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Activities of bacteriocin from *Pseudomonas putida* strain FStm2 against biofilm-forming bacteria isolated from urinary catheter

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Aims: The aim of the present study is to investigate the effect of bacteriocin from *Pseudomonas putida* FStm2 on biofilm-forming bacteria isolated from urinary catheter.
Methodology and results: In this study, 25 bacteria isolates were successfully isolated from urinary catheter. Sixteen of the isolates were urease positive. Results of crystal violet test showed that 8 isolates were active biofilm formers in microtiter plate. Seven isolates were active biofilm formers in urinary catheter filled with nutrient broth (NB) and 9 isolates formed biofilm in catheters filled with artificial urine. Bacteriocin-producing *Pseudomonas putida* strain FStm2 was isolated from shark skin. The antibacterial spectrum of the bacteriocin was determined using the well diffusion method and activity was evident against three *Burkholderia cepacia* isolates and one *Staphylococcus hominis* isolate from urinary catheter.
Conclusion, significance and impact study: Bacteriocin from *P. putida* FStm2 has good antimicrobial activity against *B. cepacia* and *S. hominis*. It can be a good candidate as anti-biofilm in combating urinary catheter infection. Further studies such as the mechanism of action of the bacteriocin against both bacterial species should be studied in detail if it is to be developed as a bacteriocide.

Keywords: Bacteriocin, biofilm, urinary catheter, *Pseudomonas putida*, antibacterial

INTRODUCTION

Urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilms on the inner or outer surfaces (Al-Mathkhury *et al.,,* 2011). According to Stickler and Zimakoff (1994), a common complication in the care of patients undergoing long-term indwelling bladder catheterization is encrustation and blockage of the catheter. The problem stems from infection by urease-producing bacteria that colonize the catheter forming extensive biofilms (Robert *et al.,,* 2009). These bacteria generate ammonia from urea, elevate the pH of urine and cause crystals of calcium and magnesium phosphate to form in the urine and the biofilm then develops on the catheter. In the latter case, painful distension of the bladder and reflux of infected urine to the kidneys can culminate in episodes of pyelonephritis, septicaemia and endotoxic shock. All available types of indwelling catheters are vulnerable to this problem (Morris *et al.,,* 1997).

Biofilm bacteria are known to be highly drug resistant and therefore very difficult to eradicate (Nickel *et al.,,* 1985 and Costerton *et al.,,* 1999). According to Stickler (2008), all types of indwelling urinary catheters, including silver-coated and those impregnated with antibiotics, can be colonized by biofilm-forming bacteria and this is an essential pathogenic feature of catheter infections. Because bacterial biofilms are inherently resistant to many antibiotics and widespread use of antibiotics contributes to resistance development, there is a need for new approaches to biofilm eradication (Jacob *et al.,,* 2009).

A new class of antimicrobial substances, termed 'natural antibiotic', may be an alternative for combating antibiotic resistance. A widely studied group of these natural antibiotics is antimicrobial peptides. In this study we have studied bacteriocin, a group of natural antimicrobial peptides or proteins with interesting potential applications as biofilm inhibitors in medical applications. The study focused on the activities of bacteriocin from *P. putida* FStm2 against biofilm-producing bacteria isolated from urinary catheter.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

Urinary catheters used in surgeries at the Universiti Kebangsaan Malaysia Medical Center were carefully collected under aseptic conditions. The catheters were transferred into sterile plastic bags places in ice and transported to the laboratory. In addition to the samples

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collected, patient details including demographic and clinical information were obtained. The period of catheter indwelling was also recorded. After withdrawal, four parts of the urinary catheter (drainage tube, urine collection bag, catheter eye-holes and lumen) were cut into pieces of 1 cm in length, flushed with 1 mL of phosphate buffered saline (PBS), placed into 5 mL Nutrient Broth (NB) and incubated at 37 °C for 24 h. Serial dilutions were made using sterile dH_2O and 0.1 mL of the sample from each dilution was plated onto nutrient agar (NA), MacConkey agar (MC), thiosulfate citrate bile salts sucrose agar (TCBS) and modified Rimler-Shott agar (mRS). The plates were incubated at 37 °C for 24 to 48 h or until colonies showed. Also, urine was collected through the urine collection bag of the urinary catheter using aseptic methods. The urine sample was centrifuged at 10 000 x*g* for 10 min at 4 °C. The pellet was resuspended in 1 mL PBS and 0.1 mL was plated onto NA, MC, TCBS and mRS. The plates were incubated at 37 °C for 24 to 48 h or until colonies showed.

Urease activity

Isolates were inoculated onto urea agar slant. The tubes were incubated at 37 °C for 24 to 48 h. After incubation the slants were observed for production of reddish pink colour indicating positive urease activity.

Microtiter plate biofilm production assay

Biofilm production by all isolates grown in NB was determined using 96-well microtiter plate as described previously by Djordjevic *et al.,* (2002), with modifications. Each isolate was grown in 10 mL of NB at 37 °C overnight. The cultures were diluted to a 0.5 McFarland standard (~10^7 cells/mL), and then 200 µL aliquots of these cultures were transferred into wells of a microtiter plate. Each isolate was tested in duplicate. Wells containing only sterile NB served as controls. The plates were incubated aerobically for 24 h at 37 °C. The culture in the well was aspirated out and plates were washed three times with 200 µL of 0.9% saline to remove non adherent cells and plates were dried in an inverted position. Each well was stained with 200 µL of 1% crystal violet solution in water for 20 min. Unbound crystal violet was aspirated out and the wells were washed three times with 200 µL 0.9% saline. Quantitative analysis of biofilm production was performed by adding 200 µL of 95% ethanol to release bound dye. One hundred microliters from each well was transferred to a new microtiter plate and the optical density (OD) of each well was measured at 595 nm using a microtiter plate reader.

Urinary catheter biofilm production assay

Biofilm formation on urinary catheter was investigated using the methods of Victoria and Tajudeen (2011) and Al-Mathkhury *et al.,,* (2011) with some modifications. Foley catheter was cut into 1 cm pieces and placed in 20 mL of NB and artificial urine containing test cultures to a final cell density of 1.5 x 10^8 CFU/mL. The artificial urine used in the experimental work was based on that formulated by Griffith *et al.,* (1976). Samples were incubated at 37 °C for 24 hours. After incubation the catheter pieces were washed twice with ddH_2O. Each piece was stained with 200 µL of 1% crystal violet solution in water for 20 min. The crystal violet was redissolved with ethanol and the absorbance at 620 nm was determined spectrophotometrically. Foley catheter pieces placed in sterile NB and artificial urine were treated as blanks.

Purification of bacteriocin

Bacteriocin-producing *Pseudomonas putida* strain FStm2 was isolated from the skin of the marine shark *Chiloscyllium griseum*. Protein extraction was conducted on cell-free culture supernatant of *P. putida* obtained after centrifugation of overnight culture for 20 min at 15000 x*g* at 4 °C. The extraction was performed according to Rajaram *et al.,* (2010). Culture supernatant was brought to 80% saturation with solid ammonium sulfate, and after stirring overnight at 4 °C, the precipitate was collected by centrifugation (GSA rotor, 6000 x*g*, 10 min, and 4 °C). The precipitate was dissolved in sodium phosphate buffer (10 mM, pH 7.0), and the suspension containing bacteriocin was desalted by dialyzing through a 2-kDa cut-off dialysis membrane (Sigma) against the same buffer for 24 h. The dialyzed suspension was centrifuged at 12,000 x*g* for 15 min at 4 °C. Further purification was carried out by gel filtration using AKTA Purifier UPC-900 (Superdex-200 column). Eluted fractions in 1 mL aliquots were checked for antimicrobial activity and the active fractions were pooled. The amount of protein was determined by Bradford method using bovine serum albumin (BSA) as standard.

Screening for bacteriocin activity

The antibacteria activity of the bacteriocin from *P. putida* FStm2 was determined using the well diffusion method. Aliquots of the bacteriocin were placed in 6 mm diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with a clinical bacteria strain isolated from the catheters. After 24 h of incubation, the zones of growth inhibition were observed.

Identification of bacteria

Probable identities of the bacteria isolated from the catheters were determined from 16S rRNA gene sequences. DNA extraction was carried out according to the conventional CTAB method described by Ausubel *et al.,*, (1992). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) a forward primer (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (5' GGT TAC CTT GTT ACG ACT 3') (Edwards *et al.,*, 1989). A final PCR reaction volume of 50 µL contained 1 µL genomic DNA template, 1 x GoTaq Flexi Buffer (Promega, USA), 2 mM $MgCl_2$ (Fermentas, USA), 1 µM of

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each forward and reverse primer, 200 µM each dNTP and 2.5 U Taq polymerase. Amplification was carried out in a Mini-cycler (MJ Research, USA). The PCR protocol used was initial heating at 95 °C for 2 min, annealing at 50 °C for 30 s, and annealing 72 °C for 45 s, followed by 22 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 45 s, with a final extension at 72 °C for 2 min. After the PCR reaction was completed, 10 µL of obtained PCR product was electrophoresed on a 1% Tris-acetate-EDTA agarose gel containing 1:10,000 gel red. A 1kb DNA ladder (Promega, USA) was also included in the run. PCR products were purified using QIAquick Purification Kit (QIAGEN, Germany) according to manufacturer's instructions. Sequencing was carried out by automatic DNA sequencing machine (ABI Prism 377) at FirstBase Laboratory, Sdn Bhd, Malaysia. Phylogenetic tree was generated by means of neighbor relationships using MEGA software version 4.0.2.

RESULTS

In this study, 25 bacterial isolates have been successfully isolated. All the 25 isolates were screened for urease activity, 16 isolates were urease positive (Table 1). Results of crystal violet test showed that 8 isolates

Table 1: Urease activity, biofilm formers and bacteriocin activity.

No. isolated	Urease activity	Biofilm (microtiter plate) (OD_{595})	Biofilm (foley catheter) (OD_{620})		Bacteriocin activity
			NB	Artificial urine	
UCa1	-	1.003 ± 0.005	0.091 ± 0.000	0.106 ± 0.000	-
UCa2	-	1.357 ± 0.380	0.084 ± 0.000	0.111 ± 0.001	-
UCa3	+	1.711 ± 0.023	0.122 ± 0.007	0.146 ± 0.011	+
UCa4	+	1.709 ± 0.057	0.119 ± 0.006	0.149 ± 0.011	-
UCa5	+	1.744 ± 0.004	0.124 ± 0.006	0.140 ± 0.003	+
UCb1	+	1.671 ± 0.099	0.126 ± 0.004	0.133 ± 0.001	+
UCb2	-	0.899 ± 0.538	0.088 ± 0.001	0.099 ± 0.001	-
UCb3	+	1.609 ± 0.177	0.125 ± 0.001	0.111 ± 0.001	-
UCb4	-	1.114 ± 0.410	0.089 ± 0.002	0.100 ± 0.001	-
UCb5	+	0.895 ± 0.311	0.104 ± 0.005	0.105 ± 0.007	-
UCb6	+	1.272 ± 0.007	0.071 ± 0.000	0.110 ± 0.001	-
UCb7	+	1.444 ± 0.001	0.089 ± 0.003	0.120 ± 0.003	-
UCb8	+	1.729 ± 0.006	0.120 ± 0.001	0.149 ± 0.001	+
UCd1	-	0.914 ± 0.371	0.109 ± 0.001	0.088 ± 0.000	-
UCd2	+	1.453 ± 0.151	0.095 ± 0.001	0.080 ± 0.002	-
UCd3	+	1.651 ± 0.008	0.121 ± 0.008	0.134 ± 0.006	-
UCd4	+	1.578 ± 0.003	0.115 ± 0.005	0.133 ± 0.008	-
UCd5	-	1.044 ± 0.260	0.140 ± 0.000	0.102 ± 0.001	-
UCd6	+	1.308 ± 0.008	0.099 ± 0.012	0.106 ± 0.001	-
UCd7	+	1.391 ± 0.002	0.080 ± 0.000	0.098 ± 0.001	-
UCe1	+	1.405 ± 0.002	0.100 ± 0.000	0.100 ± 0.003	-
UCe2	-	1.195 ± 0.349	0.098 ± 0.011	0.102 ± 0.002	-
UCe3	+	0.812 ± 0.006	0.106 ± 0.001	0.126 ± 0.001	-
UCe4	-	0.780 ± 0.051	0.081 ± 0.001	0.092 ± 0.009	-
UCe5	-	1.029 ± 0.380	0.087 ± 0.004	0.102 ± 0.003	-

(UCa3, UCa4, UCa5, UCb1, UCb3, UCb8 UCd3 and UCd4) were active biofilm formers (>1.500 OD595) on microtiter plate (Table 1). Seven isolates (UCa3, UCa5, UCb1, UCb3, UCb8, UCd3 and UCd5) were active biofilm formers (>0.120 OD620) on urinary catheter using NB and 9 isolates (UCa3, UCa4, UCa5, UCb1, UCb7, UCb8 UCd3, UCd4 and UCe3) using artificial urine (Table 1). Artificial urine showed more biofilm formers compare than NB. The lowest optical density reading for NB and artificial urine was observed to be 0.071 and 0.080, respectively. On the other hand, the highest optical density reading of the NB and artificial urine was observed to be 0.140 and 0.149, respectively. The first step in the bacteriocin purification protocol was to concentrate the active compound from the growth medium by ammonium sulfate precipitation. Approximately 152.12 AU/mg was achieved (Table 2). Further the precipitate was subjected to a gel filtration by AKTA Purifier UPC-900 (Superdex-200 column). At this stage of purification, the specific activity increased to 214.64 AU/mg (Table 2).

The bacteriocin from *P. putida* FStm2 produced bacteriocin activity against strains UCa3, UCa5, UCb1 and UCb8 (Table 1 and Figure 1). These four strains were identified as belonging to *Burkholderia cepacia* for UCa3, UCa5 and UCb8 and *Staphylococcus hominis* for UCb1 (Table 3 and Figure 2).

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Table 2: Summary of the purification of bacteriocin from *P. putida* FStm2.

Purification stage	Volume (mL)	Protein (mg/mL)	Activity (AU/mL)	Total activity (AU/mL)	Total protein (mg)	Specific activity (AU/mg)
Culture supernatant	50	0.302	51.5	2575	30.2	85.26
Ammonium sulphate precipitation (80%)	10	0.378	57.5	575	3.78	152.12
Gel filtration	1	0.282	60.1	60.1	0.28	214.64

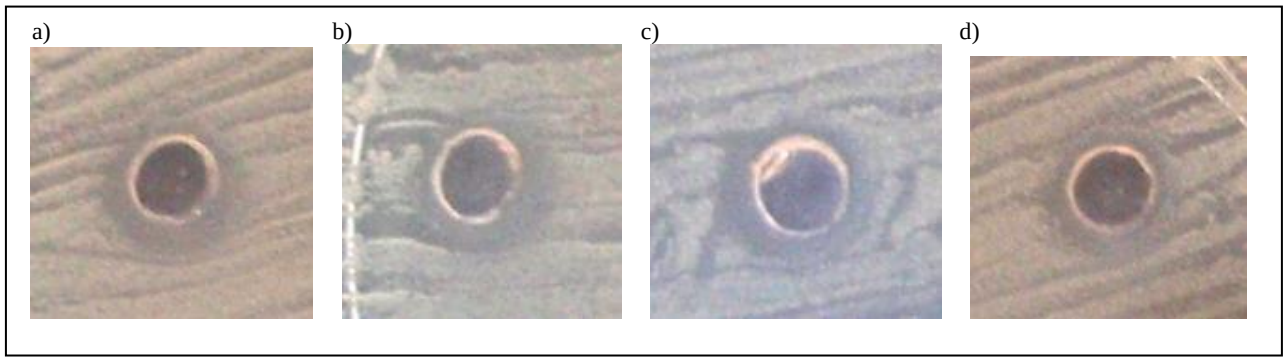

Figure 1: Screening for bacteriocin from *P. putida* FStm2 activity. (a) Bacteriocin against *B. cepacia* strain UCa3. (b) Bacteriocin against *B. cepacia* strain UCa5. (c) Bacteriocin against *S. hominis* strain UCb1. (d) Bacteriocin against *B. cepacia* strain UCb8. Arrow showed inhibition zone.

Table 3: Identification of bacteria.

No. strains	Bacteria	Max Identity (%)
UCa3	*Burkholderia cepacia*	94
UCa5	*Burkholderia cepacia*	93
UCb1	*Staphylococcus hominis*	96
UCb8	*Burkholderia cepacia*	91

DISCUSSIONS

Long-term catheterization in patients can make contaminated urine to flow through catheters for many weeks. Catheters can thus provide conducive sites for bacterial colonization leading to formation if bacterial biofilm. These biofilms can even become visible to naked eyes as coatings on the catheters surfaces. According to Ganderton *et al.,* (1992), biofilms containing 5 x 10^9 viable cells per centimeter can be found on long-term indwelling catheters removed from patients. Ohkawa *et al.,* (1990) for example, found that 21 of 28 catheters removed after 7 to 16 days were colonized by biofilm. These biofilms can form on the outer surface of the catheter around the balloon and catheter tip, and can cause trauma to the bladder and urethral epithelia. Crystals may form in the matrix of bacterial and these crystalline biofilm can have profound effects on the health and quality of life of patients. On deflation of the retention balloon crystalline debris from the biofilm can be shed into the bladder and initiate stone formation. The main complication, however, is blockage in the flow of urine through the catheter that results from the buildup of the crystalline material on the luminal surfaces.

The main sources of infection from contaminated catheter are urease-producing bacteria that form effective biofilms (Robert *et al.,* 2009). In our study 16 of the isolates obtained were urease positive. The bacterial urease generates ammonia from urea, elevating the pH of urine and biofilm (Robert *et al.,* 2009). As the pH of urine rises, crystals of calcium and magnesium phosphates are formed. The pH at which this occurs is known as the nucleation pH (pHn) (Choong *et al.,* 2001). In patients who develop infections from urease-producing bacteria, the pH of the voided urine (pHv) can thus rise above the pHn and crystallization occurs in the urine and the biofilm. The continued development of this crystalline biofilm blocks the flow of urine through the catheter (Morris *et al.,* 1999).

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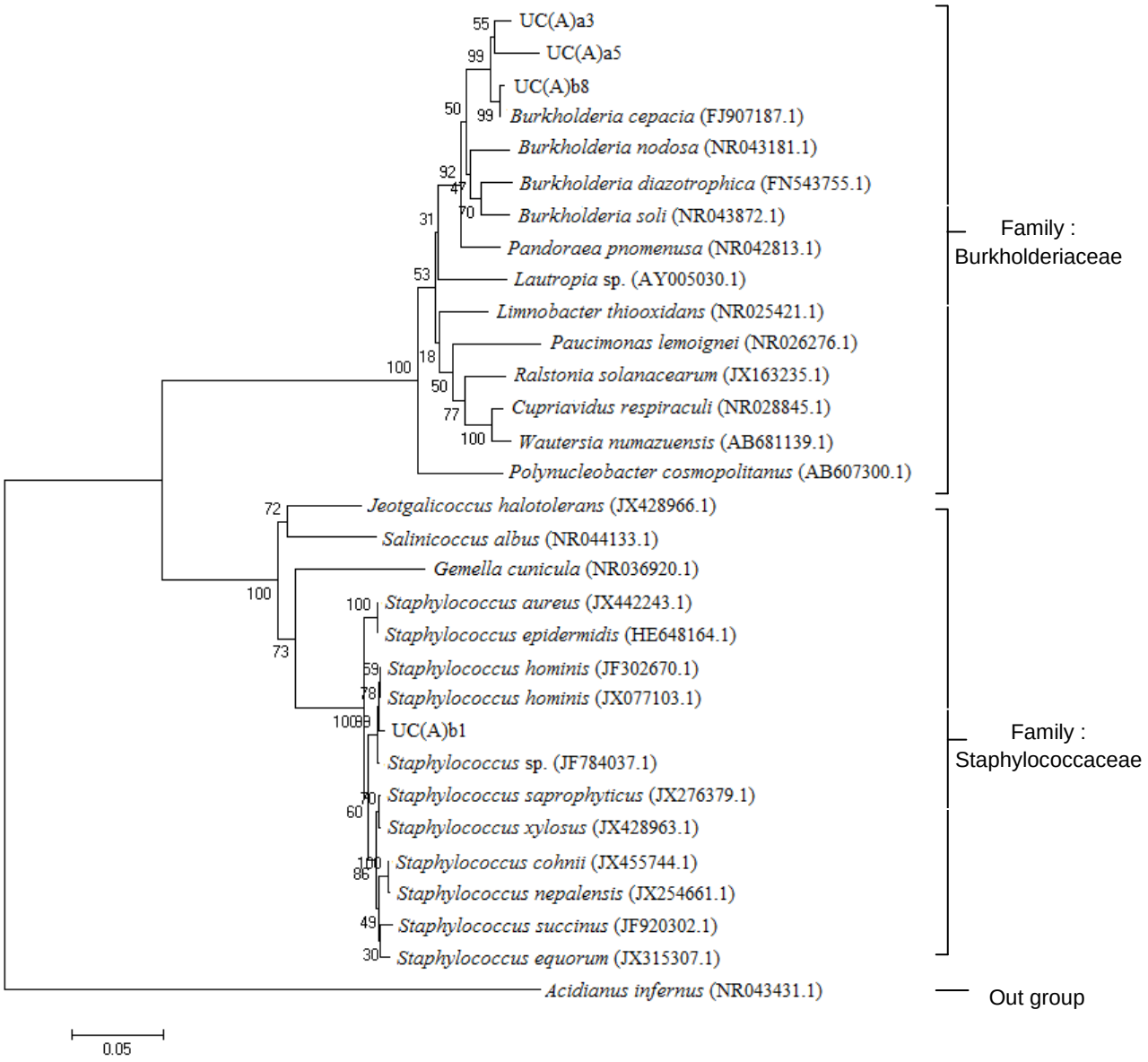

Figure 2: Phylogenetic tree produce from MEGA4 (constructed using the neighbour-joining method) which show the relationship of each culture to its nearest family member.

According to Harjai *et al.,* (2005), Di Bonaventura *et al.,* (2007) and Hoštacká and Čižnár (2007), biofilm production in vitro depends on a number of physical and chemical factors, such as composition of the cultivation media, temperature, pH, oxygen, etc. For example, phosphate, iron and glucose were reported to affect biofilm formation of *Citrobacter* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Allan *et al.,* 2002; Banin *et al.,* 2005 and Lim *et al.,* 2004). In this study, artificial urine produced more biofilm formation compared to commercial medium NB. This is similar to the findings of Oki *et al.,* (2010) that artificial urine promoted more biofilm formers compared to commercial medium TSB.

Biofilms are both persistent and highly resistant to antimicrobial treatment. It has been observed that the resistance of bacteria in biofilms to antibiotics increases compared to planktonic cells. In fact, when cells exist in biofilms, they are up to 1000 times more resistant to the effects of antimicrobial agents (Prosser *et al.,* 1987, Nickel *et al.,* 1985). For example, biofilms of a *Klebsiella pneumonia* mutant strain remained resistant to ampicillin compared to that of a planktonic culture after 24 hours of treatment (Jeff *et al.,* 2000). Anwar *et al.,* (1992) showed that treatment with levels of tobramycin far in excess of

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the MIC reduced biofilm cell counts for *P. aeruginosa* by approximately 2 log, while the same dosage provided a >8-log decrease in planktonic cells of this organism. Because bacterial biofilms are inherently resistant to many antibiotics, more work is needed to fully elucidate antibiotic resistance mechanisms in biofilms and develop new therapeutic strategies. Bacteriocin, a group of natural antimicrobial peptide or protein may be an alternative in combating antibiotic resistance.

Results of our study revealed that bacteriocin produced from *P. putida* strain FStm2 demonstrated antibiofilms activity against three isolates of *B. cepacia* and one isolate of *S. hominis* isolated from urinary catheter. According to Lynn *et al.,* (2011), *B. cepacia* is comprised of closely related species called *B. cepacia* complex, which can contaminate many finished pharmaceutical products and environments where pharmaceuticals are manufactured. *B. cepacia* may also cause catheter-related infections in patients with cancer and in those on hemodialysis. It also has been recognized as a problematic opportunistic pathogen, particularly among cystic fibrosis (CF) and chronic granulomatous disease patients (Barbara-Ann *et al.,* 2002). Kaitwatcharachai *et al.*, (2000) reported an outbreak of subclavian catheter-related *B. cepacia* bacteremia in nine patients undergoing hemodialysis. Using randomly amplified polymorphic DNA (RAPD) analysis, the bacterial isolates were clonally identical to *B. cepacia* isolated from the residue of diluted chlorhexidine-cetrimide solution used to disinfect the transfer forceps. These forceps were used to pick up cotton balls and gauze for dressing the subclavian catheter. Antibiotic therapy failed to cure the infections and all patients required catheter removal.

Quorum sensing and biofilm formation have also been observed from *B. cepacia* (Huber *et al.,* 2001). The maturation of the biofilm has been found to depend on the production of the exopolysaccharide cepacian which is known to stabilize the three-dimensional architecture of the biofilm (Monica *et al.,* 2004). According to Shawn *et al.,* (2000), *B. cepacia* bacteria are innately resistant to many common antibiotics and are able to acquire resistance against many more. Results from our study revealed that bacteriocin from *P. putida* FStm2 was active against *B. cepacia* isolated from urinary catheter. According to Parret and De Mot (2002), bacteriocins are highly potent narrow-spectrum antibacterial protein toxins that are active against bacterial closely related to the producing strain. Bacteriocin from *P. putida* FStm2 is active against *B. cepacia* probably became they are phylogeneticaly related.

Bacteriocin from *P. putida* FStm2 also demonstrated activity against *S. hominis* isolated from urinary catheter. *S. hominis* is normally found on human skin and is usually harmless, but can sometimes cause infections in people with abnormally weak immune systems. It also has been isolated from catheter (Seisdedos Elcuaz *et al.,* 2012). Studies by Karanika *et al.,* (2008) showed eighteen out of fifty *S. hominis* tested were biofilm-producers. Occasionally strains are resistant to novobiocin and may be confused with other resistant species. In addition, new subspecies are resistant to nalidixic acid, penicillin G, oxacillin, kanamycin and streptomycin. They can also be resistant to methicillin and gentamicin, and most strains were resistant to erythromycin, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole and ciprofloxacin. Although bacteriocin have activity between closely related there are also some that can act against non-related species (Klaenhammer, 1993). This is the case with the *P. putida* bacteriocin and *S. hominis*. This observation indicates that bacteriocin of *P. putida* FStm2 is broad-spectrum in term of its antibacterial activity.

CONCLUSION

From this study, it can be concluded that the bacteriocin from *P. putida* FStm2 demonstrated significant antimicrobial ability towards *B. cepacia* and *S. hominis*. It can be a good candidate as an anti-biofilm agent in combating urinary catheter infection. However, further studies that focus on the mechanism of action of bacteriocin against bacterial species are still needed for verification before they can be successfully employed as anti-biofilm agents.

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