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EFFECT OF ADAPTATION PROCESS OF *PSEUDOMONAS AERUGINOSA* TO DIDECYLDIMETHYLAMMONIUM CHLORIDE IN 2-PROPANOL ON BACTERICIDAL EFFICIENCY OF THIS ACTIVE SUBSTANCE

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ABSTRACT

Background. Microorganisms are characterized by two types of resistance innate and acquired. Innate resistance is associated with the construction of the surface structures. Wide use of active substances as antimicrobial compounds, especially in inhibitory concentrations, may promote the acquisition of bacterial resistance to these substances in the process of adaptation.
Objective. The aim of the study was to determine changes in efficiency of didecyldimethylammonium chloride in 2-propanol (CMAP) against the *Pseudomonas aeruginosa* strains, which were adapted to this active substance.
Materials and Method. Adaptation studies were conducted using two strains: *P. aeruginosa* ATCC 15442 (PA), which is used in estimation of biocide efficiency and tetracycline-resistant *P. aeruginosa* ATCC 47085 (PAO-LAC) strain. These strains were adapted to the active substance Bardac22: 50% v/v didecyldimethylammonium chloride in 20% v/v 2-propanol (CMAP) according to the National Institute of Hygiene procedure. After adaptation, obtained isolates were classified to three groups and passaged to solid media: A – strains unadapted passaged onto slant medium without active substance, control group; B – strains with adaptive resistance passaged onto slant medium with 375 mg/l CMAP; C – strains with adaptive resistance passaged onto slant medium without CMAP. Changes in susceptibility of examined strains were determined on the basis of minimum inhibitory concentrations (MICs) by broth dilution method. The minimum bactericidal concentrations (MBCs) were determined by subculture *P. aeruginosa* strains on solid media without CMAP. The efficiency of CMAP against isolates obtained after adaptation processes was evaluated by using phenol coefficient (PC).
Results. There were no differences in the adaptation process between two strains of *P. aeruginosa*: PA and PAO-LAC. Both isolates obtained after the adaptation process was characterized by approximately 6-8 fold higher MICs compared to the MICs of these strains before the adaptation. Strains passaged to a solid media characterized a variable sensitivity to CMAP. As compared to a control group A, the isolates of PA and PAO-LAC from group B and isolate PA from group C exhibited the highest and stable insensitivity (MIC from > 700 to >1000 mg/l) to 48-49 passages. Isolates from group C of PAO-LAC maintained insusceptibility up to 20th passage (MIC >375 mg/l). There were no statistically significant changes in the CMAP bactericidal efficacy against isolates of reduced sensitivity.
Conclusions. Adaptation of *P. aeruginosa* strains to didecyldimethylammonium chloride in 2-propanol does not significantly change bactericidal efficacy of this active substance against isolates with reduced sensitivity. Antibiotic-resistant strain PAO-LAC showed a similar adaptability and a similar sensitivity to the CMAP as a strain PA used to assess the effectiveness of disinfectants.

Key words: *adaptation process, Pseudomonas aeruginosa, quaternary ammonium compounds, bactericidal efficiency*

STRESZCZENIE

Wprowadzenie. Mikroorganizmy charakteryzują się dwoma rodzajami oporności wrodzoną i nabytą. Oporność wrodzona jest związana z budową ich struktur powierzchniowych. Szerokie wykorzystanie substancji czynnych, jako związków antybakteryjnych, szczególnie w stężeniach hamujących, może sprzyjać nabywaniu przez bakterie oporności na substancje czynne w procesie adaptacji.
Cel. Określenie zmian w efektywności działania chlorku didecylodimetyloamoniowego w 2-propanolu (CMAP) wobec szczepów *P. aeruginosa* zaadaptowanych do tej substancji.
Materiały i metody. Badania przeprowadzono z wykorzystaniem dwóch szczepów: *P. aeruginosa* ATCC 15442 (PA), stosowanego w badaniach oceny skuteczności preparatów dezynfekcyjnych oraz tetracyklinoopornego szcze-

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pu *P. aeruginosa* ATCC 47085 (PAO-LAC). Szczepy *P. aeruginosa* były adaptowane do badanej substancji czynnej z zastosowaniem procedury opracowanej w Państwowym Zakładzie Higieny. Po procesie adaptacji, izolaty były klasyfikowane i pasażowane na podłoża stałe: A – szczepy nieadaptowane, pasażowane na podłoża bez substancji czynnej, kontrola; B – szczepy posiadające oporność adaptacyjną, pasażowane na podłoża zawierające 375 mg/l CMAP; C – szczepy posiadające oporność adaptacyjną, pasażowane na podłoża bez CMAP. Zmiany wrażliwości na CMAP badanych szczepów określano na podstawie minimalnych stężeń hamujących (MIC) i bójczych (MBC). Skuteczność bakteriobójcza CMAP była wyznaczana z zastosowaniem współczynnika fenolowego (PC).

Wyniki. Nie zaobserwowano różnic w procesie adaptacji szczepów *P. aeruginosa*: PA i PAO-LAC. Izolaty, uzyskane po procesie adaptacji, cechowały 6-8 razy wyższe wartości MIC niż szczepy przed adaptacją. Szczepy pasażowane na podłoża stałe charakteryzowały się zmienną wrażliwością na CMAP. W porównaniu do kontrolnej grupy A, izolaty PA i PAO-LAC z grupy B oraz izolaty PA z gr C wykazywały najwyższą i stabilną niewrażliwość (od MIC > 700 do >1000 mg/l) do 48-49 pasażu. Izolaty PAO-LAC z grupy C utrzymywały niewrażliwość do 20 pasażu (MIC >375 mg/l). Nie stwierdzono natomiast istotnych statystycznie zmian w skuteczności bakteriobójczej CMAP wobec izolatów o zmniejszonej wrażliwości.

Wnioski. Adaptacja szczepów *P. aeruginosa* do CMAP nie powoduje znaczących zmian w bakteriobójczym działaniu tej substancji. Szczep antybiotykooporny wykazywał podobną zdolność adaptacji i podobną wrażliwość na CMAP jak szczep stosowany do oceny skuteczności preparatów dezynfekcyjnych.

Słowa kluczowe: *proces adaptacji, Pseudomonas aeruginosa, czwartorzędowe sole amoniowe, skuteczność bakteriobójcza*

INTRODUCTION

Bacterial resistance to biocides could be intrinsic as well as acquired. The intrinsic resistance to biocides is connected especially with the structure and impermeability of the cell envelope. Bacterial spores, mycobacteria and *Gram*-negative bacteria are generally more resistant to biocides than *Gram*-positive bacteria because of special structural composition of their cell wall [12]. This fact makes that active substances of biocides can not enter the cell and reach the target site(s). In some instances, bacteria may produce enzymes that destroy the antibacterial agents [18, 20].

Mechanisms of acquired bacterial resistance to biocides are inducted and described particularly for less reactive biocides such as quaternary ammonium compounds, chlorhexidine and triclosan. For highly reactive active substances of disinfectants like glutaraldehyde or oxidising substances such mechanisms of resistance are significantly less frequent [10, 5]. However, *Martin* et al. described higher tolerance of *Bacillus subtilis* strain isolated from an endoscope washer-disinfector to oxidising agents (peracetic acid; chlorine dioxide, hydrogen peroxide) [11].

Acquired bacterial resistance to biocides is caused by mutation, overexpression of endogenous chromosomal genes or by acquisition of genetic determinants such as plasmids or transposons harboring foreign resistance genes [19]. Acquired, but not plasmid-encoded resistance to biocides and antibiotics may occur, when bacteria are exposed to gradually increased concentrations of antimicrobials [12, 14].

Widespread usage of disinfectants active substances and antibiotics, especially in the subinhibitory concentrations, may be also responsible for acquired resistance of bacteria. Selective pressure of antimicrobial agents can reduced susceptibility of bacteria and enable them surviving and maintenance in the environment [17, 22]. The common resistance mechanisms of bacteria to biocides and antibiotics may enable surviving antibiotic--resistant bacteria [12].

P. aeruginosa is a common nosocomial pathogen, which is intrinsically resistant to multiple classes of antimicrobials [16, 1]. Antimicrobial resistance of *P. aeruginosa* is well documented both in the case to antibiotics [15] and biocides [6, 9]. Common acquired biocide-resistance and antibiotic-resistance mechanisms of *P. aeruginosa* are related to permeability of the outer and cytoplasmic membranes [3]. An important factor in the emergence of resistant strains *P. aeruginosa* is also constitutive and enhanceable efflux mechanism removing a huge range of antimicrobial agents from the cell. Active efflux in conjunction with enzymatic mechanisms of resistance can cause co-resistance between biocides and antibiotics, which may be important in the spread of pathogenic microorganisms [9]. Mechanisms of bacterial resistance to biocides as well as their association with antibiotic resistance are still little known. There is also little information about how these types of resistance change the bactericidal efficacy of disinfectants.

This study aimed how resistance of *P. aeruginosa* strains (PA and PAO-LAC) acquired by adaptation to didecyldimethylammonium chloride in 2-propanol influences the bactericidal efficiency of this active substance.

MATERIALS AND METHODS

Active substance: Bardac22 – 50% v/v didecyldimethylammonium chloride in 20% v/v 2-propanol (CMAP) was the kind gift of Lonza, Warsaw, Poland.

Bacteria: *P. aeruginosa* ATCC 15442 (PA) strain which is used in determination of biocide efficiency and tetracycline-resistant *P. aeruginosa* ATCC 47085 (PAO-LAC) were used to adaptation process to CMAP.

Preparation of inocula

The subcultures of test organisms from stock cultures of PA and (PAO-LAC) were prepared on Tryptic Soy Agar (TSA; BD Difco) slants and on LB Agar Miller (A&A Biotechnology) + 10 µg/ml tetracycline slants, respectively. Then the slants were incubated in 37°C (PA) and in 30°C (PAO-LAC) for 24 h. After incubation time a second subculture was prepare from the first subculture in the same way as above. Second subcultures were used in adaptation experiments.

Induction of biocide adaptation

CMAP adaptation experiments were performed according to National Institute of Hygiene method [7]. The general principle of this method consists of carrying out successive passages of the microbial suspension into liquid medium containing the disinfecting substance in gradually incrementing concentrations. The bacterial suspension of 6,0 x 10^8 – 9,0 x 10^8 cfu/ml density were used to the first passage on liquid medium containing CMAP. TSB (BD Difco) for PA and LB (A&A Biotechnology) for PAO-LAC were prepared with increasing concentrations of CMAP from 30 to 85 mg/l and incubate for 48 h in temperature adequate to examined strains. Subsequently, next passages were obtained by transfer microbial suspension from last three tubes containing the highest concentration of CMAP, at which bacterial growth was observed into tubes with the same and increasing concentrations of CMAP in medium. After seven of such passages adapted strains of PA and PAO-LAC to CMAP at concentration of 375 mg/l were obtained. In parallel, examined strains were passaged onto medium without tested active substance as a control. Both of microorganisms were isolated and passaged on (49 passages), according to the following scheme, classifying them into three groups: A - strains unadapted passaged onto slant medium without active substance, control group; B – strains with adaptive resistance passaged onto slant medium with 375 mg/l CMAP; C – strains with adaptive resistance passaged onto slant medium without CMAP. Obtained isolates were used to determination bactericidal efficacy of CMAP.

Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined according to the broth dilution technique as described in National Institute of Hygiene method [7]. Briefly, CMAP were prepared at 10-fold higher concentrations than those provided for the study. To tubes containing 9 ml of TSB (PA) or LB with 10 µg/ml tetracycline (PAO-LAC) was added 1 ml of aqueous solutions CMAP at the range of concentrations from 30 to 1000 mg/l, adequate to the examined groups of isolates. Each concentration was prepared in three repetitions. Subsequently, it was added 0,05 ml of the suspension of microorganisms. Tubes were incubated at 37°C (PA) or at 30°C (PAO-LAC) for 48 h. Positive and negative controls were prepared with growth medium with and without bacterial cultures, respectively. Turbidity indicates bacterial growth. The absence of bacterial growth was interpreted as inhibitory activity of CMAP in given concentration.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by sample taken from each test tube, where there was no growth in the MIC assay. The loopful (10 µl) of test sample was transferred to TSA or LB agar with 10 µg/ml tetracycline without CMAP and incubated for 72 h at 37°C (PA) or at 30°C (PAO-LAC). Concentrations of CMAP, in which the growth was not observed, act as a bactericide.

Phenol coefficient (PC) method

Dilutions in distilled water were made from 2% (w/v) phenol solution and from 0.1% and 0.5% of CMAP stock solutions, depending on the group of organisms (A, B or C of PA or of PAO-LAC). A volume of 0.5 ml of test cultures was added to test tubes containing 5 ml of each of the final dilutions of phenol or CMAP. The test culture was added at 30 s intervals. The phenol coefficient is the number obtained by dividing the numerical value of the greatest dilution of the disinfectant capable of killing microorganisms in 10 minutes by the greatest dilution of phenol showing the same results [15]. Disinfectants that are more effective than phenol have coefficient grater than 1, those that are less effective have coefficient less than 1. Results of PC were expressed as mean value and standard deviation.

Statistical analysis

Phenol coefficient results were analyzed using confidence intervals (CI). CI was determined with two-sided confidence limit and 95% confidence level. 95% confidence level indicates that tested mean value is found within the CI at the significance level of 0.05.

RESULTS

There were no differences in adaptation process to didecyldimethylammonium chloride in 2-propanol between the reference strain of PA used to assess the effectiveness of disinfectants and antibiotic-resistant

strain PAO-LAC. After successive passages on liquid media, containing increasing inhibitory concentrations of CMAP, adaptive insusceptibility increased approximately 6-8 times for both examined strains (Table 1). Parallel, control passages of both strains were performed on the medium without this active substance. There were no significant changes in sensitivity of these isolates to didecyldimethylammonium chloride in 2-propanol as a consequence of passages. MICs after 7 passages for PA and PAO-LAC amounted to 30-35 mg/l and 30-45 mg /l, respectively.

Isolates from A, B, C groups, obtained after adaptation of standard strains of PA and antibiotic-resistant strain PAO-LAC to increasing concentrations of CMAP showed different sensitivity to this substance during their passages on a solid medium. MIC and MBC values for the PA isolates from group A showed no change in susceptibility after number of passages made (Table 2). Stable sensitivity of PAO-LAC strain from group A was observed up to 20th passage, whereas at 48-49 passages an increase of insensitivity to CMAP of this strain was noted (Table 3). In the case of isolates from group B increase insusceptibility to CMAP was observed for both PA and for PAO-LAC (Table 2 and 3). The values of MIC and MBC determined for isolates from the group B after 48-49 passages were similar for both strains, and significantly higher than the values obtained for the control isolates from group A. Isolates from the group C maintained reduced susceptibility to the CMAP (PA) after 48-49 passages, while in the case of the strain PAO-LAC insusceptibility to CMAP remained stable only to the passage 20th. PAO-LAC lost adaptive insusceptibility at 48-49 passage, and the MIC and MBC reached the values characteristic for strains, which were never passaged on solid medium with CMAP (Table 3).

Efficiency of didecyldimethylammonium chloride in 2-propanol was determined on isolates obtained between 22-29 passage. The highest efficiency of CMAP achieved against isolates of group C for both PA and PAO-LAC was 10.0 ± 4.4 and 10.0 ± 5.6. The lowest efficiency was observed for isolates of group B, passaged on solid medium containing CMAP at a concentration of 375 mg/l. It amounted to 2.2 ± 1.5 in the case of PA, and 6.0 ± 3.2 in the case of PAO-LAC. Efficiency of CMAP in group A against isolates of PA and PAO-LAC was 4.0 ± 0.5 and 8.6 ± 4.3, respectively. Despite the low efficiency of CMAP in relation to isolates from group B, there were no statistically significant differences in the effectiveness of the CMAP between the tested isolates (P> 0.05).

DISCUSSION

P. aeruginosa is a nosocomial pathogen, which exhibits innate resistance to multiple antimicrobials. It spreads, especially in the presence of water, due to the

Table 1. MIC for strains of *Pseudomonas aeruginosa* ATCC 15442 (PA) and *Pseudomonas aeruginosa* ATCC 47085 (PAO-LAC) passaged in increasing concentrations of CMAP

Strain codes	MIC (mg/l) of CMAP following serial passages (P0-P7)							
	P0	P1	P2	P3	P4	P5	P6	P7
PA	45	>95	>135	>175	>215	>255	>315	>375
PAO-LAC	65	>85	>135	>175	>215	>255	>315	>375

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of didecyldimethylammonium chloride in 2-propanol determined for *P. aeruginosa* ATCC 15442 (PA) isolates

PA isolates	Passages					
	1-2		20		48-49	
	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)
Group A	30-35	35-45	35	35	35-45	45-75
Group B	>375	>375	>375	>375	800	900
Group C	>375	>375	355	355	>700	>700

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of didecyldimethylammonium chloride in 2-propanol determined for *Pseudomonas aeruginosa* ATCC 47085 (PAO-LAC) isolates

PAO-LAC isolates	Passages					
	1-2		20		48-49	
	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)
Group A	30-45	55->65	30	>100	>200	>200
Group B	>375	>375	>375	>375	>1000	>1000
Group C	>375	>375	>375	>375	45	155

low permeability of the outer membrane, which preventing penetration into the cell several active substances [3, 8]. Adaptation of *P. aeruginosa* to increasing concentrations of disinfectants active substances obtained by serial passages was described in several publications by *Abdel Malek* et al. [1, 2, 3]. Bacterial resistance to disinfectants containing quaternary ammonium compounds (QACs), e.g. benzalkonium chloride, which are widely used in both medical and food environments was describe by *Sundheim* et al. The study showed that resistance to QACs in pseudomonads is much higher than in staphylococci.

Our results are an attempt to answer the question of how adaptation of *P. aeruginosa* strains to didecyldimethylammonium chloride, belonging to QACs, decreases susceptibility of these strains and influence bactericidal efficiency of didecyldimethylammonium chloride. Many studies have focused on change in susceptibility of examined strains obtained after adaptation process [9]. Whereas there is no information as changes in the adaptive resistance affect the bactericidal effectiveness of this active substance. The derivation of the phenol coefficient as a parameter evaluating the effectiveness of the CMAP in relation to the *P. aeruginosa* isolates showed that the change in resistance obtained by the adaptation process does not significantly affect the efficiency of CMAP. Despite phenol coefficient are not quantitative method, it can be useful in estimating bactericidal efficiency of active substances of disinfectants and are still used by some researchers [15, 21]. In contrast to the minimum bactericidal doses, phenol coefficient based not only on the concentration of the active substance but also on the contact time. These parameters are important for efficient disinfection processes.

The MIC and MBC results which describe the minimum inhibitory and bactericidal concentration have a little practical use in disinfection [4], but these results allowed observing changes in the sensitivity of the tested strains caused by adaptation. Our MIC results showed that adaptation of *P. aeruginosa* strains to didecyldimethylammonium chloride increased insusceptibility of them 6-8 times in comparison to the non-adapted strains. In this case an increase of insusceptibility of PA and PAO-LAC to CMAP was insignificant, when we use the parameters of disinfection that take into account concentration and contact time as it was assessed by phenol coefficient. The reversible insusceptibility of PAO-LAC, passaged on medium without CMAP, was observed at 49 passages, what can suggest that acquired resistance may maintain only when the active substance is present. As described by *Maillard* bacteria can lose their tolerance to biocide, when they were grown in the absence of selective pressure. Many examples of this phenomenon were observed in the case of QACs [10].

However, the similar adaptive resistance of examined strains suggests that mechanisms of its acquisition may be resembled to both strains. *Pseudomonads* strains, especially wild type PAO1, are described as a strains, which have an efflux system Mex-CD-OprJ induced by disinfectant belonging to QACs [13]. Insusceptibility of examined strains to didecyldimethylammonium chloride can be caused by induction of this mechanism.

Obtained adaptive resistance to didecyldimethylammonium chloride in 2-propanol for both, PA strain used in the assessment of the effectiveness of disinfectants as well as in the case of antibiotic-resistant strain PAO--LAC may be important, when the bactericidal concentration of active substance will be reduced to MIC level.

CONCLUSIONS

1. Changes in susceptibility of *P. aeruginosa* strains to didecyldimethylammonium chloride in 2-propanol obtained by adaptation process do not significantly affect the efficacy of this active substance.
2. Antibiotic-resistant strain *Pseudomonas aeruginosa* ATCC 47085 (PAO-LAC) showed a similar ability to adaptation and a similar sensitivity to the didecyldimethylammonium chloride in 2-propanol as a *Pseudomonas aeruginosa* ATCC 15442 strain used to assess the effectiveness of disinfectants.

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Conflict of interest

The authors declare no conflict of interest.

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