

Minimum inhibitory (MIC) and minimum microbicidal concentration (MMC) of polihexanide and triclosan against antibiotic sensitive and resistant *Staphylococcus aureus* and *Escherichia coli* strains

Minimale Hemm-Konzentration (MHK) und minimale bakterizide Konzentration (MBK) von Polihexanid und Triclosan gegen Antibiotika-empfindliche und resistente *Staphylococcus aureus*- und *Escherichia coli*-Stämme

Abstract

Background: An in-vitro study was conducted investigating the anti-microbial efficacy of polihexanide and triclosan against clinical isolates and reference laboratory strains of *Staphylococcus aureus* and *Escherichia coli*.

Methods: The minimal inhibitory concentration (MIC) and the minimal microbicidal concentration (MMC) were determined following DIN 58940-81 using a micro-dilution assay and a quantitative suspension test following EN 1040. Polihexanide was tested in polyethylene glycol 4000, triclosan in aqueous solutions.

Results: Against all tested strains the MIC of polihexanide ranged between 1–2 µg/mL. For triclosan the MICs varied depending on strains ranging between 0.5 µg/mL for the reference strains and 64 µg/mL for two clinical isolates. A logRF >5 without and logRF >3 with 0.2% albumin burden was achieved at 0.6 µg/mL triclosan. One exception was *S. aureus* strain H-5-24, where a triclosan concentration of 0.6 µg/mL required 1 minute without and 10 minutes with albumin burden to achieve the same logRFs. Polihexanide achieved a logRF >5 without and logRF >3 with albumin burden at a concentration of 0.6 µg/mL within 30 sec. The exception was the North-German epidemic MRSA strain, where an application time of 5 minutes was required.

Conclusion: The clinical isolates of *E. coli* generally showed higher MICs against triclosan, both in the micro-dilution assay as well in the quantitative suspension test than comparable reference laboratory strains. For polihexanide and triclosan strain dependant susceptibility was shown. However, both antimicrobial compounds are effective when used in concentrations common in practice.

Keywords: polihexanide, triclosan, antimicrobial, inhibitory concentration, microbicidal concentration

Zusammenfassung

Hintergrund: In einer quantitativen in-vitro Studie wurde der antimikrobielle Effekt von Polihexanid und Triclosan unter identischen Verhältnissen gegen Referenzstämme sowie klinische Isolate von *Staphylococcus aureus* und *Escherichia coli* bestimmt.

Methoden: Die minimale Hemmkonzentration (MHK) und die minimale mikrobiozide Konzentration (MMK) wurden gemäß DIN 58940-81 mittels Mikro-Verdünnungs-Essay und eines quantitativen Verdünnungstests gemäß EN 1040 bestimmt. Polihexanid wurde in einer Polyethylene-Glykol 4000 Lösung, Triclosan in wässriger Lösung angesetzt.

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Ergebnisse: Die MHK von Polihexanid gegen alle getesteten Stämme lag zwischen 1–2 µg/mL. Die MHK von Triclosan war stammabhängig und lag bei 0,5 µg/mL für die getesteten Referenzstämme und bei 64 µg/mL für zwei klinischen Isolate. Gegen alle Stämme erreichte Triclosan bei einer Konzentration von 0,6 µg/mL einen logRF >5 ohne und logRF >3 mit 0,2% Albuminbelastung. Als Ausnahme erwies sich *S. aureus*-Stamm H-5-24, für den eine Triclosan-Konzentration von 0,6 µg/mL bei einer Einwirkungszeit von 1 min ohne bzw. 10 min mit Albuminbelastung erforderlich war, um dieselben logRF zu erreichen. Polihexanid erreichte bei einer Konzentration von 0,6 µg/mL einen logRF >5 ohne und logRF >3 mit Belastung bei einer Einwirkungszeit von 30 s. Hier erwies sich als Ausnahme der Norddeutsche MRSA Epidemiestamm, gegen den bei selber Konzentration eine Einwirkungszeit von 5 min erforderlich war.

Schlussfolgerungen: Die klinischen *E. coli*-Isolate erforderten höhere MHKs bei Triclosan als die untersuchten Referenzstämme. Für Polihexanid und Triclosan konnte eine stammabhängige Empfindlichkeit gezeigt werden. Beide Antiseptika werden im klinischen Einsatz jedoch regelhaft bei weit höheren Konzentrationen eingesetzt, womit die unterschiedlichen Empfindlichkeiten klinisch keine Rolle spielen sollten.

Schlüsselwörter: Polihexanid, Triclosan, antimikrobiell, minimale inhibitorische Konzentration, MHK, minimale mikrobizide Konzentration

Introduction

Due to their different chemical structure, their different use in clinical practice and their different antimicrobial capacity the matter of antibiotic resistance does not apply to antiseptics at the moment. So far, for microbicidal antiseptics no clinically relevant resistance is documented [1], [2], [3]. For microbistatic antiseptics, however, the potential risk for development of resistance can not be excluded. Examples for microbistatic antiseptics include benzalkonium chloride [4], Cetylpyridinium chloride [5], chlorhexidine and triclosan [6], [7]. For hexachlorophene [1] and chlorhexidine [8] a plasmid-coded resistance was shown already.

Furthermore, for some antiseptics and antibiotics, increased resistance against antiseptics was correlated with increased antibiotic resistance [9], [10]; however, increased antibiotic resistance has not been associated with antiseptic resistance so far. Russel et al. [11] could demonstrate in an in-vitro study that after *Pseudomonas stutzeri* was exposed to increasing concentrations of chlorhexidine after multiple passages, a stable chlorhexidine resistance was achieved. Simultaneously resistance against benzalkonium chloride, cetylpyridinium chloride and triclosan and a number of antibiotics was increased as well. For *Mycobacterium smegmatis* our study group could show a parallel increase in resistance for isoniazid and triclosan [7].

For the majority of antiseptic compounds the first step for their antimicrobial action is penetration into the bacterial cell. If this penetration is minimized or inhibited, resistance will result. Bacteria are able to alter their cell's permeability by changing its lipid content, the composition of the outer membrane proteins, plasmid-coded mucous production or by altering the affinity of their efflux pumps.

If the efflux pump has an unspecific affinity to several compounds, resistances will result. Example for an efflux pump with a broad substrate spectrum is *AcrAB* of *Escherichia coli* which is also responsible for the efflux activity of the membrane-pore-protein *TolC*, able of transporting tetracycline, chloramphenicol, fluoroquinolones, β-lactams, novobiocin, erythromycin, ethidium bromide, crystal violet and Acriflavin [12], [13].

In contrast to antibiotics where antibiograms are generated in course of routine clinical microbiological diagnostics for a broad range of antibiotics and bacteria, such routine data is not available for antiseptics and knowledge on antimicrobial efficacy is based only on selected, most often historic, literature. In light of the possibilities for development of resistance against antiseptics it seems useful to control the antimicrobial activity of frequently used antiseptics and possible changes in regular intervals.

Therefore we conducted an in-vitro study investigating the current antimicrobial efficacy of the frequently used skin, mucous membrane and wound antiseptics polihexanide and triclosan against clinical isolates and defined laboratory strains of *Staphylococcus aureus* and *E. coli*.

Methods

Tested antimicrobial compounds

For polihexanide, we used the commercially available product Lavasept® (Fresenius AG, Bad Homburg, Germany) which contains 200 µg/mL polihexanide and 10 µg/mL macrogolum 4000. Because triclosan as pure substance (Irgasan® DP 300; CAS No 88032-08-0; Ciba AG, Basel, Switzerland) is not solvable in water, we diluted

the product in dimethylsulfoxid (DMSO) and 9.2% ethanol according to the manufacturer's recommendation. In a separate pilot experiment, we could rule out that 9.2% ethanol itself possesses antimicrobial properties.

Tested clinical isolates and laboratory strains

The following bacteria were tested using the micro-dilution test: *E. coli* ATCC 25922; *E. coli* AG 100 wild type (K-12 argE3 thi-1 rpsL xyl mtl Δ (gal-uvrB) supE44) [14], [15]; *E. coli* AGT11, containing *fabI* mutation with resistance against triclosan; exchange of amino acid in the enoyl-reductase in position 93 [16]; *E. coli* AGT 11K (AcrAB::kan, deletion of the *E. coli* efflux pump AcrAB) [14]; *E. coli* AGT 11 Kan (AmarCORAB::kan, constructed by replacement of a chromosomal 1.24-kb BspHI fragment of the mar locus in AG100 by homologous recombination with the kanamycin resistance cassette (Kanr) from pKMN33; deletion of the regulator gene which activate the efflux pump) [17]; *S. aureus* ATCC 29213, MRSA strains H-5-18, H-5-19, H-5-20, H-5-24, H-5-26, H-5-27, and H-5-31 (University Clinic Bonn, Germany), MRSA epidemic strain North-Germany, Niedersachsen and Berlin (Robert Koch Institute, Wernigerode, Germany); VISA strains Ve 13985, Ve 1177/98, BK 13230, BK 1704/98, 18 A 026, and 20 A 063 (Robert Koch Institute, Wernigerode, Germany). Additionally to the micro-dilution test, *S. aureus* ATCC 29213, MRSA H-5-24, MRSA epidemic strain North-Germany, *E. coli* ATCC 25922 and *E. coli* AGT 11 were tested in a quantitative suspension test.

Culture media and neutralizing agents

As polihexanide precipitates on Mueller-Hinton agar, micro-organisms were cultured on iso-sensitest bouillon (Oxoid, Darmstadt, Germany), tryptone soya agar and broth, respectively (Oxoid, Wesel, Germany). As neutralizer for polihexanide 3% (w/v) Tween 80 (Serva, Heidelberg, Germany), 3% (w/v) saponine (Fluka, Buchs, Switzerland), 0.1% (w/v) histidine (Serva, Heidelberg, Germany), and 0.1% (w/v) cysteine (Merck, Darmstadt, Germany) was used. As neutralization of triclosan was not possible with the usual neutralization solution, we used egg yolk (sterile egg yolk diluted to 50% by sterile distilled water) instead. However, only triclosan concentrations of 0.6 μ g triclosan/L and less were possible. Therefore, this concentration also was the maximum tested concentration in the quantitative suspension test.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC was determined following DIN 58940-81 [18] using a micro-dilution assay. Briefly, using colonies from a fresh overnight culture an inoculum of 1×10^5 cfu/mL in the final medium was prepared. 50 μ l of the inoculum

were added to 50 μ l of the respective antimicrobial test-dilution and incubated at $36^\circ\text{C} \pm 1^\circ\text{C}$ for $20\text{h} \pm 2\text{h}$.

Determination of the Minimum Microbicidal Concentration (MMC)

The MMC was determined using the quantitative suspension test following Pitten et al. [19] with and without bio-burden. For each assay, 0.1 ml of the test organism (10^8 – 10^9 cfu/mL) in tryptone soya broth was transferred from a fresh overnight culture into a test tube, mixed with 1 ml distilled sterile water (test without bio-burden) or, in parallel series, with 1 ml of 0.2% bovine serum albumin (test without bio-burden), and transferred in 9 ml of the test solution. At the final time of action (30 sec, 60 sec, 5 min, 10 min, 60 min, respectively) 1 ml of the test mixture was transferred into 9 ml tryptical-soy-broth with addition of the respective neutralizer. After 5 minutes of neutralisation, serial dilutions were plated on trypticase-soy-agar. Colonies were counted after 48h. The \log_{10} reduction factor (RF) for each application time was calculated using the formula: \log_{10} (control) – \log_{10} (test sample). All experiments were performed in triplicate. Both antiseptics were tested at concentrations of 2 μ g/mL, 0.2 μ g/mL and 0.02 μ g/mL.

Statistical analysis

All assays were repeated 6-fold, and number of organisms were averaged as mean cfu/ml and expressed as \log_{10} . The \log_{10} reduction factor (logRF) was calculated as \log_{10} of the pre-value minus \log_{10} of the post-value.

Results

Microbistatic activity

For *S. aureus* no difference was observed in the antimicrobial activity of polihexanide at concentrations of 1 and 2 μ g/mL against ATCC reference strains, 10 MRSA isolates and 6 VISA strains (Table 1). Triclosan could be tested only against the ATCC strains and the epidemic North-German MRSA strain. Compared to MRSA strains the antimicrobial activity of triclosan was 512 times lower. All *E. coli* ATCC reference strains and the *E. coli* wild type AG 100 were inhibited by triclosan concentrations ranging between 0.5 and 1 μ g/mL. *E. coli* AGT 11 with a mutation in its enoylacyl-carrierprotein-reductase and *E. coli* AGT Kan11, a strain identical to *E. coli* AGT 11 but with additionally defect efflux pump regulation showed an increased MIC by the factor 128 against triclosan. For *E. coli* AGT 11K, again a strain identical to *E. coli* AGT 11 but additionally with switched-off efflux pump AcrAB triclosan resistance was decreased by 32 times (Table 2). The minimum microbistatic concentration for polihexanide was 2 μ g/mL against all tested *E. coli* strains.

Table 1: MIC ($\mu\text{g}/\text{mL}$) of polihexanide and triclosan against *S. aureus* and different MRSA and VISA-strains

Strain	Polihexanide	Triclosan
<i>S. aureus</i> ATCC 29213	1	0.125
MRSA H-5-18	1	
MRSA H-5-19 und H-5-20	1	
MRSA H-5-24	2	
MRSA H-5-26 und H-5-27	1	
MRSA H-5-31	1	
Epidemic North-German MRSA strain	2	64
VISA Ve 13985	1	
VISA Ve 1177/98	1	
VISA BK 13230	1	
VISA BK 1704/98	1	
VISA 18 A 026	1	
VISA 20 A 063	1	

Table 2: MIC ($\mu\text{g}/\text{mL}$) of polihexanide and triclosan against *E. coli*

Strain	Polihexanide	Triclosan
<i>E. coli</i> ATCC 25922	2	0.5
<i>E. coli</i> AG 100	2	1
<i>E. coli</i> AGT 11	2	64
<i>E. coli</i> AGT 11K	2	16
<i>E. coli</i> AGT Kan 11	2	64

Microbicidal activity

In the quantitative suspension test polihexanide showed superior activity than triclosan (Table 3). *S. aureus* ATCC strains did not differ in their susceptibility against triclosan as compared to the North-German epidemic MRSA strain. The minimum requirements of a logRF >5 without and logRF >3 with 0.2% albumin burden within 5 minutes application time [19] were achieved at a concentration of 0.6 $\mu\text{g}/\text{mL}$ triclosan. The exception was MRSA strain H-5-24, where a triclosan concentration of 0.6 $\mu\text{g}/\text{mL}$ was able only after 60 sec without and 10 minutes with albumin burden to achieve a logRF >5 or a logRF >3 reduction, respectively. The difference between *E. coli* ATCC 25922 and *E. coli* AGT 11 was only minute, for both strains a concentration of 0.6 $\mu\text{g}/\text{mL}$ triclosan at an application time of 60 sec was needed to achieve a logRF >5 without and a logRF >3 with albumin-burden.

Table 3: Time and concentration dependant bactericidal activity of polihexanide and triclosan

Strain	0.2% Albumin	Log reduction (log RF)															
		30 s			60 s			5 min			10 min			60 min			
		0.6	0.2	0.02	0.6	0.2	0.02	0.6	0.2	0.02	0.6	0.2	0.02	0.6	0.2	0.02	
		Polihexanide / Triclosan															
<i>S. aureus</i> ATCC	-	>5/2.6	>5/0.2	2.8/0.7	>5/0.2	2.8/0.7	>5/0.8	4.5/0.6	>5/0.9	4.6/0.8	>5/1.0	>5/0.2	4.6/0.8	>5/0.5	>5/1.0	>5/0.6	>5/0.6
	+	>5/0.8	0.3/0.1	0.4/0.1	0.4/0.2	0.4/0.1	0.4/0.4	0.4/0.3	0.5/0.4	0.3/0.4	0.5/0.6	>5/0.2	0.3/0.4	>5/0.5	0.5/0.6	>5/0.6	>5/0.6
MRSA H-5-24	-	>5/0.5	>5/0.2	4.8/0.05	>5/0.1	4.8/0.05	>5/0.1	4.8/0.0	>5/0.2	>5/0.05	>5/0.1	>5/0.2	>5/0.05	>5/0.5	>5/0.1	>5/0.6	>5/0.6
	+	>5/2.6	>5/0.2	2.8/0.7	>5/0.2	2.8/0.7	>5/0.8	4.5/0.6	>5/0.9	4.6/0.8	>5/1.0	>5/0.2	4.6/0.8	>5/0.5	>5/1.0	>5/0.6	>5/0.6
MRSA strain north Germany	-	>5/2.6	4.4/1.0	2.2/0.5	>5/1.0	2.2/0.5	>5/1.2	2.4/0.7	>5/2.4	4.4/0.6	>5/0.7	>5/2.4	4.4/0.6	>5/0.5	>5/0.7	1.0/0.6	>5/0.7
	+	2.4/2.3	0.5/0.5	0.7/0.5	0.7/0.6	0.7/0.5	0.9/0.7	0.9/0.6	0.9/0.7	0.7/0.6	0.9/0.6	>5/3.3	0.9/0.7	>5/3.8	>5/0.6	>5/0.6	>5/0.6
<i>E. coli</i> ATCC	-	>5/3.9	>5/1.9	>5/1.9	>5/2.1	>5/1.9	>5/2.1	>5/2.1	>5/2.1	>5/2.1	>5/2.1	>5/2.1	>5/2.1	>5/2.1	>5/2.4	>5/2.1	>5/2.4
	+	>5/3.4	0.9/1.8	0.9/1.7	1.0/1.8	0.9/1.7	1.3/2.0	1.1/1.9	2.9/2.2	0.9/2.1	0.9/2.1	2.9/2.2	0.9/2.1	>5/2.1	0.9/2.1	0.9/2.1	0.9/2.1
<i>E. coli</i> AGT 11	-	>5/3.0	>5/0.4	4.0/0.2	>5/0.6	4.0/0.2	>5/2.1	>5/0.3	>5/2.1	>5/0.3	>5/2.1	>5/2.1	>5/0.3	>5/2.1	>5/0.9	>5/0.3	>5/0.9
	+	>5/2.9	0.3/0.3	<0.1/0.2	0.4/1.1	<0.1/0.2	0.4/1.9	0.1/0.3	0.8/1.9	0.3/0.3	0.3/0.3	0.8/1.9	0.3/0.3	>5/2.1	0.3/0.3	0.3/0.3	0.3/0.4

Polihexanide achieved a logRF >5 without and loRF >3 with albumin burden at concentrations of 0.6 µg/mL within 30 sec, respectively, for *S. aureus*. The only exception was the North-German epidemic MRSA strain, where an application time of 5 minutes was required. MRSA H-5-24 did not differ in its susceptibility from the ATCC reference strains. *E. coli* AGT 11 was only lesser susceptible to polihexanide as compared to *E. coli* ATCC 25922 under the presence of an albumin burden. Without albumin burden a logRF >5 was achieved with a concentration of 0.2 µg/mL and an application time of 30 sec, or 0.02 µg/mL and 60 sec, respectively. With albumin burden even an application time of 1 minute was not sufficient for a concentration of 0.02 µg/mL polihexanide to achieve a logRF >3.

Discussion

In order to assess the efficacy of the tested antimicrobial compounds polihexanide and triclosan, our results must take their usual clinically used concentrations into account. Triclosan usually is used in liquid soap at concentration of 2 to 5 µg/mL, in toothpaste starting from 3 µg/mL and in hand disinfectants ranging from 2 to 20 µg/mL [7].

Wound antiseptics containing polihexanide as their active ingredient contain concentrations of 0.2 to 1 µg/mL and sanitizers 1 to 2 µg/mL. Our results showed that the required minimum concentration for triclosan and polihexanide are 0.6 µg/mL, and therefore, concentrations used in clinical still are far above the MIC demonstrated for various clinical isolates and laboratory reference strains. The focus of our study, however, was not to assess the antimicrobial efficacy in clinical practice, but rather to explore differences in susceptibility within various antibiotic susceptible strains of *S. aureus* and *E. coli* in order to detect possible induction of co-resistance against antiseptics.

The results of our study showed that the susceptibility of the epidemic North-German MRSA strain against triclosan was reduced as compared to antibiotic susceptible strains. Also MRSA H-5-24 showed reduced susceptibility against triclosan. As expected, triclosan was highly effective against the *E. coli* ATCC strain. In contrast, clinical *E. coli* isolates showed decreased susceptibility against triclosan. The mode of action of triclosan is based on the inhibition of the biosynthesis of lipid acid and enoylacyl-carrierprotein-reductase [7]. For *E. coli* to become resistant against triclosan, a single change of 1 amino acid in the reductase enzyme is sufficient resulting in a 32- to 128-fold MIC increase.

Another reason for the increased resistance of *E. coli* against triclosan could also be an increased activity of the efflux pump *AcrAB-ToIC* located in the bacterial cell membrane. *E. coli* mutants with increased efflux pump activity showed higher MIC values against various antibiotics as well as triclosan than strains with normal efflux pump activity. This correlation was also observed earlier

by McMurry et al. [14] and was explained by the fact that affected compounds are substrates of the same membrane pump *AcrAB-ToIC*. Our observation of a multiple antibiotic-resistance-phenotype combined with an increase in triclosan MIC by 1 to 2 titres in some clinical *E. coli* isolates confirms this possibility.

With one exception MICs of antibiotic resistant and susceptible strains against polihexanide did not differ showing that polihexanide has a different mode of action against bacteria [20]. Furthermore, the observed low MICs for polihexanide allow the conclusion that polihexanide is not a substrate for above efflux pumps. Based on the presented in-vitro results and clinical observations [21], it is explainable why polihexanide is a very effective wound antiseptic. Our data show that polihexanide easily is able to kill even antibiotic resistant bacteria commonly found as the cause of wound infection. Currently, it can be concluded that unlike triclosan, polihexanide is not transported outside of the bacterial cell wall by the activity of efflux pumps, and therefore is not subject to development of possible bacterial resistance. Moreover, polihexanide is integrated into the bacterial cell membrane, which it irreversibly destroys.

However, as with all antimicrobial compounds, it seems prudent to limit its use to clinically justifiable indications.

Conclusion

The clinical isolates of *E. coli* generally showed higher MICs against triclosan, both in the micro-dilution assay as well in the quantitative suspension suspension test than comparable ATCC laboratory reference strains. For polihexanide and triclosan strain dependant susceptibility was shown. However, both antimicrobial compounds are effective when used in concentrations common in practice.

List of abbreviations

ATCC – American Type Culture Collection
 logRF – log₁₀ Reduction factor
 MIC – Minimum inhibitory concentration
 MMC – Minimum microbicidal concentration
 MRSA – Methicillin Resistant *Staphylococcus aureus*
 VISA – Vancomycin intermediate sensitive *Staphylococcus aureus*

Notes

Authors' contribution

AK and PR had the idea for the study, AK and PR planned and supervised the experiments, as well drafted the manuscript and analyzed and interpreted the data. KW, TK, and SB participated in the technical design of the study and performed experiments. All authors analyzed

and interpreted the data. NOH, OA SB, and FJ participated in the study's design and coordination, helped to draft the manuscript, and analyzed and interpreted the data. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content and have read and approved the final manuscript.

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Conflicts of interest

Dr. Frank Jethon is a paid employee of Fresenius Kabi GmbH, Bad Homburg, Germany. Prof. Dr. Assadian, Dr. Bagel, Dr. Huebner, Prof. Dr. Kramer, Dr. Rudolph and Mrs. Wehse have no financial or other conflict of interest to declare.

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