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Short report

# Comparative activity of a polyhexanide–betaine solution against biofilms produced by multidrug-resistant bacteria belonging to high-risk clones

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SUMMARY

The aim of this study was to investigate the effect of polyhexanide (polyhexamethylene biguanide)–betaine (PHMB-B) compared with 2% chlorhexidine against biofilms of high-risk and/or multidrug-resistant bacterial clones. The minimum inhibitory concentrations of both biocides were determined by microdilution. The effect of PHMB-B and chlorhexidine on biofilm was evaluated by spectrophotometry and cell viability assays. At commercial concentrations, PHMB-B reduced 24 h, 48 h and 1-week biofilms of all pathogens tested. PHMB-B was more active than 2% chlorhexidine against Gram-negative bacterial 24 h and 48 h biofilms and Gram-positive bacterial 7-day biofilms. In summary, the activity of PHMB-B was superior to that of 2% chlorhexidine in those biofilms.

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## Introduction

High-risk clones of nosocomial pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are spreading worldwide with great efficiency. High-risk clones are characterized by an enhanced ability to disseminate rapidly through the hospital environment, causing outbreaks that can be difficult to control. Part of the success of these high-risk clones as nosocomial pathogens can be attributed to their ability to acquire multidrug resistance to antimicrobials, including those with broad-spectrum activity (i.e. carbapenems, colistin, or glycopeptides), although factors other than MDR acquisition, such as (hyper)virulence, have also been observed in some high-risk clones (*K. pneumoniae* and *P. aeruginosa*) [1].

Effective infection control strategies are needed to limit their spread in hospital settings. One strategy includes the use

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of antiseptics and disinfectants (sodium hypochlorite or chlorhexidine digluconate), although the activity of these biocides is variable in these high-risk clones when growing as planktonic bacteria. Bacterial biofilms are important in the pathogenesis of infections and the environmental survival of bacteria. Biofilms are involved not only in infections associated with medical devices and foreign materials, but are also important in many other types of infection, including wounds and ulcer infections [2]. Cells growing in biofilms are more tolerant to antibiotics and biocides than are planktonic cells. This is mainly because of the protection provided by the extracellular matrix secreted by the bacteria, as well as physiological and gene expression changes that occur in this setting, making antibiotic therapy inadequate [3].

Chronic wound infections constitute a suitable environment for the development or growth of bacterial biofilms. Debridement is the best method for reducing the biofilm burden in chronic wound infections, although it is unlikely to remove the biofilm completely and it is necessary to use an antiseptic irrigation solution to cleanse and hydrate the wound [4]. Chlorhexidine is one of the most widely used antiseptics in wound infections due to its wide spectrum and effectiveness against Gram-positive and Gram-negative pathogens, and its low toxicity. However, increased use of chlorhexidine in the clinical setting has led to the development of reduced susceptibility, requiring the development of new antiseptic agents.

One of these agents is polyhexanide (polyhexamethylene biguanide, PHMB), a broad-spectrum biocide with bactericidal action at very low concentrations, and good tissue tolerance. This biocide is a cationic polymer that attaches to negatively charged membrane lipids, disrupting the membrane and leading to membrane permeability. It can be used as a surface disinfectant and as an antiseptic, since it is active against Gram-negative and Gram-positive membranes, but it has minor effects on human membranes [5]. A combination of 0.1% PHMB and 0.1% betaine (Prontosan®; B. Braun Medical S.A., Rubi, Spain) is widely used to improve wound healing with good clinical results. Betaine (undecylenamidopropyl betaine) is a surfactant with antibacterial activity that reduces surface tension and increases the antimicrobial activity of PHMB. Our group has previously shown that the PHMB–betaine (PHMB-B) combination has excellent in-vitro activity against the most common multidrug-resistant (MDR) high-risk clones of both Gram-positive and Gram-negative bacteria, although that study and many others were performed on planktonic cells [6]. The in-vitro activity of the PHMB-B combination against bacterial biofilms is therefore currently unknown.

The aim of this study was to compare the in-vitro activity of a commercial 0.1% PHMB-B solution with that of a 2% chlorhexidine solution (2% CHX) used as comparator against high-risk clones of MDR nosocomial pathogens growing in biofilms at different times.

## Methods

### Strains

Six representative isolates of various high-risk or MDR bacterial clones were included: *Klebsiella pneumoniae* ST-716 producing the extended-spectrum β-lactamase (ESBL) CTX-M-15; *K. pneumoniae* ST-258 producing the carbapenemase KPC-3; and *Acinetobacter baumannii* ST-2 producing the carbapenemase OXA-23 obtained from the Reference Laboratory of the PIRASOA Program (Hospital Universitario Virgen Macarena, Seville, Spain); *Pseudomonas aeruginosa* ST-175 producing the metallo-β-lactamase VIM-2; meticillin-resistant *Staphylococcus aureus* belonging to clonal complex CC5; and *Enterococcus faecalis* belonging to clonal complex CC2. Two ATCC strains were also included as controls for biofilm formation: *K. pneumoniae* ATCC 700603 (positive control) and *S. epidermidis* ATCC 12228 (negative control) [7].

### Susceptibility testing

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 0.1% PHMB-B in aqueous solution (0.1% PHMB and 0.1% betaine) and 2% CHX in aqueous solution (Sigma–Aldrich, Madrid, Spain) were determined by microdilution, as previously described [6].

### Biofilm formation

Biofilm formation was determined on a 96-well polystyrene flat-bottom plate. Bacteria were routinely grown overnight in Mueller–Hinton broth (MHB) at 37°C under aerobic conditions using an incubator shaker. Overnight bacterial cultures were diluted at 1:100 in MHB ($10^6$ cells/mL) and 200 μL was added to wells of a 96-well flat-bottom tissue culture plate (Cellstar®; Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated for 24 h, 48 h, and 1 week at 37°C for biofilm growth. Plates were washed three times with phosphate-buffered saline (PBS) to remove planktonic cells, and 100 μL of 0.1% PHMB-B or 2% CHX solution at a final concentration equivalent to 1×MIC or the recommended commercial concentration of each product was added to each well. MHB without biocide was used as control. Biofilms were exposed to 0.1% PHMB-B or 2% CHX for 15 min and the plates were washed three times with PBS.

The effect of 0.1% PHMB-B and 2% CHX on the reduction of biofilm formation was determined by measuring (i) the amount of biofilm, and (ii) the cell viability in the biofilm after exposure to biocides. To study the amount of biofilm, adherent bacteria were fixed with 99% ethanol for 15 min and stained with 0.1% crystal violet for 30 min. Crystal violet was solubilized in 33% glacial acetic acid and the absorbance of the solubilized dye was measured at 595 nm using an Infinite® 200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland).

For cell viability assays, after biocide treatment, biofilms were washed with PBS and adherent bacteria were detached by sonication (40 kH, 2 min). Samples were then serially diluted in PBS and the numbers of viable bacteria were determined by colony counting on Mueller–Hinton agar plates. Bacteria survival data are expressed as $\log_{10}$ cfu/mL.

### Statistics

All experiments were conducted in triplicate on at least two separate days. Data were compared by Student's *t*-test using SPSS 19.0 software. $P < 0.05$ was considered statistically significant.

**Table I**
PHMB-B and chlorhexidine MIC and MBC values against strains tested

| Strain | Resistance mechanism | PHMB-B | | Chlorhexidine | | Reference |
|---|---|---|---|---|---|---|
| | | MIC (mg/L) | MBC (mg/L) | MIC (mg/L) | MBC (mg/L) | |
| *K. pneumoniae* ATCC 700603 | – | 2 | 2 | 32 | 32 | ATCC |
| *S. epidermidis* ATCC 12228 | – | 2 | 2 | 16 | 16 | ATCC |
| *K. pneumoniae* ST-716 | ESBL type CTX-M-15 | 1 | 1 | 8 | 8 | This study |
| *K. pneumoniae* ST-258 | Carbapenemase type KPC-3 | 2 | 2 | 8 | 8 | This study |
| *A. baumannii* ST-2 | Carbapenemase type OXA-23 | 4 | 4 | 8 | 8 | This study |
| *P. aeruginosa* ST-175 | Metallo-carbapenemase type VIM-2 | 8 | 8 | 16 | 16 | [6] |
| *S. aureus* CC5 | *mecA* (meticillin resistance) | 2 | 2 | 4 | 4 | [6] |
| *E. faecalis* CC2 | – | 2 | 2 | 4 | 4 | [6] |

PHMB-B, polyhexamethylene biguanide (polyhexanide); MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

## Results

### *Susceptibility testing*

Table I shows the MIC and MBC values of 0.1% PHMB-B and 2% CHX. For all tested pathogens, the range of MIC and MBC values for 0.1% PHMB-B was 1–8 mg/L, and for 2% CHX, 4–32 mg/L.

### *Activity of 0.1% PHMB-B against preformed bacterial biofilms*

Neither PHMB-B nor CHX showed any effect at concentrations equivalent to 1×MIC. Effect was observed at the commercial concentration recommended by the manufacturer (Figure 1).

*24 h biofilms*

Treatment with 0.1% PHMB-B reduced the biofilm of all isolates tested compared with controls. The reduction was >50% for all high-risk clones tested, except for *S. aureus* CC5 (biofilm reduction <10%). The reduction after treatment with 0.1% PHMB-B was significantly greater than after treatment with 2% CHX (Figure 1A). Treatment with 0.1% PHMB-B significantly reduced bacterial viability in the 24 h biofilms of all clones tested compared with the drug-free control. Compared with 2% CHX treatment, differences were observed against *K. pneumoniae* and *A. baumannii* (Figure 1B). The antimicrobial activity of both biocides was superior against Gram-positive pathogens.

*48 h biofilms*

Treatment with 2% CHX solution did not affect any of the isolates tested. Treatment with 0.1% PHMB-B significantly reduced the amount of biofilm, compared with the control and the 2% CHX solution for all pathogens tested (Figure 1C). The reduction ranged from 15% to 76% relative to the control. The lowest activity was observed against *S. aureus* CC5 biofilms. In the viability assays, 0.1% PHMB-B and 2% CHX treatments reduced the number of viable bacteria compared with the control, and 0.1% PHMB-B was more active than 2% CHX solution against *A. baumannii* ST-2 biofilms. Treatment with 0.1% PHMB-B showed superior bactericidal activity against *K. pneumoniae* ST-716, *A. baumannii* ST-2, and Gram-positive biofilms.

*7-day biofilms*

PHMB-B treatment reduced 7-day biofims of all clones tested by ≥65% relative to the control, and it was significantly more efficacious than 2% CHX in reducing the biofilm of the Gram-negative pathogens and *E. faecalis* CC2 (Figure 1E). The two biocides reduced the number of viable bacteria compared with the control. Treatment with 0.1% PHMB-B was more active than 2% CHX against 7-day *S. aureus* biofilms (Figure 1F). The antimicrobial activity of 0.1% PHMB-B was higher against *K. pneumoniae*, *A. baumannii*, and *S. aureus*.

## Discussion

Healthcare-associated infections are caused by different pathogens, including those belonging to the ESKAPE group (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species). These organisms are of particular concern to healthcare professionals because of the increasing presence of acquired resistance mechanisms in clinical isolates, which can limit treatment options [8]. Biofilms are involved in 60–70% of healthcare-associated infections and are important in human infection/colonization and environmental contamination. Nonetheless, the in-vitro activity of many biocides against bacterial biofilms remains unknown. The excellent in-vitro activity of 0.1% PHMB-B against MDR high-risk clones of important nosocomial pathogens makes it a suitable candidate for other uses in the clinical setting [6].

The aim of this study was to evaluate the in-vitro activity of 0.1% PHMB-B against biofilms formed by high-risk and/or MDR clones of significant nosocomial pathogens. With respect to the

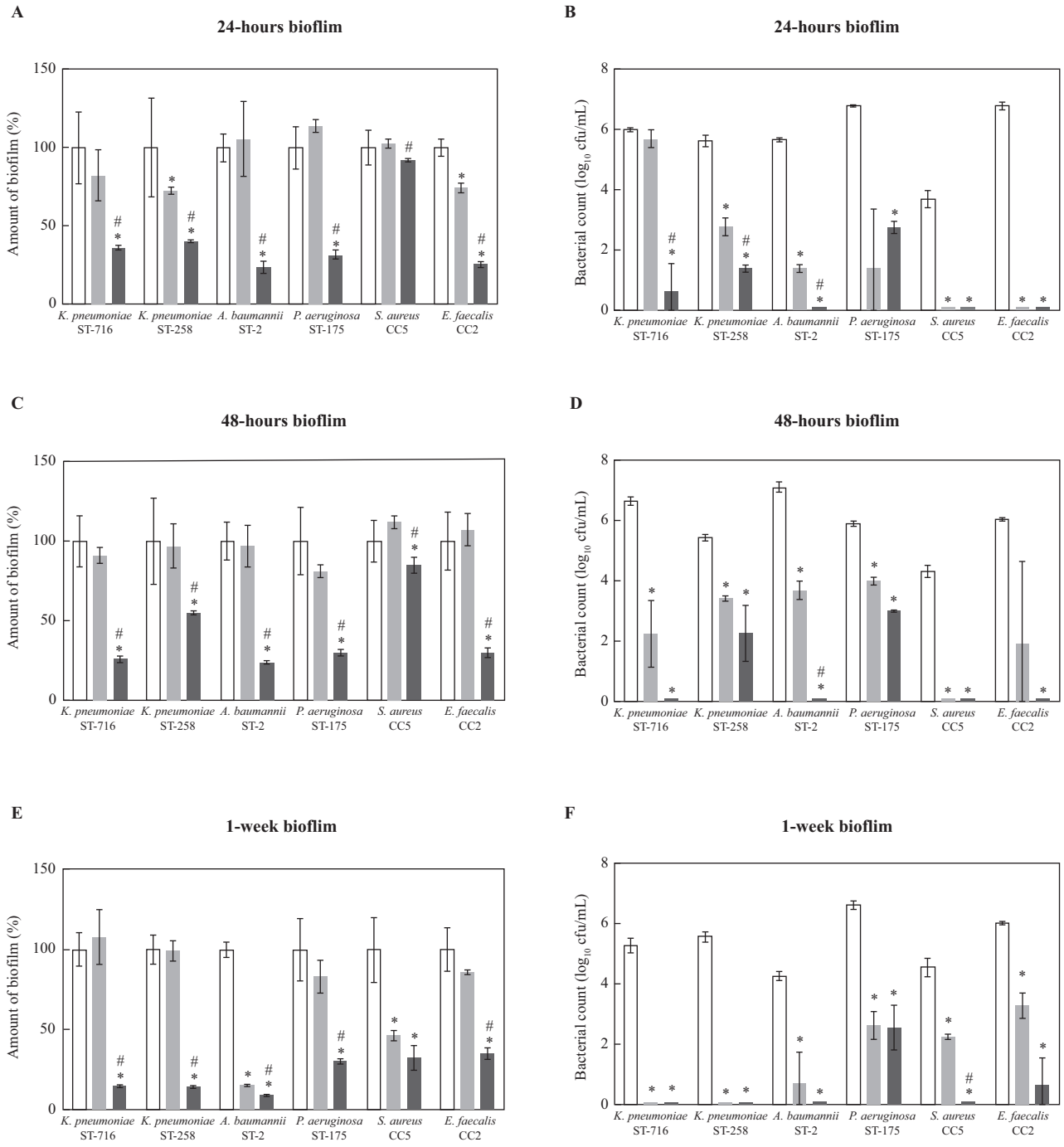


**Figure 1.** Activity of polyhexanide (dark grey bars) and chlorhexidine (light grey bars) solutions at commercial concentration against mature biofilms. Biocide effects against mature biofilms represented as mean and standard deviation of optical density (595 nm) are expressed as percentages relative to the control (white bars) (A, C, and E). Biocide antimicrobial activity represented as mean and standard deviation are expressed as $\log_{10}$ cfu/mL (B, D, and F). Values that were significantly different compared to the control values are indicated by an asterisk; and 0.1% polyhexanide solution values that were significantly different from the 2% chlorhexidine values are indicated by a hash ($P < 0.05$, Student's *t*-test, two-tailed).

MICs, 0.1% PHMB-B was shown to be more effective than 2% CHX. Our results are similar to those obtained by Koburger *et al*. who found that polyhexanide MICs were lower than the MICs of other antiseptics such as PVP–iodine and chlorhexidine digluconate [8]. MIC and MBC values for both biocides were similar, indicating that both biocides exhibit high bactericidal activity.

Treatment with 0.1% PHMB-B reduced the amount of biofilm of all high-risk clones tested, independently of biofilm age. These results correlated with its bactericidal effect (viable cell counts) against preformed biofilms. Treatment with 0.1% PHMB-B showed similar bactericidal activity against the six clones tested, except for *P. aeruginosa*, and greater activity

against 7-day biofilms. The effectiveness of 0.1% PHMB against *S. aureus* biofilms was previously described by Davis *et al.*, although only against 24 h biofilms [9]. To our knowledge, no studies have been performed against 7-day biofilms. We observed a higher antimicrobial effect, which would be partly explained by the extra antimicrobial activity of the betaine. In our study, 0.1% PHMB-B activity was greater than that of 2% CHX against 24 h, 48 h, and 7-day biofilms of both the Gram-negative and Gram-positive high-risk clones tested.

In terms of cell viability, both biocides showed similar effects against Gram-positive bacterial 24 h and 48 h biofilms, although 0.1% PHMB-B treatment showed higher bactericidal activity against *K. pneumoniae* and *A. baumannii* biofilms. The antimicrobial activity of 0.1% PHMB-B and 2% CHX against Gram-negative pathogen 7-day biofilms was similar, whereas the effect of 0.1% PHMB-B activity against Gram-positive bacteria 7-day biofilms was higher. Treatment with 0.1% PHMB-B and 2% CHX showed similar activity on *P. aeruginosa* biofilms, irrespective of the age of biofilm.

None of the biocides evaluated at commercial concentrations eliminated all the biofilms, although in some cases they killed all bacteria for some high-risk clones (Figure 1). Taking the data together, 0.1% PHMB-B was superior to 2% CHX in dismantling the biofilm and showed higher antimicrobial activity against Gram-positive and Gram-negative bacterial biofilms. Furthermore, bacterial resistance to 0.1% PHMB is not common and has not resulted in cross-resistance to 2% CHX [6].

One limitation of our study is that the experiments were conducted on monospecies biofilms which may be more susceptible to biocides than multi-species biofilms, and without interfering substances such as proteins, so they do not accurately represent clinical conditions [10]. On the other hand, our study was conducted on 24 h, 48 h, and 1-week biofilms produced by internationally successful high-risk clones in an attempt to imitate what occurs in clinical settings.

In conclusion, our results show that PHMB-B solutions at commercial concentrations and for the length of time recommended by the manufacturer were more active than 2% CHX against biofilms produced by multidrug-resistant high-risk clones of nosocomial pathogens. The clinical significance of these results should be further evaluated.

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**Conflict of interest statement**
None declared.

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