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Major Article

Evaluation of disinfectant efficacy against multidrug-resistant bacteria: A comprehensive analysis of different methods

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Background: Multidrug-resistant gram-negative bacteria (MDR-GNB) constitute a threat to health care worldwide. Disinfectants are used to prevent and control the spread of MDR-GNB in a hospital setting but their efficacy might be impaired by bacterial mechanisms that may act on both antimicrobials and disinfectants. Determination of minimum inhibitory concentrations is mainly used to determine bacterial susceptibility against disinfectants, but practical tests on surfaces might be more suitable to predict in-use conditions. Our objective was to compare and evaluate 4 different methods widely used to assess surface disinfectant efficacy.

Methods: The efficacy of benzalkonium chloride (BAC), peracetic acid (PAA), and ethanol (ETH) against multidrug-resistant *Acinetobacter*, *Pseudomonas*, and *Klebsiella* strains was assessed by minimum inhibitory concentration determinations, quantitative suspension tests, qualitative suspension tests, and carrier tests. Test results were compared to ascertain the most appropriate method.

Results: ETH, PAA, and BAC were highly effective against MDR-GNB, but we observed marked differences in efficacious concentrations (up to 100-fold) as a function of the test method applied. Minimum inhibitory concentration determination was not reliable for evaluating susceptibility or resistance to BAC.

Conclusions: Surface tests should be used to determine bacterial susceptibility against disinfectants. Moreover, suitable guidelines are needed that allow for the standardization and comparison of bactericidal values obtained by different investigators.

BACKGROUND

Multidrug-resistant gram-negative bacteria (MDR-GNB) constitute an important threat to health care worldwide.[1] Recent German surveillance data revealed a prevalence of MDR-GNB up to 3.6% in patients at hospital admission. Moreover, several reports about nosocomial outbreaks of multidrug-resistant (MDR) *Klebsiella pneumoniae* and *Acinetobacter baumannii* over long periods of time emphasize the essential role of infection prevention and control interventions.[2-9] Apart from identifying patients colonized with MDR-GNB, isolation of cases, strict barrier precautions, and the hand hygiene of health care workers, the identification and eradication of environmental reservoirs (eg, contaminated health care surfaces) are of utmost importance, as they play a significant role in pathogen transmission. Most nosocomial pathogens, including MDR-GNB, can persist on dry inanimate surfaces for months.[1,9,10] Guidelines for the control of MDR-GNB in Germany as well as in other countries have been established and include cleaning and disinfection as important interventions.[10-14] Nevertheless, more research is required with regard to strategies that ensure reliable decontamination.[1] Surface disinfectants are regularly used in infection control and should conform to standards, such as the European standards for chemical disinfectants and biocides or comparable national standards. In Germany, the "List of Disinfectants" issued by the Disinfectants Commission in the Association for Applied Hygiene (VAH) serves as the basis for selecting appropriate disinfection procedures for routine and prophylactic

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disinfection in medical areas. The VAH further provides requirements and methods for the certification of chemical disinfection procedures. The use of VAH-listed products meets the quality assurance requirements stipulated by infection control regulations at German Federal State level.

There has been justifiable concern about the efficacy of disinfectants relative to bacterial mechanisms that impair biocide action.[15-17] These mechanisms include, for example, expression of efflux pumps and lowered permeability of the bacterial outer membrane. Bacterial biocide adaptation and resistance have also been reported.[18-20] Numerous studies on the susceptibility of bacteria (including MDR bacteria) to biocides have already been conducted.[21-29] Compared to antimicrobial susceptibility testing, there is no definition of biocide resistance based on breakpoints,[15,25] but it has been defined as bacterial survival at in-use concentrations of disinfectants.[15] Many studies determined minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations to evaluate biocide susceptibility or resistance, as these are relatively simple procedures.[30,31] However, these tests do not consider that surface disinfectants applied to health care surfaces are expected to be effective within short contact times.[32] Therefore, practical tests on surfaces more closely represent in-use conditions and might be more suitable to predict disinfectant efficacy. Moreover, it has been observed that the test method used significantly influences test outcomes and thus also the interpretation of susceptibility or resistance against disinfectants.[33] Several groups of active substances are used for biocide products, including disinfectants. The present study was conducted to compare methods commonly used to evaluate surface disinfectant efficacy. The aim was to determine the most suitable method to assess bacterial resistance against surface disinfectants. The efficacy of ethanol, benzalkonium chloride, and peracetic acid against selected strains of MDR *Acinetobacter* spp, *Pseudomonas aeruginosa*, *Klebsiella* spp, and corresponding reference strains was assessed in vitro by MIC testing, qualitative and quantitative suspension tests, and practical surface tests without mechanical action. These active substances are widely used in health care settings, veterinary medicine, animal husbandry, and the food industry.

METHODS

Test organisms

Sixteen bacterial isolates comprised of *A pittii*, *A baumannii*, *P aeruginosa*, *K oxytoca*, and *K pneumoniae* were included in this study (Table 1). They were cultured from diagnostic samples during the year 2015 at the University Hospital of Leipzig, Germany. Isolates originated from different patients (except KP3 and KP4; Table 1) and several different hospital sections. Based on phenotypic resistance characteristics (ie, resistance against 3 or 4 classes of antibiotics), most of them were classified as 3MDR-GNB or 4MDR-GNB.[11] Reference and type strains were obtained from the Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *P aeruginosa* strain DSM 939 was used as a species-specific reference strain, but it is also required as a test organism by the German Association for Applied Hygiene (VAH) Disinfectants Commission.[34] As no reference strains for the genera *Klebsiella* and *Acinetobacter* are provided by the VAH, the corresponding species-specific type strains were chosen.[34] Antibiotic susceptibilities (data can be offered upon request) of all strains were determined prior to disinfectant testing using the VITEK 2 technology (bioMérieux Deutschland GmbH; Nürtingen, Germany). As the type strain of *A baumannii* (DSM 30007) was unexpectedly identified as 3MDR-GNB, a clinical *A baumannii* isolate with species-specific, wild-type resistance patterns was used as a control.

Susceptibility testing

Disinfectant testing was performed according to the VAH guidelines,[34] which are equivalent to European standards[35] but include MIC testing and qualitative suspension tests as ancillary methods. We assumed a disinfectant efficacy at concentrations below 1% with the exception of ethanol and benzalkonium chloride. Concentration ranges were chosen according to VAH guidelines and a preceding study and were adjusted stepwise where necessary.[34,36] Bacteria were grown (24 hours at 37°C) from stock cultures biweekly on Columbia sheep blood agar^{PLUS} (Oxoid GmbH; Wesel, Germany). Bacterial test suspensions contained 1.5-5.0 $\times$ 10^8 colony-forming units (CFU)/mL, except for the practical tests on steel carriers (1.5-5.0 $\times$ 10^9

Table 1
Characteristics of bacteria used for disinfectant testing

Species	Acronym	Strain number	Source	Characteristics
Acinetobacter pittii	AP1	4/1/1/20	UHL; throat swab	Species-specific, wild-type resistance pattern
	AP2	4/1/13/37	UHL; feces	3MDR-GNB
	AP3	DSM 25618, ATCC 19004	DSMZ; cerebrospinal fluid	Type strain
Acinetobacter baumannii	AB1	4/1/13/47	UHL; wound swab	4MDR-GNB, MBL
	AB2	4/1/13/38	UHL; feces	4MDR-GNB, MBL
	AB3	DSM 30007, ATCC 19606	DSMZ; urine	Type strain, 3MDR-GNB
	AB4	4/1/13/39	UHL; feces	Species-specific, wild-type resistance pattern
Pseudomonas aeruginosa	PA1	4/1/13/23	UHL; blood culture	3MDR-GNB, MBL
	PA2	4/1/13/15	UHL; feces	4MDR-GNB, MBL
	PA3	4/1/13/72	UHL; wound swab	3MDR-GNB, MBL
	PA4	DSM 939, ATCC 15442	DSMZ; animal room water bottle	Test organism for disinfectant testing
Klebsiella oxytoca	KO1	4/1/15/65B	UHL; feces	3MDR-GNB, ESBL
	KO2	4/2/4/10	UHL; urine	3MDR-GNB, ESBL
	KO3	DSM 5175, ATCC 13182	DSMZ; pharyngeal tonsil	Type strain
Klebsiella pneumoniae	KP1	4/1/9/72	UHL; feces	3MDR-GNB, ESBL
	KP2	4/2/9/76	UHL; feces	3MDR-GNB, ESBL
	KP3	4/2/9/58A	UHL; feces	3MDR-GNB, ESBL
	KP4	4/2/9/58B	UHL; feces	3MDR-GNB, ESBL
	KP5	4/1/13/30	UHL; urine	4MDR-GNB, VIM
	KP6	4/2/2/27	UHL; feces	4MDR-GNB, VIM
	KP7	DSM 30104, ATCC 13883	DSMZ; no further information	Type strain

DSMZ, Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures GmbH; *ESBL*, extended-spectrum β-lactamase; *MBL*, metallo-β-lactamase; *MDR-GNB*, multidrug-resistant gram-negative bacteria (3MDR-GNB and 4MDR-GNB resistant against 3 or 4 classes of antibiotics, respectively, according to the Commission for Hospital Hygiene and Infection Prevention[10]); *UHL*, University Hospital Leipzig; *VIM*, Verona integron-encoded metallo-β-lactamase.

CFU/mL). Ethanol (99.8%, CAS-No. 64-17-5) (AppliChem GmbH; Darmstadt, Germany), peracetic acid (15%, CAS-No. 79-21-0) (AppliChem GmbH), and benzalkonium chloride (≥99.9%, CAS-No. 63449-41-2) (Sigma-Aldrich Chemie GmbH; Munich, Germany) were tested as single active substances. Disinfectant dilutions were prepared in water of standardized hardness (WSH) immediately before testing.[34] With the exception of ethanol (ETH), serial dilutions were 2-fold. Ethanol was diluted at 1% steps (MIC testing) or 5% steps (suspension tests, carrier tests). Growth controls were conducted using WSH instead of disinfectant. The neutralizer used for quenching disinfectant activity was tryptic soy broth (TSB) (Carl Roth GmbH; Karlsruhe, Germany) containing polysorbate 80 (30 g/L), lecithin (3 g/L), L-histidine (1 g/L), and sodium thiosulfate (5 g/L).

MICs were assessed by broth macrodilution (VAH method 7).[34] Screw-cap tubes were filled with equal volumes of double-concentrated disinfectant and TSB and mixed; 50 μL of bacterial test suspensions (diluted 1:10 in TSB) were then added. After 48 hours of incubation at 37°C, bacterial growth was evaluated visually.

Bactericidal concentrations were determined by qualitative suspension tests (VAH method 8).[34] One hundred microliters of bacterial test suspensions were inoculated into 10 mL of the disinfectant dilution and gently mixed. After the respective exposure times recommended by VAH (ie, 1, 5, 15, 30, and 60 minutes) at 20°C, 100-μL aliquots were transferred to 10 mL of neutralizer, incubated for 48 hours at 37°C, and evaluated as described above.[34]

To further evaluate the bactericidal activity of disinfectants, quantitative suspension tests with and without organic load (0.3% Albumin Fraktion V bovine serum albumin [BSA] solution) (Carl Roth GmbH) were performed at 20°C. Out of the suggested exposure times, 1, 5, and 15 minutes were chosen (VAH method 9, equivalent to EN 13727).[34] Briefly, disinfectant dilutions (8 mL) were mixed with WSH or BSA (1 mL; final concentration 0.03%), and 1 mL bacterial suspension was added. After the appropriate exposure time, 500 μL were transferred to 4.5 mL of neutralizer for 5 minutes. An aliquot of 100 μL was subsequently plated on agar and incubated for 48 hours at 37°C. Bacteria were counted, and logarithmic reductions (LRs) in viable counts were calculated as described by DIN EN 13727:2014-01.[37] For sufficient bactericidal efficacy, a LR ≥ 5 in viable counts was required.[34] Biocide efficacy under practical conditions with organic load (ie, surface disinfection without mechanical action; VAH method 14.1, equivalent to EN 13697) was evaluated using stainless steel carriers (GK-Formblech GmbH; Berlin, Germany).[34] A 50-μL drop of bacterial suspension was dried (60 minutes at 37°C) on stainless steel carriers and covered with 100 μL of disinfectant for the exposure times suggested by VAH guidelines (ie, 1, 5, 15, or 30 minutes at 20°C).[34] Subsequently, carriers were transferred to 10 mL of neutralizer, and bacteria were detached using a horizontal shaking device. Bacteria were counted, and LR values were calculated as described above. In all tests, each disinfectant concentration was tested in duplicate, and all tests were independently repeated once.

Statistical analyses

Differences in bacteriostatic and bactericidal values for bacterial genera as well as for MDR-GNB strains and reference strains were evaluated using Fisher exact test. A 1-dilution difference between values was regarded as within the error of the test. The influence of exposure time and organic load was assessed using the Mann-Whitney U test. Differences were regarded as significant for $P < .05$ (2-sided). Analyses were done using IBM SPSS Statistics 22 (IBM Deutschland GmbH; Ehningen, Germany).

RESULTS

Minimum inhibitory concentrations

MICs determined after 48-hour contact times ranged from 4% to 6% (v/v) for ETH, 0.003% to 0.025% for peracetic acid (PAA), and 0.001% to 0.01% for benzalkonium chloride (BAC) (data available upon request). Among strains of a single species, MICs varied up to 4 times, but between-species differences up to 40-fold were observed. Overall, the group of *Acinetobacter* strains displayed the highest susceptibility to PAA ($P = .0001$) and BAC ($P = .0039$) compared to *Pseudomonas* and *Klebsiella* strains. *P aeruginosa* isolates were significantly less susceptible to BAC ($P = .0002$).

Qualitative suspension tests

When compared with MICs, bactericidal concentrations for ETH were up to 10 times higher in qualitative suspension tests (data available upon request). Differences among and between species were 10% ETH at maximum. PAA and BAC revealed results similar to MICs; however, up to 10 times higher values were obtained at a certain contact time (eg, strain AB 4, BAC, 1 minute). At most contact times, bactericidal PAA values differed up to 4-fold among species. When compared with all other strains, *Klebsiella* strains KO2, KP5, KP6, and KP7 revealed a significantly higher susceptibility to PAA ($P = .0002$). Moreover, *Klebsiella* strains KO2, KP6, and KP7 were eliminated by significantly lower bactericidal concentrations at the 5-minute contact time ($P = .0008$), which was also the case for strains KO2 and KP 7 at the 15-minute contact time ($P = .0048$). In tests using BAC, the group of *P aeruginosa* strains was significantly less susceptible ($P < .05$) compared with the *Klebsiella* and *Acinetobacter* strains. Increasing the contact time from 1 minute to 15 minutes had a significant influence on bactericidal PAA values ($P < .05$), and enhancing contact time overall had an influence on bactericidal BAC values ($P < .05$).

Quantitative suspension tests

Efficacious bactericidal concentrations were recorded if a LR ≥ 5 in viable counts was achieved. Overall, bactericidal values determined by this test method were mainly similar to those from qualitative suspension tests. ETH values determined at a respective contact time differed only marginally among as well as between species. Compared with all other strains, a significantly lower bactericidal PAA concentration was determined for *K oxytoca* strain KO2 without BSA and *Pseudomonas* strain PA4 with BSA at 15-minute contact times, respectively ($P = .0476$). In agreement with qualitative suspension tests, *Pseudomonas* strains were significantly less susceptible to BAC at all exposure times compared with other strains ($P < .05$). A decrease in bactericidal values up to 8-fold was noticed for single strains after increasing contact time from 1 minute to 15 minutes. Organic soiling had no significant influence on the efficacy of any of the disinfectants tested ($P > .05$). Results are given in Table 2.

Carrier test

Bactericidal ETH values were similar to those obtained from qualitative and quantitative suspension tests. Values determined for PAA and BAC were at maximum 16-fold and 64-fold higher, respectively, compared to those obtained from quantitative suspension tests (eg, PAA at 1 minute: strain KO1; BAC at 1 minute: strains AP1 and PA1) (Table 3) and exceeded 100-fold for BAC compared to qualitative suspension tests. *K pneumoniae* strain KP7 displayed the highest susceptibility ($P < .05$) to PAA. Compared to other KP strains as well as to

Table 2
Bactericidal concentrations resulting in a logarithmic reduction ≥ 5 without and with organic soiling determined by the quantitative suspension test

	Ethanol (%)						Peracetic acid (%)						Benzalkonium chloride (%)					
	1 min		5 min		15 min		1 min		5 min		15 min		1 min		5 min		15 min	
Strain	w/o	w	w/o	w	w/o	w	w/o	w	w/o	w	w/o	w	w/o	w	w/o	w	w/o	w
AP1	40	40	35	35	30	35	0.012	0.012	0.012	0.012	0.012	0.012	0.01	0.01	0.005	0.01	0.005	0.005
AP2	40	40	35	35	35	35	0.012	0.012	0.012	0.012	0.012	0.012	0.01	0.01	0.005	0.005	0.0025	0.005
AP3	**35**	**35**	**30**	**35**	**30**	**30**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.005**	**0.01**	**0.0025**	**0.005**	**0.0025**	**0.005**
AB1	40	40	35	35	35	35	0.012	0.024	0.012	0.012	0.012	0.012	0.01	0.02	0.005	0.005	0.0025	0.005
AB2	40	40	35	35	35	35	0.012	0.012	0.012	0.012	0.006	0.006	0.01	0.01	0.005	0.005	0.0025	0.005
AB3	**40**	**40**	**35**	**35**	**30**	**30**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.01**	**0.01**	**0.005**	**0.005**	**0.005**	**0.005**
AB4	**35**	**35**	**35**	**35**	**30**	**35**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.003**	**0.005**	**0.01**	**0.0025**	**0.005**	**0.0025**	**0.005**
PA1	35	35	30	30	30	30	0.006	0.006	0.006	0.006	0.003	0.003	0.08	0.08	0.04	0.04	0.04	0.04
PA2	35	35	30	30	30	30	0.012	0.012	0.006	0.006	0.006	0.003	0.08	0.08	0.04	0.04	0.04	0.04
PA3	35	35	30	30	30	30	0.012	0.012	0.006	0.006	0.006	0.003	0.08	0.08	0.04	0.04	0.04	0.04
PA4	**35**	**35**	**30**	**30**	**30**	**30**	**0.006**	**0.006**	**0.006**	**0.003**	**0.003**	**0.0015**	**0.08**	**0.08**	**0.04**	**0.08**	**0.04**	**0.04**
KO1	35	35	30	30	30	30	0.012	0.012	0.012	0.012	0.012	0.012	0.02	0.04	0.01	0.02	0.005	0.005
KO2	35	35	30	30	30	30	0.006	0.006	0.003	0.003	0.0015	0.003	0.02	0.04	0.01	0.02	0.005	0.01
KO3	**35**	**35**	**30**	**30**	**30**	**30**	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	**0.02**	**0.04**	**0.01**	**0.02**	**0.005**	**0.01**
KP1	35	35	30	30	30	30	0.012	0.012	0.006	0.006	0.006	0.006	0.02	0.04	0.01	0.02	0.01	0.01
KP2	35	35	30	30	30	30	0.012	0.012	0.006	0.006	0.006	0.006	0.02	0.04	0.01	0.02	0.01	0.01
KP3	35	35	35	35	30	30	0.012	0.012	0.006	0.006	0.006	0.006	0.02	0.04	0.01	0.02	0.01	0.02
KP4	35	35	35	35	30	30	0.006	0.012	0.006	0.006	0.003	0.006	0.02	0.04	0.01	0.02	0.01	0.01
KP5	35	35	35	30	30	30	0.006	0.006	0.006	0.006	0.006	0.006	0.02	0.02	0.01	0.01	0.005	0.005
KP6	35	35	30	30	30	30	0.006	0.006	0.006	0.006	0.006	0.006	0.02	0.02	0.01	0.01	0.01	0.005
KP7	**35**	**35**	**30**	**30**	**30**	**30**	**0.012**	**0.012**	**0.003**	**0.003**	**0.003**	**0.003**	**0.02**	**0.04**	**0.01**	**0.02**	**0.005**	**0.01**

AB, *Acinetobacter baumannii*; *AP*, *Acinetobacter pittii*; *KO*, *Klebsiella oxytoca*; *KP*, *Klebsiella pneumoniae*; *PA*, *Pseudomonas aeruginosa*; *w*, with organic soiling (ie, 0.3% BSA); *w/o*, without organic soiling.
Reference and type strains are bold.

other species, a LR ≥ 5 was achieved at up to 16 times lower PAA concentrations. Overall, the lowest PAA concentrations were needed to eliminate *P aeruginosa* strains at all contact times. Carrier tests confirmed the significantly lowest susceptibility of *P aeruginosa* strains against BAC as well as the higher sensitivity of *Acinetobacter* spp compared to *Klebsiella* spp ($P < .05$). Contact time had no pronounced effect on bactericidal concentrations, with the exception of BAC at the 1-minute contact time. Compared with all contact times, significantly higher bactericidal concentrations were needed to efficiently reduce viable counts of all strains at that time ($P < .05$). Results are displayed in Table 3.

DISCUSSION

Disinfection is essential for the control of infectious diseases. With the growing number of reports on MDR bacteria, concern has been raised regarding the development of disinfectant resistance, particularly cross-resistance to antibiotics.[38] Therefore, it is important to ensure the efficacy of chemical surface disinfectants, especially in nosocomial disease events. In the study at hand, efficacy testing was performed according to the guidelines of the VAH Disinfectants Commission, one of the most important German reference institutions for the issuance of certificates and listing of disinfection procedures.

Table 3
Disinfectant concentrations resulting in a logarithmic reduction ≥ 5 in practical tests on steel carriers with organic soiling

	Ethanol (%)				Peracetic acid (%)				Benzalkonium chloride (%)			
Strain	1 min	5 min	15 min	30 min	1 min	5 min	15 min	30 min	1 min	5 min	15 min	30 min
AP1	50	40	30	30	0.096	0.096	0.048	0.048	0.64	0.08	0.02	0.02
AP2	50	40	40	40	0.096	0.096	0.096	0.096	0.64	0.04	0.02	0.02
AP3	**40**	**30**	**30**	**30**	**0.096**	**0.096**	**0.024**	**0.024**	**0.64**	**0.08**	**0.02**	**0.02**
AB1	50	40	40	40	0.096	0.096	0.048	0.048	0.32	0.08	0.02	0.02
AB2	40	40	40	40	0.096	0.048	0.048	0.048	0.16	0.04	0.04	0.02
AB3	**40**	**40**	**30**	**30**	**0.048**	**0.048**	**0.024**	**0.024**	**0.64**	**0.04**	**0.04**	**0.02**
AB4	**50**	**40**	**40**	**40**	**0.048**	**0.048**	**0.048**	**0.048**	**0.32**	**0.08**	**0.04**	**0.02**
PA1	40	30	30	30	0.048	0.024	0.024	0.024	5.12	0.32	0.16	0.08
PA2	40	30	30	30	0.024	0.024	0.024	0.024	5.12	0.16	0.16	0.08
PA3	40	30	30	30	0.048	0.024	0.024	0.024	1.28	0.32	0.16	0.08
PA4	**40**	**40**	**30**	**30**	**0.048**	**0.024**	**0.024**	**0.024**	**5.12**	**0.64**	**0.16**	**0.16**
KO1	40	40	30	30	0.192	0.096	0.096	0.096	1.28	0.16	0.04	0.02
KO2	40	40	30	30	0.048	0.048	0.048	0.048	1.28	0.16	0.04	0.04
KO3	**40**	**40**	**30**	**30**	**0.096**	**0.096**	**0.096**	**0.096**	**2.56**	**0.16**	**0.04**	**0.04**
KP1	40	40	30	30	0.096	0.096	0.096	0.096	2.56	0.16	0.08	0.04
KP2	40	40	30	30	0.096	0.096	0.096	0.048	2.56	0.16	0.08	0.04
KP3	40	40	30	30	0.096	0.096	0.096	0.096	2.56	0.16	0.08	0.04
KP4	40	40	30	30	0.096	0.048	0.048	0.048	2.56	0.16	0.08	0.04
KP5	40	40	40	30	0.096	0.048	0.048	0.048	1.28	0.08	0.02	0.02
KP6	50	30	30	30	0.096	0.096	0.048	0.048	2.56	0.16	0.02	0.02
KP7	**40**	**30**	**30**	**30**	**0.024**	**0.012**	**0.006**	**0.006**	**2.56**	**0.16**	**0.08**	**0.04**

AB, *Acinetobacter baumannii*; *AP*, *Acinetobacter pittii*; *KO*, *Klebsiella oxytoca*; *KP*, *Klebsiella pneumoniae*; *PA*, *Pseudomonas aeruginosa*.
Reference and type strains are bold.

These guidelines are equivalent to European standards but include additional exploratory tests.[35] Test guidelines from other countries differ in, for example, reference strains or methodology.[39,40] Reference strains are recommended for disinfectant testing, but in Germany no reference values exist for standardized quality control.[34] This is mainly because testing is generally performed on commercial disinfectants, with concentrations and application processes specified by the manufacturer for a respective product. Concurrent testing of reference substances (corresponding to the class of active substances) is not compulsory for bactericidal efficacy testing according to VAH or European standards. Moreover, compared to antimicrobial susceptibility testing, there is no definition of bacterial disinfectant resistance based on breakpoints.[15,25] Resistance against biocides was defined as bacterial survival at in-use concentrations of a biocide. In addition, a bacterial strain is characterized as biocide resistant at a concentration that inactivates other strains of that organism.[15] There are numerous studies on bacterial susceptibility to biocides, including MDR isolates,[21-29] but comparison of the results is difficult or not even feasible due to differences in test methods which markedly influence test outcomes.[30,33,41] Many authors have used MIC determination, as it is routine in antibiotic susceptibility testing; however, because disinfectants are used to sufficiently reduce bacteria from surfaces within a short period of time, the assessment of minimum bactericidal concentrations at least is more trustworthy.[17,32] Moreover, values may differ vastly depending on the disinfectant and the test method used, as has been described for sodium hypochlorite, with a 500-fold difference between MIC and suspension test results, and as was also noticed for BAC in the present study.[33] Thus, practical tests on surfaces with or without mechanical action are more suitable to predict the efficacy of a disinfectant because they mimic in-use conditions.

We focused on single active substances that are classified as high-level (PAA), intermediate- to low-level (ETH), and low-level (BAC) disinfectants according to the level of inactivation reached.[42] A wide range of dilutions was tested at the same contact time to determine the efficacious range. Commercial products often contain additional ingredients that may enhance activity but allow no conclusion regarding the activity of a particular substance. Time frames from 1 minute to 60 minutes were selected according to the VAH guidelines.[34] Short contact times appear to be most suitable for routine prophylactic surface disinfection in health care; however, cleaning and disinfection measures should also be appropriate for particular risk areas (eg, intensive care units, surgical departments, isolation ward) or a specific pathogen, which may require higher concentrations and longer contact times compared to routine disinfection.[43] Users must ensure that the surface is evenly wetted by the disinfectant, which must remain in place for the duration of the required contact time (concentration/time ratio), possibly requiring reapplying the product more than once.

MICs and bactericidal concentrations determined by qualitative suspension tests revealed that these values mainly lie well below in-use concentrations of commercial products at a given contact time. Overall, our results were similar to those reported elsewhere.[24-29,44,45] Quantitative suspension test results were mainly similar to those from qualitative suspension tests. Compared to other studies using quantitative suspension tests, only a few agree with our results.[27,46-51] Up to 25-fold lower (BAC) as well as up to 33-fold higher (PAA) concentrations at identical contact times have been reported.[47,49,50] Organic soiling (ie, 0.3% BSA) had no significant influence on the efficacy of any of the disinfectants tested ($P > .05$), which is in contrast to other studies using BAC.[52,53]

Significantly higher concentrations of PAA (as much as 16-fold) and BAC (up to 64-fold) were determined in practical surface tests compared with quantitative suspension tests ($P = .00001$). This might be attributed to the fact that bacteria attached to surfaces have a different phenotype and more readily resist antimicrobial treatment than cells in suspension, which, depending on the disinfectant and attachment time, might result in higher disinfectant concentrations being necessary.[54-57] This has also been demonstrated for dry surface biofilms, which are almost ubiquitous on hospital surfaces despite regular cleaning and disinfection,[58,59] but standardized efficacy tests regarding biofilms do not exist in Germany or the European Union. These findings suggest that MIC testing or suspension tests may reveal putatively efficacious concentrations, which is supported by our results for BAC. Commercially available surface disinfectant concentrates contain up to >20 g BAC per 100 g, and the lowermost contact time usually given is 15 minutes. Most products containing BAC would sufficiently kill *Acinetobacter*, *Klebsiella*, and *P aeruginosa* strains within 15 minutes. Nevertheless, values determined at 15 minutes for all *P aeruginosa* strains and at 30 minutes for *P aeruginosa* strain PA4 exceeded in-use concentrations of certain commercial products containing BAC. These strains would have been classified as resistant even though all strains were fully susceptible based on MICs and suspension tests. Overall, similar results but lower as well as higher bactericidal values have been described by other authors.[46,60-64]

Comparing all methods applied in the current study, inter- and intraspecies susceptibility to disinfectants varied depending on the method. BAC gave concordant results throughout all of the tests in that *P aeruginosa* strains were least susceptible and *Acinetobacter* strains were most susceptible. It is interesting to note that bactericidal BAC values nevertheless differed up to more than 100-fold compared to MICs. It has been suggested that high concentrations of nutrient salts in MIC test media lower the effective concentration of BAC, thus MIC testing does not truly reflect the intrinsic activity of BAC.[65] As determined by MICs, *Acinetobacter* species had the highest susceptibility to PAA, whereas individual *Klebsiella* strains revealed the significantly highest susceptibility in suspension and carrier tests. This was also noticed for *P aeruginosa* strain PA4 for a 15-minute contact time with organic soiling in the quantitative suspension test. Differences in bactericidal concentrations were highest when comparing quantitative suspension and carrier tests. ETH values differed 10-fold at maximum between MICs and qualitative suspension tests, but overall variations among strains as well as methods were less pronounced. Culture media influence test outcomes[51,66] and may account for varying results, as MIC determination was carried out by broth macrodilution using TSB. Although no distinct differences in susceptibility against ETH, PAA, or BAC were detected between reference strains and MDR-GNB, single strains required higher bactericidal values for individual disinfectants, such as was seen for *K oxytoca* strain KO1 and PAA. Moreover, although the active substances tested here were effective against MDR-GNB, some biocides currently used in hospitals can be ineffective against nosocomial pathogens growing as wet or dry biofilms attached to surfaces. This emphasizes the need to test for disinfectant susceptibility if MDR isolates are recurring in order to prevent severe infections and fatal outcomes, as has been described elsewhere.[27,28,67,68]

CONCLUSIONS

Overall, we found that ETH, PAA, and BAC were effective against clinically relevant MDR-GNB. Nevertheless, according to carrier test results *P aeruginosa* strains used in this study could be classified as resistant to BAC depending on the product used. Strain- and species-specific differences in bactericidal concentrations were seen, but in general MDR-GNB revealed no higher insensitivity to the disinfectants tested. Marked differences were observed in the efficacious concentrations depending on the test method applied, leading to the conclusion that MIC determination might not be the best approach to evaluate susceptibility or resistance to biocides. Practical surface tests are laborious but are most suitable to test for in-use efficacy and

should be used to evaluate disinfectants used on health care surfaces. Moreover, suitable guidelines are strongly needed to ensure standardized disinfectant susceptibility testing and to allow for the comparison of results obtained by different investigators.

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