N-acetylcysteine and vancomycin alone and in combination against staphylococci biofilm

Bruna Leite, Fernanda Gomes, Poliana Melo, Clovis Souza, Pilar Teixeira, Rosário Oliveira, Elisabeth Pizzolitto*

Abstract Introduction: The ability of staphylococci to produce biofilm is an important virulence mechanism that allows bacteria both to adhere and to live on artificial surfaces and to resist to the host immune factors and antibiotics. Staphylococcal infections have become increasingly difficult to treat due their antibiotic resistance. Therefore, there is a continuous need for new and effective treatment alternatives against staphylococcal infections. The main goal of this study was to test N-acetylcysteine (NAC) and vancomycin alone and in combination against S. epidermidis and S. aureus biofilms. Methods: Biofilms were treated with NAC at minimum inhibitory concentration (MIC) and 10 × MIC concentrations and vancomycin at MIC and peak serum concentrations. Results: The use of NAC 10 × MIC alone showed a significant antibactericidal effect, promoting a 4-5 log10 CFU/mL reduction in biofilm cells. The combination of NAC 10 × MIC with vancomycin (independently of the concentration used) reduced significantly the number of biofilm cells for all strains evaluated (5-6 log10). Conclusion: N-acetylcysteine associated to vancomycin can be a potential therapeutic strategy in the treatment of infections associated to biofilms of S. epidermidis or S. aureus.

Keywords N-acetylcysteine, Vancomycin, Biofilm, Staphylococcus epidermidis, Staphylococcus aureus.

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Introduction

*Staphylococci* are the most frequent causative agents of nosocomial infections and infections on medical devices (Otto, 2008). Their ability to form biofilm on surfaces, either biotic or abiotic, seems to be critical for the establishment of infections and to contribute to their persistence by protecting cells from antibiotics and host defenses (Costerton et al., 1999; Dominguez-Herrera et al., 2012; Mah and O’Toole, 2001). *S. epidermidis* is able to form biofilms on implanted medical devices namely central venous catheter, urinary catheters, prosthetic heart valves, orthopedic devices, contact lenses, being also responsible by chronic infections such as sepsis and endocarditis (Cargill and Upton, 2009). *Staphylococcus aureus* has been recognized as a common cause of human disease (Lowy, 1998) and, as in the origin of a wide range of infections, from minor skin infections, chronic bone infections until to devastating sepsis and endocarditis (Chambers, 2005; Charles et al., 2004; Davis, 2005; Gosbell, 2005; Howden et al., 2004). *S. aureus* are also associated with infections related to artificial devices (Cramton and Gotz, 2004).

Biofilm formation is nowadays seen as the main virulence mechanism evidenced by staphylococci. The presence of polymeric matrix in biofilms is preponderant to bacterial adhesion and produces a complex structure that makes the eradication of microorganisms more difficult (Boles and Horswill, 2011; Kloos and Bannerman, 1994). Bacterial biofilms are difficult to detect in routine diagnostics and are inherently tolerant to host defenses and conventional antibiotic therapies (McCann et al., 2008). Additionally, the emergence of antibiotic-resistant bacteria and the slow progress in identifying new classes of antimicrobial agents have encouraged research into novel therapeutic strategies (Costerton et al., 2009). One of the approaches to solve this problem is the use of antimicrobial agent combinations because the development of resistance is reduced when drugs are combined (Blaser et al., 1995; Chuard et al., 1991; Gagnon et al., 1992; Lucet et al., 1990).

Moreover, combinatorial therapies are used with the aim of find a more effective alternative to antibiotics alone since combinations may allow the enhancement of the effects of individual antimicrobial agents by synergistic action.

N-acetylcysteine (NAC) is a non-antibiotic drug that has antibacterial properties (Perez-Giraldo et al., 1997). NAC affects several processes that are important for bacterial biofilm formation on stainless steel surfaces, including a drastic reduction in extracellular polysaccharide production, and thus acts as an antibiofilm substance (Olofsson et al., 2003). On the other hand, vancomycin is a glycopeptide that inhibit cell wall synthesis of *S. aureus* and other Gram-positive organisms (Pootoolal et al., 2002). Then, the aim of this work was to determine the *in vitro* effect of two antimicrobial agents with different mode of action, alone and in combination, against biofilms of *S. epidermidis* and *S. aureus*.

Methods

**Bacterial strains and growth conditions**

*S. epidermidis* (5 strains- 1, 127, 165, 9142 and 1457) and *S. aureus* (5 strains- 2, 35, 73, 173 and 174) clinical isolate strains were used in this study. The culture media used (*tryptic soy broth* (TSB), *tryptic soy agar* (TSA) and Mueller-Hinton agar) were prepared according to the manufacturer instructions. All strains were inoculated into 15 mL of TSB from TSA plates not older than 2 days and grown for 18 (± 2) h at 37 °C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 5 min at 9500 g and 4 °C), and ressuspended in TSB adjusted to 0.5 McFarland scale equivalent to 1.5 × 10⁶ cells/mL before being used in the subsequent assays. Each solution of NAC (MIC and 10 × MIC) was prepared in TSB and the solution of vancomycin (MIC and peak serum (Cerca et al., 2005)) was prepared in Milli-Q water.

**Vancomycin MIC determination: E test**

The E test was used to evaluate the susceptibility of all staphylococci strains tested to vancomycin. The cell suspension (1.5 × 10⁸ cells/mL), prepared with *tryptic soy broth* or *S. aureus*, was applied with a sterile cotton swab onto Petri plates containing Mueller-Hinton agar. The antibiotic carriers (E test strip) were then applied to each plate. Plates were incubated at 37 °C for 24 h. After incubation, an elliptical zone of growth inhibition was seen around the strip. The MIC was read from the scale at the intersection of the elliptical zone with the strip.

**N-acetylcysteine MIC determination: Microbroth dilution method**

For all strains of staphylococci, the N-acetylcysteine MIC determination was carried out in a dilution range of 0.5-64 mg/mL. The MIC was determined in 96 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 100 µL of a stock solution of NAC diluted with TSB and by adding at the end 100 µL of a cell suspension (1 × 10⁶ cells mL⁻¹). Plates were incubated at 37 °C with orbital shaking at 130 rpm for 24 h. After incubation the minimum inhibitory concentration was...
determined by identifying the lowest concentration able to inhibit bacterial growth. The controls were cells not exposed to the antimicrobial agent tested. All experiments were carried out in triplicate and repeated three times.

**Congo red agar test**

The Congo red agar is made up of brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo red dye 0.8 g/L. All cell suspensions (1.5 x 10^6 cells/mL) were seeded separately on plates containing the Congo red agar. Plates were incubated at 37 °C for 24 h. After incubation, the staphylococci that produced black colonies were considered positive slime producers, whereas the colonies that showed red color were considered negative.

**Expression of gene icaD**

**Isolation of genomic DNA**

*S. epidermidis* and *S. aureus* chromosomal DNA was extracted with Invitrogen Invitrek™ kit (Uniscience, Brazil).

**Polymerase chain reaction-PCR**

The primers used for the amplification of icaD genes were designed from the published sequence of the ica locus described by Cramton and Gotz (2004). For the detection of icaD, primers iCADF (ATG GTC AAG CCC AGA CAG AG) and iCADR (CGT GTT TTC CCC AGA CAG AG) were used for a 198 bp fragment. A 20 µL reaction volume consisted of 2.5 mM MgCl2, 200 mM of each nucleotide, 1 mM of each primer, 1.25 U of Taq polymerase and 100 ng of template DNA. Thirty cycles of amplification, each consisting of denaturation at 92 °C for 45 s, annealing at 49 °C for 45 s and elongation at 72 °C for 1 min, along with a final extension at 72 °C for 7 min were performed in a thermocycler (Eppendorf, USA). The presence and size of the amplified products were confirmed by electrophoresis on 1.5% agarose gel.

**Effect of NAC and vancomycin on biofilm cells**

Biofilms were formed in 96 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 200 µL of a cell suspension (1 x 10^6 cells/mL) in TSB supplemented with 0.25 % of glucose per well to promote biofilm formation. Plates were incubated at 37 °C with orbital shaking at 130 rpm for 24 h. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 200 µL of saline solution (0.9% NaCl; Merck). The biofilms were incubated for 24 h in fresh nutrient medium containing NAC (MIC and 10 x MIC) and vancomycin (MIC and peak serum) alone and in combination.

To assess the number of viable cells after treatment, 200 µL of saline solution were added to each well before removing the biofilm by scraping. An aliquot of 1 mL of each sample was centrifuged (for 10 min at 9500 g and 4 °C) and the pellet resuspended in 1 mL of saline solution. Next, the suspension was sonicated (20 s with 22% of amplitude; Ultrasonic Processor, Cole-Parmer, Illinois, USA) to promote biofilm disruption. The number of colony forming units (CFU) in biofilm for each strain evaluated was determined by performing 10-fold serial dilutions in saline solution and plating in TSA in triplicate and incubating for 48 h.

Biofilm controls were cells not exposed to any antimicrobial agent tested. All experiments were carried out in triplicate and repeated three times.

**Statistical Analysis**

The data from all assays were compared using one-way analysis of variance (ANOVA) by applying Tukey’s test with all calculations carried out using SPSS software (Statistical Package for the Social Sciences). Differences achieving a confidence level of 95 % were considered significant.

**Results**

Table 1 summarizes the values of vancomycin minimum inhibitory concentration obtained by E test and NAC minimum inhibitory concentration determined by the microbroth dilution method, for all the strains of staphylococci tested in this study. On the other hand, Table 2 shows the production of slime by Congo red agar and the expression of icaD gene in the strains of staphylococci evaluated. Slime production was detected in three strains (60 %) of *S. epidermidis* and in all *S. aureus* strains (100 %). The icaD gene was expressed in all strains of staphylococci evaluated.

Figures 1 and 2 present the number of viable cells (after exposure to the two antimicrobial agents tested alone) of *S. epidermidis* and *S. aureus* strains tested, respectively, expressed as log CFU/mL. The results evidenced that vancomycin alone causes no effect on the staphylococci strains evaluated, since the reduction promoted by this antibiotic was less than 3 log. On the contrary, the use of NAC alone showed to reduce the number of cells in biofilms of staphylococci strains. In fact, the use of NAC 10 x MIC reduced significantly the cells in biofilm, evidencing a reduction of circa of 4-5 log CFU/mL (p < 0.05).

Figures 3 and 4 present the number of viable cells (after the treatment with the two agents in
Table 1. Values of minimal inhibitory concentration by E test (vancomycin) and microbroth dilution method (MBD) (NAC) for the staphylococci strains studied.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of laboratory identification</th>
<th>E test (vancomycin)</th>
<th>MBD (NAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>9142</td>
<td>8 mg/mL</td>
<td>4 mg/mL</td>
</tr>
<tr>
<td></td>
<td>1457</td>
<td>8 mg/mL</td>
<td>4 mg/mL</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>4 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>3 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2</td>
<td>2 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>1.5 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>173</td>
<td>2 mg/mL</td>
<td>8 mg/mL</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>2 mg/mL</td>
<td>8 mg/mL</td>
</tr>
</tbody>
</table>

Figure 1. Effect of NAC and vancomycin (VAN) alone on biofilm cells of *S. epidermidis*. *Antimicrobial effect statistically different from untreated cells (p < 0.05).*

Figure 2. Effect of NAC and vancomycin (VAN) alone on biofilm cells of *S. aureus*. *Antimicrobial effect statistically different from untreated cells (p < 0.05).*
Table 2. Slime production determined by the method of Congo red agar and icaD gene expression.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of laboratory identification</th>
<th>Congo red agar (black or red colonies)</th>
<th>Gene expression of icaD (present or absent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>9142</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>1457</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Red</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>Red</td>
<td>Present</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>173</td>
<td>Black</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>Black</td>
<td>Present</td>
</tr>
</tbody>
</table>

Figure 3. Effect of NAC and vancomycin (VAN) in combination on biofilm cells of *S. epidermidis*. *Combinations statistically different from control (p < 0.05).

Figure 4. Effect of NAC and vancomycin (VAN) in combination on biofilm cells of *S. aureus*. *Combinations statistically different from control (p < 0.05).
combination of S. epidermidis and S. aureus strains tested, respectively, expressed as log CFU/mL. It can be observed that the combination of NAC at 10 × MIC with vancomycin (independently of the concentration used) showed a notorious effect on biofilm cells of all strains of S. epidermidis and S. aureus evaluated, promoting CFU reductions of about 5-6 log_{10} (p < 0.05).

Discussion

Bacterial cells have grown in biofilm for billions of years, as a part of their successful strategy to colonize most of this planet and most of its life forms. In fact, this is the predominant mode of growth of bacteria in natural environment. Their distinct phenotype makes them resistant to antimicrobial agents, and their matrix makes them tolerant to the antibacterial molecules and immune system cells mobilized by the host. The antimicrobial molecules must diffuse through the biofilm matrix in order to inactivate the encased cells. Thus, the extracellular polymeric substances constituting this matrix present a diffusional barrier for these molecules by influencing either the rate of transport of the molecule to the interior of the biofilm or the reaction of the antimicrobial agent with the matrix material (Donlan and Costerton, 2002; Presterl et al., 2009).

The ability of staphylococci cells to adhere and form biofilm is mediated by the production of the polysaccharide intercellular adhesin (PIA). Their synthesis is encoded by the gene product of ica locus of the operon icaADBC, being the product of this locus essential for biofilm formation and virulence of microorganisms (O’Toole et al., 2000). Arciola et al. (2001) work corroborates with the former statement, showing in their studies that the gene icaA and icaD have an important role in biofilm formation of S. epidermidis and S. aureus. The icaD gene was evaluated in this study and was reported to be expressed in all strains of S. epidermidis (100%) and S. aureus (100 %) evaluated. Another indicator of virulence is the Congo red reaction that provides information about the ability of microorganisms to produce slime (Prpic et al., 1983; Bernardi et al., 2007). This study showed that three S. epidermidis strains (9142, 1457 and 127) and all S. aureus strains evaluated were slime producers.

Several authors have reported that the development of resistance is reduced when antibiotics are combined (Moellering, 2008; Perlroth et al., 2008). Taking into consideration this notion and aiming to overcome the current problem of the emergence of resistance several researchers focused their studies in the use of combinations. This study evaluated the susceptibility of biofilm cells of S. epidermidis and S. aureus to NAC and vancomycin alone and in combination. NAC was choose due to its potential role in biofilm matrix being able to reduce the extracellular polysaccharide content of biofilm (Olofsson et al. 2003). Our results demonstrated that NAC-vancomycin exhibited a significant antimicrobial effect on biofilm cells of all strains studied. This was possibly due to the degradation of the matrix promoted by NAC which facilitated the penetration into the biofilm and the action of vancomycin being then able to reduce the viable number of bacterial cells (Dunne et al., 1993; Wilcox et al., 2001). As we have previously hypothesized this combination has a synergistic effect due to their different modes of action, acting into two different components of the biofilm namely the matrix and the cellular content. The use of vancomycin alone was ineffective against biofilm cells of the isolates studied, whereas NAC demonstrated a significant inhibitory action expressed by reductions of viability of circa 4-5 log_{10} CFU/mL (Figures 1 and 2). As described by several authors, antibiotics at MIC concentration cause no killing effect on bacteria when growing in biofilm communities (Tetz et al., 2009). Our results are in accordance with the previous researchers since NAC and vancomycin alone or in combination both at MIC concentration caused no killing effect on staphylococci cells, showing a viable cells reduction of less than 3 log_{10}. On the contrary, when exposed to NAC-vancomycin (NAC 10 × MIC combined with vancomycin independently of the concentration used) a high antibacterial activity was observed, reaching a reduction on viability cells of about 5-6 log_{10} CFU/mL. Other studies were performed with the objective to find new therapeutic strategies using combinatorial approaches and using NAC or vancomycin but no promising results were obtained. As example, Gomes and co-workers demonstrated that no synergy was observed combining farnesol with vancomycin (Gomes et al., 2011) and NAC with farnesol (Gomes et al., 2012) on biofilms of S. epidermidis. The combination of NAC and tigecycline on S. epidermidis biofilms was reported by Aslam et al. (2007) and using NAC at 20 × MIC (80 mg/mL) and tigecycline at 1000 × MIC (1 mg/mL), neither synergy nor an effective activity was also observed (reduction of less than 3 log_{10}). On the other hand and supporting our results Abbas et al. (2012) studied the effect of NAC on biofilm cells of P. aeruginosa and showed that NAC has a moderate effect against this pathogen.
Zhao and Liu (2010) also proved the inhibitory effect of this compound against the previous microorganism expressed by the antibacterial activity and detachment of biofilm cells and defend that its action is caused by competitively inhibiting cysteine utilization in bacteria or by reaction of its sulfhydryl group with bacterial cell proteins. El-Feky et al. (2009) investigated the effect of NAC (2 and 4mg/mL) in combination with ciprofloxacin (MIC and 2 × MIC) against biofilm cells of several bacteria including S. aureus, S. epidermidis, E. coli, and others. The authors concluded that NAC potentiate the therapeutic action of ciprofloxacin. This was due to the ability of NAC to degrade the extracellular polysaccharide matrix of biofilms. As general conclusion they suggest the potential use of ciprofloxacin-NAC combination as therapeutic strategy against bacterial infections mediated by biofilms.

In general, it is believed that NAC is able of acting at the level of the matrix promoting the increasing therapeutic efficacy of vancomycin, which alone had no significant effect on cells in biofilms. This activity of NAC can result in the detachment of cells, individually or in clusters, becoming the biofilm and detached cells more susceptible to the action of other antimicrobial agents and to the immune system. Thus, the role of NAC and other antimicrobial agents targeting matrix and promoting this mechanism of detachment of cells can be an important help in the eradication of biofilms associated infections.

In conclusion, the results of the present study show excellent activity in vitro of NAC (10 × MIC) alone and in combination with vancomycin (MIC or peak serum) on S. epidermidis and S. aureus biofilms. The results indicate that this combination may represents a novel therapeutic option for infections caused by staphylococci, especially by strains able to form biofilm on biotic or abiotic surfaces, because the use of antimicrobial agents in combination as described by other authors reduces the development of resistance.

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