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Efficacy of octenidine against *Pseudomonas aeruginosa* strains

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ABSTRACT: *Pseudomonas aeruginosa* is a Gram-negative bacterium causing skin and soft tissue infections, complicated urinary tract infections, blood infections, and nosocomial (hospital-acquired) infections. One of the most often used antiseptics in the skin and soft tissue infections is octenidine dihydrochloride. The aim of this study was an evaluation of octenidine activity against strains of *P. aeruginosa*. Additionally, were compared two staining methods (TTC and MTT) for confirmation of bacterial growth. The study involved eight strains of *P. aeruginosa*. In order to determine the minimum inhibitory concentration (MIC) of octenidine, the microdilution method was used. For bacterial growth detection was used staining method with 2,3,5-triphenyl-tetrazolium chloride (TTC) and with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). In the study has been demonstrated the excellent activity of octenidine against all strains of *Pseudomonas aeruginosa*. For all tested strains, MICs of octenidine were 0.00039% or 0.00078%, what is equivalent to 3.9 µg/ml and 7.8 µg/ml, respectively. In the study, test with MTT for three strains was more sensitive than a test with TTC. Concluding, octenidine is an antiseptic with high efficacy against *Pseudomonas aeruginosa* strains. Simultaneously, it was stated that a test with MTT is more sensitive than study with TTC.

Keywords: *Pseudomonas aeruginosa*; Octenidine dihydrochloride; Octenisept; Antibacterial activity; TTC; MTT.

1. INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative rod bacterium belonging to the family Pseudomonadaceae [1]. *P. aeruginosa* is oxidase-positive, non-fermenting lactose, with low nutritional requirements. It shows the ability to survive in different conditions, including a nutrient-poor environment (distilled water) and in a wide temperature range (from 4 to 44 °C). This pathogen produces a phenazine blue dye called pyocyanin [2]. *P. aeruginosa* has many virulence factors, including bacterial cell-associated lipopolysaccharides (LPS), fimbriae, flagellae, mucus, lectins, exotoxin A, exoenzymes, alkaline protease, type IV protease, type A and B elastase, neuraminidase, hemolytic and non-hemolytic phospholipase, and

pyocyanin [3]. *P. aeruginosa* is an opportunistic pathogen and has recently been classified as an ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogen. This group contains highly resistant bacteria causing nosocomial (hospital-acquired) infections [4]. The intrinsic resistance of *P. aeruginosa* to antibiotics is conditioned by three mechanisms:

1. active removal of the antibiotic from the cell (efflux system),
2. reduced permeability of the outer membrane,
3. enzymatic inactivation of the antibiotic [5].

All *P. aeruginosa* strains are naturally resistant to penicillin G, aminopenicillin, cephalosporins of the first and second generation, macrolides, tetracyclines, chloramphenicol, quinolones, sulfonamides, and trimethoprim [6, 7]. *P. aeruginosa* is highly associated with nosocomial infections and ventilator-associated pneumonia [8]. This bacterium also causes skin and soft tissue infections, complicated urinary tract infections, and blood infections [9]. European Centre for Disease Prevention and Control presented that in 2016 in Europe, 33.9% of *P. aeruginosa* strains were resistant to at least one of the antimicrobial groups under surveillance (piperacillin ± tazobactam, fluoroquinolones, ceftazidime, aminoglycosides, and carbapenems) [10]. In these studies, in Latvia, Poland, Slovakia, Hungary, Croatia, Serbia, Bulgaria and Greece, 25-50% of *P. aeruginosa* isolates were resistant to carbapenems, while in Romania more than 50% of the strains. Combined resistance to three or more antimicrobials was in 25-50% of the strains isolated in Slovakia, Romania, Croatia, Bulgaria, and Greece. The World Health Organization (WHO) has recently listed carbapenem-resistant *P. aeruginosa* as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections [11].

Octenidine dihydrochloride (OCT; 1,1'-(1,10-Decanediyl)bis(N-octyl-4(1H)-pyridinimine) dihydrochloride) is a bispyridine compound with 2 cationic active centers. This substance has a molecular formula $C_{36}H_{64}Cl_2N_4$ and molecular weight 623.826 g/mol [12]. Chemical structure of octenidine is presented in Figure 1. OCT contains antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and several viruses [13, 14]. OCT has good activity against bacteria *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and the fungus *Candida albicans* [13-19]. It acts bactericidal/fungicidal by interfering with cell walls and membranes [20]. OCT is relatively non-cytotoxic [14], and does not affect the human epithelium and the healing process [21].

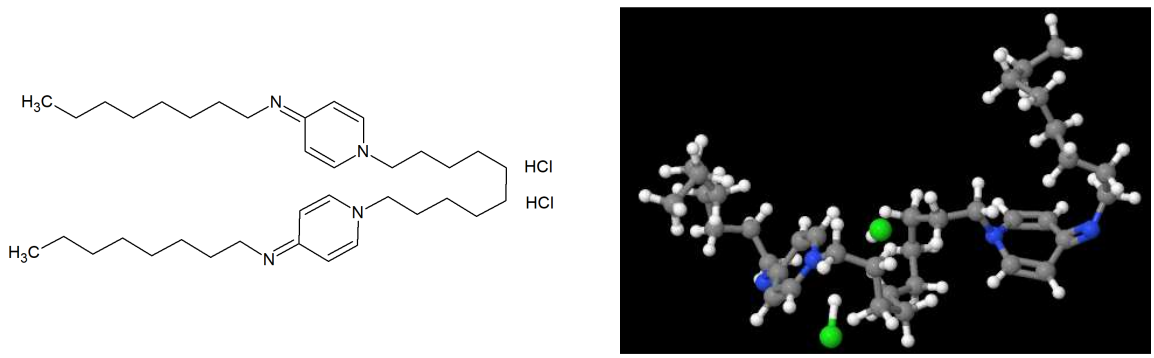

Figure 1. Chemical structure (left 2D and right 3D) of octenidine dihydrochloride.

The aim of this study was an evaluation of octenidine activity against strains of *Pseudomonas aeruginosa*. Additionally, were compared two staining methods (TTC and MTT) for bacterial growth detection.

2. MATERIALS AND METHODS

The study involved eight strains of *Pseudomonas aeruginosa*. Six isolates were from wounds as part of the service activity of the Department of Medical Microbiology, Poznań University of Medical Sciences. Two were reference strains: *P. aeruginosa* ATCC 27853 (Boston 41501) and ATCC BAA-47 (4901; PAO1). Bacteria were grown on cetrimide agar (Oxoid) for 18-24 h at 37°C.

In order to determine the minimum inhibitory concentration (MIC) of octenidine, the microdilution method was used on polystyrene 96-well plates (Nunc). The octenidine dihydrochloride (Octenisept, Schülke) was appropriately diluted on the plates to obtain concentrations ranging from 0.000195 to 0.1%. A suspension with a density of 1×10^8 cells/ml (0.5 McF) was prepared for each strain. The suspension was diluted 10-fold with TSB to obtain a bacterial suspension with a density of 1×10^7 cells/ml. 10 µl of bacterial suspension was transferred to appropriate wells (2-12) of the plate. Well no. 1 was the negative control, filled with 100 µl of Mueller Hinton broth (MHB). Well no. 2 was also the negative control but filled with 10 µl of bacterial suspension and 100 µl of disinfectant agent Aerodesin 2000 (Lysoform), containing propan-1-ol 32.5 g, ethanol 18 g, and glutaraldehyde 0.1 g. A plate with appropriate suspensions of bacterial cells was incubated for 24 hours at 37°C. Two rows of wells were prepared for each strain. 2,3,5-triphenyl-tetrazolium chloride (20 µl, 1% TTC, Sigma-Aldrich) was added to the wells of the first row. After 24 h of culture, the optical density at the wavelength λ = 495 nm was measured. In the presence of live, metabolically active microorganisms, colorless TTC is reduced to red formazan. To the wells of the second row, after 24 h culture, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (25 µl, 3% MTT, Sigma-Aldrich) was added and incubated 2 hours at 37°C. Then, the optical density at the wavelength λ = 554 nm was measured. This assay is based on the reduction of yellow tetrazolium salt (MTT) to a soluble purple/blue formazan product [21]. MIC is the lowest concentration of an antimicrobial agent in which no growth of a microorganism is observed [22]. The MIC was the concentration of the octenidine in the first well, in which after incubation, the color did not change. Each measurement was carried out in triplicate.

3. RESULTS

In the present study has been demonstrated the excellent activity of octenidine against all strains of *Pseudomonas aeruginosa*. For all tested strains, MICs of octenidine were 0.00039% or 0.00078%, what is equivalent to 3.9 µg/ml and 7.8 µg/ml, respectively. Simultaneously, it was stated the difference between studies with MTT and TTC. Test with MTT for three strains (2, 4, and 41501) was more sensitive than study with TTC (Figure 2).

4. DISCUSSION

Octenidine is an antiseptic used clinically in concentrations 0.05% (500 µg/ml) or 0.1% (1000 µg/ml). In this work is presented that bactericidal level of OCT against *Pseudomonas aeruginosa* is ranging from 0.00039% (3.9 µg/ml) to 0.00078% (7.8 µg/ml). Bartoszewicz et al. obtained for *P. aeruginosa* MICs of OCT

between 3.91 and 125 µg/ml, and MIC_{90} was 62.5 µg/ml [16]. Koburger et al. demonstrated for octenidine MIC_{24} = 2 and MIC_{48} = 8 µg/ml against *P. aeruginosa*. MICs of OCT against other bacteria are following, e.g. for *Staphylococcus aureus* 0.24-7.81 µg/ml, *S. epidermidis* 125 µg/ml, *Enterococcus faecalis* 4 µg/ml, *Esherichia coli* 2 µg/ml, *Streptococcus mutans* 120 µg/ml and Lactobacilli 15-30 µg/ml [16, 24, 25].

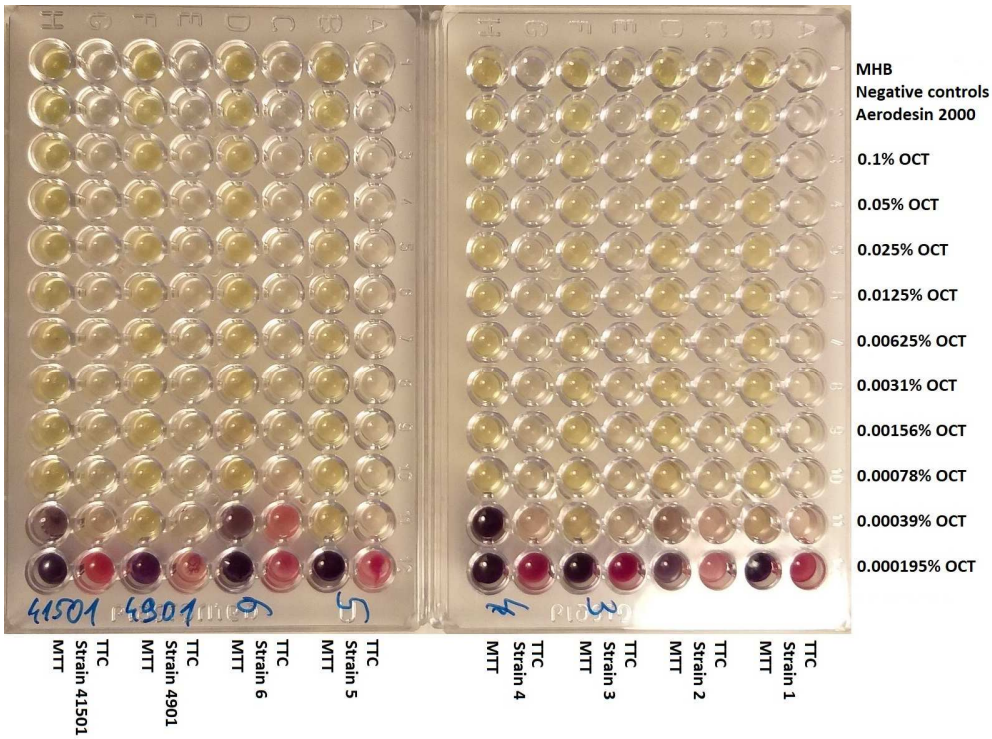

Figure 2. Study of bactericidal activity of octenidine (OCT) against eight strains of *Pseudomonas aeruginosa*. Obtained MICs are 0.00039 and 0.00078% of octenidine.

Differences between MIC levels depend, among others on whether the bacteria were tested in planktonic or biofilm form, which is less sensitive to antiseptics. Shepherd et al. presented the possibility of increased tolerance to OCT of *P. aeruginosa* [26]. Authors in seven strains of *P. aeruginosa* showed the initial MICs 4-8 µg/ml. After 12 days of adaptation study, MICs were 32-128 µg/ml. Unfortunately, the highest used OCT concentration (64 µg/ml = 0.0064%) was about 10-fold lower than the clinical doses. Moreover, at OCT level 32 µg/ml one strain did not grow, and a concentration of 64 µg/ml caused no growth in up to 3 (43%) strains [26]. The phenomenon of adaptation in such low concentrations does not seem to have any significant clinical significance. It can be stated that preparations of OCT in clinical doses remained fully effective against bacteria, especially *P. aeruginosa.* It is also extremely important that no resistance to OCT has been found so far. Such resistance has been demonstrated, among others for chlorhexidine [27-29].

Concluding, octenidine is an antiseptic with high efficacy against *Pseudomonas aeruginosa* strains. Simultaneously, it was stated that a test with MTT is more sensitive than study with TTC.

Conflict of Interest: The author declares no conflict of interest.

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