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Isolation and Biofilm Forming Ability of Bacteria Attached to Urinary Catheter Undergoing Long-Term Bladder Catheterization

Khansa M. Younis
Department of Biology / College of Science /University of Mosul

E-mail: khansaa_altaee@yahoo.com

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ABSTRACT

Catheter associated urinary tract infections are one of the most common nosocomial infections. This study aims to isolate, identify and biofilm forming ability of bacteria attached to urinary catheters particularly catheters that had been in place for prolonged periods in preventing catheter-associated infections caused by hospitalized ICU patients. Urinary catheters from (Clinical center of University Kebangsaan Malaysia) were used, one from Acute Vascular Rejection patient (Urinary catheter B), and two from Benign Prostate Hyperplasia patient (Urinary Catheter C and D).The urinary catheters and urine evaluated quantitatively and qualitatively used colony-forming unit and bacteriological assessment, respectively for each portions of urinary catheter for identifying infections. Bacterial population on agar plates showed varying density for all parts of three urinary catheters and urine samples. Altogether 100 isolates including 96 bacterial isolates and 4 yeast isolates have been successfully obtained from the three urinary catheters. Pathogens were Proteus 22 isolates followed by *E.coli* 5 isolates, Enterobacter 4 isolates, Klebsiella 3 isolates, Pseudomonas 1 isolates For UC(B), while in UC(C and D) which represent the catheters to same diseases were Pseudomonas 23 isolates showed the highest isolates followed by *E.coli* 16 isolate, Proteus 10 isolates, Enterobacter 7 isolates, Klebsiella 4 isolates, Candida 4 isolates, Bacillus 1 isolate. Results of biofilm forming on microtiter plate and sterile Foley catheter revealed that these isolates possess a high capacity for biofilm formation on the both surfaces at different degrees with delay in the initiation of biofilm formation on silicone coated Foley catheter compared to polystyrene microtiter plate. It is concluded that Gram-negative bacilli were responsible for UTI infections in our patients and almost all of the isolates have the abilities to form visible growing biofilms on both surfaces.

Keywords: Urinary catheter infection, Urinary tract infection, Biofilm formation, Bacterial quantification.

عزل وقدرة تكوين الغشاء الحيوي للبكتريا الملتصقة على القسطرة البولية المستخدمة على المدى الطويل

الملخص

تعد عدوى المسالك البولية المرتبطة بالقثطرة واحدة من أكثر حالات عدوى المستشفيات شيوعًا. تهدف هذه الدراسة الى عزل وتشخيص البكتريا الملتصقة على القسطرة البولية المستخدمة لفترة طويلة ودراسة قدرة تكوينها للغشاء الحيوي للوقاية من العدوى المرتبطة بالقثطرة للمرضى الراقدين في وحدة العناية المركزة في المستشفيات. تم استخدام ثلاث نماذج من انابيب القثطرة البولية من (المركز الصحي للجامعة الوطنية الماليزية في ماليزيا) ، أخذ النموذج الاول من مريض مصاب برفض الأوعية الدموية الحاد (قثطرة بولية B) ، واثنان آخران من مرضى تضخم البروستات الحميد (القثطرة البولية C وD). تم تقدير وتشخيص الخلايا البكتيرية المسببة

للتلوث كميا ونوعيا لكل جزء من ألاجزاء الاربعة لانبوب القثطرة البولية باستخدام الفحوصات البكتريولوجية. اظهرت الاوساط الزرعية تفاوت في اعداد البكتيريا لانابيب القثطرة البولية وعينات الادرار. تم الحصول على 100 عزلة من بينها 96 عزلة بكتيرية و 4 عزلات خميرة من النماذج الثلاث للقثطرة البولية. تضمنت العزلات من انبوب القثطرة B ،22 عزلة تابعة لجنس Proteus ، 5 عزلات *E.coli* ، 4 عزلات Enterobacter ، 3 عزلات Klebsiella وعزلة Pseudomonas 1، بينما العزلات من انبوبي القثطرة C و D والتي تمثل القثطرة لنفس المرض كانت 23 عزلة Pseudomonas ومثلت اعلى العزلات تليها 16 عزلة *E.coli* ، 10 عزلة Proteus ، 7 عزلات Enterobacter، 4 عزلات Klibsiella، 4 عزلات Candida ، عزلة Bacillus 1. بينت نتائج اختبار القدرة على تكوين الغشاء الحيوي على انبوب القثطرة البولي المعقم Foley urinary catheter واطباق 96-microtiter plates (MTP) ان معظم العزلات تمتلك قدرة عالية على تكوين الغشاء الحيوي على السطحين مع تاخر البدء بتشكيل الغشاء الحيوي على سطح انبوب القثطرة المغلف بالسليكون مقارنة باطباق MTP . استنتجت الدراسة أن العصيات سالبة الكرام كانت مسؤولة عن التهابات المسالك البولية المرتبطة بالقثطرة البولية وان معظم العزلات لديها القدرة على تشكيل الأغشية الحيوية على كلا السطحين.

INTRODUCTION

In the early stages of catheterization, infection is usually by single species of bacteria, such as *Staphylococcus epidermidis*, *Enterococcus faecalis* or *Escherichia coli* (Clayton and Stickler, 1982; Ganderton *et al*., 1992). The longer the catheter remains in place the greater the variety of organisms that accumulate in the bladder. Long-term patients commonly become infected with mixed genera of mainly Gram-negative nosocomial species, such as *Providencia stuartii*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumoniae.* In patients with permanent or long-term catheterization, the management schemes generally involve the replacement of catheters at 10–12-week periods. Infected urine can, therefore, be flowing through catheters for periods of up to 3 months in this large group. Under these conditions, bacteria colonize the catheters, particularly the lumenal surfaces, and form extensive biofilms (Sahm *et al*., 2001; Barford *et al*., 2008) for example, found that these biofilms could colonize the whole length of the catheter lumen and be up to 400 cells deep, an integral part in a polysaccharide gel-like matrix. The biofilm mode of growth is survival strategy for bacteria and enables them to tolerate a variety of environmental stresses (Costerton *et al*., 1987; Matsukawa *et al*., 2005). The colonization of catheters by these biofilms thus makes effective treatment of the infections difficult. Prevention of these infections has also proved to be problematic. Many attempts have been made to employ biocides in the management of catheters to try to prevent bacterial access to the catheterized bladder by all possible routes. It has become clear, however, that, in the long term, these strategies are generally ineffective thus facilitate colonization by drug resistant a gram-negative pathogens (Pascual *et al*., 1993; Broomfield *et al*., 2009).The purpose of the present study was to determine the attached bacteria responsible for various catheter related nosocomial infections, study their ability to form the biofilm using different surfaces.

MATERIALS AND METHODS

Patients description:

Urinary catheters (3 samples) from (Clinical center of University Kebangsaan Malaysia), used by hospitalized ICU patients were carefully collected under aseptic conditions, the researcher got the permission from the hospital before sample collection. The catheters were placed individually in a sterile plastic bag and transported immediately to the laboratory for analysis. The samples have been taken from three patients UC(B), UC(C), UC(D). All those three patients were males and their ages in 7^{th} decade, the urinary catheter (B) had been suffering from Acute Vascular Rejection (AVR) while

urinary catheter (C and D) patients were suffering from Benign Prostate Hyperplasia (BPH), those three cases had been connected by urinary catheter approximately continuous (14) days.

2- Bacterial isolation:

The treatment of urinary catheters were performed according to (Djeribi *et al*., 2012), with modification. Briefly each urinary catheter were divided into four parts represented by (Eye-holes catheter(a); Lumen(b); Draining tube(c); Urine collection bag(d) and Urine(e)), after this carefully and aseptically all parts cut into 1cm thick discs (pieces) followed by successive washes of the inner surface with a solution of sodium chloride using a tip of micropipette, washing residues and pieces inoculated on to surface of nutrient agar (NA) and MacConkey agar (MA) and the plates were then incubated at 37◦C for 24 – 48 h. Urine was taken from a catheter bag for each sample with a sterile syringe. Cultures of (urinary catheter parts and urine) were diluted using serial dilution to 3 folds dilution. From the third fold dilution 100µL was spread on prepared medium. The spread plates were incubated overnight at 37^{o} C. The developed colonies were counted in plates and the average number of colonies per three plates was determined. The numbers of total bacteria (CFU) per cm (urinary catheter pieces) or per ml (urine) was determined. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. The bacterial isolates were kept on slant nutrient agar at 4 °C.

Identification of the isolates:

All isolates were identified and determined based on Bergeys manual of determinative bacteriology (Holt *et al*., 2000), this included morphological characteristic and to check the growth pattern, different media including Eosin Methylene Blue, Mannitol Salt Agar and Cetrimide agar were used. For biochemical characteristics, oxidase test, catalase test, IMVIC tests, TSI test, H2S production, urease test were conducted.

Biofilm forming assay:

1- Microtiter Plate Biofilm Forming Assay

All 100 isolates grown in LB supplemented with 20% urea were evaluated for their biofilm forming using 96-well microtiter plate in a method that was previously described by (Djordjevic *et al*., 2002) with a few modifications. Using 10 mL of LB broth supplemented with 20% urea, each isolate was grown at 37°C overnight. Dilution of the cultures was conducted to a 0.5 McFarland standard 1.5 x 10^{7} cells/mL). Later, 200 µL aliquots of these cultures were then moved into wells of a microtiter plate. To serve as controls, some wells only had sterile LB supplemented with 20% urea. Aerobic incubation of the plates was done for 24 h at 37°C. The well culture was aspirated out before the plates were washed three times using 200 µL of 0.9% saline in order to get rid of non-adherent cells. The plates were then dried in an inverted position. Using 200 µL of 1% crystal violet solution in water, each well was stained for 20 min. Aspiration of the unbound crystal violet was done before washing the wells thrice with 200 µL 0.9% saline. Bound dye was released by the addition of 200 µL of 95% ethanol. Quantitative analysis of biofilm production was then performed. From each well, one hundred microliters was transferred to a new microtiter plate and, using a microtiter plate reader, each well's optical density (OD) was measured at 595 nm. This experiment was done in triplicate.

2-Urinary Catheter Biofilm Forming Assay

Urinary catheter biofilm forming assay was done according to (Adetunji and Isola, 2011) with some modification. The Foley catheter was cut into pieces each measuring
1 cm x 1 cm before being immersed in each well of 96-well microtiter plate that contained an LB broth supplemented with 20% urea. Following the steps of crystal violet method described in section (1)**,** the optical densities (OD) were measured. Given all tested strains and negative controls.

RESULTS AND DISCUSSION

In this study, (100) bacterial isolates have been successfully isolated. Bacterial population in (NA, MA) agar plates showed to be varying density in all parts of three urinary catheters and urine samples (Table 1).

In the present study, total isolates were recovered from isolation agar plates and the results of microscopic observation were 100 isolates including 96 bacterial isolates and 4 yeast isolates have been successfully obtained from the three urinary catheters.

Table 1: Population density of bacteria isolated from urinary catheter (B, C, D)

Urinary catheters	**Parts**	**Medium**	
		Nutrient agar	MacConkey agar
(B)	a	1.46×10^3 (CFU/cm)	6.8×10^4 (CFU/cm)
	b	0.96×10^3 (CFU/cm)	5.9×10^4 (CFU/cm)
	c	0.78×10^3 (CFU/cm)	4.3×10^4 (CFU/cm)
	d	0.79×10^3 (CFU/cm)	5.0×10^4(CFU/cm)
	e	0.79×10^3 (CFU/ml)	5.8×10^4 (CFU/ml)
(C)	a	1.48×10^4 (CFU/cm)	1.40×10^3 (CFU/cm)
	b	1.40×10^4 (CFU/cm)	1.30×10^3(CFU/cm)
	c	8.9×10^4 (CFU/cm)	6.2×10^4 (CFU/cm)
	d	9.2×10^4 (CFU/cm)	8.9×10^4 (CFU/cm)
	e	9.0×10^4 (CFU/ml)	8.6×10^4 (CFU/ml)
(D)	a	1.40×10^3 (CFU/cm)	9.6×10^4(CFU/cm)
	b	1.30×10^3 (CFU/cm)	9.0×10^4 (CFU/cm)
	c	1.00×10^3 (CFU/cm)	9.2×10^4 (CFU/cm)
	d	1.20×10^3 (CFU/cm)	9.5×10^4 (CFU/cm)
	e	1.00×10^3 (CFU/ml)	8.3×10^4 (CFU/ml)

(a)Eye-holes catheter; (b) Lumen; (c) Draining tube; (d) Urine collection bag; (e) Urine.

Table 2: Microorganisms isolated from urinary catheters and urine samples

Urinary Catheter	Isolates	No.of isolate Catheter	Urine	The total No. of isolates
UC(B)	*Proteus* spp.	20	2	22
	E.coli	3	2	5
	Enterobacter spp.	4	0	4
	Klebsiella spp.	3	0	3
	Pseudomonas spp.	1	0	1
UC(C&D)	*Pseudomonas* spp.	22	1	23
	E.coli	16	0	16
	Proteus spp.	7	3	10
	Enterobacter spp.	7	0	7
	Klebsiella spp.	4	0	4
	Candida	0	4	4
	Bacillus spp.	1	0	1

Gram's staining and biochemical tests were confirmed as 95% of them were gram negative, the most common isolated pathogens were *Proteus* spp. 22 isolates (63%) followed by *E.coli* 5 isolates (14%), *Enterobacter* spp. 4 isolates (11%), *Klebsiella* spp. 3 isolates (9%), *Pseudomonas* spp. 1 isolate (3%) for UC (B), while in UC(C and D) which represent the catheters to same disease were *Pseudomonas* spp. 23 isolates (35%) showed the highest isolates followed by *E.coli* 16 isolates (24%), *Proteus* spp. 10 isolates (15%), *Enterobacter* spp. 7 isolates (12%), *Klibsiella* spp. 4 isolates (6%), Candida 4 isolates (6%), *Bacillus* spp. 1 isolate (2%) (Table 2). These genera isolated from all parts of the urinary catheter clarify that there was no a great difference in the numbers and genera in all regions of the urinary catheter containing (Eye-holes catheter, Lumen, Draining tube, Urine collection bag).

The findings from tests of the development of biofilms utilising microtiter plates and pre-sterilised Foley urinary catheters showed that almost all of the isolates have the abilities to form visible growing biofilms on both surfaces when compared to the controls (fresh broth), though with differences in the degree of adhesions. These data clearly showed the delay in the initiation or onset of biofilm formation on silicone coated Foley catheter compared to polystyrene microtiter plate. This finding may relate to differences in adsorption patterns and adhesion, such as substratum-dependent conformational changes, seem to account for the effect. The reason for this discrepancy may relate to the more hydrophilic surfaces of microtiter plate so that it becomes easier for adherent cells to grow on the surface, otherwise silicone coated latex Foley catheter is made from a hydrophobic material that rejects moisture and the impact of this on delayed biofilm formation (Table 3). The highest percent are of biofilm formation was detected in urinary catheter B which included 63% isolates of *Proteus* spp. while *Pseudomonas* spp. was the most prevalent organism in urinary catheter C and D.

These results are incompatible with (Milan and Ivan, 2009; Savas *et al*., 2006) who found that *E. coli* was the most predominant. Early stages of catheterization, infection is usually by single species of bacteria, such as *Staphylococcus epidermidis*, *Enterococcus faecalis* or *Escherichia coli* (Gillespie *et al*., 1983). The longer the catheter remains in place the greater the variety of organisms that

accumulate in the bladder. Patients commonly become infected with mixed communities of mainly gsram-negative nosocomial species, such as *Ps. aeruginosa, Pr. mirabilis* and *Klebsiella pneumoniae.* These infections are difficult to eliminate with antibiotic therapy while the catheter remains in place (Clayton *et al*., 1982; Drekonja *et al*., 2013). In patients with permanent or long-term catheterization, management plans generally involve replacing catheters at intervals from 10 to 12 weeks. Infected urine can, therefore, be flowing through catheters for periods of up to 3 months in this large group. Under these conditions, bacteria colonize the catheters, particularly the lumenal surfaces, and form extensive biofilms (Ganderton *et al.*, 1992).

Table 3: Screening for biofilm development capability on microtiter plates and sterilised urinary catheter surface areas using microtiter plate reader at 595 nm

No.	Isolates	Microtiter plate	Foly urinary catheter
1	UC(B)a1	1.427 ± 0.070	0.137 ± 0.004
2	UC(B)a2	0.755 ± 0.003	0.166 ± 0.004
3	UC(B)a3	1.566 ± 0.004	0.171 ± 0.001
4	UC(B)a4	1.558 ± 0.002	0.208 ± 0.005
5	UC(B)a5	1.559 ± 0.002	0.171 ± 0.002
6	UC(B)a6	1.163 ± 0.004	0.119 ± 0.001
7	UC(B)a7	1.112 ± 0.004	0.135 ± 0.004
8	UC(B)a8	1.318 ± 0.003	0.185 ± 0.002
9	UC(B)a9	1.172 ± 0.002	0.168 ± 0.001
10	UC(B)b1	0.550 ± 0.001	0.154 ± 0.006
11	UC(B)b2	0.585 ± 0.001	0.162 ± 0.004
12	UC(B)b3	0.430 ± 0.005	0.160 ± 0.001
13	UC(B)b4	1.087 ± 0.000	0.199 ± 0.001
14	UC(B)b5	0.555 ± 0.001	0.064 ± 0.002
15	UC(B)b6	0.808 ± 0.001	0.274 ± 0.003
16	UC(B)c1	1.159 ± 0.004	0.192 ± 0.000
17	UC(B)c2	0.729 ± 0.000	0.119 ± 0.002
18	UC(B)c3	1.001 ± 0.000	0.102 ± 0.004
19	UC(B)c4	1.091 ± 0.009	0.100 ± 0.001
20	UC(B)c5	1.043 ± 0.060	0.182 ± 0.001
21	UC(B)c6	1.136 ± 0.001	0.122 ± 0.001
22	UC(B)c7	1.020 ± 0.005	0.103 ± 0.002
23	UC(B)c8	1.033 ± 0.000	0.104 ± 0.004
24	UC(B)d1	1.054 ± 0.013	0.113 ± 0.001
25	UC(B)d2	1.179 ± 0.139	0.216 ± 0.004
26	UC(B)d3	1.027 ± 0.004	0.196 ± 0.000
27	UC(B)d4	0.430 ± 0.002	0.182 ± 0.004
28	UC(B)d5	0.412 ± 0.004	0.182 ± 0.002
29	UC(B)d6	1.063 ± 0.016	0.103 ± 0.003
30	UC(B)d7	0.592 ± 0.004	0.171 ± 0.001
31	UC(B)d8	0.574 ± 0.006	0.166 ± 0.000
32	UC(B)e1	0.591 ± 0.013	0.197 ± 0.004
33	UC(B)e2	1.094 ± 0.001	0.101 ± 0.002
34	UC(B)e3	1.054 ± 0.003	0.198 ± 0.001
35	UC(B)e4	1.063 ± 0.009	0.214 ± 0.001

36	UC(C)a1	1.417 ± 0.011	0.118 ± 0.001
37	UC(C)a2	1.329 ± 0.003	0.110 ± 0.001
38	UC(C)a3	1.051 ± 0.064	0.106 ± 0.003
39	UC(C)a4	0.670 ± 0.001	0.173 ± 0.004
40	UC(C)a5	0.585 ± 0.001	0.180 ± 0.003
41	UC(C)a6	0.708 ± 0.003	0.161 ± 0.001
42	UC(C)a7	0.666 ± 0.001	0.068 ± 0.006
43	UC(C)a8	0.787 ± 0.001	0.161 ± 0.002
44	UC(C)a9	0.512 ± 0.010	0.157 ± 0.003
45	UC(C)b1	1.075 ± 0.006	0.199 ± 0.001
46	UC(C)b2	1.044 ± 0.017	0.102 ± 0.001
47	UC(C)b3	0.489 ± 0.005	0.161 ± 0.002
48	UC(C)b4	1.159 ± 0.025	0.112 ± 0.001
49	UC(C)b5	0.975 ± 0.010	0.105 ± 0.001
50	UC(C)b6	0.556 ± 0.006	0.196 ± 0.006
51	UC(C)b7	0.288 ± 0.004	0.157 ± 0.001
52	UC(C)b8	0.263 ± 0.002	0.257 ± 0.000
53	UC(C)c1	0.307 ± 0.001	0.157 ± 0.002
54	UC(C)c2	0.604 ± 0.014	0.161 ± 0.001
55	UC(C)c3	0.594 ± 0.018	0.173 ± 0.001
56	UC(C)c4	0.387 ± 0.002	0.051 ± 0.001
57	UC(C)c5	0.442 ± 0.007	0.153 ± 0.005
58	UC(C)c6	1.081 ± 0.012	0.200 ± 0.001
59	UC(C)c7	0.413 ± 0.000	0.207 ± 0.005
60	UC(C)c8	1.095 ± 0.060	0.102 ± 0.004
61	UC(C)d1	1.050 ± 0.007	0.191 ± 0.002
62	UC(C)d2	0.303 ± 0.000	0.161 ± 0.004
63	UC(C)d3	0.248 ± 0.001	0.162 ± 0.004
64	UC(C)d4	1.033 ± 0.008	0.103 ± 0.001
65	UC(C)d5	0.944 ± 0.006	0.185 ± 0.004
66	UC(C)d6	1.011 ± 0.001	0.193 ± 0.000
67	UC(C)d7	0.862 ± 0.069	0.185 ± 0.007
68	UC(C)d8	0.738 ± 0.004	0.260 ± 0.001
69	UC(C)e1	1.279 ± 0.008	0.107 ± 0.002
70	UC(C)e2	1.480 ± 0.006	0.121 ± 0.003
71	UC(C)e3	1.288 ± 0.023	0.115 ± 0.004
72	UC(C)e4	1.429 ± 0.023	0.119 ± 0.001

73	UC(C)e5	1.058 ± 0.003	0.199 ± 0.001
74	UC(D)a1	0.579 ± 0.001	0.156 ± 0.004
75	UC(D)a2	0.557 ± 0.000	0.261 ± 0.001
76	UC(D)a3	0.959 ± 0.001	0.160 ± 0.001
77	UC(D)a4	0.867 ± 0.005	0.161 ± 0.004
78	UC(D)a5	0.627 ± 0.002	0.151 ± 0.001
79	UC(D)a6	1.112 ± 0.013	0.199 ± 0.001
80	UC(D)a7	0.613 ± 0.007	0.249 ± 0.001
81	UC(D)b1	1.290 ± 0.000	0.111 ± 0.003
82	UC(D)b2	1.017 ± 0.000	0.100 ± 0.001
83	UC(D)b3	0.608 ± 0.001	0.164 ± 0.002
84	UC(D)b4	0.645 ± 0.002	0.249 ± 0.001
85	UC(D)b5	1.013 ± 0.063	0.107 ± 0.004
86	UC(D)b6	0.860 ± 0.016	0.159 ± 0.001
87	UC(D)c1	0.943 ± 0.004	0.166 ± 0.005
88	UC(D)c2	0.869 ± 0.013	0.167 ± 0.004
89	UC(D)c3	0.909 ± 0.008	0.167 ± 0.009
90	UC(D)c4	1.032 ± 0.654	0.155 ± 0.004
91	UC(D)d1	0.982 ± 0.053	0.108 ± 0.006
92	UC(D)d2	0.598 ± 0.057	0.169 ± 0.001
93	UC(D)d3	0.953 ± 0.008	0.195 ± 0.003
94	UC(D)d4	1.202 ± 0.092	0.106 ± 0.004
95	UC(D)d5	1.411 ± 0.003	0.119 ± 0.000
96	UC(D)d6	0.547 ± 0.067	0.151 ± 0.001
97	UC(D)d7	1.017 ± 0.016	0.099 ± 0.001
98	UC(D)d8	1.415 ± 0.006	0.119 ± 0.002
99	UC(D)e1	1.379 ± 0.071	0.120 ± 0.008
100	UC(D)e2	1.322 ± 0.066	0.125 ± 0.003
101	Control	0.007± 0.002	0.005± 0.065

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