

Effects of biocide exposure on *P. Aeruginosa*, *E. coli* and *A. Baumannii* complex isolates from hospital and household environments

Daniel Felipe Vásquez-Giraldo¹, Gerardo Andrés Libreros-Zúñiga², María del Pilar Crespo-Ortiz^{3,*}

Abstract

Background: Bacterial responses to biocide exposure and its effects on survival and persistence remain to be studied in greater detail.

Aim: To analyse the viability and survival of environmental isolates from household and hospital settings after biocide exposure.

Methods: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chlorhexidine (CHxG), benzalkonium chloride (BAC) and triclosan (TC) were determined in isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* complex and *Escherichia coli* collected from hospital and household environments. Viability was monitored after exposure and removal of biocides using agar cultures and flow cytometry.

Findings: *P. aeruginosa* isolates showed greater tolerance for all biocides tested whereas *A. baumannii* complex and *E. coli* were less tolerant. When compared with reference strains, biocide tolerance was up to 8 to 13-fold higher for TC and BAC respectively. Flow cytometry showed that biocide exposure may induce viable but non-growing states in *P. aeruginosa* and *E. coli* isolates before becoming fully replicative. Changes in the susceptibility profile in one isolate of *A. baumannii* complex were observed after biocide exposure.

Discussion: Bacteria isolates from hospital and households were able to recover after biocide exposure at bactericidal concentrations favouring persistence and spread of biocide-tolerant strains. This study reinforces that cleaning compliance should be monitored by non-culture based tests. Novel formulations in cleaning and disinfection protocols should be revisited in hospitals harbouring *P. aeruginosa* and *A. baumannii* multidrug resistant isolates.

Keywords: *A. baumannii*, flow cytometry, *P. aeruginosa*, biocidal activity, resistance.

Efectos de la exposición a biocidas en aislados ambientales de *P. Aeruginosa*, *E. coli* y del complejo *A. Baumannii* hospitalarios y de la comunidad

Resumen

Introducción: El efecto de la exposición a biocidas en las poblaciones bacterianas, su viabilidad y persistencia requieren de estudios detallados.

Objetivo: analizar la viabilidad y persistencia de bacterias de ambientes hospitalarios y domésticos posterior a la exposición a biocidas.

Materiales y Métodos: En un estudio experimental *in vitro* se determinó la concentración inhibitoria mínima (CIM) y la concentración bactericida (CBM) para clorhexidina (CHxG), cloruro de benzalconio (BAC) y triclosan (TC) en aislados de *Pseudomonas aeruginosa* (10), el complejo *Acinetobacter baumannii* (5) y *Escherichia coli* (5) obtenidos de ambientes hospitalarios y domésticos. La viabilidad y susceptibilidad bacteriana después de la exposición y remoción del biocida fue evaluada por citometría de flujo y cultivo.

Resultados: Independiente de su procedencia *P. aeruginosa* presentó mayor tolerancia a todos los biocidas. El complejo *A. baumannii* y *E. coli* fueron hasta 8 a 13 veces más tolerantes a BAC y TC que las cepas de referencia. Se observó que la exposición a biocidas altamente efectivos induce formas viables no replicativas en *P. aeruginosa* y *E. coli*. Un aislado del complejo *A. baumannii* presentó cambios en el perfil de susceptibilidad posterior a la exposición.

Discusión: Aislados tanto de ambiente hospitalario como de la comunidad pueden recuperarse después de la exposición a concentraciones bactericidas de los biocidas favoreciendo la persistencia y diseminación de bacterias no replicativas. Por lo anterior métodos alternativos al cultivo deben utilizarse en el seguimiento de protocolos de limpieza y desinfección. Los tiempos de recuperación de la viabilidad bacteriana deben tenerse en cuenta en la formulación de protocolos para erradicar y/o controlar cepas hospitalarias de *P. aeruginosa* o *A. baumannii* multirresistentes.

Palabras clave: complejo *A. baumannii*, citometría de flujo, *P. aeruginosa*, actividad biocida, resistencia.

1 Department of Microbiology and Department of Biology, Universidad del Valle, Cali, Colombia

2 Department of Microbiology, Universidad del Valle, Cali, Colombia

3 Department of Microbiology, Universidad del Valle, Cali, Colombia. Department of Biomedical Sciences, Universidad Santiago de Cali, Cali, Colombia.

* Autor para correspondencia:

Department of Biomedical Sciences, Universidad Santiago de Cali, Cali, Colombia. Department of Microbiology, Universidad del Valle. Calle 4B No 36-00. Cali, Colombia. Phone: 572-6670326. Fax 572-5542496. Correo electrónico: maria.crespo.ortiz@correounivalle.edu.co

Recibido: 30/10/2016 Recibido con modificaciones: 09/01/2017 Aceptado: 26/01/2017

Cómo citar este artículo: D.F. Vásquez-Giraldo, et al. Efectos de la exposición a biocidas en aislados ambientales de *P. Aeruginosa*, *E. coli* y del complejo *A. Baumannii* hospitalarios y de la comunidad. *Infectio* 2017; 21(4): 243-250

Introduction

Cationic antimicrobial agents, such as chlorhexidine, benzalkonium chloride and triclosan have been used as disinfectants in clinical settings and household environments for over half a century. Chlorhexidine and benzalkonium chloride are known for their residual activity and prolonged reduction in bacterial counts¹. However it is still controversial whether residual products may act as antibiotics inducing reduced bacterial susceptibility and potentially compromising the efficacy of commonly used antibacterials. Biocide tolerant bacteria are more likely to be antibiotic resistant probably due to similar mechanisms of resistance². In the hospital, cross-contamination with environmental isolates is considered a source of infections whereas in the community the use of cleaning products may exert pressure and develop tolerance in environmental bacteria^{2,3}.

Despite disinfection protocols 5% to 15% of inpatients will acquire a resistant hospital-acquired infection⁴. Cross resistance to disinfectants and antibiotics used in therapy has been documented in gram negative bacteria such as *P. aeruginosa*, *Serratia* and *A. baumannii*⁵. *P. aeruginosa* and *A. baumannii* complex usually cause opportunistic infections in intensive care unit (ICU) patients and have increased ability to adapt under adverse environmental conditions harbouring multiple determinants of resistance. These pathogens also have high propensity for clonal spread leading to environmental contamination and hospital outbreaks⁶. Despite control and hygiene programs, the risk for *P. aeruginosa* infections remains up to 30% in ICU-hospitalized patients⁷.

A. baumannii has been also reported as a frequently isolated pathogen at ICU (49%) 64.7% showing multidrug resistance⁸. One way to reduce the spread of nosocomial infections is through the strict monitoring of cleaning and disinfection protocols⁹. Most commonly used biocides have bactericidal effects at the concentrations recommended by the manufacturer, however some studies have suggested that over time, bacteria may develop resistance to these compounds, allowing their survival even at concentrations considered to be lethal². Most importantly, antibiotic resistance is no longer confined to the hospital setting. In the last decade an increase of antibiotic resistant bacteria in the community has been observed. The associated factors for resistant bacteria in outpatients are still matter of study. Despite the usage of biocides in clinical and household environments, the effects of biocide tolerance on these two settings is yet to be determined. This work aimed to analyse the effects on growth, state transitions and susceptibility induced by biocide exposure on environmental isolates from households and a healthcare setting. We focused on particularly resistant pathogens such as *A. baumannii* complex, *P. aeruginosa* and *E. coli*, that can be frequently found in nosocomial outbreaks. The isolates were characterized and the bacterial response was monitored over time after biocide exposure and removal using flow cytometry and agar cultures.

Materials and methods

Strains and bacterial isolates. A total of 128 swabs were taken from sites where biocides are usually applied in households and healthcare settings. Hospital samples were collected from a tertiary care hospital with a history of resistant *A. baumannii* complex outbreaks. Swabs from hospital and household surfaces were taken in transport media (Stuart media OXOID[®]) and cultured in nutritive agar. Bacteria were subcultured using selective culture media (Eosin Methylene Blue agar MERCK[®], and Cetrimide agar MERCK[®]) and bacterial identification was performed using biochemical tests (BD Crystal Enteric/Nonfermenter, Becton Dickinson). Identification of *Acinetobacter* spp. was to the level of *Acinetobacter baumannii* complex. A total of 20 environmental isolates were obtained: five isolates of *P. aeruginosa* and five of *A. baumannii* complex were collected from ICU environments whereas five isolates of *P. aeruginosa* and five of *Escherichia coli* were collected from environmental surfaces in households. *P. aeruginosa* ATCC 9027, *A. baumannii* ATCC 19606 and *E. coli* ATCC 25922 were used as reference strains.

Biocides. The following biocides were tested: chlorhexidine gluconate (CHxG) (Electrochemistry West SA), benzalkonium chloride (BAC) (New Chemical SAS) and triclosan (TC) (GammaQuim SA). Working solutions for CHxG and BAC were prepared at 0,5% and 0,1% in water. Triclosan was prepared at 0,5% in dimethylsulfoxide (DMSO). Toxicity of DMSO was assessed at concentrations up to 1% which was the highest test concentration.

Bacterial susceptibility. The in vitro activity of selected biocides on bacteria was tested to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)¹⁰. Briefly, bacteria suspensions were prepared in trypticase soy broth from fresh cultures. Starting inocula were adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/ml) and 100 μ l was added to 2 ml of the biocide solution (5- 8x 10^6 CFU/ml final inoculum) before being incubated for 24 h at 35 °C. The biocides were tested over a range of concentrations from 2.45 μ l/ml to 5.000 μ l/ml. The MIC was taken as the lowest concentration that prevents visible growth. For determination of the MBC, 100 μ l of the relevant dilutions (i.e, the one defined as the MIC and the next two concentrations) were cultured in trypticase soy agar and the number of colonies was counted. The MBC was defined as the lowest concentration of biocide where no bacterial growth was observed. The experiments were performed at least twice in duplicate.

Quantitative suspension test. Bactericidal efficacy of biocides was determined as described previously after several contact times^{11,12}. Briefly, 1 ml of the selected biocide (at working concentrations as above mentioned) was incubated with 100 μ l of bacterial suspension adjusted to a 0.5 MacFarland (1.5×10^7 CFU/ml final inoculum). The resulting solutions were incubated for 10, 15, 30 and 60 seconds of exposure.

At the end of the contact time 9 ml of the neutralizing agent (Tween 80: 30 g/L; thiosulfate: 5 g/L and sodium bisulphite 40% at pH 7.0) was added, mixed and then incubated for 10 min at room temperature. Thereafter, 100 µl of the neutralized test solution was cultured in nutrient agar plates. After incubation, colonies were counted and the number of recoverable bacteria was calculated. The potential effects of the neutralizing agent on the tested biocides and its bacterial toxicity were evaluated as suggested in the literature¹³. All bacterial isolates were exposed to concentrations of biocides commonly used in both, community and hospital disinfection protocols. The experiments were performed at least twice in duplicate.

Efficacy was expressed as the reduction factor (RF) when comparing the log number of colony forming units (CFU) before (CFU₀) and after the contact time (CFU_c) as follows: RF = log₁₀ CFU₀ – log₁₀ CFU_c¹⁴

Susceptibility to antibiotics. Antimicrobial susceptibility was performed by disk diffusion agar with antibiotics used for the therapy of the tested isolates, such as: gentamicin, amikacin, ceftazidime, imipenem and ciprofloxacin¹⁰. For those isolates subjected to resuscitation, the MIC values were determined using MicroScan panels (Dade International Inc.) before and after biocide exposure and recovery. Cefepime, tobramycin, meropenem and ampicillin sulbactam (for *A. baumannii* complex) were also tested. Interpretive standards published by the Clinical and Laboratory Standards Institute (CLSI) were used to categorize the isolates as susceptible, intermediate, or resistant¹⁵. Multidrug resistance was noted as non-susceptibility to at least one agent in three or more of the following classes of antimicrobials¹⁶: fluoroquinolones, aminoglycosides, carbapenems and broad spectrum cephalosporins.

Resuscitation assays. Selected isolates were exposed for 1 min (contact time with substantial bacterial reduction) at practical concentrations of biocides (i.e. 0.1% BAC, 0.5% CHxG and 0.5% TC). The cells were pelleted and resuspended in nutrient broth and then incubated at 37°C. After incubation, samples were monitored for bacterial viability every two days over seven days using flow cytometry. Bacterial populations were examined and aliquots from samples were cultured on nutritive agar for 24 hours at 37°C.

Flow cytometry analysis. Cell membrane integrity was evaluated using the nucleic acid double-staining based on SYTO9 and propidium iodide (PI)¹⁷. Bacteria were washed and resuspended in 500 µL of filtered distilled water before being labelled with 2.5 µM SYTO9 (a marker of viable and non-viable cells) and 750 nM PI (a dead cell marker) (Invitrogen™ Life Technologies). The cells were incubated for 30 min at room temperature in the dark. Fluorescence emission and light scattering were analysed in a FACS Calibur flow cytometer (Becton Dickinson). The data were measured based on logarithmic scale amplification and over 10,000 events per sample. To measure fluorescence intensity, green fluorescence

was detected through a 525 nm, 50 nm band-pass filter (FL1 channel used for SYTO9) and red fluorescence through a 670 nm long-pass filter (FL3 channel, used for PI)¹⁷. Background controls of unstained, viable and killed bacteria were included for each subset of experiments. Data were acquired in duplicate and experiments performed at least twice.

Data analysis: Cells were gated to analyse and quantify viable and non-viable bacteria. The data acquired were collected and processed using CELL Quest PRO software (BD Biosciences version 1.2) and FlowJo vX.0.7 software (Tree Star, Inc.). This work was approved by the University Hospital's Ethics Committee (approval number 200059932013).

Results

Distribution of households and hospital isolates. A total of 128 swabs were taken from sites where biocides are usually applied in household and hospital settings. *P. aeruginosa* isolates were found in kitchen drains, sinks and bathrooms from both household and clinical environments. *E. coli* was only found in household environments and *A. baumannii* complex isolates were only obtained from hospital samples, the latest were mainly isolated from dry surfaces, such as tables, handrails and dispensers (Table 1).

Biocide tolerance and antibiotic susceptibility. MIC and MBCs were used to determine the susceptibility profile to each biocide (Table 2). TC, BAC and CHxG were chosen as these biocides have found to induce changes on antimicrobial and biocide susceptibility^{2, 18}. As susceptibility breakpoints for biocides remain to be established, the isolates were classified as "more tolerant" and "less tolerant or sensitive" by comparing the MIC or MBC to that obtained for the laboratory reference strains. An isolate was considered more tolerant when the MIC or MBC was at least two fold the MIC or MBC for the reference strain (i.e one fold change) (Table 2). Overall, *P. aeruginosa* showed higher MIC and MBC values than *E. coli* and *A. baumannii* complex. For BAC, household and hospital isolates of *P. aeruginosa* were more tolerant compared to the ATCC strain with MICs ranging from 19.5 to 312.5 µg/ml and MBC from 39 to 625 µg/ml (Table 2, Figure 1). *P. aeruginosa* isolates 19D, 29H and 7H were the most tolerant (MIC 312.5 µg/ml and MBC ranging from 312 to 625 µg/ml), interestingly one isolate from a household toilet (19D) showed the highest MIC and MBC values. By contrast, *E. coli* and *A. baumannii* complex isolates showed lower MIC and MBC values oscillating between 4.9 and 78.2 µg/ml. All the *A. baumannii* complex isolates tested showed to be sensitive to BAC with MBC values lower than those of the reference strain (Table 2). All isolates were considered sensitive (less tolerant) to CHxG except one household isolate (*P. aeruginosa* 19D). When testing TC, all *E. coli* and *A. baumannii* isolates had MIC values ranging between 1.23 and 2.45 µg/ml, similar to those of the reference strain (2.45 µg/ml), however all the *E. coli* isolates had MBC values 8-fold higher than that of the reference strain and the *A. baumannii* isolates showed MBC values up to 4-fold higher than the reference (Figure 1).

Table 1. Distribution of bacterial isolates according to their environmental source

Environment	Area	Surface	Isolate	
Household	Kitchen	Dishwasher drain	<i>E. coli</i> 56D	
	Kitchen	Dishwasher drain	<i>E. coli</i> 57D	
	Kitchen	Dishwasher siphon	<i>E. coli</i> 28D	
	Kitchen	Dishwasher siphon	<i>E. coli</i> 54D	
	Kitchen	Dishwasher siphon	<i>E. coli</i> 58D	
	Toilet	Sink	<i>P. aeruginosa</i> 16D	
	Toilet	Sink	<i>P. aeruginosa</i> 19D	
	Yard	Laundry siphon	<i>P. aeruginosa</i> 15D	
	Toilet	Dishwasher siphon	<i>P. aeruginosa</i> 7D	
	Yard	Laundry siphon	<i>P. aeruginosa</i> 53D	
Hospital	Paediatrics	Cleaning room 1	<i>A. baumannii</i> 4H	
	ICU 3 adults	Patient room #3	Tables, Equipment	<i>A. baumannii</i> 37H
	ICU 3 adults	Patient room #8	Tables, Handrail	<i>A. baumannii</i> 40H*
	ICU 3 adults	Patient room #6	Tables, Dispenser	<i>A. baumannii</i> 41H
	INCU	Hall	Handrail	<i>A. baumannii</i> 70H*
	Paediatrics	Cleaning room 2	Laundry drain	<i>P. aeruginosa</i> 4H
	Paediatrics	Bathroom	Toilet	<i>P. aeruginosa</i> 3H
	Paediatrics	Lactary	Sink drain, Hand wash	<i>P. aeruginosa</i> 7H
	ICU 2 adults	Cubicle #1	Tables, Handrail	<i>P. aeruginosa</i> 30H
	ICU 2 adults	Hall	Hand wash	<i>P. aeruginosa</i> 29H*

Intermediate Care Unit (INCU), Intensive Care Unit (ICU). * Multidrug resistant isolate.

Susceptibility to antimicrobials is described in table 3. Four hospital isolates were resistant to at least one antimicrobial tested, two of them were also more tolerant to biocides (*P. aeruginosa* 29H was more tolerant to BAC and *A. baumannii* complex 41H more tolerant to TC). Three isolates were multidrug resistant: two isolates of *A. baumannii* complex (70H and 40H, an imipenem-resistant isolate) and one isolate of *P. aeruginosa* (29H). None of the isolates obtained from households was resistant to the antibiotics tested.

Efficacy of biocides. After 1 min of exposure, all biocides were bactericidal against most of the isolates (RF ≥ 2.4) except *A. baumannii* complex 4H. A 0.5% CHxG solution was highly efficient against household isolates, obtaining RFs ≥ 2 after 10 seconds of exposure. However, to obtain the same RF for *P. aeruginosa* 30H, 19D, 7H, 16D and *A. baumannii* 4H, more than 30 seconds of exposure was needed. The results obtained with the 0.1% BAC solution were similar to those reported for CHxG. As expected, a 0.5% TC solution yielded only slight reductions in cell viability in the *P. aeruginosa* isolates even after 1 min of exposure (RF 0.05-0.3).

Survival of bacteria. Six isolates were subjected to resuscitation assays, five considered more tolerant to biocides and one sensitive (Table 4). A BAC and CHxG tolerant isolate, *P. aeruginosa* 19D, (from household toilet, MBC 625 $\mu\text{g/ml}$ for BAC and 156.5 $\mu\text{g/ml}$ for CHxG) was monitored after exposure to a bactericidal concentration of CHxG. Samples obtained at day zero showed only dead cells with PI uptake and no

culture growth, however after four days the SYTO9 uptake started shifting to the right indicating an increase in the fluorescence and full recovery of viability in culture (Figure 2). Interestingly, a BAC tolerant *P. aeruginosa* 29H (from a ICU hand wash, MBC 312.5 $\mu\text{g/ml}$ for BAC and 78.2 $\mu\text{g/ml}$ for CHxG) recovered viability 2 days after bactericidal exposure to CHxG (45% as defined by the increase in SYTO 9 uptake), but remained uncultivable for 4 days suggesting that this isolate entered into a non-growing state (Figure 2). *A. baumannii* complex 40H was recovered in culture after 4 days of

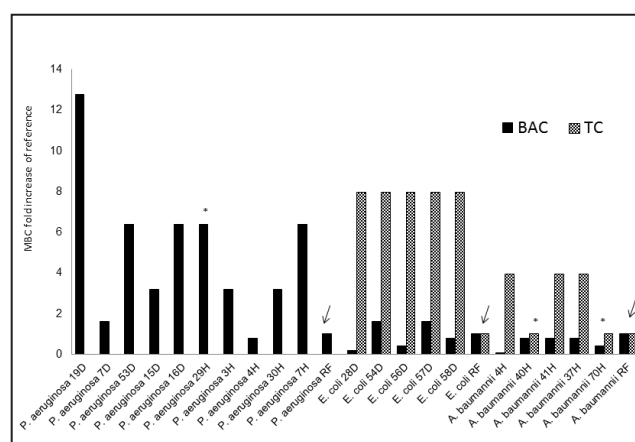


Figure 1. Biocide tolerance of *P. aeruginosa* (from household and hospital settings), *E. coli* and *A. baumannii*. MBC fold increase was calculated as the MBC value for each BAC or TC compared to that of the respective reference (RF) strain (arrows). *Multidrug resistant isolates. Reference strains were *P. aeruginosa* ATCC 9027, *E. coli* ATCC 25922 and *A. baumannii* ATCC 19606.

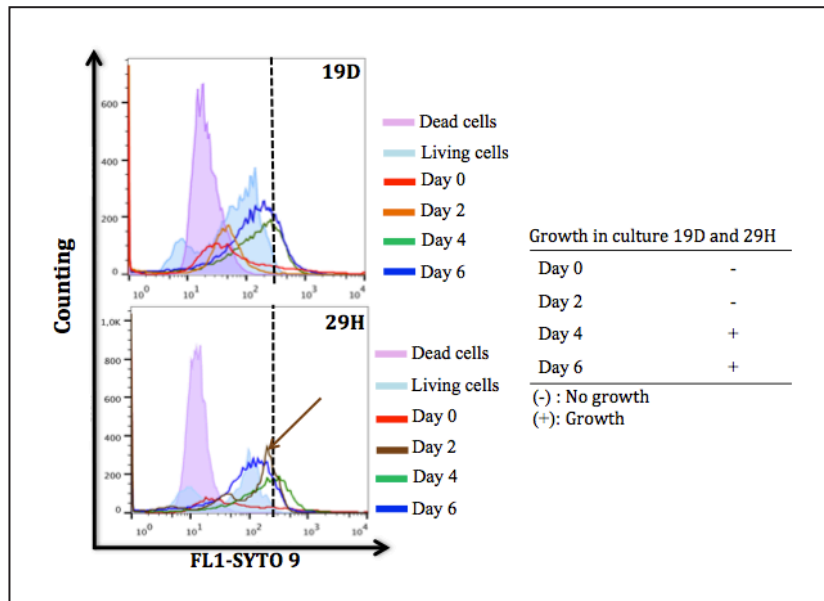


Figure 2. Cell viability of *P. aeruginosa* 19D and *P. aeruginosa* 29H after one min exposure to 0.5% CHxG. The arrow and dotted lines indicate still viable but non-growing cells.

Table 2. Biocide MIC and MBC values for hospital and household environmental isolates

Isolate	MIC(μ g/ml) /MIC fold of reference			MBC(μ g/ml)/ MBC fold of reference		
	CHxG	BAC	TC	CHxG	BAC	TC
<i>E. coli</i> 28D	2.45/<1	9.7/2	2.45/<1	2.45/<1	9.7/<1	19.5/8
<i>E. coli</i> 54D	2.45/<1	78.2/16	2.45/<1	2.45/<1	78.2/1.6	19.5/8
<i>E. coli</i> 56D	2.45/<1	19.5/4	2.45/<1	2.45/<1	19.5/<1	19.5/8
<i>E. coli</i> 57D	4.9/<1	78.2/16	2.45/<1	9.7/<1	78.2/1.6	19.5/8
<i>E. coli</i> 58D	4.9/<1	39/8	2.45/<1	9.7/<1	39/<1	19.5/8
<i>P. aeruginosa</i> 19D	39/<1	312.5/6.4	*	156.2/2	625/13	*
<i>P. aeruginosa</i> 7D	4.9/<1	39/<1	*	19.5/<1	78.2/1.6	*
<i>P. aeruginosa</i> 53D	78.2/<1	156.2/3.2	*	78.2/<1	312.5/6.4	*
<i>P. aeruginosa</i> 15D	2.45/<1	156.2/3.2	*	2.45/<1	156.2/3.2	*
<i>P. aeruginosa</i> 16D	4.9/<1	156.2/6.4	*	19.5/<1	312.5/6.4	*
<i>A. baumannii</i> 4H	39/<1	4.9/<1	2.45/<1	39/<1	4.9/<1	9.7/4
<i>A. baumannii</i> 40H	39/<1	78.2/<1	1.23/<1	78.2/<1	78.2/<1	2.45/<1
<i>A. baumannii</i> 41H	9.7/<1	39/<1	2.45/<1	9.7/<1	78.2/<1	9.7/4
<i>A. baumannii</i> 37H	4.9/<1	39/<1	2.45/<1	19.5/<1	78.2/<1	9.7/4
<i>A. baumannii</i> 70H	19.5/<1	19.5/<1	1.23/<1	19.5/<1	39/<1	2.45/<1
<i>P. aeruginosa</i> 29H	78.2/<1	312.5/6.4	*	78.2/<1	312.5/6.4	*
<i>P. aeruginosa</i> 3H	9.7/<1	156.2/3.2	*	9.7/<1	156.2/3.2	*
<i>P. aeruginosa</i> 4H	19.5/<1	19.5/<1	*	39/<1	39/<1	*
<i>P. aeruginosa</i> 30H	78.2/<1	156.2/3.2	*	78.2/<1	156.2/3.2	*
<i>P. aeruginosa</i> 7H	39/<1	312.5/6.4	*	39/<1	312.5/6.4	*
<i>A. baumannii</i> ATCC19606	78.2	98	2.45	78.2	98	2.45
<i>E. coli</i> ATCC 25922	78.2	4.9	2.45	78.2	49	2.45
<i>P. aeruginosa</i> ATCC 9027	78.2	49	*	78.2	49	*

* Intrinsic resistance to triclosan, BAC: Benzalkonium chloride, TC: Triclosan, CHxG: Chlorhexidine gluconate. MIC or MBC fold of reference was calculated as follows: biocide MIC of isolate / biocide MIC of reference strain.

Table 3. Antimicrobial susceptibility of hospital and household environmental isolates

Isolate	Antimicrobial				
	GN	AK	CAZ	IMP	CIP
<i>E. coli</i> 28D	S	S	S	S	S
<i>E. coli</i> 54D	S	S	S	S	S
<i>E. coli</i> 56D	S	S	S	S	S
<i>E. coli</i> 57D	S	S	S	S	S
<i>E. coli</i> 58D	S	S	S	S	S
<i>P. aeruginosa</i> 19D	S	S	S	S	S
<i>P. aeruginosa</i> 7D	S	S	S	S	S
<i>P. aeruginosa</i> 53D	S	S	S	S	S
<i>P. aeruginosa</i> 15D	S	S	S	S	S
<i>P. aeruginosa</i> 16D	S	S	S	S	S
<i>A. baumannii</i> 4H	S	S	S	S	S
<i>A. baumannii</i> 40H	R	I	I	R	R
<i>A. baumannii</i> 41H	R	I	R	S	S
<i>A. baumannii</i> 37H	S	S	S	S	S
<i>A. baumannii</i> 70H	R	R	R	S	R
<i>P. aeruginosa</i> 29H	R	S	R	I	R
<i>P. aeruginosa</i> 3H	S	S	S	S	S
<i>P. aeruginosa</i> 4H	S	S	S	S	S
<i>P. aeruginosa</i> 30H	S	S	S	S	S
<i>P. aeruginosa</i> 7H	S	S	S	S	S

GN: gentamycin, AK: amikacin, CAZ: cetazidime, IMP: imipenem, CIP: ciprofloxacin

exposure to BAC with a concurrent increase in SYTO 9 uptake. Non-growing bacteria were also detected in *E. coli* 28D using flow cytometry 5 days after bactericidal (625 µg/ml) exposure to BAC. *A. baumannii* complex 4H was recovered after 6 days in culture, however it became resistant to aminoglycosides after recovery and the resistant phenotype showed to be stable over time. The remaining resuscitated isolates showed no changes in the susceptibility profile.

Discussion

Limited studies have analysed and compared the response of environmental bacteria after biocide exposure in isolates from hospital and household settings. Because the lack of a clear definition for biocide "resistance", and the variability of the techniques that can be taken as reference¹⁹, in this work, we have analysed the susceptibility to biocides by comparing the MIC and MBC values of the tested isolates with laboratory strains. *A. baumannii* complex and *E. coli* isolates included in our study were considered sensitive to BAC and CHxG, whereas tolerance to TC was observed in all *E. coli* isolates from households and some of the hospital isolates of *A. baumannii* complex.

P. aeruginosa was more tolerant to BAC and TC. The MBC values of *P. aeruginosa* for BAC were considerably high being up to 13-fold higher than that of the reference strain. As

referred by other authors, TC showed no efficacy against *P. aeruginosa* isolates due to the documented intrinsic resistance²⁰. Remarkably, one isolate of *P. aeruginosa* recovered from household was highly tolerant to all the biocides tested with the highest MBC values for CHxG, BAC and TC.

Our findings agree with previous studies by Gilbert & Moore showing that *P. aeruginosa* is relatively non-sensitive to quaternary ammonium compounds due to faulty biocide penetration from the outer membrane to the cytoplasmic membrane, where the target of their antimicrobial effect is located¹. Likewise, Koburger *et al.* has shown MIC values for CHxG and TC of 32 µg/ml and >520 µg/ml respectively. The same authors showed a bactericidal effect of CHxG in *P. aeruginosa* only after 5 minutes of exposure with 500 µg/ml and tolerance to TC concentrations above 5 000 µg/ml¹¹.

The present study highlights the tolerance of *P. aeruginosa* to biocides observed in both, household and hospital environments. This may be associated with the bacterial persistence and faster recovery *P. aeruginosa* after biocide exposure. As reported by others, bacteria often have the capacity to modify themselves to several transition states known as viable non-culturable (VBNC), persisters and dormant bacteria²¹. These refer to living cells that have lost the capacity to grow on routine culture media and differ from dead cells because the latter have sustained membrane damage and loss of chromosomal and plasmidic DNA²¹. This unique survival strategy could be the key for success in adverse environmental conditions²¹. Some studies have found that processes considered normally bactericidal for bacteria could generate cells that remain in a non-replicative state²². However, injured bacteria may also remain uncultivable before dying or growing depending of the extent of damage. Therefore detection and differentiation of non-replicative states in bacteria are still matter of controversy and may require physiological markers²¹. Here, SYTO9 and PI were used to differentiate viable and dead cells based on cell membrane integrity, thus SYTO 9 has affinity for nucleic acids in cells with intact and damaged membranes and compete with PI which will only penetrate in damaged membranes. Fluorescence intensity of SYTO9 remains strong in live cells while in dead cells PI quenches the signal¹⁷. Therefore, a viable but non-growing state was considered when an increase in the SYTO 9 uptake was detected but no growth in culture was present.

This study has shown that under favourable conditions (i.e temperature and nutrient media) environmental isolates of *P. aeruginosa*, *E. coli* and *A. baumannii* complex can recover replicability, at least two days after removal of the biocide. Non-growing bacteria were detected by flow cytometry in *P. aeruginosa* after exposure to CHxG and *E. coli* after exposure to BAC. In *P. aeruginosa*, non-growing bacteria were detected in one multidrug resistant isolate from the hospital which was able to recover 48 hours faster than its counterpart from

Table 4. Susceptibility profile of resuscitated isolates

Isolate	MIC before exposure/MIC after exposure (µg/ml)/(µg/ml)								
	GN	AK	TO	CAZ	IMP	CIP	CE	ME	AS
<i>P. aeruginosa</i> 29H ^a	>8/>8	<16/<16	>8/>8	>16/>16	4/4	>2/>2	>16/>16	<1/<1	-
	R/R	S/S	R/R	R/R	I/I	R/R	R/R	S/S	-
<i>P. aeruginosa</i> 30H ^a	<4/<4	<16/<16	<1/<1	4/4	<2/<2	<1/<1	8/8	<1/2	-
	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	<8/4/>16/8
<i>P. aeruginosa</i> 19D ^b	<4/<4	<16/<16	<1/<1	4/4	<2/<2	<1/<1	8/16	<1/2	S/R
	S/S	S/S	S/S	S/S	S/S	S/S	S/I	S/S	>16/8/>16/8
<i>A. baumannii</i> 4H ^c	<4/>8	<16/>32	<1/2	4/<16	<2/<2	<1/<1	<4/>16	<1/<1	R/R
	S/R	S/R	S/S	S/R	S/S	S/S	S/R	S/S	-
<i>A. baumannii</i> 40H ^d	>8/>8	32/32	>8/>8	16/>16	>8/>8	>2/>2	>16/>16	<1/<1	
	R/R	I/I	R/R	I/R	R/R	R/R	R/R	S/S	
<i>E. coli</i> 28D ^c	<4/<4	<16/<16	<1/<1	4/4	<2/<2	<1/<1	<4/<4	<1/2	
	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	

^a BAC tolerant isolate; ^b BAC and TC tolerant isolate; ^c TC tolerant isolate; ^d Biocide sensitive isolate. Isolates recovered after exposure to CHxG are in bold the remaining isolates were recovered after BAC exposure. The box shows one isolate with susceptibility changes. GN: gentamycin, AK: amikacin, TO: tobramycin, CAZ: cetazidime, IMP imipenem, CIP: ciprofloxacin, CE: cefepime, ME: meropenem and AS: ampicillin sulbactam.

the household setting. This may suggest an increased ability of resistant hospital isolates of *P. aeruginosa* to survive and recover after biocide exposure however the precise mechanism of survival needs further investigation. One hospital isolate of *A. baumannii* complex also showed changes in antibiotic susceptibility to aminoglycosides after resuscitation. It has been suggested that biocide exposure induces gene expression related with resistance mechanisms for aminoglycosides, ciprofloxacin, carbapenems and tetracycline¹⁸.

Although in our experiments it is possible that CHxG and BAC may cause sublethal injury, the conditions used for the resuscitation assays would favour the recovery of non-replicative states rather than injured bacteria suggesting that a non-replicative state may be involved in the long term survival of *P. aeruginosa* isolates²³.

To ensure that biocides continue to be effective, the regulation of biocide usage is urgently needed. Our findings may give insights of conditions inducing the spread and persistence of community and hospital bacterial isolates and particularly those multidrug resistant strains at the hospital level. In the community, a matter of concern is the TC tolerance found in *E. coli* isolates and BAC tolerance in *P. aeruginosa*. Triclosan, a biocide that may be still part of the formulations in household products, has been found to foster proliferation of *P. aeruginosa* and induce the expression of multidrug-resistant efflux pumps²⁴. Intrinsic resistance of *P. aeruginosa* to TC is well known, but the potential transfer of the molecular mechanism of resistance to other bacteria is a concern as it may induce cross resistance to ciprofloxacin²⁴.

Despite of the efficacy of disinfectants at concentrations and contact times recommended by the manufacturer, hospital isolates often required longer contact times compared to household isolates. We found that even after one minute of bactericidal exposure, recovery of bacterial viability may be expected in hospital and household isolates and this ability to recover may be mediated by the transition to long term survival states (non-growing states) and/or biocide injured bacteria. Importantly, one isolate became resistant to antibiotics after recovery. Protocols for terminal disinfection to eradicate multidrug resistant strains should be revisited considering the recovery times for bacteria such as *P. aeruginosa* and *A. baumannii* complex and the efficacy of these protocols should be also assessed by non-culture based methods. Surveillance of biocide effects on antimicrobial or biocide susceptibility should be addressed to determine predictor markers of biocide resistance and cross-resistance over time. Furthermore, the effects of changes in biocide formulations and rotation should be also assessed.

Conflict of interest statement: None declared

Funding source: This study was supported by the Laboratory of Biological Agents at the Microbiology Department, University of Valle.

Acknowledgements

We acknowledge the laboratory staff of the Microbiology Department at University of Valle for technical assistance. We also thank Dr Christian Pallares from the Infectious Diseases Committee at the University Hospital and Diana Motta and David Garcia for technical advice.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association and the Helsinki Declaration.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors must have obtained the informed consent of the patients and/or subjects mentioned in the article. The author for correspondence must be in possession of this document.

References

- Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet. *J Appl Microbiol.* 2005;99(4):703-15.
- Wesgate R, Grasha P, Maillard J-Y. Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage. *Am J Infect Control.* 2016;44(4):458-64.
- Otter JA, Yezli S, French GL. The Role of Contaminated Surfaces in the Transmission of Nosocomial Pathogens. In: Borkow G, editor. *Use of Biocidal Surfaces for Reduction of Healthcare Acquired Infections.* Cham: Springer International Publishing; 2014. p. 27-58.
- Sydnor ERM, Perl TM. Hospital epidemiology and infection control in acute-care settings. *Clin Microbiol Rev.* 2011;24(1):141-73.
- Ortega-Morente E, Fernández-Fuentes MA, Grande-Burgos MJ, Abriouel H, Pérez-Pulido R, Gálvez A. Biocide tolerance in bacteria. *Int J Food Microbiol.* 2013;162(1):13-25.
- Weinstein RA, Hota B. Contamination, disinfection, and cross-colonization: Are hospital surfaces reservoirs for nosocomial infection? *Clin Infect Dis.* 2004;39(8):1182-9.
- Avrain L, Mertens P, Van Bambeke F. RND efflux pumps in *P. aeruginosa*: an underestimated resistance mechanism. *Antibiot Susceptibility.* 2013; 24-26.
- Ozer B, Inci M, Duran N, Kurtgoz S, Alagoz G, Pasa O, et al. Comparison of antibiotic resistance of *Acinetobacter* and *Pseudomonas aeruginosa* strains isolated from Intensive Care Units with other clinics. *Acta med Mediterr.* 2016;32(1):117-122.
- Meyer B, Cookson B. Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control? *J Hosp Infect.* 2010;76(3):200-5.
- Leber AL. *Clinical microbiology procedures handbook.* 4th edition ed: American Society for Microbiology Press; 2016.
- Koburger T, Hübner N-O, Braun M, Siebert J, Kramer A. Standardized comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine digluconate. *J Antimicrob Chemother.* 2010;65(8):1712-9.
- Antisépticos y desinfectantes químicos actividad bactericida básica. Método de prueba y Requisitos.: ICONTEC; 2003. p. 1-32.
- Fernandez-Crehuet M, Espigares M, Moreno E, Espigares E. Specificity of the neutralizers as the cause of errors in evaluating disinfectant efficacy: an assessment of triclosan. *Lett Appl Microbiol.* 2013;57(6):517-25.
- Özalp M, Ekizoğlu M, Kılıç E. Evaluation of bactericidal activity of certain glutaraldehyde-free disinfectants used in the disinfection of endoscopes and surgical devices by a quantitative suspension test. *J Faculty Pharmacy Hacettepe University, Turkey.* 2007;27(2):131-8.
- CLSI. *Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing.* Wayne,PA; 2016.
- Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect.* 2012;18(3):268-81.
- Fernández-Cuenca F, Tomás M, Caballero-Moyano F-J, Bou G, Martínez-Martínez L, Vila J, et al. Reduced susceptibility to biocides in *Acinetobacter baumannii*: association with resistance to antimicrobials, epidemiological behaviour, biological cost and effect on the expression of genes encoding porins and efflux pumps. *J Antimicrob Chemother.* 2015;70(12):3222-9.
- D'Arezzo S, Lanini S, Puro V, Ippolito G, Visca P. High-level tolerance to triclosan may play a role in *Pseudomonas aeruginosa* antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation. *BMC Res Notes.* 2012;5(1):1-6.
- Cerf O, Carpentier B, Sanders P. Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "Resistance" has different meanings. *Int J Food Microbiol.* 2010;136(3):247-54.
- Fakruddin M, Mannan KSB, Andrews S. Viable but nonculturable bacteria: Food Safety and Public Health Perspective. *ISRN Microbiol.* 2013;2013:703813.
- Zhang S, Ye C, Lin H, Lv L, Yu X. UV Disinfection induces a Vbnc state in *Escherichia coli* and *Pseudomonas aeruginosa*. *Environ Sci & Technol.* 2015;49(3):1721-8.
- Stocks SM. Mechanism and use of the commercially available viability stain, BacLight. *Cytometry A.* 2004;61(2):189-95.
- Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol.* 2014;5.
- Chuanchuen R, Karkhoff-Schweizer RR, Schweizer HP. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. *Am J Infect Control.* 2003;31(2):124-7.