Evaluation of the Bactericidal Activity of Didecyl Dimethyl Ammonium Chloride in 2-Propanol against *Pseudomonas aeruginosa* Strains with Adaptive Resistance to this Active Substance According to European Standards

This study evaluated the bactericidal activity of the cationic surf- face active compound didecyl dimethylammonium chloride in 2-propanol against the two reference strains *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* (tetracycline-resistant) as well as their isolates adapted to this active substance. Individual phases and stages of European Standards which are intended to determine the bactericidal activity of disinfectants in medical areas were used. Disinfection parameters of the tested substance as determined by suspension methods were not sufficient to achieve the required bactericidal reduction in the carrier method for *Pseudomonas aeruginosa* and all its adapted isolates. *Pseudomonas aeruginosa* (tetracycline-resistant) and its adapted isolates were more sensitive to the tested active substance when the contact time was extended from 5 to 10/15 min. Adaptive resistance of *Pseudomonas aeruginosa* strains can be abolished by using increased concentrations of the tested substance and/or extended contact time.

**Key words:** Adaptation, didecyl dimethyl ammonium chloride (DDACP), quaternary ammonium compound (QAC), in-use concentrations, *Pseudomonas aeruginosa*

**Stichwörter:** Adaption, Didecyldimethylammoniumchlorid (DDACP), quaternäre Ammoniumverbindung (QAC), Anwendungskonzentration, *Pseudomonas aeruginosa*

1 Introduction

The biocidal efficacy of chemical disinfectants is determined according to European Standards which are grouped in phases and stages. There are three stages involving different techniques and conditions for testing the efficacy of disinfectants. Phase 1 includes suspension test methods. In phase 2, there are two stages. The first stage of this phase includes suspension test methods. The second stage consists of carrier test methods. Phase 3 is not as yet used in the evaluation of the biocidal efficacy of disinfectants. Development of methods in this phase will consider the efficacy of disinfectants in their place of use [1, 2].

Currently, disinfectants ought to be tested consecutively according to phase 1 and stages 1 and 2 of phase 2. The requirements depend on the area of disinfectant application. However, biocidal efficacy studies are increasingly being limited to phase 2, stage 2. The requirements in this phase and stage are highest, because the bacterial suspension with the interfering substance is fixed to the carrier surface [1, 2].

Bactericidal activity is evaluated using reference strains that have a specific resistance to disinfectants. These strains are obligatory in evaluating biocidal efficacy. Additional test strains can be used only when the required reduction of bacteria has been obtained for obligatory strains [2].

The effectiveness of disinfectants depends not only on the type of strain, but also on the concentration and potency of the active ingredients in the formulation and the contact time between the active substance and the bacteria. The presence of inhibitors such as organic matter in the environment of disinfectant application limits the effectiveness of the disinfection [3, 4].

Determination of the concentration of a disinfectant of unknown biocidal activity can be based either on the basis...
of methods of MIC (Minimum Inhibitory Concentration)/MBC (Minimum Bactericidal Concentration), PC (Phenol Coefficient) or standards contained in phase 1 [3, 5, 6]. However, these methods are not used to determine the commercial concentrations of disinfectants (in-use concentrations) because they do not take into account the organic contamination and the attachment of the bacteria to the surface. To determine the commercial concentrations of biocide standards of phase 2 stage 2 ought to be applied. Concentrations of disinfectants determined based on the method of MIC/MBC, PC or the standards of phase 1 used in the disinfected area may have a static effect on bacteria and lead to their survival on surfaces in the environment [6]. The use of disinfectants at sub-inhibitory concentrations rather than biocidal ones may lead to the acquisition of adaptive resistance by microorganisms and thereby lead to their spread in the environment [4, 7–9].

It has been proven that the use of disinfectants in sub-inhibitory concentrations induces resistance mechanisms in (mainly Gram-negative) bacteria such as overexpression of efflux pumps and the production of enzymes degrading disinfectants [8, 10–13]. Also, bacterial modification of fatty acids and phospholipid compositions that build the cell membrane promotes the reduction of sensitivity to disinfectants of planktonic and biofilm cells [3, 7, 11]. This can hinder the combating of such cells and require the use of higher concentrations of active substances [6].

Didecyl dimethyl ammonium chloride (DDAC) is an active substance reducing surface tension which has strong bactericidal action [14]. Consequently, it is widely used for surface disinfection [9, 15]. At the same time, it is an active substance classified to quaternary ammonium compounds (QACs), to which bacterial resistance was most often ascribed [7, 16, 17].

The aim of the study was to determine the activity of didecyl dimethyl ammonium chloride in 2-propanol against P. aeruginosa strains, the numbers of surviving bacteria in each sample after 48 h incubation, in conditions specified for the tested strains, the numbers of surviving bacteria in each sample were determined and the reduction was calculated. DDACP was added to a test suspension of the bacteria to be examined. The number of cells of the tested strains in the suspension was between 1.5 \times 10^8 and 5 \times 10^9 cfu/mL. The mixture of the test suspension and DDACP at the specified concentrations was maintained at 20 °C for 5 min ± 10 s, 10 min ± 10 s and 15 min ± 10 s. After the contact time, an aliquot was neutralized for 5 min ± 10 s. Two samples of 1 mL were inoculated using the pour plate technique. After 48 h incubation, in conditions specified for the tested strains, the numbers of surviving bacteria in each sample were determined and the reduction was calculated. DDACP was considered as active if it demonstrated at least a 5 decimal lg reduction [5].

2.2 Bacterial cultures

The stock cultures of P. aeruginosa ATCC 15442 and its isolates were subcultured in Tryptone Soya Agar. Pseudomonas aeruginosa ATCC 47085 (PAO-LAC) and its isolates were subcultured in LB Miller Agar + 10 μg/mL tetracycline. In order to prepare the working cultures of these test organisms, a subculture from the stock cultures was prepared by streaking onto slopes with an appropriate medium. All strains were incubated at 37 °C, except PAO-LAC and its isolates, which were incubated at 30 °C for 24 h. Two successive subcultures were prepared. The second subcultures were used in methods of the evaluation of bactericidal activity, i.e., PN-EN 1040, PN-EN 13727 and PN-EN 14561.

2.3 PN-EN 1040

DDACP was added to a test suspension of the bacteria to be examined. The number of cells of the tested strains in the suspension was between 1.5 \times 10^8 and 5 \times 10^9 cfu/mL. The mixture of the test suspension and DDACP at the specified concentrations was maintained at 20 °C for 5 min ± 10 s, 10 min ± 10 s and 15 min ± 10 s. After the contact time, an aliquot was neutralized for 5 min ± 10 s. Two samples of 1 mL were inoculated using the pour plate technique. After 48 h incubation, in conditions specified for the tested strains, the numbers of surviving bacteria in each sample were determined and the reduction was calculated. DDACP was considered as active if it demonstrated at least a 5 decimal lg reduction [5].

2.4 PN-EN 13727

Bactericidal activity of DDACP was tested in clean conditions (0.3 g/L bovine albumin solution) at three contact times (5 min ± 10 s, 10 min ± 10 s and 15 min ± 10 s). In comparison to the concentrations of DDACP used in PN-EN 1040, the concentrations of DDACP were increased about 14 times in the active range and the 11 times in the non-active range. The number of cells in the suspension was obtained in the range required by the standard (1.5 \times 10^5 and 5 \times 10^6 cfu/mL). A tube with mixtures of the test suspension and the interfering substance was placed in a water bath at 20 °C for 2 min ± 10 s. After this time, the tested solutions were added for selected contact times. At the end of the contact times, 1 mL of each sample was transferred to a neutralizer for 10 s ± 1 s in the case of contact times of 5 and 10 min and for 5 min ± 10 s in the case of a 15-minute contact time. 1 mL samples of the test mixtures in duplicate were inoculated using the pour plate technique. After 48 h incubation, in conditions specified for the tested
strains, the numbers of surviving bacteria in each sample were determined and the reduction was calculated. DDACP was considered as active if it demonstrated at least a 5 decimal lg reduction [19].

2.5 PN-EN 14561

The test was carried out on glass carriers, on which the test suspension with the interfering substance solution (clean conditions: 0.3 g/L bovine albumin solution) was spread. The number of cells in a test suspension ranged from $1.5 \times 10^{8}$ cfu/mL to $5 \times 10^{9}$ cfu/mL. Carrier glasses with test suspensions and interfering substances were dried in a laminar chamber at room temperature for up to 60 min. After drying, each carrier was immersed in a solution of the test substance for a specified contact time (for 5 min $\pm$ 10 s, 10 min $\pm$ 10 s and 15 min $\pm$ 10 s) or in hard water intended to control the recovery of bacteria from the carrier, without the effect of the active substance. After the contact time, the glass carriers were transferred into tubes with a neutralizer and glass beads. After the neutralization time, the bacteria were shaken with glass beads to detach them from the surface of the carrier. In each sample, the number of surviving bacteria was specified. The reduction was determined by calculating the difference between the number of bacteria recovered from the carrier after hard water treatment and the number of bacteria remaining in the test after DDACP activity and neutralization. DDACP was considered as active if it demonstrated at least a 5 decimal lg reduction [20].

2.6 Statistical analysis

Tests were carried out in validated methods in one repetition. The reproducibility standard deviation was determined for each method [21]. The reproducibility standard deviation for EN 1040 was $\pm$ 0.09 lg; for EN 13727 was $\pm$ 0.15 lg and for EN 14561 was $\pm$ 0.03 lg.

3 Results

3.1 Phase 1

PA isolates from groups B and C adapted to DDACP as well as isolates from group A were insensitive to the active concentration of DDACP determined against the reference strain PA. The reduction coefficient for isolates subjected to adapta-

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**Figure 1** Bactericidal activity of DDACP expressed as reduction [lg] of the reference strain *P. aeruginosa* ATCC 15442 (PA) and the PA isolates (a; b) and reference strain *P. aeruginosa* ATCC 47085 (PAO-LAC) and the PAO-LAC isolates (c; d) unadapted (group A) and adapted to DDACP (group B and group C) at active and non-active concentrations and over contact times 5, 10 and 15 min according to PN-EN 1040: 2006. The reproducibility standard deviation of PN-EN 1040: 2006 method was $\pm$ 0.09 lg.
tion (groups B and C) and the isolates from control group A ranged from 3.93 to 4.05 and this was lower than the required \( \lg R = 5 \) [Fig. 1a]. All tested strains of PA were insensitive to the determined inactive concentration [Fig. 1b].

Tetracycline-resistant strains of PAO-LAC adapted to DDACP from groups B and C were insensitive to the tested active concentration (\( \lg R \) in the range from 4.05 to 4.12). The basic bactericidal activity was demonstrated against the reference strain PAO-LAC and against the isolates from control group A [Fig. 1c].

The PAO-LAC reference strain was susceptible to inactive DDACP concentrations in extended contact times of 10 and 15 min [Fig. 1d].

3.2 Phase 2 stage 1

The reference strain PA and its isolates from groups A and B were sensitive to increased DDACP concentrations in both active and non-active concentrations. The only exception were the isolates from group C, for which the applied active concentration fulfilled the requirements of the standard in the range of a 5 \( \lg \) reduction only within 10 and 15 min [Fig. 2a)]. Non-active concentrations of DDACP toward PA from group C met the standard assumptions regarding the requirements for inactive concentrations at 5 and 10 min. Over an extended contact time of 15 min, the reduction was as in the case of the determined active concentration [Fig. 2b].

Tetracycline-resistant strain PAO-LAC was sensitive to the tested active and non-active concentrations, both for the reference strain and unadapted isolates from group A subjected only to passage as well as in the case of isolates from groups B and C – adapted to DDACP at the contact times of 5, 10 and 15 min [Fig. 2c and 2d].

3.3 Phase 2 stage 2

The results obtained in the carrier standard of PN-EN 14561 are in contradiction to results obtained in the suspension standard of PN-EN 13727+A2: 2015-12. There was a lack of activity toward PA and its isolates from groups A and B in the case of the active and non-active concentrations used over the three contact times tested. However, active and non-active concentrations of DDACP were bactericidal against the isolates from group C at 15 min contact time [Fig. 3a and 3b].

![Figure 2](image-url)  
**Figure 2** Bactericidal activity of DDACP expressed as reduction [\( \lg \)] of the reference strain *P. aeruginosa* ATCC 15442 (PA) and the PA isolates (a; b) and reference strain *P. aeruginosa* ATCC 47085 (PAO-LAC) and the PAO-LAC isolates (c; d): unadapted (group A) and adapted to DDACP (group B and group C) at active and non-active concentrations over contact times 5, 10 and 15 min according to PN-EN 13727+A2: 2015-12. The reproducibility standard deviation of PN-EN 13727+A2: 2015-12 method was \( \pm 0.15 \lg \).
PAO-LAC was insensitive to the active concentration at a contact time of 5 min in the case of both the reference strain and isolates from individual groups. The activity of all tested isolates and the reference strain was achieved in extended contact times of 10 and 15 min at active concentrations [Fig. 3c].

Similar changes were observed in the case of non-active concentrations, which did not reduce the number of tested microorganisms to the required reduction level within 5 min and 10 min of contact against the reference PAO-LAC and the isolate from group A, which was passaged without DDACP. At 15 min contact time, the non-active concentration reduced the number of tested microorganisms to an average active level amounting to >3.40 lg [Fig. 6d].

4 Discussion

Exposure of bacteria to the active substances of disinfectants can lead to their adaptation to stressful conditions, especially if the concentrations of active substances are not bactericidal, but only bacteriostatic [6, 12, 16, 22]. Adaptation of *P. aeruginosa* to active substances of disinfectants has been widely described in the literature [10, 16, 22–24]. In the suspension tests of phase 1, the basic bactericidal action of disinfectants is determined. However, under organic loading conditions, the concentration determined in this phase of the study may be only bacteriostatic. Determination of in-use parameters of disinfectants should take place in conditions most similar to those existing in the area where disinfectants are to be used [1, 2].

This study has illustrated the differences in the activity of didecyl dimethyl ammonium chloride in 2-propanol in the individual phases of testing disinfectants against reference strains of PA and PAO-LAC and their isolates adapted to this active substance (group B; group C) as well as for isolates not adapted but only passages in parallel (group A).

In the suspension test of phase 1, the active concentration was bactericidal for PA and PAO-LAC reference strains and for group A of strain PAO-LAC. PA isolates from groups A, B and C were insensitive to the active concentration, which indicates that the presence of adaptive resistance can come from contact with DDACP (groups B and C) or can be acquired through serial passages (group A) [25].

Groups B and C isolates of PAO-LAC, as opposed to isolate from group A, showed insensitivity to the active concen-

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**Figure 3**  Bactericidal activity of DDACP expressed as reduction [lg] of the reference strain *P. aeruginosa* ATCC 15442 (PA) and the PA isolates (a, b) and reference strain *P. aeruginosa* ATCC 47085 (PAO-LAC) and the PAO-LAC isolates (c, d); unadapted (group A) and adapted to DDACP (group B and group C) at active and non-active concentrations and over contact times 5, 10 and 15 min according to PN-EN 14561: 2008. The reproducibility standard deviation of PN-EN 14561:2008 method was ± 0.05 lg.
tration of DDACP, similar to B and C isolates of PA. Tetra-
cycline-resistant reference strain PAO-LAC was more sensi-
tive to DDACP than the reference strain of PA, because the
non-active concentration was already bactericidal against it
within 10 and 15 min. In this case, biocide resistance and
antibiotic cross-resistance was not examined, but in the lit-
erature there are many examples showing that this connec-
tion exists, especially for QACs used in food processing en-
vironments [13, 15]. Loughlin et al. observed that the PAO1
strain adapted to benzalkonium chloride possesses increas-
ing resistance to chloramphenicol, which inhibits the pro-
tein synthesis, and to membrane-active polymyxin B [7].

In the suspension tests of phase 2 stage 1, the concentra-
tions of DDACP were increased due to the necessity of the
application of organic loading. It is well documented that a
heavy organic load requires a greater concentration of cat-
onic active substances such as QACs [15]. An elevated con-
centration of DDACP resulted in the abolition of the adap-
tive resistance of PA strains with groups B and A. However,
to achieve bactericidal effectiveness of this substance against
strains from group C, it was also necessary to extend the
contact time to 10–15 min. In practice also the rotation of
infectants allows avoidance of the development of bacterial
adaptation to QACs [3]. In the suspension method, the PAO-
LAC tetracycline-resistant strain and its isolates were sensi-
tive to the non-active and active DDACP concentrations over
all tested contact times. No increased resistance to DDACP
was observed because of PAO-LAC resistance to tetracycline,
albeit Bjorland et al. observed that penicillin and tetracy-
cline-resistant Staphylococcus aureus isolates recovered from
cows were more tolerant to QACs [26].

In the carrier tests of phase 2 step 2, the DDACP bacte-
ricidal activity was varied. The PA strain and its isolates
showed insensitivity to the active concentration of DDACP
differently than in the suspension test of phase 2 stage 1.
The required reduction value of bactericidal activity was only
achieved for group C of PA strain at 15 min contact time.
Unlike our results, the suspension and carrier disinfection
tests used by Thomas et al., against P. aeruginosa strains
which exhibited raised MIC for chlorhexidine diacetate and
benzalkonium chloride did not show a greater resistance of
these strains as compared to reference strains of P. aerugin-
osa [16]. In our study, the PAO-LAC strain and its isolates
were more sensitive to the active concentration of DDACP
than the PA strain and its isolates in the suspension and car-
rier tests. Bactericidal activity against these strains was
achieved at active concentrations at contact times of 10 and
15 min and at 15 min contact time at non-active concen-
trations. Increased sensitivity of the tetracycline-resistant strain
on DDACP may be associated with greater selective pressure
derived from an antibiotic and an active substance and with
energy expenditure on metabolic processes, which are re-
sponsible for the mechanisms of antibiotic resistance and
adaptation [27].

The obtained results showed that it is possible to abolish
adaptive resistance by increasing the concentration of the
tested active substance and extending the contact time.
However, the differentiation of DDACP bactericidal activity
was not only due to the adaptive resistance of microorgan-
isms to the tested active substance, but also to the test con-
ditions used [12]. The attachment of microorganisms to car-
rriers in phase 2 of stage 2 influenced the change in the
bactericidal activity of the tested active substance compared
to the activity achieved in phase 2 of stage 1. There are some
reports of changes in the sensitivity of strains attached to
the surface, whether culture medium, carrier or biofilm [9, 28,
29]. Brill et al. indicated that culturing S. aureus and P. aeur-
gina on solid medium influenced the sensitivity of these
strains to cationic active compounds such as benzalkonium
chloride, chlorhexidine digluconate and octenidine dihy-
drochloride. In suspension tests, both organisms grown on
agar medium were more sensitive to all tested biocides than
cells grown in broth [28]. In contrast, bacteria attached to
the surface, e.g., in biofilm, are more difficult to combat and
this requires the use of higher concentrations of active sub-
stances and/or longer contact times [9, 11, 15, 29]. Gerba postulated that a higher temperature also increased the ac-
tivity of QACs [15].

Kampf postulates that the high MIC values obtained for
benzalkonium chloride against environmental bacteria, in-
cluding P. aeruginosa, may mean that the parameters of dis-
fektants determined against reference strains may be ineffec-
tive in practice [22].

The conducted research indicates the necessity of using
carrier methods in determining the in-use parameters of
using disinfectants, as suspension methods may not be suf-
ficient to determine the correct disinfection parameters and
combat adaptive resistance.

5 Conclusions

1. The Pseudomonas aeruginosa strains and their adapted
isolates were found to be less sensitive to didecyl
dimethyl ammonium chloride in 2-propanol in the carrier
method than in the suspension method.
2. Adaptive resistance of Pseudomonas strains can affect
the efficiency of surface disinfection if DDACP sub-inhibi-
tory concentrations are used.
3. Correctly determined in-use parameters, i.e., the con-
centration and contact time of the disinfectant in the carrier
methods (phase 2, step 2), can contribute to the abolition
of adaptive resistance among Pseudomonas strains.
4. Reference strain Pseudomonas aeruginosa intended to ex-
amine the activity of disinfectants in European Stan-
ards should be chosen by manufacturers as obligatory
strain for determination disinfection parameters.
5. Strains with antibiotics-resistant features, e.g. tetracy-
cline-resistant Pseudomonas aeruginosa, should only be
tested as an addition because they can have higher sensitiv-
ty and the designated disinfection parameters may be too
low to act as bactericidal.

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Received: 17. 12. 2018
Revised: 04. 04. 2019

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DOI: 10.3319/113.110632
Tenside Surf. Det. 56 (2019) 4: page 287 – 293 © Carl Hanser Verlag GmbH & Co. KG
ISSN 0392-3414

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Dr hab. n. med. Aneta Nitsch-Osuch: Specialist pediatrician, epidemiologist specialist, public health specialist. Scientific interests include the issues of epidemiology, diagnosis, treatment and prevention of diseases important from the point of view of public health.

Tenside Surf. Det. 56 (2019) 4