Influence of subminimal inhibitory concentrations of some bioactive compounds on the biofilm formation ability and virulence factors of multiple drug resistant *Acinetobacter baumannii* clinical isolates

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The objective of this study was to assess the impact of subminimum inhibitory concentrations (sub-MICs) of chosen antimicrobial agents (Amikacin, imipenem, benzalkonium chloride, and chlorhexidine) and natural product (garlic) on biofilm formation ability, bacterial adherence and invasion. Susceptibility profiles of 50 non-repetitive *Acinetobacter baumannii* clinical isolates to eight antibiotics were investigated. MIC of various antibiotics, antiseptics and garlic were measured by the broth microdilution method. Quantification of biofilm formation was carried out using a microtiter plate assay. The ability of test compounds to affect the bacterial adherence and invasion was investigated using Type II pneumocyte cell line (A549) and the bacterial cells count was determined using flow cytometer. Screening for the presence of antiseptic resistant gene *qacA/B* was done using PCR. Ten isolates exhibited variable susceptibilities toward both amikacin and imipenem. The sub-MICs of benzalkonium chloride (BZC) markedly increased the biofilm formation. Additionally, amikacin, applied at sub minimal inhibitory concentrations, showed the highest induction in the bacterial adherence post treatment. Significant increase in bacterial invasion 3 h post treatment was detected upon applying BZC (616%) and imipenem (324%). BZC showed the greatest effect on the bacterial invasion. The highest impact on the bacterial invasion in case of 5 h post treatment was evident with the use of both BZC and Garlic. Results demonstrated that antibiotics, antiseptics and natural product at sub-MICs increased significantly the biofilm formation ability, bacterial adherence and invasion of *A. baumannii* clinical isolates, therefore careful consideration of sub-MIC effects is mandatory before their use.

**Key words:** *A. baumannii*, Sub-MICs, biofilm formation, bacterial adherence and invasion, *qac A/B*

**INTRODUCTION**

Multiple drug resistant *Acinetobacter baumannii* is now known as one of the most opportunistic pathogens for the health care centers throughout the world, though it was regarded as a low-virulence bacterium before (Peleg et
al., 2008; Antunes et al., 2014). It has been involved in numerous health-care associated infections integrated with high morbidity and/or mortality rates, involving ventilator-associated pneumonia, wound infections, bloodstream infections and meningitis (Peleg et al., 2008). The resistance to many antimicrobial agents and biofilms formation ability on both biotic and abiotic surfaces play principal part in A. baumannii pathogenicity whereas multiple clinical strains have the ability to attach their planktonic cells to different hydrophilic or hydrophobic substrata; hospital equipment, indwelling medical devices and bronchial epithelial cells, and growing as biofilms (Longo et al., 2014).

Chlorhexidine is a cationic biguanide and benzalkonium is a nitrogen-based quaternary ammonium compound; they work similarly by affecting the cell membrane, producing lysis of cytoplasmic material (Poole et al., 2002). The RND-type MexCD-OprJ multidrug efflux pump is induced by sub-inhibitory concentrations of disinfectants as benzalkonium chloride or chlorhexidine (Morita et al., 2003).

Among the numerous medicinal plants, garlic has an antimicrobial feature used for centuries to combat infectious diseases, emphasizing the significance of search for natural antimicrobial drugs (Wojdylo et al., 2007).

Multiple researches had revealed its efficacy and broad spectrum antimicrobial activity against many species of bacteria, viruses, parasites, protozoan and fungi (Jaber and Al-Mosawi, 2007). Although, as previously mentioned, antibiotics reduce bacterial biofilm formation, conversely, subminimal inhibitory concentrations (subMICs) of some antibiotics can even induce its formation as it allows susceptible strains to continue to grow, which sometimes results in a reduced growth rate compared with the growth rate that is observed in the absence of the drug, and thus may play a significant role in colonization and progression to the development of acute or chronic infection (Hoffman et al., 2005; Kaplan, 2011; Nucleo et al., 2009).

With the fact that data about the effect of subminimal inhibitory concentrations of antibiotics, antiseptics and natural product on the biofilm formation ability and virulence factors of A. baumannii are scarce, this study aimed to investigate the effect of sub-MICs of selected antimicrobial agents (amikacin, imipenem, benzalkonium chloride, and chlorhexidine) and natural product (garlic) on biofilm formation ability, bacterial adherence and invasion and the dissemination of resistance between multiple drug resistant A. baumannii recovered from hospitalized patients.

MATERIALS AND METHODS

Bacterial strains

Fifty non-duplicate A. baumannii strains collected from various clinical specimens recovered from hospitalized patients, admitted to El Demerdash hospital (Ain shams, Egypt) and Arab contractor's medical center (Cairo, Egypt) in the period from January 2015 to March 2016 were included in the study. Identification of isolated organisms was performed by conventional biochemical reactions and confirmed by API 20NE (bioMérieux, Marcy l’Etoile, France). All strains were grown in Luria–Bertani (LB) broth with aeration or on TSB agar plates at 37°C. When appropriate, LB plates were supplemented with 25 µg chloramphenicol ml⁻¹ (Sigma) to select for A. baumannii strains. Strains were stored as glycerol stocks at −80°C for further analyses.

Cell line

Type II pneumocyte cell line A549 derived from a human lung carcinoma (LGC Standards, United Kingdom) were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen-Spain). The cells were seeded 24 h in 24 well plates prior to infection with A. baumannii strains and incubated at CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) till confluency.

Antimicrobial susceptibility testing

Susceptibility of the isolates to the following antibacterial agents amikacin, meropenem, cefotaxime, imipenem, doxycycline, levofloxacin, Gentamicin and trimethoprim-sulfamethoxazole were tested by the Kirby-Bauer disc diffusion method (Bauer et al.,1966) using disks (Oxoid ltd., Basin Stoke, Hants, England) on Mueller Hinton agar and interpreted as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2015).

Determination of MIC

The MIC of various antibiotics against A. baumannii strains was determined using a broth dilution method. A broth microdilution assay was performed using serial 2-fold dilutions of each antibiotic in TSB (LabM, UK). The range of antimicrobial concentrations used were 0.152 to 312.5 μg/ml (amikacin, smithkline beecham. Egypt L.L.C) and 0.152 to 312.5 μg/ml (imipenem, MERCK and Co. - USA). The MICs of antibiotics were determined and the breakpoints used for the antibiotics tested were in accordance with CLSI performance standards for antimicrobial susceptibility testing (CLSI, 2015).

Antiseptic susceptibility

Both antiseptics chlorhexidine (CLX) and benzalkonium chloride (BZK) were purchased from Sigma Aldrich (St Louis, MO, USA). MICs of antiseptics were determined by the broth microdilution method according to CLSI (CLSI, 2015). Since there was no

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standard breakpoint available for antiseptics against *A. baumannii*, 2 fold dilutions from 12.5 to 0.0122% w/v (benzalkonium chloride) and 0.076 to 156.25 µg/ml (chlorhexidine) were tested.

Standard bacterial concentration of 0.5 Mc Farland standards (1.5 x 10⁸ CFU/mL) was used. Briefly, 100 µl of stock antiseptics was added to well one. Then 50 µl of TSB was added to all wells. Upon mixing well, 50 µl was transferred to next well and continued until last well. Fifty microliters of bacterial suspension was added from well one to twelve in a 96 well plate. Susceptibility was interpreted based on the turbidity on the inoculum after incubation at 37°C for 24 h (Babaei et al., 2015).

**Determination of MIC for garlic extract**

Garlic extract was prepared according to Rasmussen et al. (2005), where 150 g of garlic cloves was shredded with kitchen blender with 300 ml of toluene (Sigma Aldrich, St Louis, MO, USA) and left overnight, then the suspension was filtered through Whatman no. 1 filter paper. Sterile water was added as 150 ml and the mixture was stirred for 24 h at room temperature, where two phases were formed. The aqueous phase was separated from the organic phase and allowed to evaporate using rotavap (Heidolph, USA) evaporator to obtain 100 mg dried garlic extract, which dissolved in 2ml saline so that the stock concentration was 50000 µg/ml and filtered using 0.22 nm syringe filter.

MIC for garlic extract was determined by the broth microdilution method according to CLSI (2015). A two-fold dilution was tested from 12500 to 98 µg/ml and a standard bacterial concentration of 0.5 McFarland standard (1.5 x 10⁸ CFU/mL) was used. Susceptibility was interpreted based on the turbidity on the inoculum after incubation at 37°C for 24 h (Rasmussen et al., 2005).

**Biofilm formation assay**

The ability of *A. baumannii* strains to form biofilms was tested using a modification of the Calgary biofilm method. Briefly, bacterial strains were grown overnight at 37°C and were diluted in sterile saline to a concentration of 0.5 McFarland standard. Fifty microliter volumes of each culture were added to wells of a 96-well plate with 50 µl of subMICs of each antimicrobial agent. Plates were inoculated with 100 µl well of TSB and incubated at 37°C for 24 h.

The medium and planktonic cells were removed and the biofilms were washed three times with 200 µl phosphate buffer saline (PBS) using gentle pipetting then, the plates were left for air drying. Biofilms were then stained using 100 µl well 0.1 % (v/v) crystal violet solutions for 20 min. The plates were washed under slowly running tap water to remove excess free dye and the water drained by tapping onto paper towels. This process was repeated several times until the water run clear. The plate was then air-dried and the biofilm was destained by adding 200 µl well 95% ethanol for 30 min at room temperature. The optical density (OD) was measured at 540 nm using Microplate Reader (BioTek-USA) (Wand et al., 2012).

**Bacterial adherence assay**

The impact of the chosen bioactive compounds on the bacterial adherence was assessed, where Human lung epithelial cells was counted as 1.1 x 10⁵ cells/ml and cultured in 24 well cell culture plates. On confluency growth, media was discarded and 50 µl of prepared bacterial suspension adjusted at 0.5 McFarland (MOI = 74:1) were added to each well as well as 50 µl of subMICs of the antimicrobial agents, DMEM was added as 100 µl and the plates were incubated in 5% CO₂ incubator at 37°C for 1h.

Post incubation period, the non-adherent bacterial cells were removed by washing plates five times with Phosphate buffer saline (PBS). Cells were lysed using 100 µl 0.1% triton X-100 at 37°C for 30 min. PBS was added to all wells as 500 µl/well. The flow cytometer (Invitrogen™ Attune™ NXT Thermo Fisher Scientific) was used to count the bacterial cells in each well and compared to negative control (Letourneau et al., 2011).

**Bacterial invasion assay**

Twenty-four well A549 pre-cultured plates were treated with sub MIC concentrations of antimicrobial agents and incubated at 37°C for 3 and 5 h interval. In post incubation period, plates were washed five times with PBS Gentamicin (Sigma-Aldrich, St Louis, MO, USA).

300 µg/ml prepared was inoculated as 300 µl/well and plates were incubated at 37°C for 2 h. Gentamicin was discarded and cells were washed five times with PBS, lysed with 0.1% tritonX100 and incubated at 37°C for 30 min. PBS was inoculated as 500 µl/well and the bacterial count was determined in each well using flow cytometer and compared to negative control (Choi et al., 2008).

**PCR assay for qacA/B**

Total genomic DNA was extracted using Genomic DNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer’s procedure, PCR mixture contained 1 µl forward qacA/B primer ATTCATTAGTGGCCCTTGGC and 1 µl reverse qacA/B primer TGGCCCTTCTTATAGGTTT (Sidhu et al., 2002) (10 p mole/µl) is added to 12.5 µl Master Mix. 1 µl of DNA extract were added in a total volume of 25 µl. The conditions for each cycle were denature for 1 min at 95°C, annealing for 1 min at 53°C and primer extension for 2 min at 72°C with reaction mixture incubated at 72°C for 10 min.

Negative and positive controls were involved in PCR experiment. The PCR products were separated by electrophoresis in 1% agarose gel (Fermentas, Lithuania) and visualized under UV light.

**Statistical analysis**

All experiments were repeated three independent times and results were represented as mean ± standard deviation. The statistical significance of the data was determined by the Student’s t-test. Level of statistical significance was set at p ≤ 0.05.

**RESULTS**

**Antibiotic susceptibility test**

The resistance rates of *A. baumannii* to antibiotics are shown in Table 1. High multi-resistance to antibiotics tested was observed among the isolates. Among the 8 antimicrobial agents tested, the frequencies of resistances were as follows: cefotaxime (98%), meropenem (88%), doxycycline (88%), imipenem and levofloxacin (86%), gentamycin (84%), trimethoprim-sulfamethoxazole (74%) and amikacin (72%).

These results also shows that resistance to
carbapenems (imipenem) is significantly associated with resistance to aminoglycosides (amikacin) in *A. baumannii* isolates, where 6% of the isolates were sensitive to both antibiotics, 28% of the isolates were sensitive to one antibiotic and resistant to the other. On the other hand, 66% of the isolates were resistant to both antibiotics suggesting an associated resistance possibly present between imipenem and amikacin.

Out of the fifty isolates, ten isolates were found to have variation in their susceptibility toward both Amikacin and Imipenem where six isolates were amikacin sensitive imipenem resistant (ASIR) and four were amikacin resistant imipenem sensitive (ARIS); therefore these 2 groups were chosen to perform the following assays on them.

**MIC detection**

MIC was done by broth microdilution and the results of the tested antimicrobials were quite variable in the range of 1.22 to 78.13 µg/ml (imipenem); 4.88 to 312.5 µg/ml (amikacin); 4.88 to 39.06 µg/ml (chlorohexidine); 390.6 to 3125 µg/ml (garlic) and 0.78 to 3.125 % w/v (benzalkonium chloride) (Table 2).

**Sub-MICs for selected antimicrobial agents**

Based on the MICs obtained, the subMIC for each antimicrobial was calculated where the 3/4 MIC was considered as subMIC (Table 3). The SubMIC values of the antimicrobials tested were quite variable in the following ranges: 0.92 to 58.59 µl/ml (IMP); 3.66 to 234.38 µl/ml (AMK); 3.66 to 29.30 µl/ml (CHX); 293 to 2344 µl/ml (garlic) and 0.59 to 3.2.34 µl/ml (BZC).

**Effect of sub-MIC of antimicrobials on biofilm formation ability of *A. baumannii***

There was statistical significance increase in the biofilm formation upon addition of the subMIC concentrations of benzalkonium chloride by 354% for ASIR group and
Table 3. SubMICs obtained for the selected isolates against the 5 chosen antimicrobials.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Garlic (µg/ml)</th>
<th>CHX (µg/ml)</th>
<th>AMK (µg/ml)</th>
<th>IMP (µg/ml)</th>
<th>BZC (%w/v)</th>
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<tbody>
<tr>
<td>304</td>
<td>586</td>
<td>7.32</td>
<td>29.32</td>
<td>3.66</td>
<td>0.59</td>
</tr>
<tr>
<td>307</td>
<td>2344</td>
<td>3.66</td>
<td>234.38</td>
<td>0.92</td>
<td>0.59</td>
</tr>
<tr>
<td>310</td>
<td>2344</td>
<td>3.66</td>
<td>234.38</td>
<td>0.92</td>
<td>0.59</td>
</tr>
<tr>
<td>311</td>
<td>1172</td>
<td>7.32</td>
<td>29.32</td>
<td>7.32</td>
<td>0.59</td>
</tr>
<tr>
<td>315</td>
<td>293</td>
<td>3.66</td>
<td>7.32</td>
<td>14.65</td>
<td>0.59</td>
</tr>
<tr>
<td>316</td>
<td>586</td>
<td>29.30</td>
<td>7.32</td>
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Figure 1. The effect of subMICs of different antimicrobials and garlic extract on the biofilm formation ability of ASIR and ARIS A. baumannii isolates.

657% in ARIS group compared with control treatment (Figure 1).

Effect of Sub-MIC of antimicrobial agents on bacterial adherence

Induction in bacterial adherence was detected in ASIR group in the order of 70, 216, 160, 117 and 67% post treatment with benzalkonium chloride, amikacin, imipenem, chlorhexidine and garlic extract compared to control, respectively. Additionally, marked increase in bacterial adherence by 63, 213, 24, 92 and 21%, respectively was also observed with the ARIS group. The highest induction in bacterial adherence was observed with the use of amikacin, where it significantly increased the ability of ARIS and ASIR groups to adhere to A549 cells by 213 and 216%, respectively (Figure 2).

Effect of sub-MIC of antimicrobials on the bacterial invasion

Tested antimicrobials as well as garlic extract, markedly increased bacterial invasion 3 h post treatment where it was found that, benzalkonium chloride and imipenem increased the bacterial invasion by 616 and 324%
respectively in ASIR group compared with control treatment. The greatest effect on bacterial invasion in ARIS group was observed upon applying benzalkonium chloride where it significantly increased invasion by 1108% compared to untreated isolates (P<0.05) (Figure 3).

A low increase in the bacterial invasion to A549 cells was detected 5 h post treatment with the tested antimicrobials; the highest increase was observed in case of amikacin in ASIR group (122%) compared to control, whereas the effect of garlic, BZC and amikacin on the bacterial invasion in the ARIS group were as follows: 234, 177 and 107%, respectively (P<0.05) [Figure 4].

**PCR assay for qacA/B**

Genotypic analysis of qacA/B gene by PCR revealed the amplification of 217 bp fragment in 6 isolates (307, 310, 311, 316, 317 and 340) out of 10 A. baumannii isolates.
DISCUSSION

Sub-MIC antibiotic concentrations generally have a range of important downstream effects, which produces clinically relevant alterations in bacterial behaviour as increased drug resistance. The induction of biofilm formation and the expression of virulence genes; antibiotics at sub-MIC levels are widely distributed in in vivo and ex vivo environments having the ability of both enriching for resistant bacteria and selecting for de novo resistance (Andersson and Hughes, 2014).

In this context, the current study aimed to evaluate the effect of subminimum inhibitory concentrations of commonly used biocides (chlorhexidine and BZC), antibiotics (amikacin and imipenem) and natural product (garlic) on A. baumannii virulence factors and the emergence of resistance among clinical isolates.

In the current study, fifty MDR A. baumannii strains were isolated from different clinical specimens during the study period. High multi-resistance to antibiotics tested representing four antibiotic classes (fluoroquinolones, aminoglycosides, cephalosporins, and beta-lactam/beta-lactamase inhibitor combinations) was observed among all the clinical isolates included in the study. This high capability of A. baumannii to acquire resistance to different classes of antimicrobial agents could be rationalized by up-regulation of intrinsic resistance mechanisms besides gaining determinants of resistance (Nowak et al., 2014). Carbenapens are often used in combination with aminoglycosides in treatment of A. baumannii infections due to their evident synergistic bactericidal activity (Nowak et al., 2014).

The results of antibiotic susceptibility revealed an association in the resistance to carbenapens (imipenem) and aminoglycosides (amikacin), where 66% of the isolates were resistant to both antibiotics, indicating that a cross resistance might exist between imipenem and amikacin. Concurrent resistance was also observed between carbenapen and aminoglycoside among multidrug-resistant A. baumannii clinical isolates in previous studies (Nowak et al., 2014; Cao et al., 2013). Ten isolates were found to have variation in their susceptibility toward amikacin and imipenem in which 6 out of the 10 isolates were amikacin sensitive and imipenem resistant (ASIR) while 4 isolates were amikacin resistant and imipenem sensitive (ARIS), therefore the biofilm formation assay and virulence factors experiments (bacterial adhesion and invasion) were performed on those 10 isolates represented in both groups ASIR and ARIS. Amikacin and imipenem represented the antibiotics, benzalkonium chloride and chlorhexidine were examples of biocides and the garlic were chosen as an example of natural product.

Broth microdilution assay was used to detect the minimum inhibitory concentrations of the chosen antimicrobial agents against the ten selected isolates. The MIC values of the antimicrobials tested were quite variable in the following ranges: 1.22 to 78.13 µl/ml (imipenem); 4.88 to 312.5 µl/ml (amikacin); 4.88 to 39.06 µl/ml (chlorhexidine); 390.6 to 3125 µl/ml (garlic) and 0.78 to 3.125 µl/ml (BZC) (Table 2). In case of amikacin, 2/10 isolates (20%) were resistant with MIC value (312.5 µg/ml) whereas 5 isolates (50%) were sensitive with MIC value (4.88 µg/ml) as well as 2 isolates which showed an intermediate resistance (39.1 µg/ml). Six isolates (60%) were resistant to imipenem with MIC value (78.1µg/ml) whereas 2 (20%) isolates were sensitive with MIC value (1.22 µg/ml) as well as 2 isolates which showed an
intermediate resistance (9.7μg/ml). The 10 selected A. baumannii isolates included in our study showed changeable susceptibility to the used biocides (Chlorhexidine and BZC) which are considered as substrates for efflux pumps. A competitive advantage in isolates that are more resistant to biocides may be suggested due to variability, since they may be more easily selected for in the presence of biocides than those that are more sensitive to them (Naparstek et al., 2012). The Sub-MIC concentrations in the current study were calculated to assess their impact on biofilm formation ability and bacterial adhesion and invasion of the isolates, where 25% below MIC values was considered as sub-MIC as the concentration below this most probably of low effect might be difficult to get significant conclusion, also trends in hospitals to use disinfectants for several times a day which allow high concentration to be the major seen all over the day.

A. baumannii clinical isolates have been noticed to possess a powerful ability and form biofilms (Rodriguez-Bano et al., 2008). Biofilm formation becomes even more uncontrolled in response to sub-inhibitory concentrations of antibiotics, a situation normally encountered as a direct consequence of low-dose therapy (Kaplan, 2011). We determined the efficacy of commonly used antibiotics and other bioactive compounds at their sub-MIC concentration on biofilm formation ability of A. baumannii. Our results showed that sub-MIC of BZC significantly induced the biofilm formation (p<0.05) in the two tested groups ASIR by 354% and ARIS by 657% compared to control treatment. Similar findings were found by Machado et al. (2011) which reported that, the significant increase of biofilm mass due to BZC pressure (5 folds higher) regardless the strain or the number of strains that generate the biofilm are considered as unexpected result (Machado et al., 2012). Biofilm formation was also increased without any effect onto planktonic growth in a study conducted on S. epidermidis where exposure of S. epidermidis to chlorhexidine at 1/2, 1/4 and 1/8 MIC, or BZC at 1/8, 1/16 and 1/32 MIC increased biofilm formation (Houari and Di Martino, 2007). Since the majority of A. baumannii infections affect the pulmonary system (Wong et al., 2017), the epithelial A549 cell line was selected as a model system for testing adherence to, invasion of A. baumannii isolates in vitro.

The results of bacterial adherence assay showed that the Sub-MIC of BZC, amikacin, imipenem, chlorhexidine and garlic extract have significantly induced the bacterial adherence in the ASIR by 71, 216, 164, 117 and 67% respectively compared with control treatment whereas the increase in bacterial adherence in case of ARIS group were as follows 64, 214, 25, 92 and 21% respectively compared with control treatment which has statistically significant result. Gentamicin survival assay was used to test the effect of sub-MIC of chosen antimicrobial agents on the bacterial invasion to A549 cells. There was a significant increase in the bacterial invasion 3 h post treatment in both groups, in case of ASIR isolates there was an increase with BZC, amikacin, imipenem, chlorhexidine and garlic extract by 616, 96, 325, 55 and 110% respectively compared with control treatment and in ARIS isolates there was an increase by 1108, 119, 102, 24 and 26% respectively compared with control treatment. They recorded unexpected very high induction (616 to 1108%) of the bacterial adherence; 3 h post treatment was evident with the use of BZC and could be explained as it might affect the permeability of cell lines causing increase in the uptake of the bacterial cells. It was previously reported that garlic extract could reduce the bacterial invasion due to its quorum sensing blocking properties (Persson et al., 2005). This was not the case in our study where an increase in bacterial invasion was observed with using garlic extract which may be explained by the difference in garlic concentration tested in both studies.

It is also important to point out that the recorded decline in the number of bacterial cells that invade A549 cells following prolonged incubation might be due to the destructive effect of Acinetobacter on A549 cells leading to externalization of intracellular bacteria. Therefore it is recommended that cytotoxic assays should be considered in future studies. The antiseptic resistance gene qacA/B was tested in the 10 isolates by PCR, the resistance gene was present in 6 out of 10 isolates suggesting the presence of other resistance mechanisms such as overexpression of genes, encoding some efflux pumps (AdeB and AmvA) and reduced expression of genes coding for some porins (OmpA and CarO) in the tested isolates with absence of qac A/B. However, the small isolates number used did not allow the evaluation of the emergence of disinfectant resistance genes over time, or assessing the relationship of disinfectant resistance genes and antibiotic resistance.

**Conclusion**

The study showed that sub-MICs of antibiotics and antiseptics can influence bacterial virulence by increasing bacterial biofilm formation, adhesion and invasion. Therefore, the effective concentration of antimicrobials is an essential determinant to overcome resistance as the improper use of these agents could enhance bacterial infection instead of eradicating it.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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