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Characteristics of biofilms formed on non-tunneled hemodialysis catheters

J.V. Sathish[a], Malleshappa Pavan[b,*]

[a] *Department of Microbiology, Adichunchanagiri Institute of Medical Sciences, B.G. Nagara, Karnataka State, India*
[b] *Division of Nephrology, Department of Medicine, Adichunchanagiri Institute of Medical Sciences, B.G. Nagara, Karnataka State, India*

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Biofilm;
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Abstract
Background: Microbial biofilms are mechanisms used by microorganisms that cause chronic infections in humans. In hemodialysis patients with catheter-related bacteremia, *Staphylococcus aureus* is an independent risk factor for both infectious complications and failure of bacteremia treatment. We analyzed the characteristics of biofilms formed by these *Staphylococcus* species on non-tunneled hemodialysis catheters.
Patients and methods: A total of 50 adult patients with end-stage renal disease receiving hemodialysis through non-tunneled catheters, whose catheters were removed for catheter-related bacteremia, were studied.
Results: Catheter cultures were positive in only 32 patients and staphylococcal biofilm was found in 25 patients. All biofilm producers were *S. aureus*. In tissue culture plate method, 2 were strong biofilm producers, 15 were moderate biofilm producers and 5 isolates were considered as weak biofilm producers. In tube method, there were no strong biofilm producers, 12 were moderate biofilm producers and 13 were weak biofilm producers. In Congo red agar method there were no strong biofilm producers, 10 were moderate biofilm producers and 15 isolates were weak biofilm producers.
Conclusion: Our study shows that *S. aureus* is the most common bacteria isolated from patients with catheter-related bacteremia. *S. aureus* is the predominant microorganism responsible for biofilm formation in the non-tunneled HD catheters. Tissue culture plate method is more sensitive to detect biofilm formation by *S. aureus*.

* Corresponding author.
E-mail address: dr_pavanm@yahoo.co.in (M. Pavan).

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PALABRAS CLAVE
Biopelícula;
Bacteriemia asociada a catéter;
Hemodiálisis;
S. aureus

Características de las biopelículas formadas sobre los catéteres de hemodiálisis no tunelizados

Resumen
Antecedentes: Las biopelículas microbianas son mecanismos utilizados por los microorganismos que causan infecciones crónicas en el ser humano. En los pacientes en hemodiálisis que presentan una bacteriemia asociada al catéter, *S. aureus* constituye un factor de riesgo independiente, tanto para las complicaciones infecciosas como para el fracaso terapéutico del tratamiento de la bacteriemia. Hemos analizado las características de las biopelículas formadas por estas especies de estafilococos sobre los catéteres de hemodiálisis no tunelizados.
Pacientes y métodos: Se estudiaron a 50 pacientes adultos con enfermedad renal terminal tratados con hemodiálisis mediante catéteres no tunelizados, en los que se retiraron los catéteres a causa de una bacteriemia asociada a los mismos.
Resultados: Los cultivos de los catéteres fueron positivos en tan solo 32 pacientes y se observó una biopelícula estafilocócica en 25 pacientes. Todos los gérmenes productores de biopelícula fueron *S. aureus*. En el examen con el método de placa de cultivo tisular, 2 de ellos fueron productores intensos de biopelícula y 15 fueron productores moderados, mientras que 5 cepas se consideraron productoras débiles de biopelícula. En el método de tubo, no hubo ningún productor intenso de biopelícula, 12 cepas fueron productoras moderadas y 13 fueron productoras débiles. En el método agar rojo Congo no hubo ningún productor intenso de biopelícula, 10 cepas fueron productoras moderadas y 15 fueron productoras débiles.
Conclusión: Nuestro estudio pone de relieve que *S. aureus* es la bacteria aislada con más frecuencia en los pacientes con bacteriemia asociada a catéter. *S. aureus* es el microorganismo predominante responsable de la formación de biopelícula en los catéteres de hemodiálisis no tunelizados. El método de placa de cultivo tisular es más sensible a la detección de la formación de biopelícula por parte de *S. aureus*.

Introduction

Microbial biofilms are mechanisms used by microorganisms that cause chronic infections in humans.[1] Biofilms are associated with many diseases including cystic fibrosis, endocarditis, osteomyelitis, and nosocomial diseases related to central venous catheters, urinary catheters, prosthetic heart valves and orthopedic devices.[2] Microbial biofilms develop when microorganisms adhere to surfaces and produce extracellular polymers that provide structural matrix and facilitate adhesion. The surface may be living tissue or non-living material. Biofilms on indwelling medical devices may be composed of bacteria or yeasts. *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), viridans streptococci, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are commonly isolated from such biofilms.[2] Biofilm-associated bacteria behave differently from planktonic (freely suspended) bacteria with regard to growth rate and antibiotic resistance. Therefore, biofilms pose a public health problem.

Staphylococcus species comprising *S. aureus* and *S. epidermidis* are associated with implantable medical device related infections that are often difficult to treat.[3] Staphylococcal virulence is caused by a complex process that involves cell-to-cell communication through the release of and response to chemical signals in a process called quorum sensing.[4] Staphylococcal biofilm depends on the production of a polysaccharide intercellular adhesin (PIA) encoded by the *ica* operon comprising *icaA*, *icaB*, *icaC* and *icaD* genes.[5]

In chronic kidney disease, it is estimated that 25% of the patients use catheters as vascular access.[6] The use of tunneled dialysis catheter is an important source of infection with an incidence of bacteremia reported as 0.8–5.5/1000 catheter days.[7] Staphylococcal tunneled dialysis catheter infection, particularly with *S. aureus*, is highly prevalent in a hospital setting. Infection with either coagulase-negative *Staphylococcus* or *S. aureus* accounts for 40–81% of cases of catheter-related infections in reported studies.[8] In India, non-tunneled catheters are commonly used as a source of temporary vascular access for the initiation of hemodialysis in end-stage renal disease patients. This study analyzed the characteristics of biofilms formed by these *Staphylococcus* species on non-tunneled hemodialysis catheters.

Aims and objectives

1. To determine the rate of biofilm production by *S. aureus* and *S. epidermidis* isolated from non-tunneled HD catheters.
2. To compare the reliability of tissue culture plate (TCP) method, tube method (TM) and Congo red agar (CRA) method to detect the biofilm produced by staphylococci.

Materials and methods

- The study was conducted in the Department of Microbiology and Nephrology.

- Non-tunneled HD catheters were collected from both outpatients and inpatients.
- Catheter-related bacteremia was defined as the presence of bacteremia in an HD patient with a non-tunneled catheter and in whom no other obvious source of infection was evident.
- Peripheral blood cultures were obtained from patients with catheter-related bacteremia before starting systemic antibiotic therapy.
- The decision to remove the HD catheter was made by the Nephrologist after obtaining peripheral blood culture reports.
- The catheters were removed by a Nephrologist under strict aseptic precautions, after taking informed consent from the patients.
- Catheter cultures were obtained from the surfaces of the removed HD catheters.

Specimen collection

The tip of the catheter is rolled across the surface of a blood agar plate and the resulting colonies are counted after overnight incubation. A statistical association of >15 CFU with catheter-associated sepsis was established.[9]

Identification of *Staphylococc*

Staphylococcus spp. isolated from HD catheters were identified using standard procedure.[10] The phenotypic characteristics tested were colony morphology, Gram staining, catalase test, coagulase test, mannitol fermentation and novobiocin sensitivity test.

Gram-positive cocci of about 1 μm diameter arranged in irregular clusters, catalase positive, coagulase positive, fermenting mannitol were considered *S. aureus*. Gram-positive cocci of about 1 μm diameter arranged in irregular clusters, catalase positive, coagulase negative, mannitol non-fermenter and novobiocin sensitive were considered *S. epidermidis*.

Biofilm detection

Detection of biofilm formation was done by the following three methods:

1. Tissue culture plate method (TCP)

   Bacteria were inoculated into tryptic soy broth (Hi media, Mumbai) and incubated at 37 °C for 18 h in a stationary condition and diluted 1 in 100 with fresh medium.[11] Individual wells of tissue culture plate were filled with 0.2 ml aliquots of the diluted cultures and only broth (control) to check sterility and non-specific binding of media.

   The tissue culture plates were incubated at 37 °C for 24 h. The content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic bacteria. Biofilm formed by adherent organisms in plates was fixed with sodium acetate solution (2%, w/v) and stained with crystal violet (0.1, w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro-ELISA autoreader at a wavelength of 570 nm (OD_{570}). These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Classification of bacterial adherence by TCP method.

Mean OD values	Adherence	Biofilm formation
<0.120	None	Non/weak
0.120–0.240	Moderate	Moderate
>0.240	Strong	High

2. Tube method

   Tube method described by Christensen et al. was used.[12] The tube containing tryptic soy broth was inoculated with a loopful of bacteria from overnight culture plates and incubated at 37 °C for 24 h. The tubes were decanted and washed with phosphate buffered saline (PBS pH 7.2) and dried. The dried tubes were stained with crystal violet (0.1%). Excess stain was removed and the tubes were washed with deionized water. The tubes were then dried in inverted position and observed for biofilm formation.

   Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was considered negative.

   The tubes were examined and the amount of biofilm formation was scored as 0 – absent, 1 – weak, 2 – moderate, and 3 – strong.

3. Congo red agar (CRA) method

   This is an alternative method for screening biofilm formation by *Staphylococcus* spp.[13] The medium contains sucrose, 50 g; Congo red, 0.8 g; agar, 20 g and brain heart infusion broth, 1000 ml. The chemicals were purchased from Hi Media, Mumbai. Congo red was prepared as concentrated aqueous solution and autoclaved at 131 °C for 15 min separately from other medium constituents and was added when the medium had cooled to 55 °C. Plates were inoculated and incubated aerobically at 37 °C for 24–48 h.

   Positive results were indicated by black colonies with a dry crystalline consistency. Pink colonies indicate weak slime production. Darkening of colonies with the absence of a dry crystalline colonial morphology indicates an intermediate result. The experiment was performed in triplicate and repeated three times.

   In all experiments, *S. epidermidis* ATCC 35984 (high slime producer) and *S. epidermidis* ATCC 12228 (non-slime producer) were used as controls.

Statistics

Statistical analysis of the results was done using the Wilcoxon Signed Rank, Kruskal–Wallis test, and chi-square test, and p values <0.05 were considered significant.

Table 1 Patient characteristics.

	ESRD patients with catheter-related bacteremia
Number	50
Age (years)	54 ± 15
Sex – M/F	38/12
BMI (kg/m^2)	22.4 ± 2.1
History of diabetes mellitus	41 (50)
Duration of catheter (days)	25 ± 12
Hemoglobin (g/dL)	9.7 ± 0.64

Table 2 Organisms responsible for hemodialysis catheter-related bacteremia.

Organism	Number (n = 50)	Percentage (%)
Gram-positive bacteria	41	82
Staphylococcus aureus	25	
Staphylococcus epidermidis	11	
Gram-negative bacteria	6	12
Polymicrobial	3	6

Results

A total of 50 adult patients with ESRD receiving HD through non-tunneled HD catheter whose catheters were removed for catheter-related bacteremia were studied.

Patient's demographic data and clinical information are shown in Table 1.

Blood cultures were positive in all 50 patients. Gram-positive organisms were isolated in 82%, Gram-negative bacteria in 12% and both Gram-positive and Gram-negative in 6% of the cultures (Table 2). *S. aureus* was the most common pathogen (61%), followed by *S. epidermidis* (27%).

Catheter cultures were positive in only 32 patients. Staphylococcal biofilm was found in 25 patients. The efficiency of tissue culture plate (TCP) method, tube method and Congo red agar (CRA) method in the detection of staphylococcal biofilm was compared in Fig. 1. In the TCP method, two were strong biofilm producers, 15 were moderate biofilm producers and 5 isolates were considered as weak biofilm producers. In the tube method, there were no strong biofilm producers, 12 were moderate biofilm producers and 13 were weak biofilm producers. In the CRA method also there were no strong biofilm producers, 10 were moderate biofilm producers and 15 isolates were weak biofilm producers. All biofilm producers were *S. aureus*.

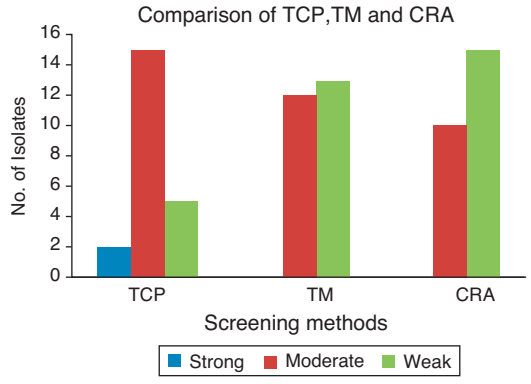

Figure 1 Comparison of TCP, TM and CRA for the detection of biofilm by *Staphylococcus aureus*.

Discussion

We studied biofilm formation on non-tunneled HD catheters from patients with bacteremia. Staphylococci are the commonly isolated bacteria from these non-tunneled HD catheters. *S. aureus* was the predominant bacteria responsible for the biofilm formation in our study. Our study also shows that TCP method is superior to TM and CRA methods in the detection of biofilm by staphylococci.

Several interrelated factors have been proposed to participate in the pathogenesis of biofilm formation. Impaired host immunity in end-stage renal disease, caused by neutrophil dysfunction in the setting of iron overload, hyperparathyroidism and retention of uremic solutes, has been implicated.[14] Four pathogenic pathways have been incriminated in the development of catheter-related bloodstream infections, and include, in order of descending frequency: colonization of the cutaneous catheter tract and tip with skin flora; intraluminal colonization due to contamination of the catheter hub; hematogenous seeding to the catheter from another focus of infection; and very rarely, intraluminal contamination of the catheter with solvent/infusate.[14] Passerini et al. detected biofilms in 100% of CVCs removed from 26 intensive care unit patients; bacteria were present in the biofilms of 88% of CVCs.[15] In our study catheter culture was positive in only 32 patients. All patients with bacteremia had received systemic antibiotics for a few days prior to catheter removal. Subsequently, when the catheter was removed and processed, catheter cultures were positive in only 32 patients.

An observational study of 114 episodes of hemodialysis catheter-related bacteremia revealed that 70.7% were associated with a Gram-positive organism only, 17.9% with a Gram-negative organism only, 9.8% with both Gram-positive and Gram-negative organisms, and 1.6% with an acid-fast organism.[16] Similar to this study, our study also shows that Gram-positive organisms were isolated in 76%, Gram-negative bacteria in 20% and both Gram-positive and Gram-negative in 6% of the blood cultures. The organisms responsible for dialysis catheter-related bacteremia are Gram-positive in two-thirds of the cases, predominantly *S. aureus* and *S. epidermidis*.[17,18] Other causative bacterial agents are enterococci and Gram-negative rods.[19] In our study, *S. aureus* was the predominant bacteria isolated in blood cultures followed by *S. epidermidis*.

We tested 25 catheter isolates of staphylococci by three in vitro screening procedures for their ability to form biofilm. In the TCP method, of 25 strains of *S. aureus*, two isolates displayed a strong biofilm positive phenotype. This was in agreement with observations of other investigators in which only few or no biofilm producing isolates could be detected using this medium.[20] On the other hand, supplementation of TSB media with different sugars such

as glucose and sucrose exhibited biofilm formation in more number of strains. By TCP method we can discriminate better between moderate and weak biofilm producing staphylococci, which correlates well with previous studies.[20]

The tube method correlated well with the TCP method for five moderately biofilm producing isolates, but it was difficult to discriminate between weak and biofilm negative isolates due to the variability in observing results. Consequently high variability was observed and classification of biofilm as positive and negative was difficult by the tube method. These results are consistent with findings of a previous study.[21] Tube method cannot be recommended as a general screening test to detect biofilm production by bacteria.

In CRA method, out of 10 moderately biofilm positive isolates showed no correlation with TCP and TM. Based on our results we are unable to recommend the CRA method for detection of biofilm formation by staphylococci. The results of the present study indicate that the TCP method is more sensitive and superior to the TM and CRA methods for biofilm detection. This is in agreement with previous reports.[22] It was also found to be an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of staphylococci.

Our study has certain limitations. First, only 50 patients were studied. This is a small number considering the high prevalence of catheter-related bacteremia among the HD patients. Second, catheter cultures were positive in only 32 patients, owing to the use of systemic antibiotics prior to catheter removal. Third, only patients with catheter-related bacteremias were studied. Fourth, this is only an observational study.

Conclusion

Our study shows that *S. aureus* is the most common bacteria isolated from patients with catheter-related bacteremia. *S. aureus* is the predominant microorganism responsible for biofilm formation in the non-tunneled HD catheters. The TCP method is more sensitive to detect biofilm formation by *S. aureus*.

Conflict of interest

The authors declare no conflict of interest.

References

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284:1318–22.
2. Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis. 2001;7:1–6.
3. Acqua GD, Giacometti A, Cirioni O. Suppression of drug-resistant staphylococcal infections by the quorum-sensing inhibitor RNA III-inhibiting peptide. J Infect Dis. 2004;190:318–20.
4. Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol. 2001;55:165–99.
5. Heilmann C, Schweitzer O, Gerke C, Vanittana Kom N, Mack D, Gotz F. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol. 1996;20:1083–91.
6. Rodríguez-Aranda A, Alcazar JM, Sanz F, García-Martín F, Otero JR, Aguado JM, et al. Endoluminal colonization as a risk factor for coagulase-negative staphylococcal catheter-related bloodstream infections in haemodialysis patients. Nephrol Dial Transplant. 2011;26:948–55.
7. Langer JM, Cohen RM, Berns JS, Chittams J, Cooper ET, Trerotola SO. *Staphylococcus*-infected tunneled dialysis catheters: is over-the-wire exchange an appropriate management option? Cardiovasc Intervent Radiol. 2011, http://dx.doi.org/10.1007/s00270-011-0180-4.
8. Beathard GA, Urbanes A. Infection associated with tunneled hemodialysis catheters. Semin Dial. 2008;21:528–38.
9. Washington Jr CW, Stephen DA, William MJ, Koneman EW, Procop GW, Paul CS, et al. Chapter 2, Guidelines for the collection, transport, processing, analysis, and reporting of cultures from specific specimen sources. In: Koneman's colour atlas and text book of diagnostic microbiology. 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2006. p. 105.
10. Baird D. *Staphylococcus*-cluster forming Gram-positive cocci. In: Collee JG, Fraser AG, Marmias BP, Simmons A, editors. Mackie and McCartney practical medical microbiology. 14th ed. New York: Churchill Livingstone; 1996. p. 246–8.
11. Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase negative staphylococci to plastic tissue cultures: a quantitative model for the adherence of staphylococci to medical device. J Clin Microbiol. 1995;22:996–1006.
12. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surface. Infect Immun. 1982;37:318–26.
13. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989;42:872–4.
14. Jaber BL. Bacterial infections in hemodialysis patients: pathogenesis and prevention. Kidney Int. 2005;67:2508–19.
15. Passerini L, Lam K, Costerton JW, King EG. Biofilms on indwelling vascular catheters. Crit Care Med. 1992;20:665–73.
16. Beathard GA. Management of bacteremia associated with tunneled-cuffed hemodialysis catheters. J Am Soc Nephrol. 1999;10:1045–9.
17. Tanriover B, Carlton D, Saddekni S. Bacteremia associated with tunneled dialysis catheters: comparison of two treatment strategies. Kidney Int. 2000;57:2151–5.
18. Hoen B, Paul-Dauphin A, Hestin D, Kessler M. EPIBACDIAL: a multicenter prospective study of risk factors for bacteremia in chronic hemodialysis patients. J Am Soc Nephrol. 1998;9:869–76.
19. Nassar GM, Ayus JC. Infectious complications of the hemodialysis access. Kidney Int. 2001;60:1–13.
20. Ludwicka A, Switalski LM, Lundin A, Pulverer G, Wadstrom T. Bioluminescent assays for measurement of bacterial attachment to polyethylene. J Microbiol Methods. 1985;4:169–77.
21. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun. 1982;37:318–26.
22. Mathur T, Sin ghal S, Khan S, Upadhyay DJ, Fatura T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an elevation of three different screening methods. Indian J Med Microbiol. 2006;24:25–9.