

Original Investigation

In Vitro Activity of Linezolid, Daptomycin and N-acetylcysteine Agents on Staphylococcus Aureus Biofilms in the Ventriculoperitoneal Shunt Model

Tuba KURUOGLU¹, Mehmet Emin ONGER², Derya BAYIRLI TURAN³, Enis KURUOGLU⁴

¹Ondokuz Mayıs University, School of Medicine, Department of Clinical Microbiology and Infectious Diseases, Samsun, Turkey

²Ondokuz Mayıs University, School of Medicine, Department of Histology and Embryology, Samsun, Turkey

³Yeni Yuzyl University, School of Medicine, Department of Clinical Microbiology and Infectious Diseases, Istanbul, Turkey

⁴Ondokuz Mayıs University, School of Medicine, Department of Neurosurgery, Samsun, Turkey

ABSTRACT

AIM: To examine the effects of N-acetylcysteine (NAC) alone and in combination with linezolid (LIN) and daptomycin (DAPT) on methicillin-sensitive *Staphylococcus aureus* (MSSA) biofilm formation.

MATERIAL and METHODS: Twelve groups (each containing six molds) of standard ventriculoperitoneal shunts were infected with MSSA. By using microbiological and electron microscopic evaluation methods, NAC was evaluated, alone and in combination with DAPT and LIN, in terms of preventing and eliminating biofilm capacity. The effect of NAC alone and in combination with DAPT and LIN were shown by microbial counts and electron microscopic observation.

RESULTS: There was no significant difference in biofilm formation in shunts after different antibiotic treatments. However, the combination of NAC and DAPT had the highest bactericidal effects of all the groups.

CONCLUSION: The resistance of bacteria and the dose-dependent effects of antibiotics can be considered.

KEYWORDS: Biofilm, Daptomycin, Linezolid, N-acetylcysteine, Scanning electron microscope

INTRODUCTION

Staphylococcus aureus infections are a major problem in hospital settings, especially in patients with permanent devices (17). Most serious infections, especially catheter-related ones, are caused by biofilm-producing strains (7,8,11). In the present study, the activities of daptomycin (DAPT), linezolid (LIN), and N-acetylcysteine (NAC) against *Staphylococcus aureus* were investigated in an in vitro ventriculoperitoneal (VP) shunting model. Shunting of cerebrospinal fluid (CSF) to the peritoneal cavity, also known as VP shunting, is generally preferred for hydrocephalus treatment because the technique is simple, and placement of the distal catheter to the peritoneal cavity can be performed quickly. However, some common

complications can be observed after VP shunting, such as shunt infection, disconnection, distal catheter migration, or any combination of these (16,26).

Biofilm, defined as complex communities of microbial species embedded in self-produced biopolymer extracellular polymeric substances (EPSs), which are gross structures resembling mushroom and pillar-like structures of cells, is a type of bacterial group behavior. There are thousands of bacteria in a mature biofilm, a great number of which grow in matrix-enclosed biofilms adherent to surfaces in all nutrient-sufficient ecosystems. Biofilms seem to have both structural and metabolic heterogeneity, which enables them to resist stresses from either host defense systems or antimicrobial agents.



Corresponding author: Mehmet Emin ONGER

E-mail: mehmetemin.onger@gmail.com

Because they are surrounded by EPSs, biofilms are difficult to disintegrate. Currently, scientists are interested in bacterial biofilm studies, the number of which has been increasing at a fast pace. A great many detachment processes force the separation of bacterial cells from biofilms, which occurs as a result of complex molecular events in biofilm cells (9,29). Biofilms mature in three steps: attachment and colonization, growth and proliferation, and the extensive production of EPSs. Following these steps, cell detachment becomes the central process in the spread of infection to other body parts (1). Blocking tissue destruction, resistance to bacteria, and developing chemicals that might prevent initial adherence are approaches that can be used to prevent these infections (4,27). At this point, this protection is critical for the pathogenicity of most bacteria, especially *Staphylococcus aureus* (13).

Staphylococcus aureus is one of the common causes of infections that occur in surgical shunt operations because the active microorganism is unknown, and the biofilm layer causes the bacteria to become resistant to treatment. Two-stage revision surgery is the classical method in *Staphylococcus aureus* treatment (30). However, in the case of an unknown agent or the absence of suitable antibiotic treatment, this method can also be unsuccessful. In order to prevent biofilm formation and to eradicate biofilm-associated bacteria, such as *Staphylococcus aureus*, various antimicrobial or non-antimicrobial agents, such as NAC, have been researched (19,20).

In the light of all the facts mentioned above, the purpose of this study was to examine the effect of NAC alone and in combination with each of LIN and DAPT on both adherence and preformed biofilms. At this point, morphological changes occurring in *Staphylococcus aureus* on related biofilms were investigated using scanning electron microscopy (SEM). Furthermore, for full clarification of the effects of those agents on the biofilms involved, there is a need for further studies, especially at the molecular level.

■ MATERIAL and METHODS

The study consisted of 12 groups (each containing six molds) formed using methicillin-sensitive *Staphylococcus aureus* (MSSA) strains as follows:

Group I (+): MSSA infected only group

Group II (+): MSSA infected + NAC (6 mg/ml) given group

Group III (+): MSSA infected + DAPT (2 mg/ml) given group

Group IV (+): MSSA infected + DAPT (2 mg/ml) + NAC (6 mg/ml) given group

Group V (+): MSSA infected + LIN (2 mg/ml) given group

Group VI (+): MSSA infected + NAC (6 mg/ml) + LIN (2 mg/ml) given group

Group I (-): MSSA infected only group

Group II (-): MSSA infected + NAC (6 mg/ml) given group

Group III (-): MSSA infected + DAPT (2 mg/ml) given group

Group IV (-): MSSA infected + DAPT (2 mg/ml) + NAC (6 mg/ml) given group

Group V (-): MSSA infected + LIN (2 mg/ml) given group

Group VI (-): MSSA infected+ NAC (6 mg/ml) + LIN (2 mg/ml) given group

(+) indicates the antibiotic-impregnated catheters and (-) shows the non-antibiotic-containing catheters.

All shunt material samples were infected with *Staphylococcus aureus* strain ATCC 48300. A suspension of *Staphylococcus aureus* ATCC 48300 with turbidity equivalent to a 0.5 McFarland-standard ($\sim 10^8$ cfu/mL) was prepared in tryptic soy broth (TSB; Merck, Darmstadt, Germany) supplemented with 0.5% glucose, and molds were inoculated in this solution at 37°C for 48 hours. After the incubation, they were placed into sterile saline solution to wash and remove planktonic cells for one minute. Then, these were separated into 12 groups. Then, related plates belonging to the same group were placed into new sterile containers with tops, which contained 10 mL sterile saline solution, and vortexed for 30 seconds. Thereby, the biofilm layer of bacterial colonies was removed from the shunt surface. For counting bacterial colonies in the biofilm layer, we removed 10 μ L of these solutions and cultured them on agar medium plates for 24 hours at 37°C. Finally, at the end of the incubation period, plates were checked for growth, and colonies were counted.

SEM Analysis

Biofilm formation on the material surface was also confirmed by SEM on a JSM-7001F STEM (JEOL Ltd., Tokyo, Japan). Samples were critical-point dried and coated with gold-palladium. Then, these were evaluated for biofilm. The eight selected areas were investigated at 10,000 \times magnification. A histological scoring system was employed according to our previous work (21).

Statistical Analysis

For the statistical analysis, SPSS Ver. 21 software (IBM Corp., Armonk, NY) was used. Data were expressed as mean \pm SD and subjected to statistical analysis. Data were normally distributed in the present study; therefore, differences between groups were assessed using parametric ANOVA followed by post hoc analysis with Tukey's HSD test to identify individual group differences. Differences were regarded as statistically significant at $p < 0.05$.

■ RESULTS

In the present study, when the colonies in the biofilm layer were counted, significant differences were found between groups. A significant decrease ($p < 0.01$) was found in the microbial count of Groups III (-) and IV (-) compared to Groups I (-), II (-) and Group V (-), VI (-). However, no significant differences between Groups I (-) and II (-) nor between Groups V (-) and VI (-) ($p > 0.05$) were observed in either of the non-antibiotic-containing catheters. In addition, there was a significant increase ($p < 0.05$) in the microbial count of Group II (+) compared to that of Group I (+) in the antibiotic-impregnated

catheters. Similar to the groups with non-antibiotic-containing catheters, no significant difference between Group V (+) and Group VI (+) was observed in the antibiotic-impregnated catheters ($p>0.05$). Moreover, a significant decrease ($p<0.01$) was observed in the microbial count of Group III (+) and IV (+)

compared to Group I (+), II (+) and Group V (+), VI (+) (Figure 1A, B).

In contrast, when histopathological analysis was conducted using SEM observation (Figures 2, 3 and Table I), there was no statistically significant difference ($p>0.05$) between any of the groups.

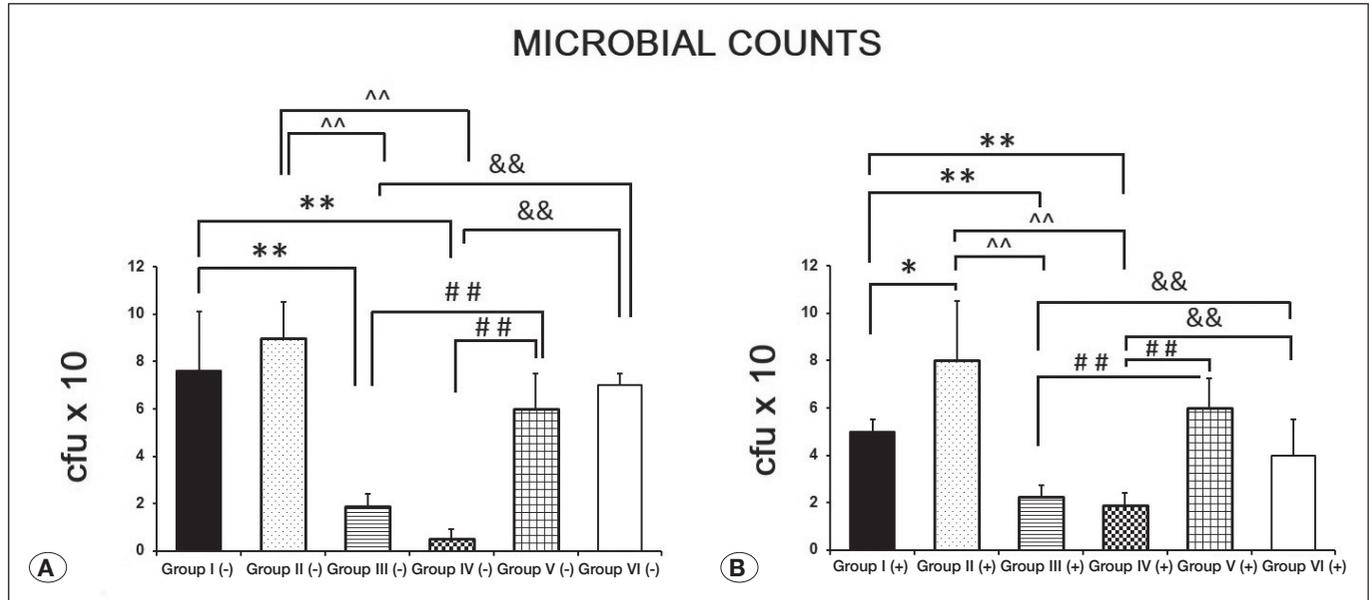


Figure 1: Bacterial counts of the groups (cfu × 10). The bacterial counts of the non-antibiotic containing catheters in **A** and the bacterial counts of the antibiotic-impregnated catheters in **B**.

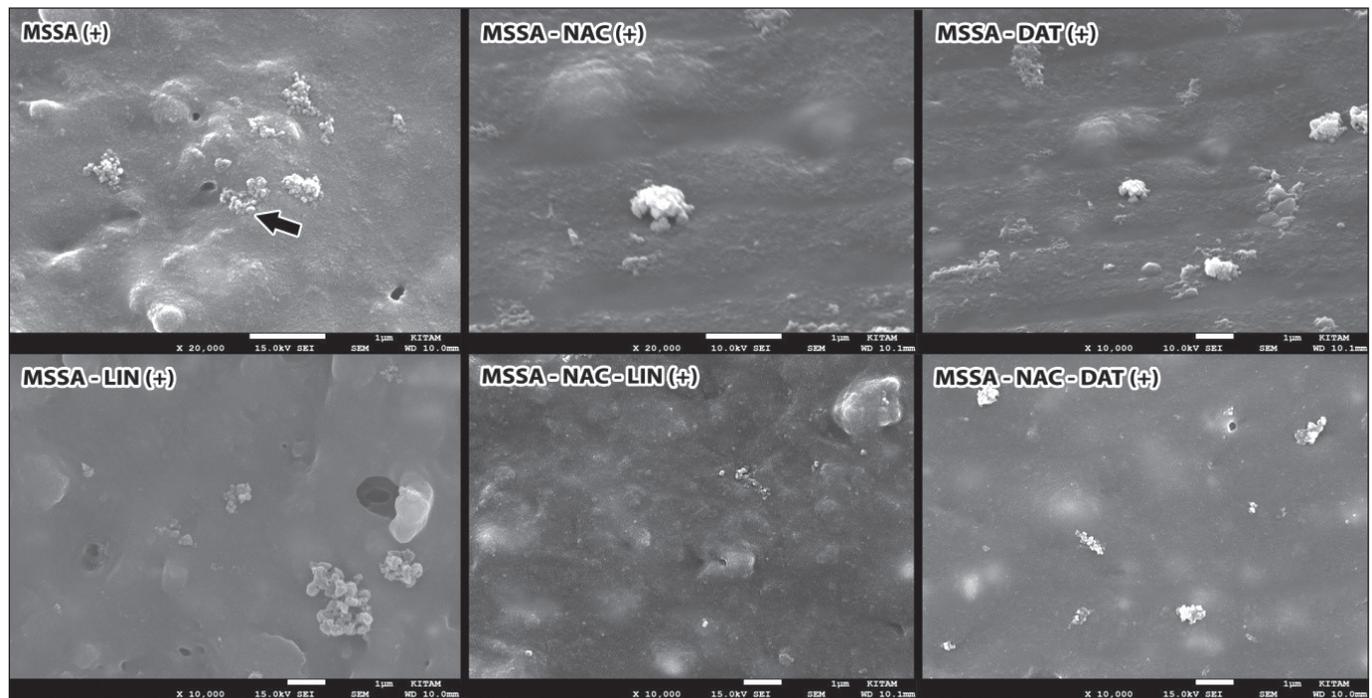


Figure 2: SEM images of antibiotic-impregnated catheters belonging to the different groups in the present study. The histopathologic photomicrograph shows the in vitro activity of LIN, DAPT, and NAC agents on *Staphylococcus aureus* biofilms in the ventriculoperitoneal shunt model. Finally, the microbial counts ($p>0.05$) in the present study were supported by SEM images. Arrow refers to *Staphylococcus aureus* cells.

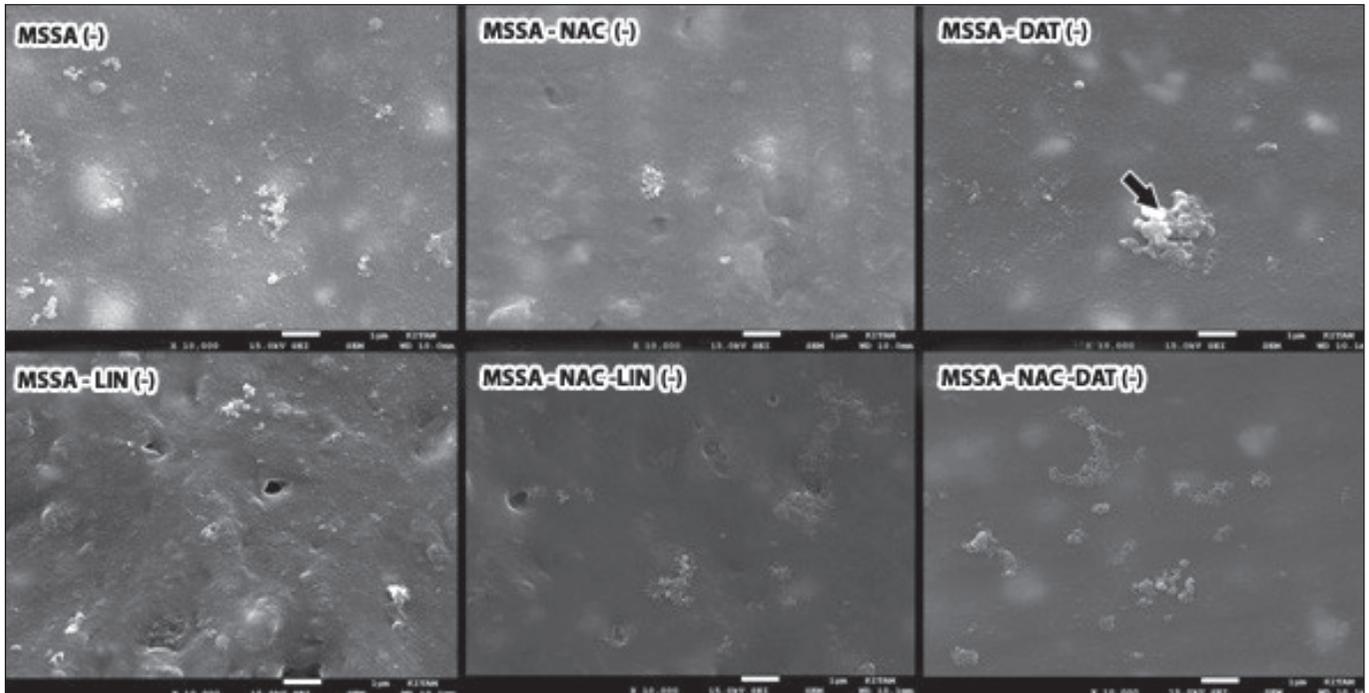


Figure 3: SEM images of non-antibiotic-impregnated catheters belonging to the different groups in the present study. The histopathologic photomicrograph shows the in vitro activity of LIN, DAPT, and NAC agents on *Staphylococcus aureus* biofilms in the ventriculoperitoneal shunt model. Finally, the microbial counts ($p>0.05$) in the present study were supported by SEM images. Arrow refers to *Staphylococcus aureus* cells.

Table I: Histopathological and Microbiological Findings among the Groups

	Bacteria (<i>Staphylococcus aureus</i>) (Mean±SD)	
	Cell counts (SEM)	Colony counts (Mean cfu × 10)
Group I (+)	1.50 ± 0.58	7.6 ± 2.5
Group II (+)	2.50 ± 0.58	9 ± 1.5
Group III (+)	0.60 ± 0.50	1.9 ± 0.5
Group IV (+)	0.01 ± 0.01	0.50 ± 0.40
Group V (+)	1.62 ± 0.52	6 ± 1.5
Group VI (+)	2.00 ± 0.01	7 ± 0.5
Group I (-)	1.65 ± 0.58	5 ± 0.5
Group II (-)	3.20 ± 0.45	8 ± 2.5
Group III (-)	0.75 ± 0.50	2.25 ± 0.5
Group IV (-)	0.49 ± 0.75	1.9 ± 0.5
Group V (-)	2.00 ± 0.01	6 ± 1.5
Group VI (-)	2.80 ± 0.58	4 ± 1.5

DISCUSSION

Infection is one of the common complications of shunt treatment. Although shunt infection rates reach 20% in some series, recently reported shunt infection rates vary from 5 to 10%. Furthermore, 90% of all shunt infections are seen within the first month of shunt placement (3).

When considered in the light of the literature, *Staphylococcus epidermidis* (40%–60%) and *Staphylococcus aureus* (20%–30%) are frequently responsible for VP shunt infections. In addition, streptococci, gram-negative bacilli, and fungi are less frequently detected microorganisms (14,24). In the literature, intraoperative contamination from skin flora has been cited as the most probable cause of infection (3,14). In order to decrease the infection rate, all precautions should be taken to decrease the contamination risk in the preoperative period and during the operation. While the efficacy of prophylactic antibiotic use in shunt surgery is debated, it is recommended that prophylactic antibiotics be used an hour before surgery (23), and that the surgical area be covered with sterile plastic transparent drapes which adhere to the skin (28). While they are also debated, some studies report that it would be useful in preventing infections to use cleansing solutions with antibiotics during surgery, to keep the shunt system in water with antibiotics, to have the patient among the first cases in the surgery list (to schedule the surgery in the early hours of the morning), and to limit the number of staff in the surgery room (14,28). Three different approaches can be applied in cases of shunt infection. These are antibiotic treatment without removing the shunt, antibiotic treatment

after removing the shunt, and antibiotic treatment after removing the shunt and placing external ventricular drainage (3,14,24,25). In their study Schreffler et al. compared these three methods and showed that the best result was obtained with antibiotic treatment started after removing the shunt and placing external ventricular drainage (25).

In this study, we detected antimicrobial and antibiofilm effects of NAC alone and in combination with LIN and DAPT against *Staphylococcus aureus* in shunt infections. Although there are some studies indicating antimicrobial and antibiofilm effects of those agents (6,18), to the best of our knowledge, there are no studies demonstrating those effects on *Staphylococcus aureus* biofilms in shunts.

In order to find out the morphological changes in *Staphylococcus aureus* cells and the changes in the intensity of biofilm formed on a shunt surface in the presence of those agents, SEM was used. The first approach outlined in the literature is quantitative evaluation of shunt samples using SEM. Thus, our study is the first to highlight new application and evaluation methods from those perspectives. A biofilm is a microbial group of one or more bacteria with self-reproduction capacity (5). Developmental stages of colony formation and biofilm maturation start following the interaction of a bacterium with a biological surface (12).

Daptomycin has been widely used in the treatment of *Staphylococcus aureus* bacteremia in skin infections in recent years. Gram-positive infections do not respond to conventional therapy with daptomycin. This should be considered outside of specific and rigid indications (15). When daptomycin is administered systemically, it passes poorly into the cerebrospinal fluid. However, it shows higher bactericidal activity in intraventricular administration. In addition, daptomycin has a longer half-life than vancomycin administered intraventricularly (10). However, there are limited clinical data in the literature regarding intraventricular/intravenous administration of daptomycin in central nervous system infections. In such cases, linezolid has been successfully used in systemic therapy. In addition, this antibiotic does not show bactericidal activity, and the risk of side effects increases with prolonged use (10).

The use of NAC has been suggested by a great number of studies as an alternative pharmacological approach to controlling bacterial biofilm growth. These studies have attributed its antibiofilm property to a number of factors such as inhibition of bacterial adherence and reduction of the extracellular polysaccharide matrix production (6,20,22). N-acetylcysteine is also a precursor of glutathione, which is involved in reactive oxygen species balance and homeostasis (2).

Previous studies have shown that NAC in combination with different antibiotics significantly promoted their permeability to the deepest layers of the biofilm with safety and efficacy (6). In contrast, in our study, NAC had no effect on either bacterial adherence or biofilm eradication, whether alone or in combination with LIN and DAPT. Moreover, more studies with different amounts of chemicals that include different microbial species belonging to both gram-positive and gram-negative

families need to be investigated. In the present study, no significant difference between SEM images of the groups was observed. It is questionable whether the bacteria are dead or alive in the SEM images. In this regard, advanced techniques are needed.

■ CONCLUSION

According to the bacterial counts obtained in the present study, when the combination of NAC and DAPT was administered, bacterial growth was suppressed. In addition, significant increases in the bactericidal effect were observed in the groups in which DAPT was administered in both non-antibiotic-containing and antibiotic-impregnated groups. The combination of DAPT and NAC had the highest antibiofilm effect of all the treatments. The absence of any bactericidal effect in the NAC-administered groups can be interpreted as a dose-dependent effect against MSSA strains. High doses of NAC should be studied in the shunt-related cerebrospinal fluid infections. In other respects, resistance phenotypes are also considered in the treatment of shunt-related infections.

■ REFERENCES

1. Abd El-Baky RM, Abo El Ela DMM, Gad GFM: N-acetylcysteine inhibits and eradicates candida albicans biofilms. American Journal of Infectious Diseases and Microbiology 2(5):122-130, 2014
2. Asevedo E, Mendes AC, Berk M, Brietzke E: Systematic review of N-acetylcysteine in the treatment of addictions. Rev Bras Psiquiatr 36:168-175, 2014
3. Boran BO, Kizilcay G, Bozbuga M: Ventriculoperitoneal shunt dysfunction. Turk Neurosurg 15:148-151, 2005
4. Braxton EE Jr, Ehrlich GD, Hall-Stoodley L, Stoodley P, Veeh R, Fux C, Hu FZ, Quigley M, Post JC: Role of biofilms in neurosurgical device-related infections. Neurosurg Rev 28:249-255, 2005
5. Costerton JW: Overview of microbial biofilms. J Ind Microbiol 15:137-140, 1995
6. Dinicola S, De Grazia S, Carlomagno G, Pintucci JP: N-acetylcysteine as powerful molecule to destroy bacterial biofilms. A systematic review. Eur Rev Med Pharmacol Sci 18:2942-2948, 2014
7. Donlan RM: Role of biofilms in antimicrobial resistance. ASAIO J 46:S47-52, 2000
8. Donlan RM, Murga R, Bell M, Toscano CM, Carr JH, Novicki TJ, Zuckerman C, Corey LC, Miller JM: Protocol for detection of biofilms on needleless connectors attached to central venous catheters. J Clin Microbiol 39:750-753, 2001
9. Dunne WM Jr: Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev 15:155-166, 2002
10. Elvy J, Porter D, Brown E: Treatment of external ventricular drain-associated ventriculitis caused by Enterococcus faecalis with intraventricular daptomycin. J Antimicrob Chemother 61:461-462, 2008
11. Gotz F: Staphylococcus and biofilms. Mol Microbiol 43:1367-1378, 2002

12. Heindl JE, Wang Y, Heckel BC, Mohari B, Feirer N, Fuqua C: Mechanisms and regulation of surface interactions and biofilm formation in *Agrobacterium*. *Front Plant Sci* 5:176, 2014
13. LaPlante KL, Mermel LA: In vitro activity of daptomycin and vancomycin lock solutions on staphylococcal biofilms in a central venous catheter model. *Nephrol Dial Transplant* 22:2239-2246, 2007
14. Lee M, Leahu D, Weiner HL, Abbott R, Wisoff JH, Epstein FJ: Complications of fourth-ventricular shunts. *Pediatr Neurosurg* 22:309-313; discussion 314, 1995
15. Lowman W, Coetzee J, Perovic O; National Antimicrobial Committee (NAC) a sub-committee of SASCM: SASCM guideline for daptomycin use in South Africa-2017 update. *South Afr J Infect Dis* 32:77-81, 2017
16. McGirt MJ, Buck DW 2nd, Sciubba D, Woodworth GF, Carson B, Weingart J, Jallo G: Adjustable vs set-pressure valves decrease the risk of proximal shunt obstruction in the treatment of pediatric hydrocephalus. *Childs Nerv Syst* 23:289-295, 2007
17. Mermel LA, Farr BM, Sherertz RJ, Raad II, O'Grady N, Harris JS, Craven DE; Infectious Diseases Society of America; American College of Critical Care Medicine, Society for Healthcare Epidemiology of America: Guidelines for the management of intravascular catheter-related infections. *J Intraven Nurs* 24:180-205, 2001
18. Munoz-Egea MC, Garcia-Pedrazuela M, Mahillo-Fernandez I, Esteban J: Effect of antibiotics and antibiofilm agents in the ultrastructure and development of biofilms developed by nonpigmented rapidly growing mycobacteria. *Microb Drug Resist* 22:1-6, 2016
19. Oddera S, Silvestri M, Sacco O, Eftimiadi C, Rossi GA: N-acetylcysteine enhances in vitro the intracellular killing of *Staphylococcus Aureus* by human alveolar macrophages and blood polymorphonuclear leukocytes and partially protects phagocytes from self-killing. *J Lab Clin Med* 124:293-301, 1994
20. Olofsson AC, Hermansson M, Elwing H: N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. *Appl Environ Microbiol* 69:4814-4822, 2003
21. Onger ME, Gocer H, Emir D, Kaplan S: N-acetylcysteine eradicates *Pseudomonas Aeruginosa* biofilms in bone cement. *Scanning* 38:766-770, 2016
22. Rasmussen K, Nikrad J, Reilly C, Li Y, Jones RS: N-Acetyl-L-cysteine effects on multi-species oral biofilm formation and bacterial ecology. *Lett Appl Microbiol* 62:30-38, 2016
23. Rieder MJ, Frewen TC, Del Maestro RF, Coyle A, Lovell S: The effect of cephalothin prophylaxis on postoperative ventriculoperitoneal shunt infections. *CMAJ* 136:935-938, 1987
24. Sacar S, Turgut H, Toprak S, Cirak B, Coskun E, Yilmaz O, Tekin K: A retrospective study of central nervous system shunt infections diagnosed in a university hospital during a 4-year period. *BMC Infect Dis* 6:43, 2006
25. Schreffler RT, Schreffler AJ, Wittler RR: Treatment of cerebrospinal fluid shunt infections: A decision analysis. *Pediatr Infect Dis J* 21:632-636, 2002
26. Simon TD, Hall M, Riva-Cambrin J, Albert JE, Jeffries HE, Lafleur B, Dean JM, Kestle JR; Hydrocephalus Clinical Research Network: Infection rates following initial cerebrospinal fluid shunt placement across pediatric hospitals in the United States. *Clinical article. J Neurosurg Pediatr* 4:156-165, 2009
27. Souza MC, dos Santos LS, Sousa LP, Faria YV, Ramos JN, Sabbadini PS, da Santos CS, Nagao PE, Vieira VV, Gomes DL, Hirata Junior R, Mattos-Guaraldi AL: Biofilm formation and fibrinogen and fibronectin binding activities by *Corynebacterium pseudodiphtheriticum* invasive strains. *Antonie Van Leeuwenhoek* 107:1387-1399, 2015
28. Tabara Z, Forrest DM: Colonisation of CSF shunts: Preventive measures. *Z Kinderchir* 37:156-158, 1982
29. Wang Z, Gao M, Wei J, Ma K, Zhang J, Yang Y, Yu S: Extracellular polymeric substances, microbial activity and microbial community of biofilm and suspended sludge at different divalent cadmium concentrations. *Bioresour Technol* 205:213-221, 2016
30. Yoon YC, Lakhota D, Oh JK, Moon JG, Prashant K, Shon WY: Is two-stage reimplantation effective for virulent pathogenic infection in a periprosthetic hip? A retrospective analysis. *World J Orthop* 6:712-718, 2015