In vitro Activity of some Antimicrobial Agents against Intact and Disrupted Biofilms of Staphylococci in the Indwelling Vascular Catheter Patients

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Abstract: Biofilm-associated infections are becoming more common and occur largely because of the increase usage of indwelling medical devices (for example, the catheter). Bacterial biofilms, which are micro-colonies encased in extracellular polysaccharide material (slime) are the sources of many bacterial infections which is so difficult to respond to routine treatments, also, shed cells or disrupted parts of the biofilm may enter the circulation causing serious and very hard to treat infections. The activity of some antimicrobial agents against intact biofilms and shed cells/disrupted biofilms is largely unknown, in Egypt. So, direct swab samples were collected from 328 patients in the Catheter Unit-in National Heart Institute in Imbaba-Giza-who undergo treatment by Catheter operation. We studied in vitro susceptibility of intact and disrupted biofilms of thirty eight clinical isolates of methicillin-resistant and methicillin-susceptible of both, Staphylococcus aureus (MRSA and MSSA) and Staphylococcus epidermidis (MSSE and MRSE) to Chloramphenicol, Gentamicin, Ciprofloxacin, Rifampicin, Erythromycin, Tetracycline, Clindamycin and Vancomycin and compared it with that of (planktonic) bacteria and also determined the (MICs, MBCs and MBECs) for both biofilm and planktonic forms. Results indicated that Ciprofloxacin and Vancomycin were more sensitive for all strains in both planktonic and biofilm forms of bacteria than other antibiotics used in this study. In conclusion infections by biofilm form of bacteria must have good attention by Physicians in following up and treatment of indwelling medical devices patients especially in Egypt.

Key words: Biofilm - Staphylococcus - Slime producer - Catheter

INTRODUCTION

Because they shield themselves in slime, bacteria associated with biofilms are much more difficult to kill than free-floating organisms [1]. The identity of the organism and the slime production test predicted the clinical significance of blood isolates of coagulase-negative staphylococci with an overall accuracy of 89% [2]. In a study by Christensen et al. [3] 60% of clinically significant bloodstream isolates of coagulase-negative staphylococci produced slime, as did 37% of contaminants.

Once encased in a biofilm, bacteria become recalcitrant to immune surveillance and also, antibiotic therapy becomes of limited value. [4]. Staphylococcus epidermidis is the most important member of the coagulase-negative staphylococci, which are normal micro-flora on the human skin and mucous membranes, but have attracted considerable attention as dangerous nosocomial pathogens. There are increasingly frequent reports about the devastating complications of indwelling medical device-related infections by S. epidermidis, which prolong disease and result in higher morbidity and mortality, especially in immuno-compromised patients. Particularly, S. epidermidis plays an important role in foreign body infections, such as catheter-related infections, prosthetic-valve endocarditis, prosthetic joint infections and peritoneal dialysis-related infections. Biofilm formation has long been recognized as a key virulence determinant of S. epidermidis. Bacteria in a biofilm are more resistant to attacks by mechanisms of innate host defense and antibiotics, which is based on the specific biofilm structure and metabolism [5].

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Antibiotics are generally not effective against organisms in exopolysaccharides biofilms. Bacterial populations form a biofilm by adhering to surfaces using pili and exopolysaccharides. The cells become enveloped in a matrix of hydrated exopolysaccharides termed a glycocalyx. The glycocalyx matrix modifies the environment of the adherent cells by concentrating nutrients and protecting the cells from surfactants, biocides and phagocytic cells [6].

Bacteria growing in biofilms cause a wide range of human infections. Biofilm bacteria are resistant to antimicrobics at levels 500 to 5,000 times higher than those needed to kill non-biofilm bacteria. The most effective way to eradicate such infections is the removal of colonized foreign bodies; but removal carries significant morbidity, cost and occasionally, mortality [7].

Microscopic observations show that catheter biofilm-associated bacteria form polymicrobial microcolonies that are embedded within an amorphous, protective extracellular matrix [8].

Catheter-related blood stream infection (CRBSI), results from both, the intraluminal contamination with transfer of planktonic bacteria to the blood and also the release of bacteria within biofilms. Microorganisms embedded in a biofilm encounter a unique microenvironment with higher cell density, growth rates and gene transcription versus planktonic components, resulting in bloodstream infections (23.2% of bacterial infections of the central line-associated infections in intensive care units for 1986 to 1989 and 19.5% for 1990 to 1995). This finding suggested that, these organisms must not be completely inhibited by surfaces conditioned with whole blood [12].

The predominant microorganisms associated with CVC-related infections are *Staphylococcus epidermidis* and *Staphylococcus aureus* whereas they are often found in biofilms upon removal of the devices [13].

Microbial infections are the most serious complications associated with indwelling central venous catheters (CVCs). A catheter lock solution that is both antibacterial and antithrombotic is needed [9].

Despite concerted efforts to treat biofilm infections with antibiotic therapy, the physical removal of an infected medical device is often necessary which carries an additional economic and health cost. The resistance of bacterial cells in a biofilm to antibiotics does not seem to depend on traditional mechanisms of antibiotic resistance. Although it is not yet clear how biofilms resist antimicrobial agents, a possible explanation has been suggested by several authors who assume that biofilms
present a diffusional barrier to antibiotics. However, it seems that this mechanism can only partially explain the increased resistance phenotype generally present in clinically relevant biofilms. Other mechanisms have been suggested, including slow growth of the cells within the biofilm, activation of the general stress response, emergence of a biofilm-specific phenotype and persister cells. Resistance is reportedly up to 1000-fold greater in the antibiotic susceptibilities of planktonic bacteria with bacterial cells in biofilms, but a reliable method to compare cells. Resistance seems that this mechanism can only partially explain the emergence of a biofilm-specific phenotype and persister biofilm, activation of the general stress response, suggested, including slow growth of the cells within the biofilms. Other mechanisms have been associated bacterial biofilms are generally quite resistant to antibiotic treatment. Despite decades of research in this area, treatment options are limited. A factor contributing to this unmet need is the lack of a standardized method for determining the drug susceptibility of bacterial biofilms. Several methods are available, but are limited by long processing times, incompatibility with high throughput, expensive reagents or equipment, or the method measures mass instead of viability. There are numerous differences in biofilm structure, growth and regulation in S. aureus and S. epidermidis [10].

Biofilm associated infections are difficult to treat due to the inherent antibiotic resistance of the sessile bacteria. A number of factors contribute to this resistance such as a slow growth rate, failure of the agent to penetrate the biofilm, physiological changes and gene expression or repression due to the biofilm mode of growth. Other factors such as age of the biofilms, production of extracellular polymeric substance (EPS) and presence of biomaterials also play a role in decreasing susceptibility of the bacteria within the biofilms to antimicrobial agents. Routinely, the diagnostic laboratories report the susceptibilities done on planktonic bacteria only. Although many studies have focused on the antimicrobial susceptibility of bacteria grown in biofilms, none of these studies included bacteria that disrupted from the biofilms. Disruption of the biofilm can occur during the removal of colonized catheters or during fluid infusion through them. The result is the entrance of bacteria or groups of bacteria shed from the biofilm into circulation causing bloodstream infections [13].

The present work aimed to isolate and identify the microbial species which cause the bloodstream infection in the indwelling vascular catheter patients in Egypt. Cultivate and form the biofilm form of the isolated Staphylococcal species in vitro to study on, the activity of different antibiotics and the minimum inhibitory concentration of these antibiotics in vitro.

MATERIALS AND METHODS

Antibiotics: (All Antibiotics used for MICs, MBCs and MBECs were obtained from Sigma: St. Louis, Missouri, USA. Except Ciprofloxacin were obtained from Bayer: Sp A, Milan, Italy).

Chloramphenicol (C), Clindamycin (Cd), Erythromycin (E), Gentamicin (G), Rifampicin (R), Tetracycline (T), Vancomycin (Va) (Sigma), Ciprofloxacin (Cf) (Bayer). The working solutions of these antibiotics were prepared in CAMHB at a concentration of 128 mg/L (for Biofilm) and 32 mg/L (for Planktonic) and from these working solutions serial twofold dilutions were made in CAMHB in the wells of the 96-well plate. (Antibiotics were prepared as stock solutions of 128 mg/L and were stored at-80°C.).

Microorganisms: Standard reference strains for S. aureus, ATCC No. 29213 for MSSA, ATCC No. 33591 for MRSA and S. epidermidis strains, ATCC No. 12228 (For NSP), ATCC No. 12228-ica (For SP) for MSSE and ATCC No. 35984 (RP62 A) for MRSE were used in this study as controls which proposed for quality control use by the National Committee for Clinical Laboratory Standards (NCCLS).

Fifty clinical staph isolates [38 were (SP) and 12 were (NSP)] with blood stream infections each of S. aureus MRSA, MSSA and S. epidermidis MRSE and MSSE were collected from 328 patients undergoing treatment by cardiovascular catheters as investigated and provided by the Catheter Unit-in National Heart Institute In Imbaba-were used in this study. These isolates were screened for biofilm formation on 96-polystyrene micro-liter plates. The isolates were grown in Trypticase soy broth (TSB; (Lab. M., U.K.)), which was also used in the reaction vessel to initiate biofilm formation. Bacterial counts were done on Trypticase-soy agar (TSA). Antibiotic susceptibility screening and recovery of viable biofilm organisms were carried out in cation-adjusted Mueller-Hinton broth (CAMHB). All 38 SP isolates were used for biofilm formations and subsequent antibiotic studies, Isolates were already resistant to antibiotics in the micro-broth dilution test. They were also distinguished by the colony morphology in Congo red agar [15]. The Congo red agar colony morphology was verified in each biofilm test, to confirm the absence of SP to NSP reversions. S. aureus and S. epidermidis strains were evaluated for methicillin resistance (Oxacillin and methicillin discs test, Hi-media-India).
Biofilm Formation and Quantification: Following the procedure steps described by MBEC Bioproducts, Calgary, AB, Canada [16] to form the Biofilm, (The inoculum of the growth medium of slime producer (SP) bacterial culture requiring about 18 to 24h growth at 37°C in TSB 2% and containing about 1 x 10^7 cells (cfu)/mL). Biofilm formation was carried out at 35°C and 95% relative humidity on a Shaker (Grant Instruments, Cambridge, Ltd., England,) such that fluid flowed along the channels of the CBD (Calgary Biofilm Device), generating the required shear force across all pegs. Biofilms were developed for 24 and 72h growth time, the growth medium in the trough were used as inoculums of planktonic bacteria for 24 and 72h growth time and then discarded (as biofilms were formed and adhered to the sides of the pins of plate cover lid). For planktonic antibiotic assay, 20 µL of the growth medium of slime producer (SP) bacterial culture from the trough prescribed above were added under aseptic conditions to each well of a tissue culture-treated polystyrene 96-well plate (cell well tissue culture treated polystyrene plates; MBEC Bioproducts, Calgary, AB, Canada), containing 180 µLs of growth medium (TSB 2%) and 200 µL of the particular antibiotic dilution in Mueller-Hintonbroth were added to one column of another 96-well plate and serial dilutions were then formed as 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/mL in total volume of each well 200 µL after adding 20 µL of the above diluted growth medium for antibiotic assay of planktonic bacteria. The mixture was maintained at 37°C for 24h. After antibiotic exposures to 24 and 72h planktonic growth time, viable bacteria were detected at this stages and verified by plate count after transferring 100 µL of the mixture to a trypticase-soy agar plate) [17]. The minimal biofilm eradication concentration (MBEC) was defined as the minimal concentration of antibiotic required to eradicate the biofilm. MICs were determined according to the 2003 guidelines of NCCLS [18]. The concentration of antibiotic required to prevent the growth of a planktonic population was also derived from the CBD either by obtaining plate counts or by measuring the turbidity at 650 nm after incubation of the isolate in antibiotics for 24 h. In this case the MIC (CBD) was defined as the lowest concentration of antibiotic in which a planktonic bacterial population could not be established by shedding of bacteria from the biofilm [16]. MICs and MBCs were obtained for the SP and the NSP variant of each isolate, using the micro-broth dilution method for bacterial suspensions [19]. MBC was defined as a 99.99% reduction of cell viability with respect to that of the initial inoculum. To facilitate antibiotic comparisons between the biofilm and the planktonic micro-broth dilution assays, antibiotics were diluted in all cases in the standard Mueller-Hinton medium, which is recommended for the latter assay [19]. This resulted in avoiding possible side effects caused by interactions of the various media with the antibiotic [19]. Using biofilm assays, an antibiotic concentration study was carried out, involving 8 antibiotics with 24h exposure time and biofilms of 24 and 72h of age. The concentrations used represented a wide concentration range: 4 x MBC (calculated from the MBC of the isolate with the highest MBC among the four isolates tested; this concentration purposefully exceeded the MBC value obtained in the planktonic micro-broth dilution assay, knowing the resistance of biofilm bacteria of killing, observed at 1 x MBC in preliminary experiments and in previous work) [20]; 128 mg/L (a high concentration exceeding 4 x MBC in most cases and also used in other biofilm studies) [20].

Scanning Electron Microscopy: Pegs were broken from the lid and allowed to air dry overnight. Samples were then fixed with 2.5% glutaraldehyde in phosphate-buffered saline (0.2 M; pH 7.4) and were prepared for scanning electron microscopy (SEM) on a (Joel JXA 840A-Electron Probe-Micro-analyzer) scanning electron microscope as reported previously [21, 16].

Statistical Analysis: Statistical presentation and analysis of the present study was conducted, using Chi-square by SPSS V17.
RESULTS

It was found that the source of infection is the skin of the patients. The +ve Catheter tip isolates after the cardiovascular catheters (CVCs) operations were 138 +ve by different bacterial infections of 328 (168 male and 160 female) patients investigated in this study their age were between 20-70 years old, all patients undergoing treatment by cardiovascular catheters. We just focused only on 50 clinical isolates of Staphylococci (15.2%), differentiated into slime producer (11.6%) and non-slime producer (3.6%), Staph aureus constitute (5.5%) and Staph epidermidis (9.7%) differentiated as [(4 (SP) and 2 (NSP)] MSSA, [9 (SP) and 3 (NSP)] MRSA, [3 (SP) and 2 (NSP)] MSSE and [22 (SP) and 5 (NSP)] MRSE) to determine their susceptibility or resistance for killing by (8) Antibiotics. Slime producer (SP) staph bacteria were differentiated from the Non-slime producing (NSP) staph bacteria and confirmed by growing on the Congo red agar media. The most prevalent Age of getting infections in Males and Females were the age between 41-50 years old, The +ve SP Staph aureus infected male patients were (7.9%) while the +ve SP Staph epidermidis were (18.4%) and The +ve SP Staph aureus infected female patients were (13.1%) while the +ve SP Staph epidermidis were (13.1%). (The ratios with respect to 38 (SP) clinical isolates).

Scanning Electron Microscopy (SEM) of Untreated Biofilms: Before determining the antibiotic effect on biofilm bacteria, SEM was used to visualize the formation of the biofilms and their characteristics of growth and compare it with the planktonic-grown bacteria, It was observed that biofilms had a relatively uniform thickness throughout an extensive biofilm matrix in the well (Images 2,3,5 and 6), whereas Planktonic-grown bacteria were associated with the presence of clumps or aggregates of different sizes, bacteria being unevenly distributed which contained abundant inclusion material.(Images 1 and 4).

The Qualitative Susceptibility Test of Antibiotics Proved That:
For Planktonic Form: Ciprofloxacin and Erythromycin were sensitive for all MSSA, MRSA, MSSE and MRSE, but Vancomycin was sensitive for MSSA, MSSE and MRSE and Chloramphenicol was sensitive for MSSA, MRSA, and MRSE.

For Biofilm and Disrupted Biofilm: Ciprofloxacin and Vancomycin were sensitive for all MSSA, MRSA, MSSE and MRSE, but Rifampicin was sensitive for MSSA and MRSA only and Clindamycin was sensitive for MSSE only, Gentamycin was sensitive for MSSE and MRSE only whereas Tetracycline, Erythromycin and Chloramphenicol had non-significant effects on cell viability of biofilm.

MICs and MBCs for Cells in Suspension: The MICs and the MBCs (for planktonic form of bacteria) and the MICs and the MBECs (for biofilm and disrupted biofilm forms of bacteria), even for SP versus NSP variants of each clinical isolate obtained with micro-broth dilution method for cells in suspension were similar (differences not exceeding one dilution) and therefore a single value is provided per isolate-antibiotic combination.

Antibiotic Concentration Study on 96-well Plate Biofilms after 24h Exposure: The effect of the antibiotics on cell viability in biofilms using TSB was determined by growing on blood agar plate using the pool of data on the thirty eight SP isolates under study. The biofilm test applied had
a high repeatability in all cases, with non-significant differences between replicate wells, both within and between test data.

In suspensions, all isolates were susceptible to all antibiotics tested. At MICs, the antibiotics showed very little effect on the viability of bacteria within the biofilms (intact or disrupted). At higher concentrations (16, 32, 64 and 128 µg/ml), the biofilms of all isolates were of same susceptible to the antibiotics compared to disrupted biofilms (Figures 2, 3, 4 and 5).

Ciprofloxacin and Vancomycin were sensitive for all MSSA, MRSA, MSSE and MRSE but Rifampicin was sensitive for MSSA and MRSA only and Clindamycin was sensitive for MSSE only, Gentamycin was sensitive for MSSE and MRSE only whereas Erythromycin and Chloramphenicol had low-sensitivity effects but Tetracycline had non-significant effects on cell viability of biofilm. Ciprofloxacin and Vancomycin showed the best activity against cells of the biofilms and the disrupted biofilms at concentrations above MICs.
Fig. 2: The MIC & MBC & MBEC Values of 24 Hours & 72 Hours of the three phases Planktonic, Biofilm & Disrupted Biofilm Forms of MSSA.
Fig. 3: The MIC & MBC & MBEC Values of 24 Hours & 72 Hours of the three phases Planktonic, Biofilm & Disrupted Biofilm Forms of MRSA.
Fig. 4: The MIC & MBC & MBEC Values of 24 Hours & 72 Hours of the three phases Planktonic, Biofilm & Disrupted Biofilm Forms of MSSE.
Fig. 5: The MIC & MBC & MBEC Values of 24 Hours & 72 Hours of the three phases Planktonic, Biofilm & Disrupted Biofilm Forms of MRSE.
They were also more active than other antibiotics used against biofilms of both *S. aureus* and *S. epidermidis* at 16 and 32 µg/ml respectively. The effect of different antibiotics used in this study on the viability of disrupted biofilms to that of intact biofilms were approximately the same for the isolates with each antibiotic concentration (Figures 2, 3, 4 and 5). The ratio values were similar for the used antibiotics at MICs. At other concentrations, the highest viability ratio was observed with Tetracycline and the lowest with Ciprofloxacin. The viability tests demonstrated resistance of the intact biofilms to antibiotics used indicated by large number of viable cells and also, resistance of the disrupted biofilm compared to the planktonic cells. It is also clear that the disrupted biofilm consists of clumps of larger size compared to that of the planktonic cells.

**DISCUSSION**

Coagulase-negative staphylococci and *S. aureus* (mostly methicillin-resistant) are among the leading causes of nosocomial blood stream infections in the USA [22] with a crude mortality of 21-25%, respectively [23]. *S. epidermidis* is a common cause of blood stream infections associated with indwelling medical devices. Three phases of bacteria were used in this study; planktonic, biofilms and disrupted biofilms. Planktonic cells were used for determination of MICs and MBCs. Biofilm associated infections are more resistant to the effects of antimicrobial agents [24-26]. Bacteria are shed through biofilm disruption, which may result in entrance of biofilm pieces into circulation causing systemic infections.

All isolates in suspension were susceptible to the antibiotics as determined by NCCLS guidelines. Ciprofloxacin and Vancomycin were capable of killing 99.9% of the all clinical isolates species of bacteria MSSA, MRSA, MSSE and MRSE in suspension (Planktonic form) at concentrations up to 4 µg/ml, Erythromycin, Rifampicin were capable of killing MSSA and MRSA but Chlindamycin killing MSSE while Gentamicin, Chloromphenicol and Tetracycline antibiotics did not show such effect even at the maximum concentration used.

At the MICs, the antibiotics exerted little effect on the viability of the intact and disrupted biofilms. As in suspension for Planktonic form, The MIC<sub>90</sub>s were 2-4 times lower than the MBCs and 16-32 times lower than the MBEcs (for biofilm) for Ciprofloxacin, Rifampicin, Chlindamycin and Chloromphenicol, but The MIC<sub>90</sub>s were 2 times lower than the MBCs and 8-16 times lower than the MBEcs (for biofilm) for Vancomycin, Erythromycin, Gentamicin and Tetracycline. At higher concentrations, the intact biofilms were with same resistant as the disrupted biofilms. For better comparison, the viability ratios of the disrupted to the intact biofilms were calculated at different concentrations of the antibiotics. Tetracycline was less efficient in killing bacterial cells in intact or disrupted biofilms which explains its highest viability ratio. Ciprofloxacin with the lowest viability ratio was more active against the cells of the disrupted biofilms at concentrations above MICs for both *S. aureus* and *S. epidermidis*. It was also more active than vancomycin against biofilms of both (Methicillin Resistance) *S. aureus* and *S. epidermidis* at 16 and 32 µg/ml respectively. On the other hand, Vancomycin at 8 and 16 µg/ml was more active against the biofilms of MSSA, MRSA respectively and *S. epidermidis* (MSSE) but not MRSE than other antibiotics used in this study.

It has been reported that vancomycin accumulates at high concentration in the biofilms of Gram positive bacteria, especially *S. epidermidis* [27]. This may be attributed to the ability of glycopeptides to bind to exopolysaccharides produced by the bacteria. However, such high concentrations of vancomycin or Ciprofloxacin are not achievable in clinical practice.

In general, our data show that Ciprofloxacin is more active than vancomycin against the intact and the disrupted biofilms and there was small difference between methicillin-susceptible and methicillin resistant staphylococci.

We conclude that the difficulty in treating the infections related to indwelling medical devices may be due to converting of the free floating bacteria to the high resistance biofilm form of bacteria and the lack of eradication of the cells in the Biofilm phase as they were treated simply as if they were free floating bacteria.

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