MICROBIAL DRUG RESISTANCE
Volume 20, Number 2, 2014

DOI: 10.1089/mdr.2013.0113

Activity of the Antiseptic Polyhexanide Against Gram-Negative Bacteria

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The activity of the antiseptic polyhexanide was tested against 250 gram-negative clinical isolates, that is, 50 isolates each of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, and *Haemophilus influenzae*. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by using a serial broth microdilution technique according to DIN 58940. Time-kill studies were performed for reference stains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 4352, *P. aeruginosa* ATCC 15442, *M. catarrhalis* ATCC 43617, and *H. influenzae* ATCC 49247. All tested isolates had MICs and MBCs within a range of 1–32 mg/L and were regarded as susceptible to polyhexanide. The highest values were found for *P. aeruginosa* and *H. influenzae* with MICs and MBCs of 32 mg/L. Addition of up to 4% albumin to the test medium did not change MICs and MBCs. Time-kill studies of the reference strains showed reduction rates from 3 $\log_{10}$ colony forming units (CFU)/ml to more than 5 $\log_{10}$ CFU/ml for 200 and 400 mg/L polyhexanide within 5–30 min. Testing of polyhexanide in combination with antibiotics showed indifference with amoxicillin, cefotaxime, imipenem, gentamicin, and ciprofloxacin; no antagonism was found. As no resistance and no antagonism with antibiotics were detected, polyhexanide is regarded as suitable agent for topical eradication of gram-negative bacteria.

Introduction

Gram-negative bacteria, like *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, and *Haemophilus influenzae*, are important pathogens of local infections in clinical practice. Testing a few strains, the antiseptic polyhexanide showed activity to gram-negative bacteria.[8,14,17] To obtain more detailed information about the activity of polyhexanide, 50 clinical isolates each of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *M. catarrhalis*, and *H. influenzae* were tested quantitatively. The susceptibility of the strains to various antibiotic agents was determined to assess a possible correlation of polyhexanide activity and special antibiotic resistance patterns. In addition to single-substance susceptibility analysis, the strains were tested against combinations of polyhexanide and various antibiotic agents to detect potential synergistic or antagonistic effects of simultaneous administration of the antiseptic and antibiotics. These data clearly indicate the suitability of polyhexanide for the topical eradication of gram-negative bacteria.

Materials and Methods

Strains

Two-hundred fifty isolates, that is, isolates of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *M. catarrhalis*, and *H. influenzae*, were collected from different patients of the university hospital of Rostock and Synlab Laboratories Kassel (FRG). The strains were identified by standard techniques and stored at −70°C using the Protect System (Technical Service Consultants Limited). Each isolate was thawed and subcultured on Mueller–Hinton agar before testing; isolates of *H. influenzae* were subcultured on Mueller–Hinton agar with HTM supplement (Oxoid).

Determination of the activity of polyhexanide

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by serial broth microdilution tests according to DIN[2,3] using Mueller–Hinton broth (MHB; Merck), supplemented with calcium chloride (final Ca^{++} concentration 50 mg/L) and

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magnesium chloride (final Mg^{++} concentration 25 mg/L). For isolates of *H. influenzae* MHB was HTM supplemented. Incubation of *H. influenzae* was performed in a 10% CO_2/ 20% O_2 atmosphere. Polyhexanide was obtained from Fresenius Kabi. The stock solution contained 200 mg. It was diluted in the test broth to final polyhexanide concentrations of 0.125–256 mg/L.

The final inoculum of the strains was 5×10^5 colony forming units (CFU)/ml. The MIC was ascertained after 24 hr of incubation at 36°C as the lowest concentration of the antiseptic preventing visible growth. For the determination of MBCs, a 10-µl aliquot from wells nearby the threshold for turbidity of the MIC plate was transferred after incubation of 6 hr at 36°C onto blood agar plates in duplicate. The MBC was defined as the lowest antiseptic concentration preventing visible regrowth.

Additional testing was performed for two selected isolates of each species, which were also used for combination studies. This included MIC determinations after incubation of 48 hr and MBC determinations with subcultivation after 3 and 24 hr. Tests using broth supplemented with 0.2% and 4% bovine albumin (Sigma) were also performed for these strains.

E. coli ATCC 25922 and *P. aeruginosa* ATCC 15442 were tested repeatedly as quality control.

Time-kill studies

Killing rates of polyhexanide against the reference strains *E. coli* ATCC 25922, *K. pneumonia* ATCC 4352, *P. aeruginosa* ATCC 15442, *M. catarrhalis* ATCC 43617, and *H. influenzae* ATCC 49247 were determined using supplemented MHB. Stock solutions were diluted in Ca^{++}- and Mg^{++}-supplemented MHB with addition of 3% tween 80, 0.1% cysteine, 0.1% histidine, and 3% saponin to inactivate polyhexanide. The final concentrations of polyhexanide were 200 and 400 mg/L, corresponding to the therapeutic concentrations. The final density of strain stock solutions immediately prior to the experimental series was about 10^8 CFU/ml.

One hundred microliters of such a polyhexanide-containing stock suspension was transferred to 9.9 ml MHB (1:100). Two 1:10 serial dilution steps (1 ml of bacterial suspension to 9 ml of MHB) followed after 1, 3, 5, 10, 15, and 30 min and 1, 2, 6, 24, and 48 hr exposure time. From each dilution step, 100 µl was transferred onto Mueller–Hinton agar plates. After 24 hr of incubation at 36°C, plates with growth of 30–300 colonies were counted and the CFU/ml was calculated. Each experiment was performed in duplicate at two independent occasions (biological replicates). Values therefore represent means of two experiments.

Antibiotic susceptibility testing

Antibiotic susceptibility and the MICs of strains used for combination testing were determined by using the micronaut system (Merlin).

E. coli, *M. catarrhalis*, and *H. influenzae* were tested against amoxicillin, ampicillin/sulbactam, cefotaxime, cefaclor, cefpodoxime, ceftazidime, imipenem, meropenem, gentamicin, doxycycline, cotrimoxazole, levofloxacin, and ciprofloxacin. For *K. pneumoniae* the same antibiotics were used with the exception of amoxicillin and ampicillin/sulbactam. *P. aeruginosa* was tested against piperacillin, piperacillin/tazobactam, cefsulodin, ceftazidime, imipenem, meropenem, gentamicin, amikacin, levofloxacin, and ciprofloxacin.

Analytical grade powders of amoxicillin, cefotaxime, imipenem, gentamicin, and ciprofloxacin were kindly provided by their manufacturers for combination studies.

Testing of strains for production of extended β-lactamase (ESBL) or AmpC β-lactamase was performed by using MASTDISCS™ ID (MAST Diagnostica).

Combination studies

In vitro interactions of 400 mg/L polyhexanide with ampicillin, cefotaxime, imipenem, gentamicin, and ciprofloxacin were investigated by time-kill kinetics. Ten strains, two of each species, with high MICs within the susceptible range of the antibiotic according to EUCAST[16] were chosen. The aim was mainly to detect a possible antagonism, if a high dose of the antibiotic is administered. In detail the MICs (mg/L) were as follows (abbreviations see Table 2):

for *E. coli* and AM/GM/CT/I/CI: 8/2/1/0.5/0.25 and 8/1/0.5/0.5/0.5,
for *K. pneumoniae* and GM/CT/I/CI: 4/0.25/4/0.5 and 2/0.25/2/0.25,
for *P. aeruginosa* and GM/I/CI: 4/4/0.5 and 4/4/0.25,
for *M. catarrhalis* and CT/I/CI: 0.25/0.5/0.015 and 0.125/0.25/0.015,
for *H. influenzae* and AM/CT/I/CI: 0.5/0.125/0.5/0.125 and 0.25/0.125/0.25/0.125.

Antibiotics were added in concentrations of 1/4× MIC and 2× MIC.

The transfer to agar plates was performed after 1, 5, and 30 min and 1, 3, 6, and 24 hr. Sterility controls were also included.

Synergy according to time-killing curves was defined as a reduction in the mean $\log_{10}$ CFU/ml bacterial counts of ≥2.0 at any time during the experiment when the time–concentration killing curves generated with the combination of polyhexanide and antibiotic were compared with those generated with the antiseptic alone. Antagonism was defined as an increase in the colony counts of ≥2 $\log_{10}$ CFU/ml, and synergism was defined as a decrease of ≥2 $\log_{10}$ CFU/ml, respectively. Indifference was defined as difference of 0 to <2 in the mean $\log_{10}$ CFU/ml bacterial counts.[5,11]

Results

As a median of five determinations, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 4352, and *M. catarrhalis* ATCC 43617 had polyhexanide MICs and MBCs of 2 mg/L; *P. aeruginosa* ATCC 15442 and *H. influenzae* ATCC 49247 had polyhexanide MICs and MBCs of 8 mg/L. The maximum deviation from test to test was one dilution step. All tested isolates had polyhexanide MICs and MBCs within a range of 1–32 mg/L (Table 1) and were regarded as susceptible to this substance. The highest values were found for *P. aeruginosa* and *H. influenzae* with MICs and MBCs of 32 mg/L.

Bovine serum albumin did not influence the MICs and MBCs of polyhexanide in inactivation experiments with the 10 selected strains.

Table 1. Minimal Inhibitory Concentrations and Minimal Bactericidal Concentrations of Polyhexanide for Gram-Negative Bacteria

	MIC (mg/L)								*MBC (mg/L)*							
Species	*1*	*2*	*4*	*8*	*16*	*32*	MIC_{50}	MIC_{90}	*1*	*2*	*4*	*8*	*16*	*32*	MBC_{50}	MBC_{90}
Escherichia coli	13	33	4				2	2	13	33	3	1			2	2
Klebsiella pneumoniae	26	11	13				1	2	26	11	13				1	2
Pseudomonas aeruginosa			23	24	2	1	8	8			22	25	1	2	8	8
Moraxella catarrhalis	21	23	6				2	4	21	23	6				2	4
Haemophilus influenzae			10	14	19	7	16	32			9	15	18	8	16	32

MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

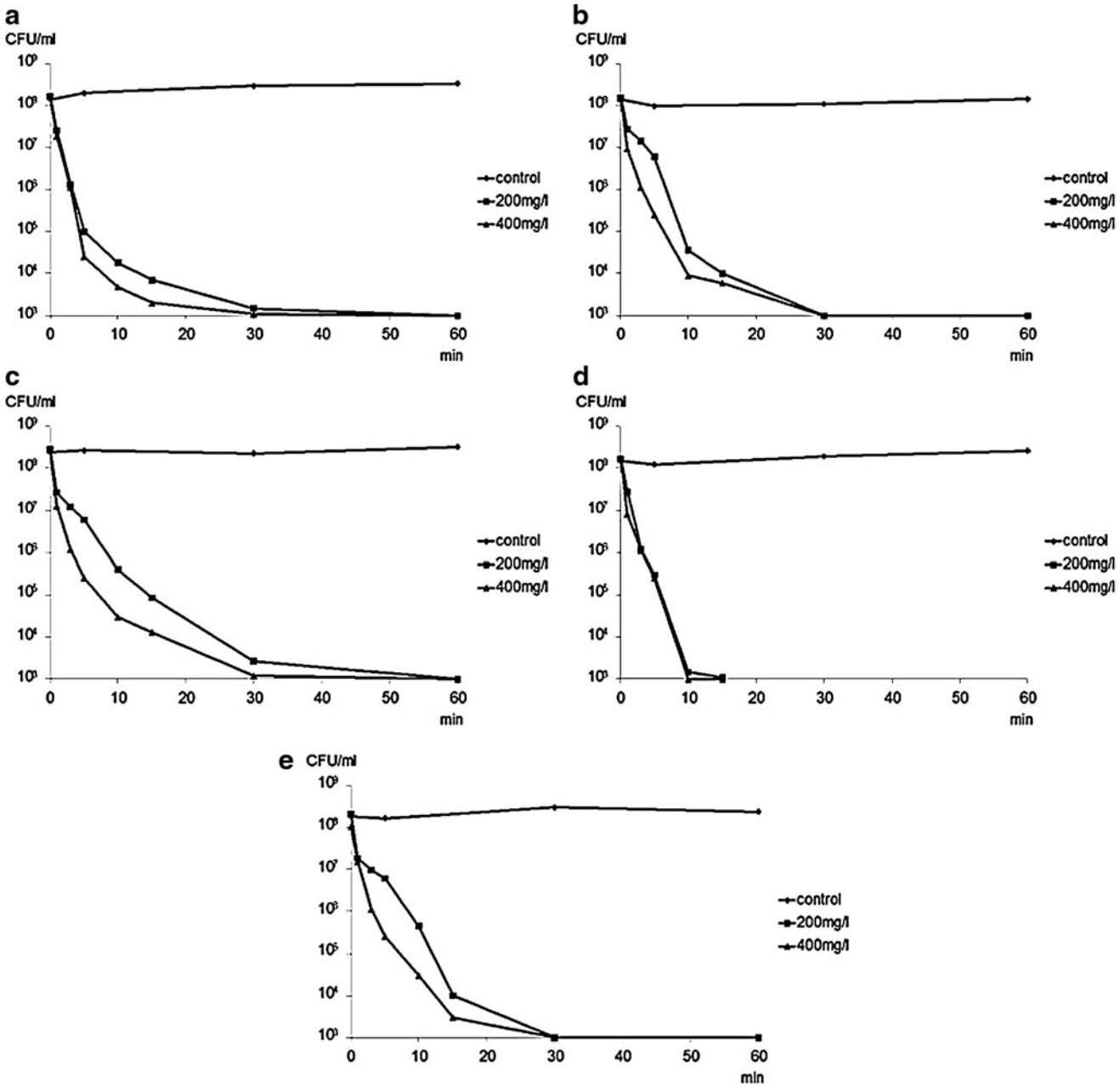

FIG. 1. Killing curves. **(a)** Polyhexanide against *Escherichia coli* ATCC 25922. **(b)** Polyhexanide against *Klebsiella pneumoniae* ATCC 4382. **(c)** Polyhexanide against *Pseudomonas aeruginosa* ATCC 15442. **(d)** Polyhexanide against *Moraxella catarrhalis* ATCC 43617. **(e)** Polyhexanide against *Haemophilus influenzae* ATCC 49247.

In these strains, the MBC was also determined after 3 and 24 hr of exposure to polyhexanide. The maximal MBC difference comprised one dilution step in comparison to the standard exposure time of 6 hr. Extension of exposure periods to 48 hr did not change MIC values obtained after 24-hr exposure.

The killing curves of the reference strains are shown in Figure 1. The reduction rate after exposure to 400 mg/L was slightly higher as compared with 200 mg/L. In all cases the detection limit was reached after 1 hr.

Several different patterns of resistance were found in the clinical isolates (Table 2). One *E. coli* strain expressed AmpC β-lactamase; another *E. coli* and two *K. pneumoniae* strains produced extended spectrum β-lactamase (ESBL). However, there was no link between resistance to antibiotics and the MICs and MBCs of polyhexanide.

Combination testing yielded indifference between antibiotic and polyhexanide action.

Discussion

For a successful application of antiseptics, these substances must show bactericidal effects. So far, for polyhexanide, this feature was demonstrated for only a few selected gram-negative reference strains.[8,14,17] A concentration of 10 mg/L polyhexanide was bactericidal for some strains of *E. coli*[1]; in that study, novel interaction mechanisms with nucleic acids were found. Previous studies had shown that the antimicrobial

Table 2. Patterns of Antibiotics Tested Resistant or Intermediate for Gram-Negative Bacteria

E. coli		K. pneumoniae		P. aeruginosa		M. catarrhalis		H. influenzae	
Antibiotic	n	Antibiotic	n	Antibiotic	n	Antibiotic	n	Antibiotic	n
None	7	None	18	None	9	AM	29	None	23
AM (i)	4	CE	6	PI, PT (i)	3	None	7	AM	4
AM	3	D, ST	4	GM (i)	3	ST	5	ST	4
AM, D	3	CE, D	4	PI, PT, CS, CA, I, M, GM, AN, LV, CI	3	ST (i)	3	GM	3
AM, ST	3	D	3	PI, PT (i), I, M	3	AM, ST	3	ST (i)	2
AM, D, ST	3	ST	2	GM	2	AM, ST (i)	2	AM, ST	2
D, ST	3	LV	2	LV, CI	2	AM, GM	1	GM (i), ST	2
D	2	CC, D, ST, LV, CI	2	I, M, CI, LV	2			AM (i)	1
AM, AS, CE, D	2	GM (i)	1	PI	1			CC (i)	1
AM, AS, CE, D, ST, LV, CI	2	CC, D, ST	1	I	1			D (i)	1
AM, AS (i)	1	GM, LV, CI	1	LV	1			AM (i), CE	1
AM, AS	1	ST, LV, CI	1	PI (i), PT (i)	1			AM, GM	1
AM, AS, CC	1	CC, ST, LV, CI	1	PI, PT	1			CC (i), ST	1
AM, AS, ST	1	D, ST, LV, CI	1	I, M	1			GM (i), D (i)	1
AM, LV, CI	1	CC, CP, D, LV, CI	1	I, GM	1			AM (i), AS (i), D	1
AM, AS (i), D, ST	1	CT, CE, CP, CA, D, ST, LV (i), CI	1	GM, AN	1			AM (i), AS (i), ST	1
AM, AS, LV, CI	1	CT, CE, CP, CA, D, ST, LV, CI	1	LV (i), CI (i)	1			AM (i), AS (i), CE, ST	1
AM, GM, D, ST	1			LV, CI (i)	1				
AM, D, LV, CI	1			PI, PT, GM	1				
AM, AS, CT, CE, CP	1			GM (i), CI, LV	1				
AM, AS, CE, D, ST	1			GM, CI (I), LV	1				
AM, AS, GM (i), D, ST	1			GM, CI, LV	1				
AM, AS (i), ST, LV, CI	1			CS, CA, I, M	1				
AM, AS, D, LV, CI	1			I, GM, CI (i), LV	1				
AM, GM, D, LV, CI	1			GM, AN, LV, CI	1				
AM, D, ST, LV, CI	1			PI, PT, CS, CA (i), GM (I)	1				
AM, AS, GM, ST, LV, CI	1			PI, PT, CS, CA, I, M	1				
AM, GM, D, ST, LV, CI	1			I, M, GM, AN, LV (i), CI	1				
				CS, CA, I, M, GM, AN, LV, CI	1				
				PI, PT, CS, CA, I, GM, AN, LV, CI	1				
				PI, PT (i), CS, CA, I, M, GM, AN, LV, CI	1				
Total	50		50		50		50		50

AM, amoxicillin; AS, ampicillin/sulbactam; PI, piperacillin; PT, piperacillin/tazobactam; CT, cefotaxime; CE, cefaclor; CP, cefpodoxime; CS, cefsulodin; CA, ceftazidime; I, imipenem; M, meropenem; GM, gentamicin; AN, amikacin; D, doxycycline; ST, cotrimoxazole; LV, levofloxacin; CI, ciprofloxacin; (i), intermediate.

action is based on the binding of the cationic molecules to the anionically charged bacteria membrane surfaces, leading to membrane rupture and denaturation of proteins.[6,7,13]

In the current study, we determined the MICs and MBCs of polyhexanide for gram-negative bacteria with a standardized susceptibility test. The obtained values did not exceed 32 mg/L. The highest values were determined for *P. aeruginosa* followed by *H. influenzae*. No tolerant strains with differences between MIC and MBC values of two orders of magnitude or more were observed. The presence of albumin did not alter the activity of the antiseptic.

According to the application instructions of the manufacturer, the concentration of polyhexanide should be 0.1–0.2% of the stock solution in therapeutic preparations, like liquids with 0.9% sodium chloride or gels with hydroxyethylcellulose. This corresponds to final concentrations of 200–400 mg/L polyhexanide. Polyethylene glycol is added in therapeutic preparations to lower the surface tension of the solution for improving the wetting of wound surfaces.[18] Therefore, based on the present as well as on recent data, therapeutic preparations of polyhexanide have concentrations higher than needed for bactericidal activity *in vitro*.

Time-kill studies of the reference strains showed reduction rates of 3 to more than 5 $\log_{10}$ CFU/ml within 5–30 min using therapeutic concentrations. These results are consistent with a previous publication,[17] in which a reduction of up to more than 5 $\log_{10}$ CFU/ml in the first 30 min for some reference strains of gram-negative bacteria was described. Similar reduction rates were also observed under lower concentrations of polyhexanide (12.5–100 mg/L), but needed longer incubation times (6 and 24 hr).[14]

Another important aspect of antiseptics is not only their ability to kill or inhibit susceptible microorganisms but also the rate of resistance. Unfortunately, the literature presents only few data concerning *in vitro* susceptibility of gram-negative bacteria against antiseptics. To detect the occurrence of resistance, usually a large number of stains must be tested. The chemically related antiseptic chlorhexidine was found to be effective against hospital isolates of gram-negative bacteria.[4] On the other hand, an increase of MICs of chlorhexidine was observed for gram-negative bacteria with rising rates of resistance. Further, the occurrence of chlorhexidine resistance was associated with multiple resistances to antibiotics.[9,12,15] In this study, an extended range of MICs and MBCs was observed comparing the values of the reference strains. However, it was still small and regarded as within the biological variability. No resistance of gram-negative bacteria against polyhexanide was found. Further, the MIC and MBC values of polyhexanide were not associated with specific antibiotic resistance patterns, suggesting that there is no resistance against polyhexanide similar to that reported for chlorhexidine.

Another important aspect is that clinical infected wounds usually require systemic antibiotic treatment, if classical clinical signs or symptoms of inflammation are observed.[10] Our study clearly showed the absence of antagonistic effects between polyhexanide and antibiotics, which makes the combination of antibiotics and polyhexanide possible.

In conclusion, both the absence of polyhexanide-resistant strains and cross-reactivity with antibiotics confirms the suitability of wound infections with gram-negative bacteria alone or in combination with appropriate antibiotics. This feature could become more important with the increasing prevalence of multiresistant bacteria throughout the hospitals worldwide.

Disclosure Statement

There are no conflicts of interest. This study has not been funded.

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