

Susceptibility of catheter-related *Klebsiella pneumoniae* strains to quaternary ammonium compounds under biofilm and planktonic conditions

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ABSTRACT

Background: The aim of this study was to evaluate the susceptibility of catheter-related *Klebsiella pneumoniae* isolates to two biocides (benzalkonium chloride and Deconex) under biofilm and planktonic conditions.

Methods: A total of 85 strains of *K. pneumoniae* were isolated from catheters of inpatients hospitalized in four hospitals in Kerman, Iran. Susceptibility to antibiotics and biocides under biofilm and planktonic growths was performed using the microdilution method. Antibiofilm activity of the biocides was determined by microtiter assay. Biofilm eradication was carried out at different periods of time. The presence of *cepA* and *qacEΔ1* genes were detected by polymerase chain reaction (PCR).

Results: We found that 15% (n = 12) of the isolates showed strong biofilm activity, 40% (n = 35) displayed moderate activity, 30% (n = 26) demonstrated weak activity, and 15% (n = 12) showed no attachment to microtiter wells. Both the biocides had profound inhibitory activities on planktonic cells (average minimum inhibitory concentration [MIC] 0.06 ± 0.2 mg/ml for Deconex and 0.03 ± 0.1 mg/ml for benzalkonium chloride). They exerted the least antibiofilm activity at a sub-MIC concentration of 0.015 mg/ml. The isolates that formed high biofilm also harboured the *cepA* gene. Furthermore, a considerable increase in MIC to piperacillin/tazobactam, tetracycline, and cefotaxime was observed for cells grown in biofilm conditions for 24 hours, but all the isolates were sensitive to colistin and tigecycline. These differences were statistically significant, with a p-value of < 0.05. Most of the biofilms were eradicated from the microtiter plate within 30 minutes' exposure to these biocides.

Conclusions: As the data indicates, benzalkonium chloride and Deconex have good potential as hospital disinfectants for catheter-related infections caused by *K. pneumoniae* in planktonic conditions. Antimicrobial stewardship programs must be performed weekly in our hospitals to improve the quality of antimicrobial use, reduce the use of antibiotics, and shorten the length of hospital stay without increasing mortality rates.

KEYWORDS

Klebsiella pneumoniae; biocides; biofilm; PCR; hospital hygiene

INTRODUCTION

Klebsiella pneumoniae is a gram-negative opportunistic pathogen, which is responsible for 10% of all hospital-acquired infections [1, 2]. This bacterium causes important diseases such as pneumonia, septicemia, urinary tract infections, wounds and intensive care unit infections in immunocompromised patients, diabetic patients, and still-born infants [3]. In recent years, the emergence of *K. pneumoniae* resistant to both antibiotics as well as biocides (disinfectants) has caused serious concern for infectious diseases specialists around the world [4]. It has been reported that about 60% to 70% of *Klebsiella* infections in hospitals are resistant to more than three classes

of antibiotics [5]. Studies of pneumonia in Chinese hospitals revealed a pathogen unlike any the investigators had previously seen in healthcare settings. It was hyper-virulent *K. pneumoniae* harbouring various antibiotic resistance genes, a combination that causes severe, quick-developing, and deadly infections that are nearly impossible to treat with currently available drugs [6]. Both infections and antibiotic resistances in *K. pneumoniae* are often associated with the formation of biofilm. A biofilm is broadly considered a population of microorganisms grown on a surface or interface and embedded in a matrix of extracellular polymeric substances [7]. This will provide effective resistance

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against large molecules such as antimicrobial agents, lysozyme, and biocides [8]. It has been estimated that biofilms can tolerate antimicrobial agents (disinfectants, antibiotics, surfactants) at concentrations of ten to 1,000 times that needed to inactivate genetically equivalent planktonic bacteria [8, 9]. The presence of catheters favours biofilm formation by providing an inert surface for the attachment of bacterial cells, thereby enhancing microbial colonization and the development of biofilm [10, 11]. At present, there are few studies describing *K. pneumoniae* isolates resistant to benzalkonium chloride and Deconex through biofilm formation [12]. Reports suggest that, in the presence of benzalkonium chloride, biofilm development of *K. pneumoniae* was inhibited fourfold and the compound's MIC value was reduced by one-eighth [13].

The primary aim of this study was to evaluate the susceptibility of *K. pneumoniae* to antibiotics, benzalkonium chloride, and Deconex under biofilm and planktonic conditions. Furthermore, we studied the prevalence of quaternary ammonium compound-resistant genes (*qacEΔ1* and *cepA*) in these isolates.

METHODS

Bacterial samples and identification

A total of 85 non-duplicate strains of *K. pneumoniae* were isolated from catheter-associated specimens of 345 patients hospitalized in four referral hospitals in Kerman (southeastern Iran). Sampling was carried out from February to November 2015. For each isolate, patient demographic data and culture site were obtained. The samples were collected from intravascular and urinary tract central catheters peripherally inserted by a trained laboratory technician that were inoculated into 5 ml Stuart transport medium (Merck Group, Darmstadt, Germany) and transferred to our lab within 24 hours of collection. The identification process was carried out based on biochemical and conventional diagnostic tests for *Enterobacteriaceae*, as described by Kilian and Bülow (1979) [14].

Compound sources and analysis

Biocides, benzalkonium chloride as standard concentrate 50% w/v solution, under trade name BC50, and Deconex were purchased from NIPCO chemical group (Tehran, Iran).

Determination of susceptibility to biocides under planktonic conditions

The MIC and minimum bactericidal concentration (MBC) of Deconex and benzalkonium chloride against isolates were determined with a broth microdilution test, explained in detail in a previous report [15]. In brief, 10 ml of 32 mg/ml stock solutions of benzalkonium chloride and Deconex were prepared and diluted to 0.015 mg/ml. 50 μ l of each dilution was added to 96-well microtiter plates containing 150 μ l Luria-Bertani broth. To this preparation, 50 μ l of the bacterial sample at 1×10^6 colony-forming units (CFU)/ml was added. To prevent bacterial aggregations, the microtiter plates were incubated under shaking conditions (100 RPM) for 24 hours at 37° C. MIC was defined as the lowest concentration of the above compounds that

inhibits bacterial growth (no visible growth). A loopful of MIC was then streaked into Muller-Hinton agar and checked for grown colonies after 24 hours' incubation at 37° C. The number of colonies formed was considered the MBC. The MBC was defined as the lowest concentration of biocides killing at least 99.9% of the initial inoculums. Simultaneously, *E. coli* ATCC 25922 was used as a control strain.

Biofilm formation under static conditions

Biofilm formation was quantified by the microtiter method, as described by O'Toole et al. (2000) [9]. In this method, $1:100$ diluted *K. pneumoniae* isolates at a concentration of 1×10^6 CFU/ml were inoculated into a 96-well microtiter plate containing 100 μ l of fresh Tryptic Soy Broth (TSB) (BioMérieux, Marcy-l'Étoile, France). Growths were monitored after 24 hours' incubation at 37° C. Non-adherent cells were aseptically aspirated with pasture pipettes and washed with 300 μ l of phosphate-buffered saline (PBS) solution (pH 7.2) to remove any remaining suspended cells. The biofilm was subsequently stained with the addition of 150 μ l of 0.2% safranin (Merck Group, Darmstadt, Germany) for 30 minutes. The stain was removed by thorough washing, once with PBS at a pH of 7.2 and then with distilled water. The wells containing biofilm matrix were kept at room temperature or at 60° C until dry. Quantification of cells in the biofilms was carried out by solubilization of dye with 300 μ l of glacial acetic acid. Optical density at 480 nm was then measured for each well [15]. All mentioned experiments were performed in duplicate. *Pseudomonas aeruginosa* PAO1 was used as positive control for biofilm formation study.

MIC of antibiotics under planktonic and biofilm conditions

Susceptibility of 12 strong biofilm-forming *K. pneumoniae* isolates to different antibiotics was determined under planktonic and biofilm conditions using the microdilution method [16]. In the case of planktonic conditions, the microtiter plates were incubated under shaking at 100 RPM for 18 hours. In case of biofilm, cells attached to the wall of the microtiter wells after 24 hours were scraped in 1 ml of phosphate buffer (pH 7.2). The suspensions were then diluted to 1×10^6 CFU/ml using 5 ml of D/W. 10 μ l of bacterial suspensions was added to each microtiter well containing 100 μ l of sterile TSB medium and 50 μ l different concentrations of antibiotics (0.5-516 μ g/ml). The following antibiotics were used in this study: tigecycline, colistin, ceftazidime, imipenem, amikacin, cefotaxime, ceftoxitin, gentamicin, ciprofloxacin, trimethoprim/sulfamethoxazole, piperacillin/tazobactam, tetracycline, and chloramphenicol (Mast Group Ltd., Bootle, Merseyside, UK). *E. coli* ATCC 25922 was selected as a quality control strain in this antimicrobial susceptibility study.

Biofilm inhibitory activities of

Deconex and benzalkonium chloride

The antibiofilm activities of Deconex and benzalkonium chloride were evaluated as described here. Briefly, microtiter plates containing 180 μ l TSB medium were prepared. 10

μL of bacterial suspension at 10×10^6 CFU/ml and $10 \mu\text{L}$ Deconex and benzalkonium chloride preparation at a sub-MIC concentration of 0.03 mg/ml were added to each well. The sub-MIC was defined as the highest concentration of the antimicrobial compounds that bacterial cells can grow and showing visible turbidity. The microtiter plates were kept at 37°C for 24 hours and the amount of biofilm formed in each well was measured as described above. Prior to the experiments, we measured the growth rate and viable count of each isolate in the absence and presence of biocides. The adherence efficiency was calculated as described by Stepanović et al. (2007) [17].

Determination of biofilm eradication time

To determine biofilm eradication time, we decanted the contents of a microtiter plate with 24-hour biofilm treated with biocides for contact times of five, 15, 30, and 60 minutes. Microplate wells were then rinsed gently with sterile water and stained. The bound dye was re-solubilized in 33% glacial acetic acid. Absorbance was read as described by Leary et al. (2017) [18]. The combination of benzalkonium chloride at a concentration of 0.3 mg/ml and Deconex at a concentration of 0.06 mg/ml was also used for biofilm eradication.

Detection of *qacEΔ1* and *cepA* genes by PCR

For PCR assays, the genomic DNA of each isolate was extracted using the boiling method and subjected to phenol/chloroform treatment. The cell lysate was centrifuged at 8,000 RPM for ten minutes and used to detect quaternary ammonium compounds

(QACs) (*qacEΔ1*, *cepA*) genes with the PCR technique using a set of primer sequences F: 5-GCCCTACACAAATTGGGAGA-3 and R: 5-CTGCGGTACCACTGCCACAA-3 specific for the *qacEΔ1* gene; and primer pairs F: 5-CAACTCCTTCGCCTATCCCG-3 and R: 5-TCAGGTCAGACCAAACGGCG-3 specific for the *cepA* gene [19]. The PCR assay was carried out using a temperature gradient thermal cycler (Biometra-T300, Göttingen, Germany). The amplification program consisted of an initial denaturation at 95°C for 300 seconds, 95°C for 30 cycles for 30 seconds, annealing 60°C for 35 seconds, extension at 72°C for 40 seconds, and final extension at 72°C for 300 seconds. The 100 bp DNA Ladder was used as a molecular weight standard.

Statistical analysis

The SPSS program version 17.0 for Windows (SPSS, Chicago, IL, U.S.A.) was used for statistical analysis. Data are presented as the mean \pm standard error of the mean. For all tests, a two-sided *p*-value < 0.05 was considered significant.

RESULTS

Biofilm quantification revealed that 15% of the isolates showed strong biofilm activity, 40% showed moderate activity, 30% showed weak activity, and 15% showed no biofilm activity. However, no significant antibiofilm activity of benzalkonium chloride and Deconex were detected after exposure of the isolates to a sub-MIC concentration of 0.05 mg/ml ($p \leq 0.05$), as shown in Figure 1. Further analysis of the susceptibility of *K. pneumoniae* to antibiotics grown under planktonic and

FIGURE 1: Biofilm formation and the effect of benzalkonium chloride and Deconex on biofilm in *K. pneumoniae* multidrug-resistant isolates. No statistical significance in the amount of biofilm masses was observed in the presence and absence of these agents.

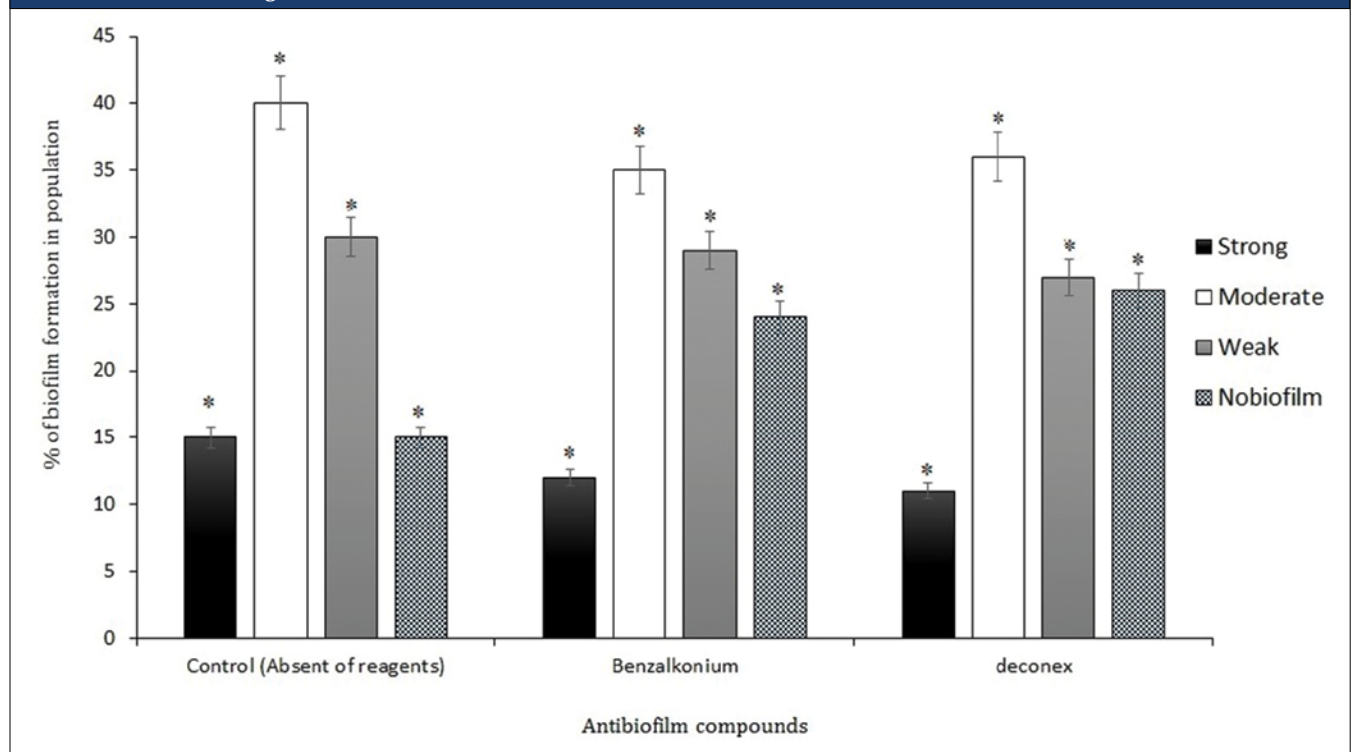
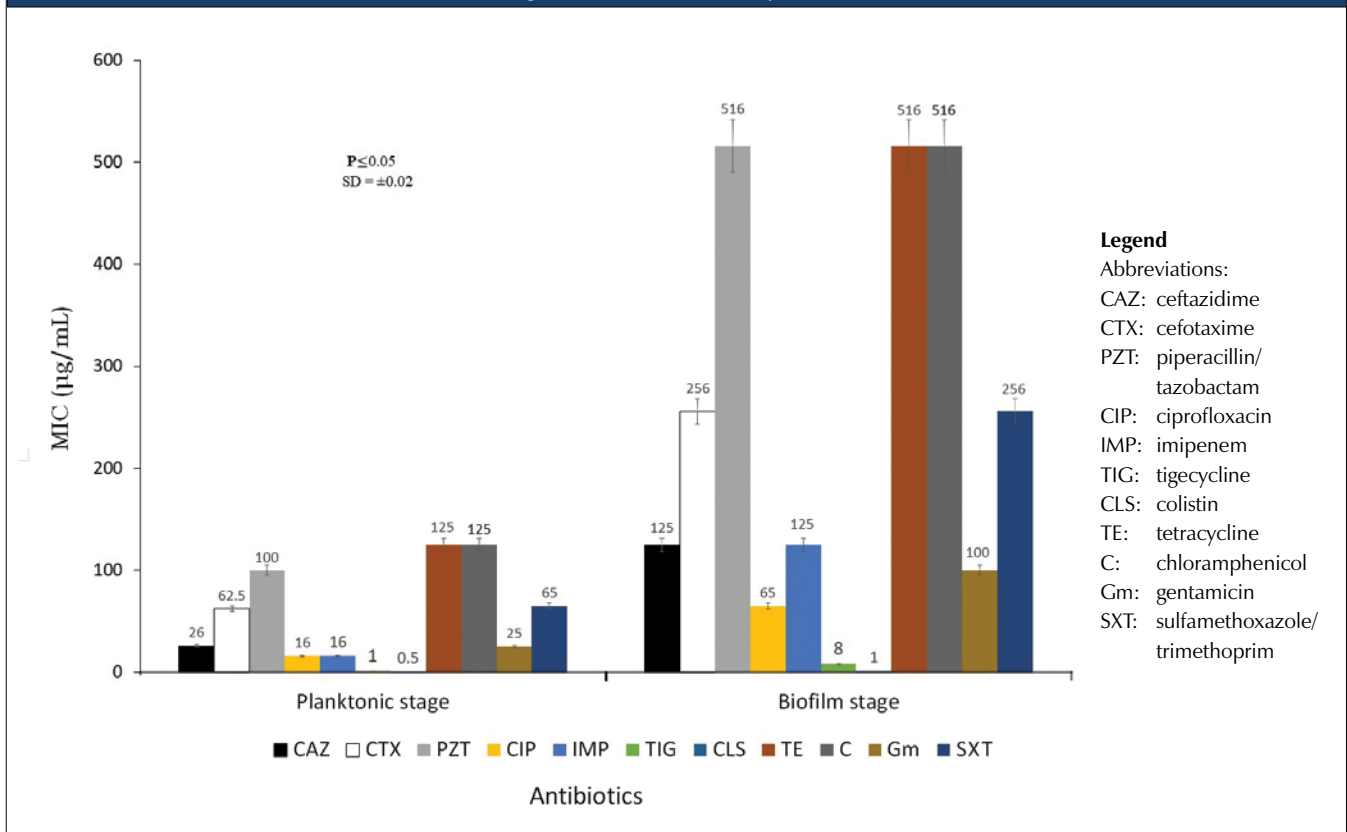


FIGURE 2: Antibiotic susceptibility of *K. pneumoniae* under biofilm and planktonic conditions. MIC was defined as the lowest concentration of antibiotics that inhibits growth (visible turbidity) after 24 hours of incubation.



biofilm conditions (Figure 2) revealed a significant increase in the MIC level for the piperacillin/tazobactam, tetracycline, chloramphenicol, trimethoprim/sulfamethoxazole, and cefotaxime antibiotics for cells taken from 24 hour biofilm. Resistance to imipenem and ceftazidime increased more than fourfold; however, in the case of tigecycline and colistin, the MIC did not differ under planktonic and biofilm growths (Figure 2). These differences were statistically significant, with a p -value of < 0.05 . To assess the antimicrobial activity of benzalkonium chloride and Deconex, we performed MIC and MBC tests using different concentrations of the above biocides. More than 58.8% and 35.2% of the isolates exhibited an MIC of 0.015 mg/ml to benzalkonium chloride and Deconex under planktonic conditions, while 17.6% and 18.8% of the isolates showed an MIC of 0.03 mg/ml to the above compounds. We detected one isolate with an MBC of 1 mg/ml to benzalkonium chloride (isolate 53) and one isolate with an MIC of 4 mg/ml to Deconex (isolate 43), respectively.

The results of survival curves of the planktonic cells and biofilm eradication time in the presence of biocides are illustrated in Figures 3a and 3b. Here, benzalkonium chloride at a concentration of 0.03 mg/ml and Deconex at a concentration of 0.06 mg/ml reduced bacterial population to 3 log₁₀ units within four hours of exposure (Figure 3a). Further exposure of planktonic cells to biocides indicated a continuous drop of viable cells with no detectable CFUs after

eight hours of incubation. Furthermore, we challenged biofilms with benzalkonium chloride at concentrations of 0.03 mg/ml and Deconex at concentrations of 0.06 mg/ml, respectively. A minimum biofilm eradication time assay revealed that both biocides were not effective against preformed biofilm at the recommended time of five minutes, but that they eradicated the biofilm masses from microplate wells after 30 minutes' exposure (Figure 3b). In addition, combinations of benzalkonium chloride at a concentration of 0.03 mg/ml and Deconex at a concentration of 0.06 mg/ml had a synergistic effect and removed most of the biofilm from the microtiter wells within five minutes of incubation (Figure 3b). PCR analysis showed the presence of *cepA* in 15% of the isolates showing strong biofilm, while the *qacEA1* gene was absent in this study.

DISCUSSION

The U.S. Centers for Disease Control and Prevention alone records nearly 560,000 catheter-related urinary tract infections in U.S. hospitals [20]. Catheterization increases the risk of developing bacteriuria by about 3% to 6% per day. Almost 50% of short-term catheterized patients acquire infections within this period, whereas the risk of infection from long-term catheters is 100% [21].

In the present study, we selectively isolated *K. pneumoniae* strains from catheters in four hospitals and exposed them

FIGURE 3a: Survival of *K. pneumoniae* in the presence of benzalkonium chloride and Deconex. The above results are the average of two replicate experiments.

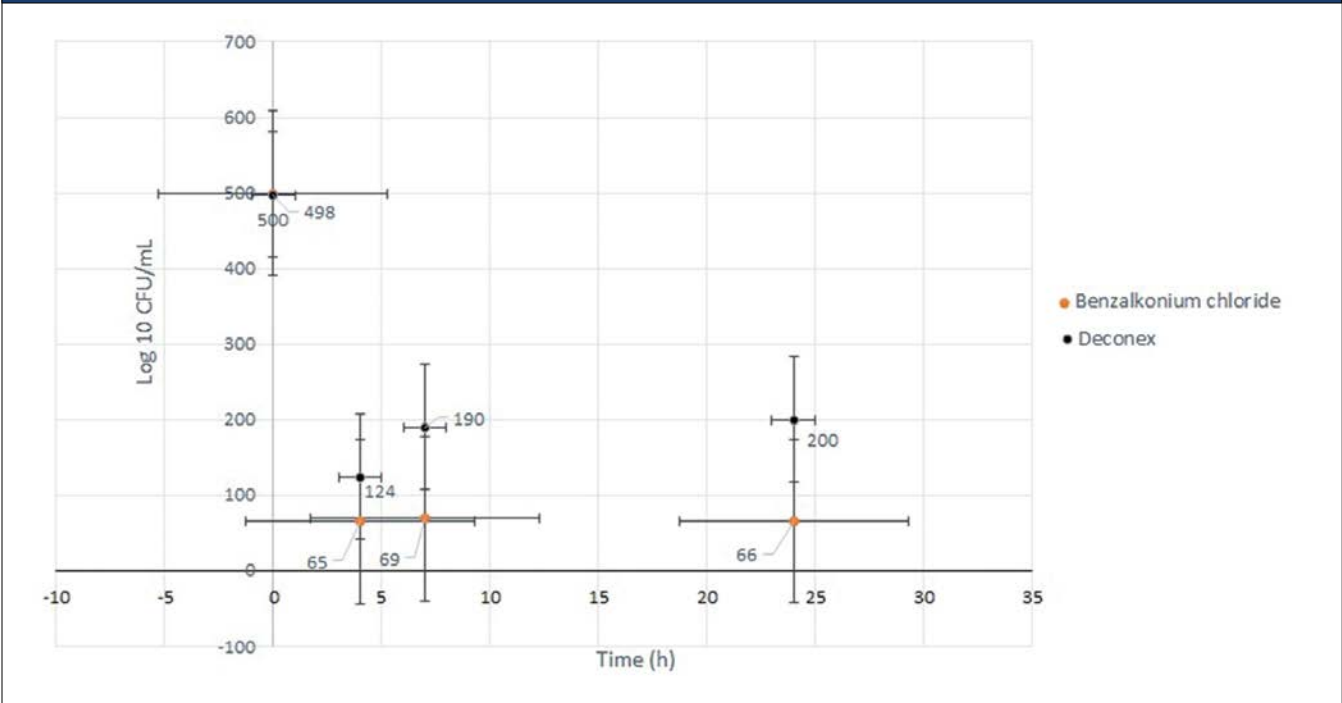
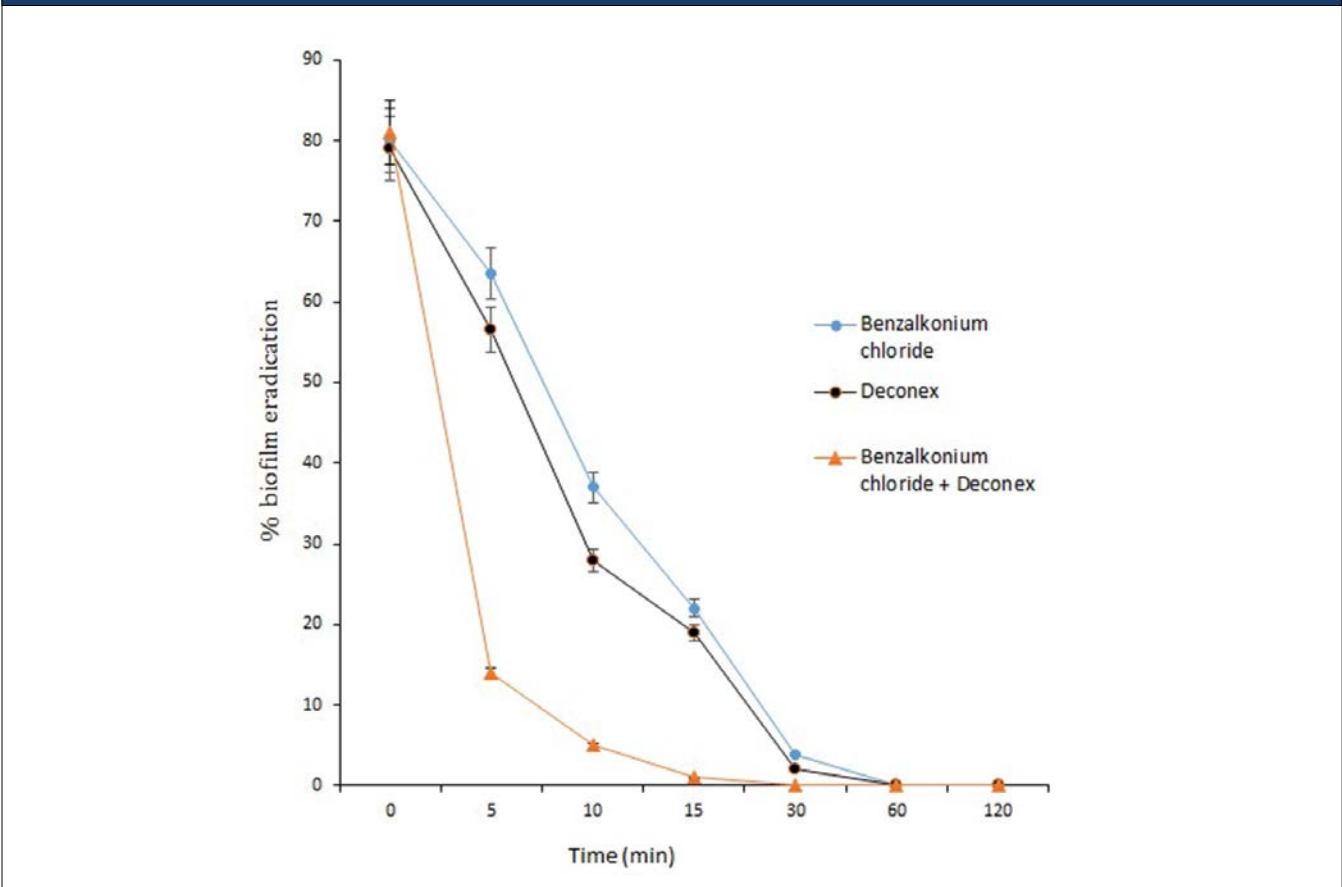


FIGURE 3b: Eradication of biofilm in the presence of benzalkonium chloride and Deconex at different time intervals. Mean values and standard deviation of the compounds are indicated by the error bar.



to different concentrations of Deconex and benzalkonium chloride. Both compounds act on the cell membrane, causing the dissociation of the bilayer membrane and the disruption of cell permeability. Our results show a high rate of sensitivity to these compounds by strains of *K. pneumoniae* (average MIC to Deconex of 0.06 ± 0.2 mg/ml and benzalkonium chloride of 0.03 ± 0.01 mg/ml). However, the compounds did not exert biofilm inhibitory activity. A recent study revealed that bacterial adherence to the benzalkonium chloride-impregnated catheters was significantly reduced compared with control catheters containing no antimicrobial agent ($p < 0.01$) [22]. Furthermore, we found a direct relationship between biofilm formation and high MIC values to antibiotics, which highlights how current planktonic-based antimicrobial susceptibility tests are often misleading [23]. This increased resistance occurs mainly in mature biofilms and is attributed to the formation of antibiotic-tolerant subpopulations in the deeper layers of biofilms in combination with impaired molecule diffusion [24]. A fourfold increase in MIC to imipenem in biofilm-grown cells was unexpected; Chen et al. (2014) [25] reported that imipenem displayed potent activity against established *K. pneumoniae* biofilms under both static and flow conditions in vitro, while Vuotto et al. (2017) [26] demonstrated that the association of biofilm production and imipenem resistance in *K. pneumoniae* was statistically significant. Recently, research has been directed at understanding why antibiotics do not effectively penetrate biofilms (specific antibiotics are required at higher dosages to treat biofilms) [27].

There was no significant relationship between antibiotic resistance and resistance to biocides in our study. On the contrary, many isolates were susceptible to biocides but resistant to antibiotics. We propose that hand hygiene, contact precautions, active patient screening, and disinfecting hospital equipment and catheter-related devices with these compounds at concentrations of 0.03 mg/ml can effectively reduce the transmission of infections caused by *K. pneumoniae* planktonic cells but not biofilm conditions. A population-based study on bloodstream infections caused by *K. pneumoniae* from 2000 to 2007 in Canada, a setting with low prevalence of antimicrobial resistance, showed an increase of the burden of disease during the last decade and a case fatality rate of 19% [28].

Our data also suggest that for resistance to QACs, the presence of both *qacEΔ1* and *cepA* genes is essential. The absence of the *qacEΔ1* gene led to sensitivity to these biocides. One study performed in Tehran hospitals investigated the susceptibility of 26 isolates of *K. pneumoniae* to a hospital biocide in the presence of *qacEΔ1* and *cepA* genes [19]. The *qacEΔ1* and *cepA* genes were detected in 26 and 19 biocide-resistant isolates. The results suggest that susceptibility to biocide in clinical isolates of *K. pneumoniae* is directly related to the presence of both *qacEΔ1* and *cepA* genes. Similarly, it has been shown that 50%, 49%, and 53% of *K. pneumoniae* strains had reduced susceptibility to chlorhexidine and benzalkonium chloride. The antiseptic-resistant genes *cepA* and *qacEΔ1* were found in 56, 34, and one isolates, respectively [19].

CONCLUSION

Based on the above data, we concluded that benzalkonium chloride and Deconex antiseptics are very good at controlling infections caused by *K. pneumoniae* planktonic cells and could be included in the antimicrobial stewardship program of our hospitals.

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