Resistance to antibiotics and biocides among non-fermenting Gram-negative bacteria

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Objective To investigate the antibiotic and biocide susceptibilities of clinical isolates of rarely encountered Gram-negative, non-fermenting bacteria.

Methods Thirty Gram-negative non-fermenting bacterial strains were isolated from blood cultures of oncology patients. These were studied for their resistance to 11 antibiotics. Their susceptibilities to seven biocides used in hospitals were also examined.

Results Isolates of *Stenotrophomonas maltophilia* and *Ochrobactrum anthropi* were generally resistant to at least five of the antibiotics, whereas isolates of *Comamonas acidivorans*, *Flavobacterium oryzihabitans*, *Aeromonas hydrophila*, *Sphingobacterium spiritivorum*, *Acinetobacter junii* and *Acinetobacter lwoffi* were generally sensitive to at least nine of the antibiotics. Trovafloxacin and trimethoprim–sulfamethoxazole were the most effective antibacterial agents tested, with 0% and 7%, respectively, of isolates being resistant, whereas 63% of isolates were resistant to aztreonam. Some isolates, sensitive to meropenem and/or ceftazidime in vitro, possessed very high MBC/MIC ratios for these β-lactams. Two out of three biocides used in hospital pharmacies showed lethal activity towards all strains tested when used at less than one-third of their recommended in-use concentration. Proceine 40 failed to give a 5 log reduction in bacterial cell number for the isolates tested when used at its 'in-use' concentration. A concentration of >500 mg/L chlorhexidine was required to achieve a 5 log reduction for the same isolates.

Conclusions We have examined the antibiotic susceptibilities of non-fermenting Gram-negative bacterial strains isolated from immunocompromised patients. Despite being sensitive to certain antibiotics in vitro, some isolates were still able to cause serious bacteremia. We have also reported for the first time the susceptibilities of non-fermenting Gram-negative bacteria to common biocides used in hospital infection control, and have shown that some strains are able to persist at the 'in-use' concentration of particular biocides. It is therefore important to study further this particular group of organisms, and, in particular, to examine whether there exists a link between resistance to antibiotics and resistance to biocides.

Keywords Non-fermenting Gram-negative bacteria, antibiotic and biocide susceptibilities

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INTRODUCTION

The use of antibiotics, in particular β-lactams, has increased dramatically in recent years, both therapeutically once infection has occurred, and prophylactically, particularly for immunocompromised patients. Bacteria can resist the action of antibiotics in many ways [1–3], including (1) the presence of an enzyme that inactivates the antibiotic, (2) modification of the target of the antibiotic, thus reducing binding of the antibiotic to the target, (3) reduced uptake of the antibiotic, and (4) active efflux of the antibiotic. Resistance to an antibiotic, whatever the mechanism, poses a major problem for therapeutic regimens and treatment of infection.

The development and persistence of antibiotic resistance in bacteria is an ongoing problem which has been the subject of a great deal of research. By contrast, biocide (collectively disinfectant, antiseptic and preservative) resistance is an emerging problem which is only now attracting greater interest [4,5]. Resistance to these agents displayed by Gram-negative bacteria is often intrinsic, whereby cellular impermeability or an efflux

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system(s) prevents sufficiently high concentrations of the biocide reaching target sites within the cell [4]. Resistance may also be mediated by mutation or acquisition of genetic elements such as plasmids, integrons or transposons [6].

Non-fermenting Gram-negative bacteria (NFGNB), such as *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Alcaligenes* spp., *Aeromonas* spp., *Chryseobacterium* spp., *Ochrobactrum anthropi*, *Comamonas acidivorans*, and *Sphingobacterium spiritivorum*, have been regarded historically as either environmental contaminants or organisms of low pathogenicity, and hence not clinically significant. However, their emergence as significant causes of nosocomial bacteremia has more recently been noted, particularly with regard to immunocompromised patients [7–13]. Many reports describe the antimicrobial susceptibilities of NFGNB to various antibiotics [8,9,14–21], whereas very little is known about the susceptibility of these organisms to biocides. We therefore decided to study the susceptibility profiles of clinical isolates of NFGNB for both clinically relevant antibiotics used in therapy and compounds used as biocides.

MATERIALS AND METHODS

Clinical isolates of NFGNB

Thirty isolates of NFGNB have been isolated from blood cultures of oncology patients over the past 10 years. The NFGNB isolates were identified using a commercial biochemical profile kit (Api20NE; bioMérieux, Marcy L'Etoile, France) according to the manufacturer's instructions, supported by 16S rRNA DNA sequencing. Primers used for amplification were 'rDNA' forward (5′-TCAGATTTGAACGCTGGCGGCA-3′) and reverse (5′-CGTATTACCGCGGCTGCTGCCAC-3′). These primers bind to highly conserved sequences in the 16S rRNA gene and amplify a 500-bp fragment of the gene where the majority of sequence variation is known to occur in different Gram-negative bacteria. Subsequent sequences were analyzed using the Ribosomal Database Project (Michigan State University, USA).

Determination of the minimum inhibitory concentration (MIC) of an antibiotic

The MIC was determined on Mueller–Hinton agar (Oxoid, Basingstoke, UK) using E test strips according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

Determination of the minimal bactericidal concentration (MBC) of an antibiotic

The MBC was determined by the macrobroth dilution method. Cultures grown to mid-log phase were diluted 1 : 100 in fresh IsoSensitest broth containing antibiotic at 1×, 2×, 4× and 8× the MIC (as determined in IsoSensitest broth). These cultures were incubated overnight at 37 °C with shaking. On the following day, 100 μL was taken from the cultures where growth was not observed and plated on antibiotic-free IsoSensitest agar. These plates were incubated overnight at 37 °C. The number of colonies on the agar plates was counted, and the MBC was noted as the lowest concentration of antibiotic capable of killing 99.9% of the cells in the innoculum.

Determination of MICs of biocides

The MICs of chlorhexidine diacetate (Sigma, Poole, UK), benzalkonium chloride (ICN Biochemicals, Cleveland, Ohio, USA), phenol, Klericide A containing a bisbiguanide and a quaternary ammonium compound (Shield Medicare Ltd, Farnham, UK), Klericide B containing ClO_2 and a quaternary ammonium compound (Shield Medicare), Proceine 40 (Agma Plc, Haltworth, UK), broadly equivalent to a quaternary ammonium compound, and Texpure (Texwipe Co., Upper Saddle River, New Jersey, USA), a detergent, were determined using the agar incorporation method. MICs were also determined for all of the biocides, except phenol and Texpure, with ethylenediamine tetra-acetic acid (EDTA) (Sigma) added to the agar at concentrations of 1, 0.5 and 0.1 mM. An overnight culture incubated at 30 °C in nutrient broth (Oxoid) was diluted 1 : 10 in sterile water and used as the innoculum. A Denley multipoint inoculator was used to spot 1 μL of the bacterial suspension on nutrient agar (Oxoid) containing dilutions of the appropriate biocide. Nutrient agar lacking biocide was used as a control. Plates were incubated at 30 °C and growth was checked at 24 and 48 h. The MIC was defined as the lowest concentration of biocide which inhibited the growth of single colonies.

Bactericidal activity of biocides

A quantitative Suspension Test [22] was used to assess the log reduction of bacterial cell numbers. When Klericide A and B were assessed, concentrations in sterile water of 0.5%, 1.0%, 5.0% and 10% of the 'in-use' concentration were used. Cells from 10 mL of a culture grown overnight were harvested at 4000 *g* for 10 min. The cell pellet was resuspended in 5 mL of sterile water to produce a suspension of approximately 5×10^8 CFU/mL. One milliliter of the suspension was then mixed with 9 mL of the biocide solution and left for 5 min. One hundred microliters of the suspension was removed, and the biocide was neutralized in 900 μL of appropriate neutralizer. For Klericide A this was a 5% Tween (Sigma), 0.75% azolectin (Sigma) mixture, and for Klericide B, 0.5% sodium thiosulfate (Fisher Scientific, Loughborough, UK). The neutralized cell suspension was then serially diluted five more times in neutralizer. From each dilution, 10-μL samples were dropped in

triplicate on nutrient agar and allowed to dry before incubation at 30 °C for 24 h. After incubation, an appropriate dilution was chosen for counting. The test was repeated to simulate 'dirty' conditions by mixing the test suspension with a 3% (w/v) bovine serum albumin (BSA; Sigma) solution for 2 min before inoculating 2 mL of the mixture into 8 mL of the biocide solution. BSA is used to mimic organic soiling, and hence the 'dirty' test simulates practical 'in-use' conditions [22]. Suspension tests were also carried out for Proceine 40 and chlorhexidine diacetate, the neutralizer used for these being the same as that for Klericide A. A further test with Proceine 40 was carried out, where samples were also removed and neutralized at 60-s intervals after mixing of the cell suspension and biocide. In all cases, the tests were carried out in quadruplicate. The microbicidal effect (ME) was determined by subtracting the log number of survivors from the log number of cells at time 0.

RESULTS

MICs and MBCs of antibiotics

The results of MIC testing of 11 antibiotics against the 30 NFGNB isolates are shown in Table 1. Each value has been designated resistant, intermediate, or sensitive (Table 1), according to the NCCLS breakpoints for the non-Enterobacteriaceae [23].

Different non-fermentative species differed in their susceptibilities to the antibiotics tested. Isolates of *Stenotrophomonas maltophilia* and *O. anthropi* were generally resistant to at least five of the antibiotics, whereas isolates of *Comamonas acidivorans*, *Flavobacterium oryzihabitans*, *Aeromonas hydrophila*, *Sphingobacterium spiritivorum*, *Acinetobacter junii* and *Acinetobacter lwoffi* were generally sensitive to at least nine of the antibiotics. The overall percentage of resistant isolates for each of the antibiotics is as follows: aztreonam 63%, cefotaxime 60%, piperacillin–tazobactam 37%, meropenem 27%, gentamicin 26%, ceftazidime 23%, cefepime 23%, tetracycline 16%, chloramphenicol 10%, trimethoprim–sulfamethoxazole 7%, and trovafloxacin 0%.

A number of isolates with low MICs for meropenem and ceftazidime were chosen at random for further analysis. Given that the patients from whom these isolates were obtained had received multiple antibiotic therapies, including β-lactams, it was decided to determine the MBCs of meropenem and ceftazidime for some of these strains. The MBCs of meropenem and ceftazidime were determined for isolates N528 (*Comamonas acidivorans*), J611a (*Chryseomonas luteola*), and O91 (*Acinetobacter junii*), and of meropenem for isolate N217 I (*O. anthropi*) using a broth dilution method. In all cases, the MBC/MIC ratio was >8, meaning that the concentrations of these antibiotics required to kill the organisms are more than eight-fold greater than those required to inhibit growth.

MICs of biocides

MICs of a subset of 14 isolates of NFGNB were determined for chlorhexidine diacetate and benzalkonium chloride in the presence and absence of EDTA (Figure 1). Of the strains with MICs >100 mg chlorhexidine diacetate/L, *Aeromonas hydrophila* P881 had an MIC of 250 mg/L and four had MICs of 175 mg/L (Table 2). *Alcaligenes* sp. J370 and *Sphingobacterium spiritivorum* P780 I were the most sensitive strains (MICs of 10 mg/L) to chlorhexidine diacetate. None of the strains tested had an MIC of >100 mg/L for benzalkonium chloride, and strain P780 I was again the most sensitive to this antibacterial (MIC 10 mg/L). In all cases, the inclusion of EDTA (0.5 mM) reduced the MICs of chlorhexidine diacetate and benzalkonium chloride (Figure 1), and this effect was enhanced (data not shown) at higher EDTA concentrations. The MICs for phenol were spread over a large range from 125 to 1500 mg/L.

The results for the commercial biocide preparations tested are expressed as the percentage of their 'in-use' concentration (Table 2). The Klericides come pre-diluted and ready for use. However, Proceine 40 and Texpure require dilution, and are used at 0.6% (v/v) and 3% (v/v) dilutions of the concentrate provided, respectively. Klericide A had MICs between 2% and 9%, with MICs greater than 5% against the majority of the strains tested. Klericide B produced considerably lower MICs, with an MIC of 4% or less against 11 of the 14 strains tested. The highest MIC of Klericide B was 6% for strains *Acinetobacter baumannii* F301 I and *Alcaligenes* sp. J370. Strain P780 I was most sensitive to Klericide A and B (MICs 2% and 0.5%, respectively). Proceine 40 and Texpure showed higher MICs; Proceine 40 had MICs >4% against all but two of the strains, and

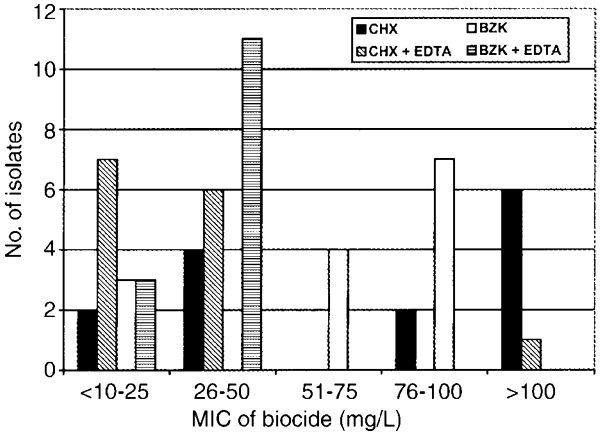

Figure 1 MICs of chlorhexidine diacetate (CHX) and benzalkonium chloride (BZK), with and without the addition of EDTA at 0.5 mM, for 14 non-fermenting Gram-negative bacilli isolates.

Table1 MICs (mg/L) of 10 antibiotics against 30 non-fermenting Gram-negative bacilli isolates, as determined by E test

	CTZ	CEF	CTX	MER	AZT	PIP/TAZ	GENT	TROV	TRI/SUL	TET	CHL
Alcaligenes spp.											
F136	12 (I)	2 (S)	16 (I)	0.38 (S)	48 (R)	3 (S)	24 (R)	0.094 (S)	6 (R)	2 (S)	24 (I)
J370	3 (S)	4 (S)	> 16 (R)	0.125 (S)	> 256 (R)	0.5 (S)	2 (S)	1.5 (S)	0.016 (S)	0.75 (S)	6 (S)
Comamonas acidivorans											
N528	0.5 (S)	6 (S)	2 (S)	0.094 (S)	3 (S)	1.5 (S)	> 256 (R)	0.25 (S)	0.094 (S)	3 (S)	12 (I)
Chryseomonas luteola											
J611a	1.5 (S)	1 (S)	1 (S)	0.38 (S)	8 (S)	8 (R)	16 (R)	0.38 (S)	0.094 (S)	8 (S)	3 (S)
P559 II	8 (S)	1.5 (S)	> 16 (R)	> 32 (R)	> 256 (R)	2 (S)	4 (S)	0.012 (S)	0.094 (S)	8 (S)	6 (S)
Chryseobacterium indologenes											
P567 A	8 (S)	1.5 (S)	> 16 (R)	> 32 (R)	> 256 (R)	3 (S)	6 (S)	0.012 (S)	0.094 (S)	8 (S)	8 (S)
N217 II	2 (S)	0.38 (S)	> 16 (R)	> 32 (R)	> 256 (R)	2 (S)	8 (S)	0.016 (S)	0.125 (S)	16 (R)	16 (I)
P198 A	2 (S)	0.38 (S)	> 16 (R)	> 32 (R)	> 256 (R)	2 (S)	8 (S)	0.016 (S)	0.125 (S)	16 (R)	16 (I)
Chryseobacterium meningosepticum											
O70	2 (S)	0.5 (S)	> 16 (R)	8 (I)	> 256 (R)	2 (S)	8 (S)	0.023 (S)	0.19 (S)	12 (I)	16 (I)
Ochrobactrum anthropi											
N217 I	> 256 (R)	64 (R)	> 16 (R)	0.25 (S)	> 256 (R)	> 256 (R)	1.5 (S)	0.5 (S)	0.094 (S)	0.25 (S)	16 (I)
N531 II	> 256 (R)	64 (R)	> 16 (R)	0.25 (S)	> 256 (R)	> 256 (R)	1.5 (S)	0.5 (S)	0.094 (S)	0.25 (S)	16 (I)
P566 II	> 256 (R)	64 (R)	> 16 (R)	0.25 (S)	> 256 (R)	> 256 (R)	1.5 (S)	0.5 (S)	0.094 (S)	0.25 (S)	16 (I)
Sphingobacterium spiritivorum											
N546 I	1.5 (S)	1.5 (S)	0.75 (S)	0.064 (S)	> 256 (R)	1.5 (S)	128 (R)	0.006 (S)	0.19 (S)	0.25 (S)	1.5 (S)
O749	1.5 (S)	1.5 (S)	0.75 (S)	0.064 (S)	> 256 (R)	1.5 (S)	128 (R)	0.006 (S)	0.19 (S)	0.25 (S)	1.5 (S)
P780 I	1.5 (S)	1.5 (S)	0.75 (S)	0.064 (S)	> 256 (R)	1.5 (S)	128 (R)	0.006 (S)	0.19 (S)	0.25 (S)	1.5 (S)
Flavimonas oryzihabitans											
8854	0.5 (S)	0.19 (S)	2 (S)	0.004 (S)	12 (I)	1.5 (S)	0.047 (S)	0.047 (S)	0.38 (S)	0.75 (S)	4 (S)
Aeromonas hydrophila											
P881	0.19 (S)	0.047 (S)	< 0.25 (S)	0.023 (S)	0.016 (S)	1.5 (S)	0.5 (S)	0.032 (S)	0.38 (S)	0.5 (S)	0.75 (S)
P211 I	0.25 (S)	0.064 (S)	< 0.25 (S)	0.023 (S)	0.016 (S)	0.75 (S)	0.38 (S)	0.047 (S)	0.5 (S)	0.5 (S)	0.75 (S)
J610	> 256 (R)	> 256 (R)	> 16 (R)	0.094 (S)	> 256 (R)	1 (S)	0.064 (S)	0.016 (S)	0.003 (S)	0.25 (S)	4 (S)
P478 A	0.25 (S)	0.047 (S)	< 0.25 (S)	0.016 (S)	0.016 (S)	0.075 (S)	0.038 (S)	0.047 (S)	0.5 (S)	0.5 (S)	0.75 (S)
Acinetobacter baumannii											
K277	4 (S)	4 (S)	> 16 (R)	0.5 (S)	24 (I)	8 (R)	0.25 (S)	0.023 (S)	0.19 (S)	3 (S)	128 (R)
F301 I	12 (I)	2 (S)	> 16 (R)	0.38 (S)	48 (R)	2 (S)	24 (R)	0.064 (S)	8 (R)	2 (S)	12 (I)
J686 II	6 (S)	4 (S)	> 16 (R)	0.5 (S)	24 (I)	8 (R)	0.25 (S)	0.032 (S)	0.19 (S)	3 (S)	128 (R)
P558 Ib	4 (S)	3 (S)	> 16 (R)	0.38 (S)	12 (I)	4 (R)	0.25 (S)	0.047 (S)	0.38 (S)	2 (S)	24 (I)
Acinetobacter junii											
O91	3 (S)	0.5 (S)	3 (S)	0.094 (S)	12 (I)	< 0.016 (S)	0.094 (S)	0.012 (S)	0.008 (S)	0.75 (S)	1.5 (S)
Acinetobacter lwoffi											
O948	3 (S)	0.5 (S)	3 (S)	0.094 (S)	12 (I)	< 0.016 (S)	0.125 (S)	0.012 (S)	0.008 (S)	0.75 (S)	1 (S)
Stenotrophomonas maltophilia											
G478	96 (R)	32 (R)	> 16 (R)	> 32 (R)	> 256 (R)	48 (R)	4 (S)	1 (S)	0.125 (S)	48 (R)	6 (S)
N531	1.5 (S)	8 (S)	> 16 (R)	> 32 (R)	> 256 (R)	24 (R)	12 (I)	0.094 (S)	0.094 (S)	6 (S)	3 (S)
K279a	96 (R)	32 (R)	> 16 (R)	> 32 (R)	> 256 (R)	192 (R)	4 (S)	1.5 (S)	0.125 (S)	64 (R)	8 (S)
J675 I	256 (R)	> 256 (R)	> 16 (R)	> 32 (R)	> 256 (R)	> 256 (R)	24 (R)	0.19 (S)	0.064 (S)	12 (I)	12 (I)

CTZ, ceftazidime; CEF, cefepime; CTX, cefotaxime; MER, meropenem; AZT, aztreonam; PIP/TAZ, piperacillin–tazobactam; GENT, gentamicin; TROV, trovafloxacin; TRI/SUL, trimethoprim–sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol. NCCLS breakpoints for non-Enterobacteriaceae [23] have been used to determined whether an MIC is resistant (R), intermediate (I), or sensitive (S).

Table 2 MICs of seven biocides against 14 non-fermenting Gram-negative bacilli isolates

Identity	Strain	KI A (%)	KI B (%)	Proceine 40 (%)	Texpure (%)	CHX (mg/L)	BZK (mg/L)	Phenol (mg/L)
Alcaligenes spp.	F136	6	4	5	33.3	175	25	1250
Alcaligenes spp.	J370	5	6	4	33.3	10	75	250
Comamonas acidivorans	N528	5	2	9	33.3	50	100	1250
Chryseomonas luteola	J611a	9	3	6	50	80	100	1250
Chryseobacterium indologenes	P567a	4	3	4	50	175	100	1000
Ochrobactrum anthropi	N217I	6	1	4	50	30	10	750
Sphingobacterium spiritivorum	P780 I	2	0.5	1	50	10	10	1500
Aeromonas hydrophilia	P881	3	2	4	50	250	100	1250
Acinetobacter baumannii	K277	6	3–4	4	33.3	125	50	1250
Acinetobacter baumannii	F301 I	8	6	5	10	175	100	1000
Stenotrophomonas maltophilia	G478	8	5	6	33.3	175	100	1000
Stenotrophomonas maltophilia	J675 I	8	3	6	50	30	100	1000
Chryseobacterium meningosepticum	O70	3	1	3	33.3	80	75	1000
Flavimonas oryzihabitans	8854	9	4	6	33.3	30	75	1250

MICs for Klericide A (KI A), Klericide B (KI B), Proceine 40 and Texpure are expressed as a percentage of their 'in-use' concentration. MICs for chlorhexidine (CHX), benzalkonium chloride (BZK) and phenol are expressed as mg/L.

Texpure had MICs of 33.3% or more against 13 of the 14 strains tested.

The addition of 0.5 mM EDTA reduced the MICs of Proceine 40 and Klericides A and B to 2% or less, with the exception of *Aeromonas hydrophila* P881, against which Proceine 40 had an MIC of 5% with each concentration of EDTA used.

Microbicidal effects of biocides

At low concentrations (0.5% and 1.0% of the 'in-use' concentration) in the absence of organic material, Klericides A and B had comparable activities against the four strains tested, as demonstrated by the log reduction of bacterial cell number (Tables 3 and 4). The performance of both biocides, at most concentrations, was impaired in the presence of 3% BSA. Higher concentrations of Klericide B were more effective than Klericide A, especially under 'clean' conditions. Under our laboratory test conditions, Proceine 40 failed to give a 5 log reduction in bacterial cell numbers after a 5-min exposure for isolates *Acinetobacter baumannii* F301 I (log reduction of 3.935 ± 0.872) and *Stenotrophomonas maltophilia* G478 (log reduction of 4.225 ± 0.143) when it was used at its suggested 'in-use' concentration (0.6%) for floor cleaning. Further tests with *Acinetobacter baumannii* F301 I over a 5-min period, with samples taken at 60-s intervals, showed the reduction in viable cell number to be almost linear over the 5-min period following exposure to Proceine 40 (data not shown). Strains of *Acinetobacter baumannii* (F301 I) and *Stenotrophomonas maltophilia* (G478) required a concentration of >500 mg/L chlorhexidine diacetate to give a 5 log reduction in bacterial cell number following a 5-min exposure.

DISCUSSION

The NFGNB isolates in this study were obtained from blood cultures of oncology and bone marrow transplant patients. The patients from whom the strains were isolated were generally immunocompromised due to chemotherapy, and had intravenous central lines. Many patients had recurrent bacteremia, despite receiving multiple antibiotic therapies, which included meropenem, ceftazidime and piperacillin–tazobactam. Owing to the increasing isolation of NFGNB from immunocompromised patients [7–13], it is important to determine the antibiotics to which these organisms are truly susceptible. Results of the determination of the MICs of a number of antibiotics against the 30 NFGNB isolates showed different susceptibilities in different species, generally in line with those in previous reports [8,9,14–21]. Our results indicate that several of the isolates were sensitive to certain antibiotics in vitro, despite their abilities to still cause bacteremia after antibiotic therapy. To examine this finding further, the MBC values of four isolates, each of which has a low MIC for meropenem and/or ceftazidime, were determined. All of these isolates were found to have high MBC/MIC ratios for these β-lactams. If the concentration of antibiotic given to the patient is below the MBC, then the antibiotic will only temporarily inhibit the growth of any susceptible microorganisms. When the antibiotic is withdrawn, the possibility exists that the organisms may re-proliferate, potentially causing prolonged infection. Organisms that, in vitro, appear to be sensitive to those β-lactams used clinically may thus cause serious bacteremias. This phenomenon has been observed in other cases of *O. anthropi* bacteremia [24].

In addition to studying the antibiotic susceptibilities of these NFGNB strains, their susceptibilities to biocides were also

Table 3 Microbicidal effect of Klericide A (KI A), shown as log reduction in bacterial cell number after 5-min exposure, in 'clean' (0% BSA) and 'dirty' (3% BSA) conditions; mean ± standard deviation

	0.5% KI A		1.0% KI A		5.0% KI A		10.0% KI A	
	0% BSA	3% BSA	0% BSA	3% BSA	0% BSA	3% BSA	0% BSA	3% BSA
Chryseobacterium indologenes P567 A	2.310 ± 0.229	2.553 ± 0.079	2.638 ± 0.116	2.703 ± 0.420	> 6.0 ± 0	4.503 ± 0.170	NM	4.678 ± 0.441
Stenotrophomonas maltophilia G478	2.888 ± 0.527	1.060 ± 0.133	3.445 ± 0.246	1.783 ± 0.835	5.913 ± 0.118	3.578 ± 0.098	NM	> 6.0 ± 0
Stenotrophomonas maltophilia J675 I	0.330 ± 0.253	0.480 ± 0.197	1.340 ± 0.489	0.990 ± 0.093	3.363 ± 0.109	2.385 ± 0.270	> 6.0 ± 0	5.313 ± 0.581
Acinetobacter baumannii F301 I	3.305 ± 0.482	1.443 ± 0.334	3.978 ± 0.031	2.033 ± 0.129	> 6.0 ± 0	5.923 ± 0.092	NM	NM

Concentrations of Klericide A refer to percentages of 'in-use' concentration. NM, not measured.

Table 4 Microbicidal effect of Klericide B (KI B), shown as log reduction in bacterial cell number after 5-min exposure, in 'clean' (0% BSA) and 'dirty' (3% BSA) conditions; mean ± standard deviation

	0.5% KI B		1.0% KI B		5.0% KI B		10.0% KI B	
	0% BSA	3% BSA	0% BSA	3% BSA	0% BSA	3% BSA	0% BSA	3% BSA
Chryseobacterium indologenes P567 A	2.443 ± 0.095	1.825 ± 0.321	3.370 ± 0.318	2.270 ± 0.644	> 6.0 ± 0	5.293 ± 0.165	NM	NM
Stenotrophomonas maltophilia G478	1.270 ± 0.074	0.660 ± 0.105	2.720 ± 0.764	1.080 ± 0.255	> 6.0 ± 0	4.685 ± 1.562	NM	> 6.0 ± 0
Stenotrophomonas maltophilia J675 I	1.280 ± 0.159	0.528 ± 0.199	3.200 ± 0.181	1.093 ± 0.162	> 6.0 ± 0	4.115 ± 0.535	NM	> 6.0 ± 0
Acinetobacter baumannii F301 I	2.050 ± 0.078	1.710 ± 0.557	3.130 ± 0.168	2.882 ± 0.099	> 6.0 ± 0	> 6.0 ± 0	NM	NM

Concentrations of Klericide B refer to percentages of 'in-use' concentration. NM, not measured.

determined. There are two views on how biocide resistance should be defined: (1) increase in minimum inhibitory concentration (MIC) or an MIC higher than that of a reference strain; and (2) a point at which bacteria are non-susceptible to the 'in-use' concentration of the biocide. MICs for the bisbiguanide chlorhexidine diacetate for some strains are higher than those (10–50 mg/L) reported to be inhibitory to Gram-negative bacteria [5], with nine of the 14 strains having MICs of 50 mg/L or more. These relatively high levels of chlorhexidine diacetate non-susceptibility can be attributed to the failure of the biocide to reach its target sites within the cell [25]. This value is still significantly lower than the MIC of 800 mg/L found for *Proteus mirabilis and Pseudomonas aeruginosa* isolated from catheters washed out with chlorhexidine diacetate [26]. MICs for a quaternary ammonium compound (bezalkonium chloride) for all isolates were less than 100 mg/L. Pseudomonad species were termed resistant if they were able to grow in broth containing 200 mg benzalkonium chloride/L [27]. MICs of phenol varied considerably (125–1500 mg/L) but were below the concentration normally used (5000 mg/L) as a preservative.

Klericide B is stated as being the more effective of the two Klericide products, because of its stabilized ClO_2 component. Our MIC data support this view; 11 of 14 strains were inhibited by a concentration of 4% or less for Klericide B, whereas most of the strains tested had MICs of $>5\%$ for Klericide A. MICs for the floor cleaners Proceine 40 and Texpure were higher than those obtained for Klericides A and B, the latter agents usually being used on more critical work surfaces such as aseptic preparation isolators or laminar airflow cabinets. Texpure is a detergent-based cleaner, and the MICs obtained of $\geq 33.3\%$ show that it is a less efficacious antimicrobial product than the others tested. However, under certain conditions of use, surface-active properties may contribute to the in situ effectiveness of a biocide, by physically removing bacteria from a surface. In all cases, MICs were reduced significantly by the addition of EDTA, which potentiates entry of the biocide into the target cell by increasing the permeability of the outer membrane [4]. This is most markedly demonstrated by the reduction of the Proceine 40 and chlorhexidine diacetate MICs.

Bactericidal testing was undertaken by a method based on the European Suspension Test (EST). Although the EST has been shown to be subject to variability [28], it remains a useful test to discriminate between highly effective (ME $>$ 5 log reduction in bacterial cell number after 5 min) and ineffective biocides [29]. Klericide A and B produced >5 log reduction in 5 min when tested at 10.0% of their in-use concentrations in clean conditions, and only when 3% BSA was added did Klericide A fail to do so against two of the strains tested (*Chryseobacterium indologenes* P567 A and *Stenotrophomonas maltophilia* J675 I). Conversely, Proceine 40 consistently failed to give a 5 log reduction in bacterial cell number for isolates F301 I (*Acinetobacter baumannii*) and G478 (*Stenotrophomonas maltophilia*) when it was tested at its recommended 'in-use' concentration for floor disinfection. However, like Texpure, Proceine 40 has surface-active characteristics that may contribute to its in situ activity. For isolates F301 I and G478, the concentration of chlorhexidine diacetate required to achieve a 5 log reduction in bacterial cell number was >500 mg/L.

There have been a number of investigations into possible links between biocide and antibiotic resistance [26,30–33]. With emerging pathogens showing resistance to many antibiotics, it is of interest to examine whether they also show reduced susceptibility to biocides. If this were found to be the case, then investigating the extent to which the two factors are linked is of prime clinical significance, given the possibility of biocides selecting antibiotic-resistant organisms. Further investigations with a larger number of these NFGNB strains may provide greater insight into a possible link.

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