

Comparative in vitro Efficacies of Colistin-Levofloxacin, Colistin-Tigecycline and Tigecycline-Levofloxacin based Catheter Lock Solutions on Eradication of *Acinetobacter baumannii* Biofilms

Berna Ozbek* and Emel Mataraci

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Istanbul University, 34452 Beyazit, Istanbul, Turkey
Tel. +90 212 440 00 00, Fax +90 212 440 02 57; E-Mail bernaozbek@hotmail.com

Aims: Although the central venous catheter (CVC)s play an essential role in patient care, catheter-related bacteraemia (CRB) infections are an important problem in patients, putting them at increased risk for morbidity and mortality [1, 2]. The aim of this study was to determine the in-vitro stability and efficacy of colistin-levofloxacin, colistin-tigecycline and tigecycline-levofloxacin combinations based catheter lock solutions against biofilm embedded *Acinetobacter baumannii* strains.

Methods: MICs of strains used were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [26]. *A. baumannii*-1 was isolated from patients with CRB. *A. baumannii* ATCC 19606 which known to form biofilm was used throughout the study to verify the accuracy of the microdilution test procedure and also lock solution studies. These two strains were confirmed for biofilm-forming ability. Briefly, 1 µL of an overnight culture was inoculated into 100 µL of fresh Trypticase soy broth (TSB) with %1 glucose in each well of a 96-well tissue culture plate. After incubation at 37°C for 24 hours, added 125 µL of 99% methanol in water in each well of microtiter plate then bacteria were stained with 125 µL of 0.5% crystal violet for 20 min. The plate then was washed with deionized water, the biofilm-bound dye was eluted with 95% ethanol, and the absorbance was measured at 550 nm.

The compatibility and efficacy of antibiotic lock solutions at 400xMIC was tested in an in-vitro catheter biofilm model against *A. baumannii* strains at 24h, 48h, 72h and 96h [3]. Statistical analysis was performed with GraphPad Prism 5.0

Results: Each tested antibiotic combination at 400xMIC was physically compatible each other. When colistin-levofloxacin, colistin-tigecycline and tigecycline-levofloxacin combinations were tested, each combination demonstrated potent bactericidal activity against the clinically isolated *A. baumannii* strain. Especially, the lock solution including colistin-levofloxacin was the unique agent that could eradicate both clinical and ATCC strains of *A. baumannii* embedded in the catheter biofilm model.

Conclusion: Catheter lock solution containing colistin-levofloxacin at 400xMIC may have the most promising adjuvant for treating or preventing biofilm-producing catheter infections caused by *A. baumannii*. Furthermore, clinical trials are required to access the role of our findings in the management of CRB.

Keywords Gram negative, catheter related infection

1. Introduction

A central venous catheter (CVC) which placed percutaneously into the internal jugular, subclavian, or femoral veins give fluids, nutrition products, chemotherapy, blood, medications or to do medical tests quickly. They can remain for weeks or months, and some patients receive treatment through the line several times a day. Their using have dramatically increased during the last decade worldwide. It is estimated that about 50% of all patients admitted to hospitals receive intravenous therapy, this situation creating a large population at risk for local and systemic bacteremia infections [1]. Catheter-related bacteremia (CRB) infections associated with the insertion and maintenance of CVC are among the most common and dangerous complications that can occur. CRB has a substantially effect enhances the morbidity, mortality, duration of hospitalization, and overall cost of health care [2, 3, 4]. According to CDC, each year in the United States, CVCs may cause an estimated 80.000 CRB infections in intensive care unit (ICU)s and between 12 and 25% of patients could die because of CRB and many others have prolonged hospitalization [5, 6]. To reduce the incidence of intravascular CRB infections, specific guidelines comprising strategies for prevention have been established [5, 7, 8]. According to the guidelines that removal of the catheter is the most effective treatment for catheter-related infections, especially in patients with severe sepsis or septic shock. However, removing the infected CVC is not always possible; not easy to perform, entertain risk for patient, other access sites may not be available and this process increase costs [9]. Moreover, most central venous catheters can develop a bacterial biofilm as early as 24 hours after placement [10]. Studies on this subject showed that scanning electron microscopy demonstrates the presence of a biofilm coating the inner lumen in 80% of indwelling central vein catheters within days of their replacement [11, 12]. In this circumstances, instead of the removal of the device, the antibiotic lock technique (ALT) is recommended as an effective therapeutic option for CRB [7, 8]. The ALT which was first reported by Messing et al [13] is based on instillation of high concentrations of antimicrobial agent into the lumens of infected CVCs for extended periods to

overcome the relative antimicrobial resistance of biofilm bacteria [14, 15, 16, 17]. Although, most investigations have focused on Gram positive bacteria CVC infections, Gram-negative are also important pathogens in catheter-related infections [18, 19, 20]. Gram negative bacteria accounted for 19% and 21% of CRB reported to CDC [21] and the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) database, respectively [22]. As well as, *A. baumannii* is one of the important organisms that causes catheter-related infections and often difficult-to-eradicate bloodstream infections [18, 23]. Additionally Seifert et al. showed that, patients with *A. baumannii* bacteremia, 91% were hospitalized in an intensive care unit, 99% had indwelling vascular catheters [24] and biofilm has been increasingly recognized as an antibiotic resistance mechanism in *A. baumannii* [25].

In the present study, we examined the in vitro activity of ALT using colistin sulfat, tigecycline, levofloxacin alone or in combination to determine the activities on biofilms embedded *A. baumannii*.

2. Materials and methods

2.1. Bacterial strains used in this study

Two strains of *A. baumannii* were used in this study: *A. baumannii* ATCC 19606 (AB-19606) are known to form biofilms. *A. baumannii* AB-1, obtained from an ICU patient with a CVC, was isolated from both the removed CVC tip, which produced a positive culture defined as ≥ 15 CFU and the peripheral blood. These strains were confirmed for biofilm-forming ability.

2.2. Determination of MICs

The MIC was determined by the broth microdilution method according to the CLSI [26]. *A. baumannii* ATCC 19606 was used as control.

2.3. Tissue Culture Plate (TCP) Method

These two strains were confirmed for biofilm-forming ability. Briefly, 1 μ L of an overnight culture was inoculated into 100 μ L of fresh TSB with %1 glucose in each well of a 96-well tissue culture plate. After incubation at 37°C for 24 hours, added 125 μ L of 99% methanol in water in each well of microtiter plate then bacteria were stained with 125 μ L of 0.5% crystal violet for 20 min. The plate then was washed with deionized water, the biofilm-bound dye was eluted with 95% ethanol, and the absorbance was measured at 550 nm.

2.4. In vitro antibiotic lock model

One-centimeter segments of 7-French, triple-lumen, central venous catheters (Cook, Inc., Bloomington, IN) were incubated in bacterial suspensions that contained 10^6 CFU/ml of bacteria in Trypticase soy broth to allow biofilm formation. After incubation at 37°C for 24 h, segments were removed and excess broth was shaken off. Three catheter segments were rinsed and cultured to obtain a baseline value, and the remaining segments (three replicates per condition) were suspended for 24, 48, 72 and 96 h at 37°C in one of the following treatment solutions: colistin sulfat, tigecycline, levofloxacin, alone, and levofloxacin-colistin, levofloxacin-tigecycline, tigecycline-colistin. Phosphate-buffered saline (PBS) was used as a control. Colistin, tigecycline, levofloxacin were used all lock study at concentrations of 400 X MIC, both alone or in combination for the organisms tested in the planktonic phase. Then catheter segments were removed, washed ten times with PBS to remove planktonic bacteria. These sections were individually sonicated for 10 min and vortexed for 30 s in 1 ml of PBS. After successive dilutions, if needed, all original sonication fluid were inoculated onto Trypticase soy agar plates (limit of detection, < 10 CFU) and the plates were incubated at 37°C overnight [27]. The median colony count of the three 1-cm sections was considered the representative value for that segments. The experiments were repeated three times, and the mean values for the biofilm bacteria were compared between groups for each antibiotic alone or in combinations. The CFU per centimeter values of catheters for different groups were compared by One-way ANOVA followed by Bonferroni's multiple comparison test (GraphPad Software Inc., San Diego, Calif., USA), and a P value of < 0.05 indicated significance.

2.5. Drug stability and compatibility

Each solution was incubated 96 h, and then evaluated for physical compatibility by particulate formation, colour change, or gas evolution. Antibiotic solutions tested also demonstrated compatibility with no significant physical or chemical interaction [15].

2.6. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, Calif., USA). One-way ANOVA followed by Bonferroni's multiple comparison test was performed to examine the change in the CFU counts at 96 h of each antibiotic alone or in combinations. In the results, $p < 0.05$ was considered to be significant.

3. Results

The MICs of the antimicrobial agents for each of the planktonic forms of *A. baumannii* are shown in Table 1. AB-19606 was susceptible to all antibiotics tested in this study, whereas AB-1 was resistant to levofloxacin and colistin.

Each tested antibiotic combination at 400xMIC was physically compatible each other. Levofloxacin at 400 x MIC completely eradicated the AB-19606 and AB-1 biofilm bacteria within three days and four days, respectively (Figure a, b). Tigecycline alone eradicated any of the two biofilm bacteria within 4 days. However, its reduction of live cell count of the biofilm was found significant compare to control ($p < 0.05$) and demonstrated potent bactericidal activity against two *A. baumannii* strains at 72 h. Our two *A. baumannii* catheter biofilms were not sterile within 4 days of exposure to 400 x MIC colistin alone. Moreover this antibiotic at 400 x MIC could able to kill both biofilm strains, AB-19606 and AB-1, almost 2 log, compare to control ($p < 0.05$). In particular, the catheter related biofilms by AB-19606 or AB-1 were sterilized after 2 day of exposure to levofloxacin-tigecycline combinations. The lock solution including levofloxacin- tigecycline combination was the unique agent that could eradicate both clinical and ATCC strains of *A. baumannii* embedded in the catheter biofilm model at 48h.

Although levofloxacin-colistin combination showed bactericidal effect against AB-19606 and AB-1 within two days, same combinations did not eradicated two tested strains within 4 days.

While tigecycline-colistin combination eradicated AB-1 at 48 h. Although, AB-19606 catheter biofilms were not sterile within 4 days of exposure to this combination. Although this combination did not eradicated AB-1 at 96 h, it only killed all 19606 bacteria almost 3 log within 3 days of the lock period, compare to control ($p < 0.05$). While *A. baumannii* AB-1 was successfully eradicated by tigecycline-colistin combinations at 48 h, AB-19606 was not sterilized by this combination until 96 h. On the other hand, this combination killed almost 3 log of all 19606 bacteria within 3 days of the lock period, compare to control ($p < 0.05$).

4. Discussion

Although CVC are increasingly being used to save life for majority of patients in ICUs in order to receive medicine and fluids [7], the use of CVC may result in serious bloodstream infections. Several studies on biofilm formation in *A. baumannii* indicate that antimicrobial therapy of biofilm-forming bacteria is more challenging [28, 29, 30]. This ability will make treatment of CRB infections more difficult and this fact should be taken into consideration when treatment protocols are established. Under the circumstances, the guidelines recommended that the patients whom CVC that cannot be removed, should be treated for 2 weeks with systemic and antibiotic lock therapy suffer from Gram-negative bacteraemia [7, 31]. According to clinical study by Krishnasami, the antibiotic lock protocol therapy was found successful in achieving a clinical and bacteriologic cure of dialysis catheter associated bacteremia in 64.5% of the patients without requiring catheter replacement [20]. Similarly, Funalleras_et all suggests that ALT combined with systemic antibiotic supplied a 95% cure rate for 37 patients with Gram-negative bacteraemia [32].

According to our results, levofloxacin alone was found significantly more effective than tigecycline or colistin inhibiting *A. baumannii* organisms embedded in biofilm ($p < 0.05$). The finding displays that levofloxacin at 400 x MIC may be an options to instilled in the catheter in situ for a sufficient dwell time to prevent colonization and biofilm formation or to eliminate the biofilm embedded microorganisms. For this reason, levofloxacin including lock solution could be seen as an adjunct therapeutic option in the catheter for a sufficient dwell time so as to prevent *A. baumannii* colonization and biofilm formation or to eliminate the biofilm-embedded microorganisms. Additionally, the use of tigecycline or colistin alone as antibiotic lock solutions is associated with a reduction in the viable bacterial density of the biofilm, compared with the control ($p < 0.05$). Still, they were not effective enough to eliminate the microbial burden of *A. baumannii* colonization in biofilms.

When colistin-levofloxacin, colistin-tigecycline and tigecycline-levofloxacin combinations were tested, each combination demonstrated potent bactericidal activity against both the clinically isolated *A. baumannii* and AB-19606. Especially, the lock solution including levofloxacin-tigecycline was the unique agent that could eradicate both clinical and ATCC strains of *A. baumannii* embedded in the catheter biofilm model at 48h.

In conclusion, antibiotics to be included in the ALT regimen should be active against organisms embedded in biofilm. Levofloxacin was significantly more active than tigecycline and colistin against *A. baumannii* embedded in biofilm. Additionally tigecycline alone significantly reduced the CFU of all tested bacteria, ≥ 4 log for 96 h, so it could be considered as an adjunct therapeutic option for treatment or prevention of catheter-related infections with *A. baumannii*. Combination of levofloxacin with tigecycline fastened and enhance antibacterial effect of ALT. It was

associated with the prevent emergence of resistant organisms and ultimately effective to eradicate *A. baumannii* colonization in biofilm. Hence, it can firstly state that it could serve as an antibiotic lock solution against *A. baumannii*. The findings of this study may have important information for the optimal combination and timing of the ALT against *A. baumannii*.

5. Funding

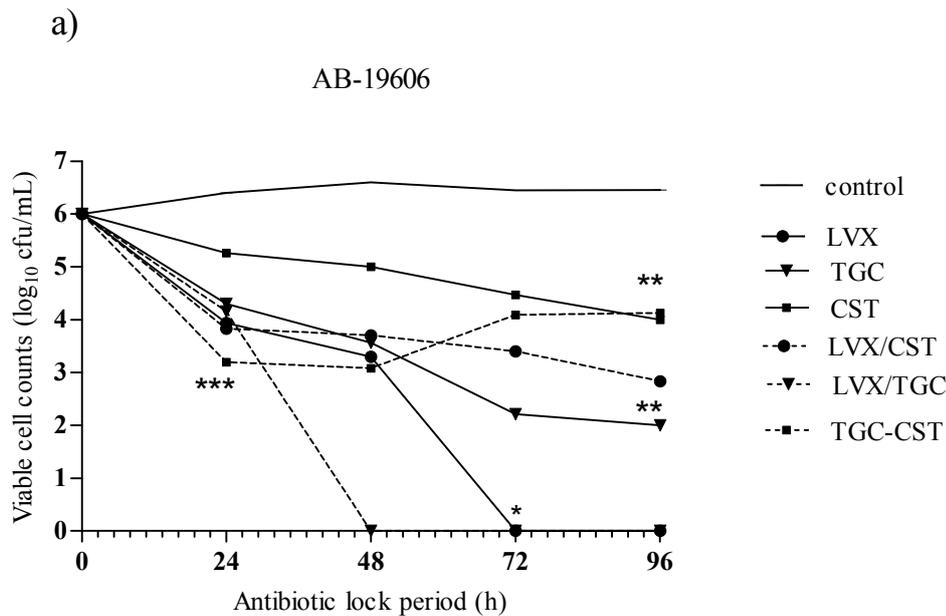
This work was supported by a grant from the Research Fund of the University of Istanbul. Project number: 4566.

Table 1 MICs of antimicrobial agents for *A. baumannii* strains

	MIC mg/ml		
	Colistin	Tigecycline	Levofloxacin
<i>A. baumannii</i> ATCC 19606	0.5	2	1
<i>A. baumannii</i> AB-1	8	4	8

CLSI breakpoints for susceptibility and resistance to levofloxacin are ≤ 2 and ≥ 8 mg/L, respectively, and for susceptibility and resistance to colistin are ≤ 2 and ≥ 4 mg/L, respectively. The FDA-approved breakpoints for Enterobacteriaceae susceptibility and resistance to tigecycline are ≤ 2 and ≥ 8 mg/L, respectively.

^aNo tigecycline breakpoints for *A. baumannii* are provided by the CLSI.



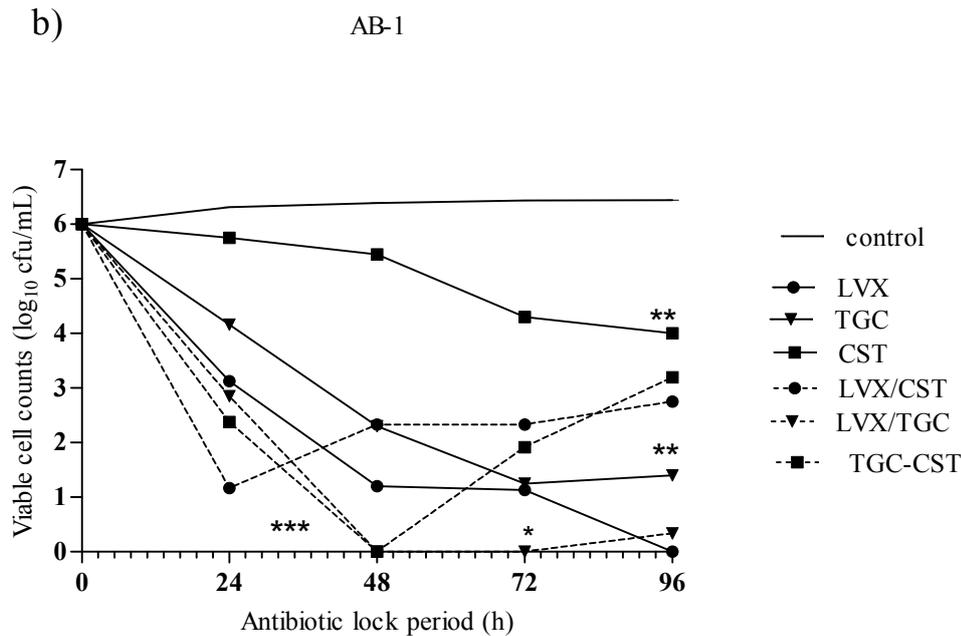


Fig. 1 In-vitro activity of ALT, including tested agents- levofloxacin (LVX), tigecycline (TGC), colistin (CST), and their combinations against biofilms formed by a representative *A. baumannii* strain, AB-19606 (a), and a clinical strain, AB-1(b). *Levofloxacin alone was significantly more effective than tigecycline or colistin at inhibiting *A. baumannii* organisms embedded in biofilm ($p < 0.05$), **tigecycline or colistin alone reduced the viable bacterial density of the biofilm, compared with the control ($p < 0.05$), *** antibiotic combinations used in ALT showed enhanced antimicrobial activity for *A. baumannii* biofilms, compare with each antibiotic used alone ($p > 0.05$)

References

- [1] Crnich CJ, Maki DG. Intravascular device infection. In: Carrico R, ed: *APIC Text of Infection Control and Epidemiology*. Washington, DC: Association for Professionals in Infection Control and Epidemiology, 2009, 24.1–24.22.
- [2] Digiovine B, Chenoweth C, Watts C, Higgins M. The attributable mortality and costs of primary nosocomial bloodstream infections in the intensive care unit. *Am J Respir Crit Care Med*. 1999; 160: 976–81.
- [3] Pittet D, Tarara D, Wenzel RP. Nosocomial bloodstream infection in critically ill patients. Excess length of stay, extra costs, and attributable mortality. *JAMA* 1991; 271: 1598–601.
- [4] Smith RL, Meixler SM, Simberkoff MS. Excess mortality in critically ill patients with nosocomial bloodstream infections. *Chest* 1991; 100: 164–7.
- [5] Mermel LA. Prevention of intravascular catheter-related infections (Erratum: *Ann Intern Med* 2000;133:395). *Ann Intern Med* 2000; 132: 391–402.
- [6] Maki DG, Kluger DM, Crnich CJ. The risk of bloodstream infection in adults with different intravascular devices: a systematic review of 200 published prospective studies. *Mayo Clin Proc* 2006; 132: 391–402.
- [7] 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. *Clin Infect Dis* 2001; 32: 1249–72.
- [8] O'Grady NP, Alexander M, Dellinger EP, Gerberding JL, Heard SO, Maki DG, Masur H, McCormick RD, Mermel LA, Pearson ML, Raad II, Randolph A, Weinstein RA. Guidelines for the prevention of intravascular catheter-related infections. Centers for Disease Control and Prevention. *MMWR Recomm Rep* 2002; 51: 1–29.
- [9] Toltzis P. Antibiotic lock technique to reduce central venous catheter-related bacteremia. *Pediatr Infect Dis J* 2006; 25: 449–50.
- [10] Raad I, Costerton W, Sabharwal U, Sacilowski M, Anaissie E, Bodey GP. Ultrastructural analysis of indwelling vascular catheters: a quantitative relationship between luminal colonization and duration of placement. *J Infect Dis* 1993; 168: 400–7.
- [11] Passerini L, Lam K, Costerton JW, King EG. Biofilms on indwelling vascular catheters. *Crit Care Med* 1992; 20: 665–73.
- [12] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 21: 1318–22.
- [13] Messing B, Peitra-Cohen S, Debure A, Beliah M, Bernier JJ. Antibiotic-lock technique: a new approach to optimal therapy for catheter-related sepsis in home-parenteral nutrition patients. *J Paren Enteral Nutrit* 1988; 12: 185–9.
- [14] Berrington A, Gould FK. Use of antibiotic locks to treat colonized central venous catheters. *J Antimicrob Chemother* 2001; 48: 597–603.
- [15] Bestul MB, Vandenbussche HL. Antibiotic lock technique: review of the literature. *Pharmacotherapy* 2005; 25: 211–27.
- [16] Bagnall-Reeb H. Evidence for the use of the antibiotic lock technique. *J Infus Nurs* 2004; 27: 118–22.

- [17] Fortún J, Grill F, Martín-Dávila P, Blázquez J, Tato M, Sánchez-Corral J, García-San Miguel L, Moreno S. Treatment of long-term intravascular catheter-related bacteraemia with antibiotic-lock therapy *J Antimicrob Chemother* 2006; 58: 816–21.
- [18] Hanna H, Afif C, Alakech B. Central venous catheter-related bacteremia due to gram-negative bacilli: significance of catheter removal in preventing relapse. *Infect Control Hosp Epidemiol* 2004; 25: 646–9.
- [19] Fätkenheuer G, Cornely O, Seifert H. Clinical management of catheter-related infections. *Clin Microbiol Infect* 2002; 8: 545–50.
- [20] Krishnasami Z, Carlton D, Bimbo L, Taylor ME, Balkovetz DF, Barker J, Allon M. Management of hemodialysis catheter-related bacteremia with an adjunctive antibiotic lock solution. *Kidney Int* 2002; 61: 1136–42.
- [21] Gaynes R, Edwards JR. Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* 2005; 41: 848–54.
- [22] Wisplinghoff H, Bischoff T, Tallent SM. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 2004; 39: 309–17.
- [23] Levin AS, Barone AA, Penço J. Intravenous colistin as therapy for nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Clin Infect Dis* 1999; 28: 1008–11.
- [24] Seifert H, Strate A, Pulverer G. Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. *Medicine (Baltimore)* 1995; 74: 340–9.
- [25] Rice LB. Challenges in Identifying new antimicrobial agents effective for treating infections with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Clinical Infectious Diseases* 2006; 43:S100–5.
- [26] Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically-Eighth Edition: Approved Standard M07-A8. CLSI, Wayne, PA, USA, 2008.
- [27] Ozbek B, Mataracı E. In vitro effectiveness of colistin, tigecycline and levofloxacin alone and combined with clarithromycin and/or heparin as lock solutions against embedded *Acinetobacter baumannii* strains. *J Antimicrob Chemother* 2013; 68: 827–830.
- [28] Davies, D. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov.* 2003 2:114-122.
- [29] Lewis, K. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* 2001; 45:999-1007.
- [30] O'Toole, G.A. Microbiology: a resistance switch. *Nature* 2002; 416:695-696.
- [31] Ashby MJ, Neale JE, Knott SJ, Critchley IA. Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. *J Antimicrob Chemother* 1994; 33: 443–52.
- [32] Funalleras G, Fernández-Hidalgo N, Borrego A, Almirante B, Planes AM, Rodríguez D, Ruiz I, Pahissa A. Effectiveness of antibiotic-lock therapy for long-term catheter-related bacteremia due to Gram-negative bacilli: a prospective observational study. *Clin Infect Dis* 2011; 53: e129–32.