterial growth in Table 3. However, there were no bacteria in the catheters inserted for 2 days. Further, weak biofilm production was detected in the isolates from short term catheter inserted for 7 days and the mid-term catheter inserted for 8 days in Table 3.

All these results obtained through the present study are supported by different studies carried out by the researchers [10]. When the duration exceeds more than 7 days, free single-cell planktonic bacteria are getting adopted to persist in the catheter environment, as well as start to form a multicellular bacterial community for their long-term survival [11, 12]. Among the screened colonies, biofilm production was detected in most of the bacterial isolates extracted from long-term catheters inserted for 30 days into the patient's bladder which revealed that catheterization for a longer period is a likely contributory factor for biofilm production in Table 3.

Identification of Bacterial Isolates

Moreover, catheter-associated bacterial isolates were able to identify in the preliminary screening based on the specific biochemical methods used for the present study together with the Gram staining. The Biochemical test results of a few of the isolated colonies were shown in Table 4.

Mostly, gram-negative rod-shaped bacteria were identified in the short term, midterm, and long-term catheters. These isolates were confirmed as *E. coli* by indole test and negative results for oxidase test which are widely used methods for differentiating *E. coli* with *Pseudomonas aeruginosa*. Next to the *E. coli*, gram-positive cocci forms were identified in long-term catheters. They were confirmed as *Staphylococcus sp.* as it showed positive results for the catalase test which is the commonly used method to differentiate *Staphylococci* with gram-positive *Streptococci*. Moreover, the cocci were confirmed as *Staphylococcus aureus* as it showed positive results for the coagulase test in Table 4.

Table 3. Detection of Biofilm production by selected bacterial isolates using three different standard methods

Catheter information		Bacterial colonies	Biofilm detection method				
Days	Duration		Tube Method	Congo Red Method	Tissue Culture Plate	Avg OD	Stand Error
2	S	NSG					
2	S	NSG					
2	S	BS21; BS22; BS23; BS24	Negative	Negative	Negative	0.020	0.118
5	S	CS51; CS52; CS53; CS54	Negative	Negative	Negative	0.033	0.116
5	S	DS51; DS52; DS53; DS54	Negative	Negative	Negative	0.033	0.007
6	S	ES61; ES62; ES63; ES64	Negative	Negative	Negative	0.020	0.116
7	S	FS71; FS72; FS73; FS74	Negative	Negative	Negative	0.033	0.008
7	S	GS71; GS72; GS73; GS74	Positive +	Positive +	Positive +	0.049	0.0003
8	M	HM81; HM82; HM83; HM84	Positive +	Positive +	Positive +	0.048	0.0003
30	L	MLX1; MLX2;	Positive +++	Positive +++	Positive +++	0.17	0.004
		MLX3; MLX4	Negative	Negative	Negative	0.030	0.007
30	L	NLX1; NLX2;	Positive +++	Positive +++	Positive +++	0.16	0.003
		NLX3; NLX4	Negative	Negative	Negative	0.030	0.008
30	L	OLX1; OLX2; OLX3; OLX4	Positive +++	Positive +++	Positive +++	0.16	0.003
30	L	PLX1; PLX2; PLX3; PLX4	Negative	Negative	Negative	0.021	0.012

Short term : S; Mid term : M; Long term : L; No Significant Bacterial Growth : NSG ; Weak biofilm production: + ; Moderate biofilm production: ++; Strong biofilm production: + + +, Average Optical Density: Avg OD

Table 4. Morphological and Biochemical characters of few isolated bacterial colonies

Bacterial colonies	Morphological tests		Biochemical tests				Identified bacteria
	Gram Stain	Shape	Catalase test	Oxidase test	Coagulase test	Indole test	
BS21	Negative	Short rods	-	-	-	+	<i>E. coli</i>
CS51	Negative	Short rods	-	-	-	+	<i>E. coli</i>
DS51	Negative	Short rods	-	-	-	+	<i>E. coli</i>
ES61	Negative	Short rods	-	-	-	+	<i>E. coli</i>
FS71	Negative	Short rods	-	-	-	+	<i>E. coli</i>
GS71	Negative	Short rods	-	-	-	+	<i>E. coli</i>
HM81	Negative	Short rods	-	-	-	+	<i>E. coli</i>
MLX1	Negative	Short rods	-	-	-	+	<i>E. coli</i>
MLX3	Positive	cocci	+	-	+	-	<i>S. aureus</i>
MLX2	Negative	Short rods	-	-	-	+	<i>E. coli</i>
NLX1	Negative	Short rods	-	-	-	+	<i>E. coli</i>
NLX3	Positive	cocci	+	-	+	-	<i>S. aureus</i>
OLX1	Negative	Short rods	-	-	-	+	<i>E. coli</i>
NLX4	Positive	cocci	+	-	+	-	<i>S. aureus</i>
PLX1	Negative	Short rods	-	-	-	+	<i>E. coli</i>

+ Positive result - Negative result

According to the results, the main pathogen found in the present study was *E. coli* (Table 5 & 6) and it was found to be the highest in number than the other species detected in the present study which was identified as *Staphylococcus aureus*. The study reported by Alves [4] and Sayal [10] also revealed similar results as *E. coli* was the most isolated pathogen in urinary catheters. The reason could be that *E. coli* is one of the major biofilm producers and it has been recorded as a causative organism in most serious nosocomial infections worldwide [4, 13].

Table 5. Identified biofilm-producing bacteria in short and mid-term Catheters

Catheter information		Bacterial colonies	Identified bacteria	Biofilm Production
Days	Duration			
2	S	NSG	-	-
2	S	NSG	-	-
2	S	BS21; BS22; BS23; BS24	<i>E.coli</i>	Negative
5	S	CS51; CS52; CS53; CS54	<i>E.coli</i>	Negative
5	S	DS51; DS52; DS53; DS54	<i>E.coli</i>	Negative
6	S	ES61; ES62; ES63; ES64	<i>E.coli</i>	Negative
7	S	FS71; FS72; FS73; FS74	<i>E.coli</i>	Negative
7	S	GS71; GS72; GS73; GS74	<i>E.coli</i>	Positive
8	M	HM81; HM82; HM83; HM84	<i>E.coli</i>	Positive

Table 6. Identified biofilm producing bacteria in long term catheters

Catheter information		Bacterial colonies	Identified bacteria	Biofilm Production
Days	Duration			
30	L	MLX1; MLX2;	<i>E.coli</i>	Positive
		MLX3; MLX4	<i>S.aureus</i>	Negative
30	L	NLX1; NLX2;	<i>E.coli</i>	Positive
		NLX3; NLX4	<i>S.aureus</i>	Negative
30	L	OLX1; OLX2; OLX3; OLX4	<i>E.coli</i>	Positive
30	L	PLX1; PLX2; PLX3; PLX4	<i>E.coli</i>	Negative

Furthermore, the identification of bacterial isolates in relation to the duration of catheter usage is shown in below figure 3. Among the screened colonies, *E. coli* was found in 24 colonies isolated from catheters used for ≤ 7 days as well as 20 colonies isolated from catheters used for > 7 days. *E. coli* was the most common bacteria found among the screened colonies isolated from both short-term and long-term catheters. Interestingly,

S. aureus was identified in the colonies isolated from catheters used for > 7 days only.

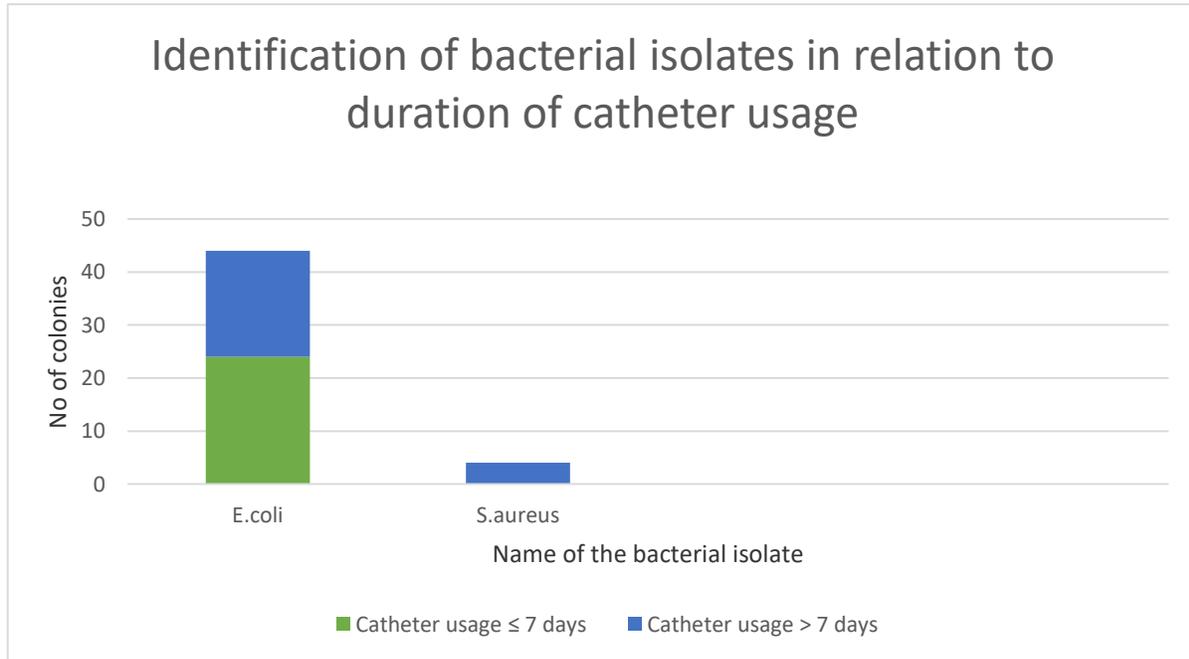


Figure 3. Identification of bacterial isolates in relation to the duration of catheter usage

Biofilm Producing Bacteria

Biofilm production of isolates in catheters used for ≤ 7 days and >7 days are shown in figure 4 and figure 5 respectively. Among the 44 colonies screened, 83% of colonies that were isolated from catheters used for ≤ 7 days were detected as negative for biofilm production. Whereas 60% of colonies isolated from the catheters inserted for > 7 days were detected as positive for biofilm production.

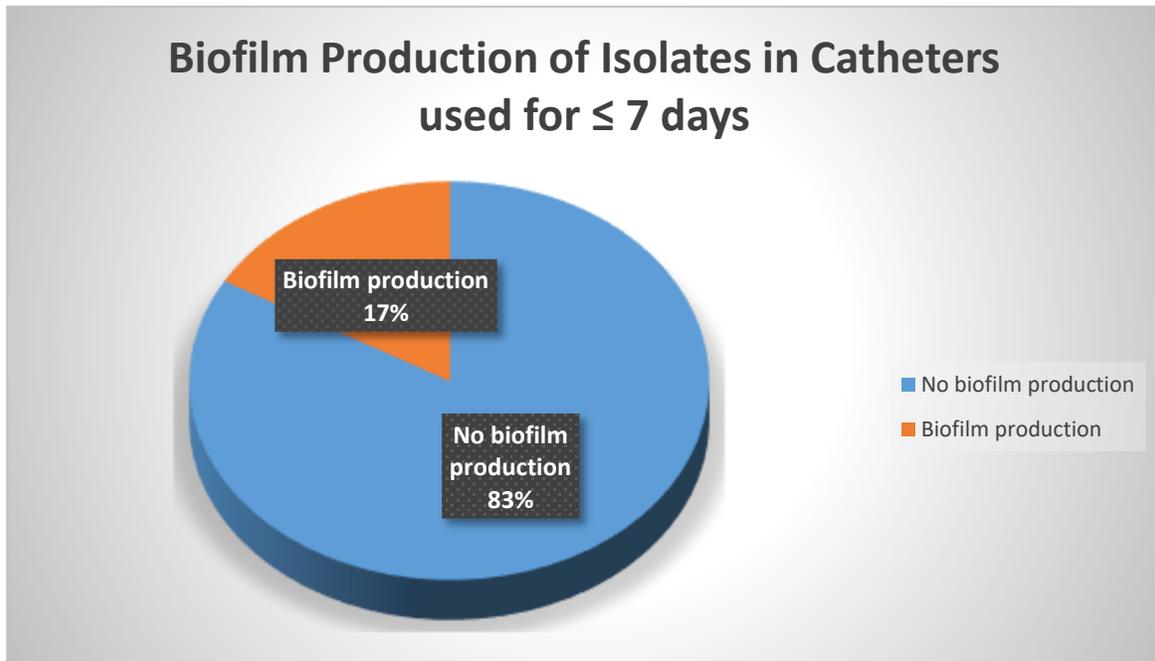


Figure 4. Biofilm production of isolates in catheters used for ≤ 7 days

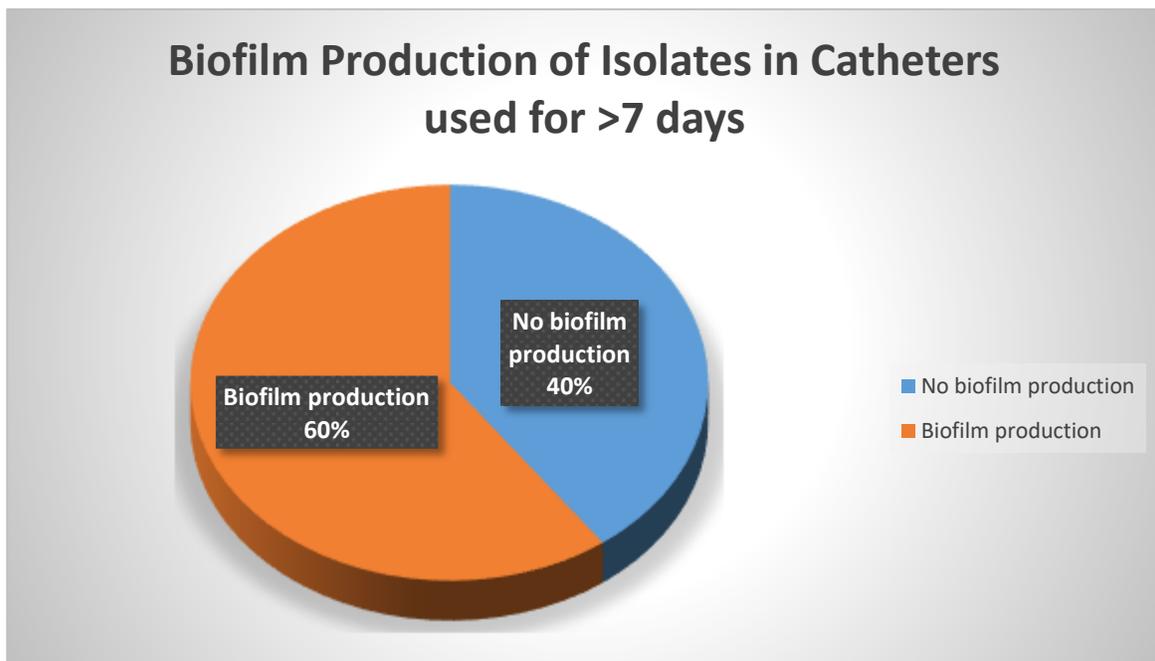


Figure 5. Biofilm production of isolates in catheters used for >7 days

Analysis of Bacterial Isolates in Catheters

Based on the results obtained in the present study, a comparison was done between the bacterial isolates identified from the long-term and short-term catheters. Bacterial isolates extracted from the short-term catheters were found to be *E.coli* and mostly those were non-biofilm producers (Table 5). However, bacterial isolates extracted from the long term were mostly biofilm producers. Among the isolates identified as *E. coli*, the 67% of the isolates were found to be biofilm producers extracted from long-term catheters (Table 6). It indicates that the tendency of biofilm production increases with the duration of catheterization. In long-term catheters, *Staphylococcus aureus* was also identified along with *E. coli* indicating the development of a diverse bacterial population in long-term catheters. Even though *Staphylococcus aureus* was identified in long-term catheters, none of them were biofilm producers. The reason could be that some microbial species have greater potential to produce biofilms such as *E. coli*, whereas others can be only passive members of the biofilm community [14] such as *Staphylococcus aureus*. Though *Staphylococcus aureus* is identified as a strong biofilm producer in most of the studies, some mutant strains do not have the ability to form biofilms. Methicillin resistant *S. aureus* is capable of biofilm production, whereas methicillin-susceptible strains are impaired in biofilm production [15, 16]. However, the test results need to be tested further using molecular techniques at the gene level to identify the strain.

Moreover, the present study clearly indicates that the survival and diverse biofilm-producing bacteria in long-term urinary catheters may be the potential risk for chronic bacterial infections in long-term catheterized patients [17].

Conclusion

In summary, the current data revealed that *E. coli* is the most prevalent bacteria in both short-term and long-term catheters based on biochemical identification. Further, those bacteria were confirmed as biofilm producers mainly detected in long-term catheters. However, isolates identified as *Staphylococcus aureus* in the long-term catheters were found to be non-biofilm producers. Further, the present study concludes that the amount of biofilm production increases in the bacteria isolated from long-term catheters than the short-term catheters.

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Conflicts of Interest

The authors declare no conflict of interest.

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