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The Effectiveness of the Antimic® Biocide against Nosocomial Bacteria Specified by Different Standard Methods

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ABSTRACT

The effectiveness of Antimic® (3-(trimethoxysilyl)-propyl, cocodimethylammonium chloride) against different nosocomial pathogens was evaluated. Despite the fact that Antimic® biocide is a recommended compound for disinfecting areas, there is no published data about the antibacterial activity of this formulation against nosocomial pathogens (*Acinetobacter baumanii*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*). The minimum inhibitory/minimum bactericidal effective concentrations for test bacteria were determined. The bactericidal activity of determined dosages was evaluated using the culture-based quantitative suspension test (British Standards BS EN 1276) for 1 and 5-minute contact period, under clean and dirty conditions. Antibacterial activity was also confirmed by fluorescence staining. The biocide was found to be effective at 25 and 50 mg/L concentrations at contact times of 1 and 5 minute, with ≥ 5 log reduction in all bacteria. According to fluorescent microscopic examinations similar bacteria reduction was determined as ≥ 5 log reduction. The results indicated that Antimic® compound meets the requirements of EN 1276 against the tested bacteria. Moreover, Antimic® biocide provide an advantage by not promoting the viable but non culturable state in the tested bacteria and removing the tested bacteria successfully. The results showed that the execution of different microbiological growth and/or antibacterial activity monitoring tests, simultaneously, provide information about the optimum concentration and contact time of a biocide.

Keywords: Nosocomial pathogens, Antimic®, antibacterial activity, MIC, MBC

INTRODUCTION

In recent years nosocomial infections [hospital associated/acquired infections (HAI)] have been recognised as a serious safety issue for both patients and health care providers. According to the World Health Organization (WHO), nosocomial infection is defined as: "an infection developing in patients during the process of care in a health-care facility which was not present or incubating at the time of admission." This definiton includes infections which can be acquired in the hospital but appear after discharge from hospital, and also occupational-related infections among medical staff (1).

Nosocomial infections occur worldwide as an important healthcare problem both in developed and developing countries. Of every 100 hospitalized patients, 7 in developed and 10 in developing countries will acquire at least one of the health care-associated infections (2,3). At any given time, nosocomial infection prevalence is 5%-12% and 5.7%-19.1% in high-income and in low- and middle-income countries, respectively (3).

Nosocomial infections, cause functional impairment and emotional stress in patients due to increased lengths of stay in hospitals (1,3). It should be noted that HAIs result in increased healthcare costs. The greatest contributor to this cost is increased length of stays for infected patients; also indirect costs due to lost work is also considerable. Most importantly of all, these infections increase morbidity and are a major cause of death (1,3,4).

Patient susceptibility, environmental factors, contaminated environmental sites, microbial agent type and bacterial resistance to antimicrobials can influence the development of nosocomial infections (1). Surveillance studies have proved that many nosocomial infections are caused by antimicrobi-

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al-resistant organisms (4). In general, the agents which are related to nosocomial infections include *Acinetobacter* spp., *Streptococcus* spp., *Staphylococcus aureus* and coagulase-negative staphylococci, enterococci, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Legionella* spp., and Enterobacteriaceae family members including *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumonia* and *Serratia marcescens* (5,6). In fact, the methicillin resistant *Staphylococcus aureus* (MRSA) infection is now used as a measure of hygiene in hospitals, since they resist desiccation and can survive in hospital dust for up to a year (1,7-10)

Various measures for controlling and preventing infection, such as the usage of masks and gloves, appropriate hand hygiene and the application of basic precautions are recommended by the authorities (3). However, it has been proven that pathogens such as VRE and MRSA can be transmitted from contaminated surfaces to caregivers' hands (9). Epidemiologic studies have shown that previously colonized places with MRSA, VRE or *Acinetobacter baumanii* are at significant risk of acquiring these organisms (8).

For this reason, HAI prevention/control measures should involve the use of effective biocides (4). Therefore, considering the limited treatment options and the mortality rate, the eradication of the cause of the infection with the correct agent, correct dose and contact time at the infection source points will provide a permanent and effective solution.

There are various reports on cross- and co-resistance to biocidal compounds and antibiotics (4). Therefore, alternative biocidal compounds are being investigated. One of the new durable alternative compounds 3-(trimethoxysilyl)-propyl, cocodimethylammonium chloride (Antimic®), has alkoxy silane functional groups to form covalent bonds at the molecular level and thus, provide antibacterial features to the applied surface. While Antimic®'s hydrophobic long chains approach the lipid membrane, the positively charged quaternary region of the Antimic® breaks down the cell membrane, leading to the death of the bacteria (11).

Four different trimethoxysilyl quaternary ammonium chloride compounds were studied in detailed by the Environmetal Protection Agency (EPA) and were reported as non migrating and non toxic features. (12). Therefore, they prevent antimicrobial resistance and do not cause cross contamination.

Although Antimic® compound is recommended for disinfection, there is no published report on the antibacterial activity of this compound against different nosocomial pathogens. Therefore, in this study the inhibitory characteristics of Antimic® biocide were investigated against *Acinetobacter baumanii*, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* pathogens.

MATERIALS AND METHODS

Test Organisms and Culture Conditions

Acinetobacter baumanii (ATCC 19606), vancomycin-resistant *Enterococcus faecium* (ATCC 51299) and methicillin-resistant *Staphylococcus aureus* (ATCC 33591) organisms were obtained American Type Culture Collection. Test organisms were maintained in phosphate buffer and glycerin suspension separately and stored at - 86°C in cryotubes. Freeze-dried cultures were not subcultured more than 3 times in order to prevent mutations and not affect the resistance of the organisms against antimicrobials (13). For experimental use, freeze-dried cultures of the organisms were grown on tryptone soya agar (Oxoid) at 37°C. After 24 hours, cells were harvested and a suspension was prepared turbidimetrically to a 1.5-5.0 10^8 cfu ml^{-1} concentration in Cl_2-free sterile tap water and used in the experiments.

Biocide

Different dosages of Antimic® biocide (10.000-1 mg/L), were prepared in sterile distilled water. Effective dosages for suspended bacteria were determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

Determination of Minimum Inhibitory and Minimum Bactericidal Concentration

Broth macrodilution MIC tests were carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (14,15). Briefly, 1 mL of $5x10^5$ cfu mL^{-1} bacterial suspensions were added to tubes containing different concentrations of biocide. After incubation at 37°C for 24 h, the first tube in the biocide/bacteria suspension series without signs of visible growth was considered as the MIC. To determine MBC values, 10 µL samples from each tube showing no visible growth were spread on Mueller Hinton agar plates (Oxoid) and incubated at 37°C for 24 h. MBCs of the compound were determined as the lowest concentration at which no colony formed. The MIC and MBC values were determined in three independent experiments.

Determination of Biocidal Activity with a Quantitative Test Method

The bactericidal activity of the effective dosages (50, 25, 20, 10 mg/L), determined by MIC and MBC tests, was assessed by the culture-based phase 2/step1 quantitative suspension test (BS EN 1276:2009). According to BS EN 1276 (2009) experiments were carried out under clean and dirty conditions for each organism and dosages at contact times of 1 and 5-minute. In this study, tests were undertaken in triplicate.

Briefly, an 8.0 mL biocide sample diluted in standard hard water was added to bovine serum albumin at a final 0.03% (w/v) and 0.3% (w/v) concentrations to represent clean and dirty conditions, respectively. To these tubes 1.0 mL bacterial suspension were added.

After a contact time of 1 and 5 minutes, 1.0 mL of the test blend was pipetted into 8.0 mL neutralizer and 1.0 mL deionized water, After 5 minutes of neutralization. 1.0 mL of test mixture were pour plated, in triplicate with tryptone soya agar. Plates were incubated at 37°C for 48 h prior to counting.

Three control (validation) groups were conducted in parallel for each test:

Validation A. The test was conducted with the addition of 8.0 mL sterile standard hard water in place of the biocide solution to ensure that there was no biocidal activity of the other experimental parameters.

Validation B. The test was conducted with the addition of 8.0 mL neutralizer and 1.0 ml water to the bacterial suspension to ensure that the neutralizer solution did not have biocidal activity.

Validation C. The test was conducted with the addition of 1.0 mL bacterial suspension to the neutralized biocide to ensure that the biocide had been neutralized.

Determination of Antibacterial Activity by Culture Independent (CTC/DAPI Flourescence Staining) Method

At contact times of 1 and 5 minutes, the number of respiring and total cells in biocide exposed and unexposed (control) samples was detected by staining CTC and DAPI, according to a modified technique of Rodriguez et al. (16,17). 900 µL of samples were incubated with CTC redox dye solution (at 5 mM concentration) at 28 °C for 4 hours, in the dark. After that, samples were counterstained with 1.0 µg/mL DAPI for 1 h. Cells were subsequently harvested, after incubation, by vacuum filtration onto black polycarbonate filters (0.2 µm pore size, Millipore, USA). The air-dried filters were mounted with non-fluorescent immersion oil and coverslipped, and stained cells were enumerated microscopically.

Microscope slides were examined using a Nikon 80i epiflouresence microscope. For statistical evaluation, the number of microorganisms was estimated from counts of at least 20 randomly chosen fields (at x 1,000) per sample. The number of microorganisms present in the sample is calculated by applying the following conversion formula:

$$N = \frac{S \times n}{C \times V} \times D$$

where N, microorganism counts per milliliter; S, real filtration area; n, average number of microorganisms per field of vision; C, real microscopic range area; V, filtered sample volume; D, sample dilution.

Following the manufacturer's instructions, respiring cells showing red CTC formazan crystals were considered live cells, while blue cells stained by DAPI were considered dead. Results were expressed as the log number of corresponding bacteria per sample.

Statistical Analysis

The data was analysed by using the Graphpad prism 7. A comparison of biocide exposed and control samples were analyzed using Student's t-test. Differences were considered significant when $p<0.05$.

RESULTS

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory and minimum bactericidal concentrations of Antimic® biocide values against important nosocomial bacteria can be seen in Table 1.

Determination of Biocidal Activity with a Quantitative Test Method

Following MIC and MBC determinations, the bactericidal activity of the effective dosages for each test organisms were further tested by (50-10 mg/L), culture-based standard quantitative suspension test (BS EN 1276:2009). Experiments were performed with the presence of inhibitory substances that simulate the organic load or conditions comparable to the practical use of the product. To simulate dirty and clean conditions, 0.3% or 0.03% (w/v) bovine serum albumin, was used respectively.

According to BS EN 1276 standard (2009), the reduction in culturable bacteria was calculated by subtracting the log of the colony count after biocidal activity (Na) from the log of the initial count in the test chamber ($Nx10^{-1}$). Compounds needed to achieve a five log reduction in culturable colony counts, to pass the test,

The 50 mg/L biocide dosage was effective against all tested bacteria, at each time of contact (1 and 5 minutes), in dirty and clean conditions (Table 2-4).

The lower dosage (25 mg/L) of Antimic also passed against *Acinetobacter baumanii* and vancomycin-resistant *Enterococcus faecium* for both clean and dirty conditions (Table 2, 4). This dosage was also effective against methicillin-resistant *Staphylococcus aureus,* but only after 5 minutes of exposure, in clean conditions (Table 3).

With the 25 mg/L dosage the highest bacterial reduction was achieved against vancomycin-resistant *Enterococcus faecium* bacteria, for this reason, the lower concentrations than this (20 and 10 mg/L) were tested only against *Enterococcus faecium* bacteria.

The 20 and 10 mg/L dosages achieved a 5 log reduction as required by EN 1276, after 5 minutes of exposure, under both clean and dirty conditions (Table 4).

The differences between Antimic® treated and untreated samples were statistically significant ($p<0.05$).

Determination of Antibacterial Activity by Flourescence Staining Method

The log reduction of flourescence stained bacteria counts after Antimic® treatment was found to be similar to the culture based colony counts.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in mg/L, exhibited by Antimic® biocide against nosocomial bacteria

Microorganism	MIC mg/L	MBC mg/L
Acinetobacter baumanii (ATCC 19606)	5	50
Methicillin-resistant *Staphylococcus aureus* (ATCC 33591)	20	25
Vancomycin-resistant *Enterococcus faecium* (ATCC 51299)	5	50

*Mean results are expressed as three independent experiments in triplicate. Significant differences between each treatment and microorganism are indicated as $p<0.05$.

Table 2. Bactericidal activity of Antimic® biocide against *Acinetobacter baumanii* (ATCC 19606) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.10±0.02	1 minute	> 5.96±0.02	> 5.96±0.01
		5 minutes	> 5.96±0.04	> 5.96±0.03
25 mg/L		1 minute	> 5.96±0.04	> 5.96±0.01
		5 minutes	> 5.96±0.03	> 5.96±0.01

Table 3. Bactericidal activity of Antimic® biocide against methicillin-resistant *Staphylococcus aureus* (ATCC 33591) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.44±0.06	1 minute	5.49±0.01	5.04±0.04
		5 minutes	> 6.30±0.02	5.39±0.04
25 mg/L		1 minute	4.26±0.04	4.07±0.03
		5 minutes	5.57±0.03	4.00±0.02

Table 4. Bactericidal activity of Antimic® biocide against vancomycin-resistant *Enterococcus faecium* (ATCC 51299) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.44±0.06	1 minute	> 6.44±0.04	> 6.44±0.01
		5 minutes	> 6.44±0.06	> 6.44±0.04
25 mg/L		1 minute	5.09±0.01	5.05±0.05
		5 minutes	> 6.44±0.01	6.09±0.01
20 mg/L		1 minute	5.02±0.01	4.26±0.04
		5 minutes	5.70±0.01	5.18±0.02
10 mg/L		1 minute	4.69±0.02	4.02±0.02
		5 minutes	5.06±0.04	5.05±0.05

As regards the 50 mg/L biocide dosage a > 5 log reduction was achieved at each time of contact (1 and 5 minutes), in dirty and clean conditions (Figure 1-3).

With the 25 mg/L dosage > 5 log reductions against all bacteria were found at all contact times under dirty and clean conditions; except MRSA bacteria, at 1 minute contact time under dirty and clean conditions, and also at 5 minute contact time in dirty conditions (Figure 1-3) .

20 mg/L and 10 mg/L dosages achieved a 5 log reduction, after 5 minute of exposure, under both clean and dirty conditions (Figure 3), unlike the cultural-based colony counting results.

DISCUSSION

Nosocomial infections can be defined as those occuring in individuals, within 48 hours after entering a health facility (1,18,19). Hospital infections are signs of the service quality of inpatient treatment institutions. Those infections have critical importance, because of the prolongation of treatment duration, loss of work power and productivity, cost increase, and most importantly, increase in morbidity and mortality (1,3,4,9).

According to a survey conducted by the WHO in 55 countries, at any given time 1.4 million people suffer from hospital-acquired infections worldwide (1,9). Annual financial losses due to nos-

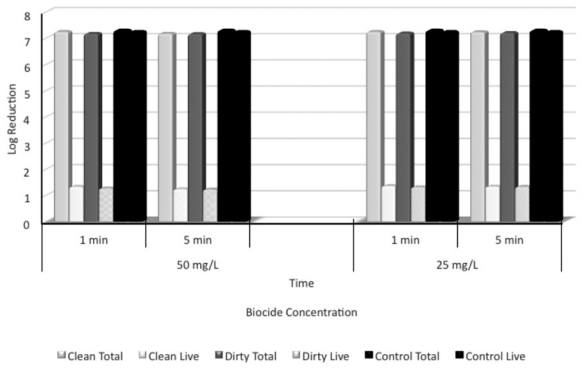

Figure 1. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated *Acinetobacter baumanii* (ATCC 19606)

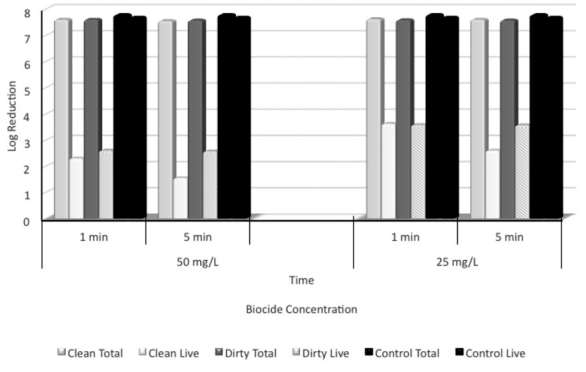

Figure 2. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated methicillin-resistant *Staphylococcus aureus* (ATCC 33591)

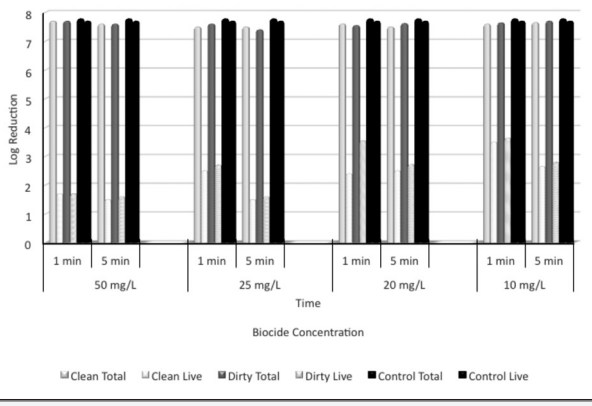

Figure 3. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated vancomycin-resistant *Enterococcus faecium* (ATCC 51299)

ocomial infections are estimated at approximately €7 billion in Europe and US$ 6.5 billion in the USA (3). The results of such studies reveal the importance of gaining a better understanding into the prevention of these infections.

The risk factors for the nosocomial infections may be divided into two broad categories: intrinsic and extrinsic factors. Age, immunity, nutrition of the patient and underlying disease conditions constitute intrinsic risk factors. Extrinsic risk factors are composed of factors related to the health care institution such as the architectural structure of the hospital, failure to observe the asepsis/isolation procedures, and lack of attention to hand washing, disinfection and sterilization (1,20-22).

Surveillance studies have shown that many nosocomial infections are caused by antimicrobial-resistant organisms (4). In many scenarios, one of the major reasons for cross-contamination is due to bacterial adhesion of resistant microorganisms from commonly touched places and materials in the hospital (23,24). Since the hospital environment serves as an important reservoir for these pathogens, the eradication of the cause of infection with an effective biocidal agent is a major challenge for the the control of hospital infections (1,5,9,23-25). Thus, in the current study, the inhibitory characteristics of Antimic® biocide were investigated against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, *Acinetobacter baumanii* pathogens.

Moreover, the incorrect and extensive usage of antimicrobials results in the developed resistance (26), thus, effective dosages of biocides should be determined in the laboratory. The MIC and MBC values are helpful parameters to assess the bacteriostatic and bactericidal activity of a biocide, respectively (25). In the current study, MIC and MBC values were between 5-50 mg/L concentration. The highest MIC value was 20 mg/L against MRSA bacteria, which has a particular facility for nosocomial transmission, while the lowest MBC value (25 mg/L) was determined against the same bacteria.

On the other hand, it should be noted that MIC/MBC tests were conducted under nutrient-rich conditions, where high organic material concentrations may interfere with the biocidal action of the substance (4). Therefore, at the second stage of the study (quantitative suspension test, EN1276), effective dosages of biocide against test bacteria were tested with the presence of inhibitory substances that simulate the organic load or conditions comparable to the practical use of the product. The organic material concentration in biocide solutions in EN 1276 tests was lower than in MIC testing. To mimic dirty and clean conditions, 0.3% or 0.03% (w/v) bovine serum albumi, was used, respectively. According to the results, the tested biocide was found to be effective at 25 and 50 mg/L concentrations at contact times of 1 and 5 minutes, with ≥ 5 log reduction in all test bacteria.

Nevertheless, currently, actively used standards are based only on colony count or conventional culture methods. On the other hand, bacteria can enter the viable but non culturable (VBNC) phase as a response to biocidal treatment, which cannot be detected with conventional culture methods and retain sits virulence, posing a public health risk (27,28). In the current study, fluorescent microscopic examinations were carried out to evaluate VBNC state of the bacteria after exposure to Antimic®. Similar bacteria reduction with the culture was

determined. This study indicates the importance of performing *in vitro* biocidal activity tests in relevant simulations. In this regard, the main objective of the current study is to determine the effective biocide dosages, to ensure that those dosages are used in practical conditions. Thus, the emergence of new resistant microorganism strains may be prevented.

The tested biocide is an alternative, ideal disinfectant for hospitals and household facilities, since it is i) safe for the environment and humans, ii) noncytotoxic, iii) stable even at elevated temperatures, iv) readily biodegradable v) has no risk of inducing bacterial resistance vi) non-corrosive vii) does not have harmful effects on materials.

As a conclusion, to prevent nosocomial infections and possible risks associated with resistant microorganisms, specific biocides should be evaluated under simulated conditions. Antimic® biocide provide an advantage by not promoting the VBNC state in the tested bacteria and removing the tested bacteria successfully. Approaches involving producing antimicrobial surfaces and/or furniture may be explored for further investigation into *in vitro* tests for the prediction of the compound's durability of biocidal activity.

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