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Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage

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Key Words:
Resistance
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Background: In this study we assessed the propensity of biocide exposure in the development of antimicrobial resistance in bacteria.

Methods: Our protocol is based on reporting changes in established antimicrobial susceptibility profiles in biocides and antibiotics after during use exposure to a product. The during use exposure reflects worse conditions of product use during application. It differs from the term low concentration, which usually reflects a concentration below the minimal inhibitory concentration, but not necessarily a concentration that occurs in practice.

Results: Our results showed that exposure to triclosan (0.0004%) was associated with a high risk of developing resistance and cross-resistance in *Staphylococcus aureus* and *Escherichia coli*. This was not observed with exposure to chlorhexidine (0.00005%) or a hydrogen peroxide-based biocidal product (in during use conditions). Interestingly, exposure to a low concentration of hydrogen peroxide (0.001%) carried a risk of emerging resistance to antibiotics if the presence of the oxidizing agent was maintained. We observed a number of unstable clinical resistances to antibiotics after exposure to the cationic biocide and oxidizing agent, notably to tobramycin and ticarcillin-clavulanic acid.

Conclusions: Using a decision tree based on the change in antimicrobial susceptibility test results, we were able to provide information on the effect of biocide exposure on the development of bacterial resistance to antimicrobials. Such information should address the call from the U.S. Food and Drug Administration and European Union Biocidal Products Regulation for manufacturers to provide information on antimicrobial resistance and cross-resistance in bacteria after the use of their product.

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In January 2013, the U.S. Food and Drug Administration proposed a rule to determine the safety and effectiveness of antibacterial soap (<http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm378542.htm>), whereby manufacturers of antibacterial hand soaps and body washes need to demonstrate that their products are safe for long-term daily use. This rule is based on the concern that long-term exposure to certain active ingredients, such as triclosan (TRI), may be associated with bacterial resistance and therefore pose a health risk.^{1,2} This proposed rule echoes the European Biocidal Product regulation (effective from September 1, 2013; articles 19-b/ii, 37, and 47-1/b), which asks

manufacturers to provide information on the antimicrobial resistance associated with their biocidal products.³ This follows a number of European reports on the association of biocides with antimicrobial resistance.^{4,5} Because of the increased use of biocidal products worldwide for a mounting number of applications, particularly domiciliary ones (eg, washing up liquid, surfaces, stationary, textiles), it is not surprising that biocidal products used at a low concentration, for example after dilution, or released in the environment at low concentrations, produce a selective pressure for bacteria to express resistance mechanisms.^{1,2,4,6-9} In 2010, the European Scientific Committee on Emerging and Newly Identified Health Risks reported on the dearth of information concerning biocide exposure on the development of antimicrobial resistance in bacteria⁵ and in particular the need for a standard protocol that could measure the ability of a biocide to induce or select for antimicrobial resistance in bacteria. Recently, a protocol reflective of the in use conditions of biocides was proposed.⁷ Knapp et al reported on the use of this protocol to determine the effect of exposure to

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chlorhexidine, benzalkonium chloride, and 3 biocidal products to *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *B lata*, *Klebsiella pneumoniae*, and 2 *Salmonella enterica* serovar Typhimurium strains.^{10,11}

Bacterial resistance to cationic agents (eg, biguanides, quaternary ammonium compounds) and phenolics (eg, TRI) has been widely reported^{2,4,6,7,9,11-14} and is often perceived to present a higher risk for the development of bacterial resistance to antimicrobials. A number of resistance mechanisms to these biocides have been described, including overexpression of efflux and changes in bacterial surface.^{7,15} Bacterial resistance to highly reactive biocides such as alkylating and oxidizing agents has also been reported.^{6,16,17} An outbreak of *Mycobacterium massiliense* in particular showed for the first time a clinical isolate, with resistance to glutaraldehyde and all the frontline antimycobacterial antimicrobials, causing significant public concern.¹⁷

In this study we explored the use of a predictive protocol⁷ to determine changes in the antimicrobial susceptibility profile of *Staphylococcus aureus* and *Escherichia coli* when exposed to TRI, chlorhexidine gluconate solution (CHG), hydrogen peroxide, and a hydrogen peroxide-based product.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and storage of cultures

One representative gram-positive and 1 gram-negative bacteria were selected for testing against 1 formulated biocidal product and 3 biocides. The bacterial strains chosen were *S aureus* (NCIMB 9518) and *E coli* (NCIMB 8545).^{18,19} Both bacteria are commonly used in standard efficacy test protocols. Liquid cultures of all strains were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 37°C ± 1°C for 16-24 hours. Strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at -80°C ± 1°C and restricted to a maximum of 2 subcultures from the original freezer stock prior to exposure to a given biocide. Test inocula were prepared from harvesting an overnight TSB culture centrifuged at 5,000 g for 10 minutes and resuspended in deionized water (diH₂O).

Formulations, actives, and neutralizer

A hydrogen peroxide-based foaming lotion for hand disinfection (Oxy BAC F31 RO 1331; DEB Group, Denby, UK) was tested at 1% and 0.001% H₂O₂ (final concentration). Three unformulated biocides, TRI (0.0004% in 5% dimethyl sulfoxide [DMSO]), CHG (0.00005%), and hydrogen peroxide (0.001%), were also used. All biocides were neutralized with 5 g/L sodium thiosulfate. Neutralizer toxicity and efficacy to quench the biocides were tested as described by Knapp et al¹⁰ and confirmed (data not shown).

Antimicrobial susceptibility testing

The protocol to evaluate the effect of biocide exposure on the susceptibility profile and stability of bacterial isolates has been described.^{7,11} Briefly, it consists of 3 parts: (1) an initial background antimicrobial susceptibility profile of test bacteria before biocide exposure, (2) exposure of test bacteria to during use concentration of test biocide or biocidal products, and (3) determination of antimicrobial susceptibility profile of biocide-exposed bacteria and stability profile of any change in antimicrobial susceptibility. During use exposure reflects the worst-case scenario during product usage by customers, notably dilution of product and lengthy contact time. It differs from the term low concentration, which usually reflects a concentration below the minimal inhibitory concentration (MIC), but not necessarily a concentration that occurs in practice.

The manufacturer guidelines for during use exposure conditions of the biocidal product were used.

Suspension testing and exposure to microbicide

Bacterial exposure to biocides and biocidal products was carried out in suspension using the British Standards Institute suspension test protocol.¹⁸ Briefly, bacterial suspensions in diH₂O produced from overnight cultures were standardized to 1 × 10⁸ colony forming units/mL through optical density measurement. Suspensions were used within 15 minutes of preparation. One milliliter of standardized suspension was added to 9 mL of the appropriate concentration of a biocide-product (diluted in diH₂O) at 1.25 times the required concentration for a 30-second, 5-minute, and 24-hour exposure. Then 1 mL of this suspension was removed and added to 9 mL of neutralizer. After neutralization, suspensions were centrifuged at 5,000 g for 10 minutes, and the supernatant was discarded. The remaining cells were then used in further antimicrobial susceptibility testing experiments. Concentrations of biocide tested were as follows: Oxy BAC F31 RO 1331 1% and 0.001%, unformulated H₂O₂ 0.001%, TRI 0.0004%, and CHG 0.00005%. The 1% concentration of the formulated product corresponded to the during use concentration, whereas the lower concentrations for the oxidizing agents and the cationic biocides corresponded to a concentration that resulted in a 1 log₁₀ reduction in colony forming units per milliliter, leaving sufficient survivors for further antimicrobial susceptibility testing.

MIC and minimal bactericidal concentration

The MIC of each biocide was determined before and after biocide exposure with the British Standards Institute protocol.¹⁹ To determine the minimal bactericidal concentration (MBC), 20 µL of suspension was removed from each well of the MIC microtiter plate where no bacterial growth was observed and the 2 lowest biocide concentrations at which growth was observed, and they were plated onto a tryptone soy agar plate containing 10% neutralizer. After 24 hours of incubation at 37°C, the MBC was defined as the lowest biocide concentration where no bacterial growth was observed.¹¹

Antibiotic susceptibility testing

The susceptibility of both bacteria to the following antibiotics was determined before and after biocide exposure using the European Committee on Antimicrobial Susceptibility Testing disk diffusion protocol:²⁰ ampicillin (10 µg), ciprofloxacin (1 µg), ceftazidime (30 µg), tobramycin (10 µg), ticarcillin-clavulanic acid (75:10 µg), and gentamicin (10 µg). These antibiotics were selected because of their use as therapeutic agents in the treatment of infections with the organisms chosen for this study.

Phenotype stability testing

The stability of observed changes in antimicrobial susceptibility profile was investigated by 24 hours subculturing of surviving bacteria in TSB with or without a biocide; the exposure concentrations previously described were used.¹¹ Changes in the antimicrobial susceptibility profile were measured using the protocol previously described following 1, 5, and 10 subcultures. A check of culture purity was performed at each stage.

Reproducibility

Tests were carried out in triplicate on 3 separate occasions. No statistical analysis was conducted on antibiotic breakpoints because only the clinical resistance breakpoint given by European Committee on Antimicrobial Susceptibility Testing²⁰ was of interest. Likewise, no statistical analysis was performed on the MIC-MBC data. Here a significant change in the susceptibility profile corresponding to

a >15-fold difference was used as a breakpoint. Further justification is given in the text.

RESULTS

One oxidizing formulation and 3 biocides were evaluated against 2 commonly used bacteria in standard efficacy test protocols. TRI was used as a positive control because, according to the literature, a change in the antimicrobial susceptibility profile could be expected using the bisphenol.^{2,4} The mean MIC and MBC for each bacteria before and after exposure to TRI (0.0004%) and CHG (0.00005%), unformulated H₂O₂ (0.001%), and Oxy BAC (0.001% and 1%) are presented in Tables 1 and 2. To ease the interpretation of the results, the fold change in susceptibility is presented in Tables 1 and 2. This corresponds to the difference in susceptibility (MIC or MBC) before and after exposure to the biocides. Exposure of *S aureus* to TRI (0.0004%) resulted in significant increases in antimicrobial insusceptibility, particularly after 5 minutes of contact with, for example, a 69- and 74-fold increase in both MIC and MBC, respectively. Such an increase in MIC and MBC was not observed with *S aureus* exposure to TRI for 24 hours. A significant increase in MIC only (>30-fold change) was observed after *E coli* exposure to the bisphenol. Exposure of either bacteria to CHG (0.00005%), H₂O₂ (0.001%), and Oxy BAC (0.001%) did not result in changes in the susceptibility profile (<2-fold change) (Tables 1 and 2). A 30-second exposure of both bacteria to Oxy BAC (1%) did not result in changes in the susceptibility profile, and exposure beyond 30 seconds resulted in no viable bacteria recovered (Table 2). To determine whether or not observed changes in the biocide susceptibility profile were stable, 24-hour exposed bacteria were propagated in 24-hour subculture in the presence or not of biocide and biocidal products and retested for their antimicrobial susceptibility profile after 1, 5, and 10 subcultures (Table 3). *E coli* exposed to TRI (0.0004%) and passage with or without the bisphenol retained a high MIC for 10 passages. The propagation of *E coli* in TRI resulted in a 163-fold increase in MIC after the first passage and then in a 32- and 16-fold increase in MIC after the 5th and 10th passages, respectively. There was no change in the *S aureus* susceptibility profile, except for an unstable increase in MBC after the first passage with or without TRI (Table 3). The other biocides did not alter the antimicrobial susceptibility profile of both bacteria, and passaging them in the presence or not of biocides did not result in any changes either; however, *S aureus* exposed to Oxy BAC (0.001%) presented a 16-fold

increase in MIC after the 10th passage. The differences in results in passaging in biocide-free broth (Table 3) resulted from the fact that the bacteria that were passaged were isolated from the different biocides in the first place. As such, the results from the biocide-free broth are not directly comparable.

A clinical change in antibiotic susceptibility profile was observed after bacterial exposure to the biocide and biocidal product, and such change in susceptibility was stable after some biocide exposure (Table 4). A 30-second and 5-minute exposure of *S aureus* to TRI (0.0004%) induced clinical resistance to ciprofloxacin, but a 24-hour exposure to the bisphenol did not alter the antibiotic susceptibility profile. Passaging bacteria exposed to TRI (0.0004%) for 24 hours resulted in a stable resistance to ampicillin whether the subculturing was performed in the presence of TRI or not. A 24-hour exposure to CHG (0.00005%) resulted in *E coli* exhibiting an unstable clinical resistance to tobramycin. Other clinical resistance to antibiotics was observed in *E coli*, but these were not stable (Table 4). Of particular interest was the observation of *S aureus* stable clinical resistance to ciprofloxacin, particularly in the presence of H₂O₂ (0.001%). A 24-hour exposure of *E coli* in H₂O₂ (0.001%) also resulted in an unstable resistance to ampicillin. Exposure to the formulated product at 0.001% resulted in stable resistance to ampicillin in *E coli* after 5 subcultures in the presence of the formulation (Table 4).

A larger number of results were produced after the execution of our protocols, and the practical significance and implication of the results needs to be considered. By creating a decision tree reflecting every step of the protocol in terms of change in susceptibility profile, a clearer understanding of the interpretation of the results can be obtained (Fig 1). Every step is followed by a yes or no question and leads to a clear observation of the risk resulting from the biocide and biocidal product exposure. Here, for all results combined, exposure to CHG (0.00005%) and Oxy BAC (1%) resulted in no significant change in antimicrobial biocide susceptibility profile and no stable change in antibiotic susceptibility profile. This exposure to these biocide and biocidal products at the concentration tested is deemed not to present a risk for emerging bacterial resistance (Fig 1). On the other hand, exposure to TRI (0.0004%) resulted in stable antimicrobial susceptibility changes to both antibiotics and biocides; with that in mind, exposure to TRI at this concentration is associated with a significant risk in bacteria developing resistance and cross-resistance (Fig 1). Exposure to H₂O₂ (0.001%) resulted in a change in the antibiotic susceptibility profile after passaging

Table 1
Mean MIC and MBC for both bacteria before and after exposure to triclosan (0.0004%) or chlorhexidine (0.00005%)

	Pre-exposure		Exposure: 30 s		Exposure: 5 min		Exposure: 24 h	
	MIC, %	MBC, %	MIC, %	MBC, %	MIC, %	MBC, %	MIC, %	MBC, %
Triclosan (0.0004%)								
<i>Staphylococcus aureus</i>	0.0009	0.0017	0.0047	0.1250	0.0625	0.1250	0.0079	0.0031
	± 0.0000	± 0.0000	± 0.0027	± 0.000	± 0.0000	± 0.000	± 0.0000	± 0.0000
Fold change in susceptibility			5	74	69	74	9	2
<i>Escherichia coli</i>	0.0002	0.0017	0.0065	0.0065	0.0078	0.0078	0.0063	0.0063
	± 0.0000	± 0.0000	± 0.0023	± 0.0023	± 0.0000	± 0.0000	± 0.0000	± 0.0000
Fold change in susceptibility			33	4	39	4	32	4
Chlorhexidine (0.00005%)								
<i>S aureus</i>	0.0004	0.0078	0.0009	0.0156	0.0006	0.0020	0.0020	0.0020
	± 0.0000	± 0.0003	± 0.0027	± 0.0000	± 0.0003	± 0.0000	± 0.0000	± 0.0000
Fold change in susceptibility			2	2	<2	-4	5	-4
<i>E coli</i>	0.0007	0.0078	0.0006	0.0026	0.0039	0.0052	0.0026	0.0039
	± 0.0003	± 0.0000	± 0.0003	± 0.0000	± 0.0034	± 0.0022	± 0.0011	± 0.0000
Fold change in susceptibility			0	-3	6	<-2	4	-2

NOTE. Values are mean ± SD or as otherwise indicated. A negative value denotes an increase in susceptibility. Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.

Table 2
Mean MIC and MBC for both bacteria before and after exposure to hydrogen peroxide (0.001%) or Oxy BAC (0.001% and 1%)

	Pre-exposure		Exposure: 30 s		Exposure: 5 min		Exposure: 24 h	
	MIC, %	MBC, %	MIC, %	MBC, %	MIC, %	MBC, %	MIC, %	MBC, %
Hydrogen peroxide (0.001%)								
<i>Staphylococcus aureus</i>	0.160 ± 0.000	0.160 ± 0.000	0.042 ± 0.000	0.083 ± 0.000	0.042 ± 0.000	0.083 ± 0.000	>0.300 ± 0.000	>0.300 ± 0.000
Fold change in susceptibility			-4	-2	-4	-4	>2	>2
<i>Escherichia coli</i>	0.083 ± 0.000	0.083 ± 0.000	0.042 ± 0.000	0.042 ± 0.000	0.042 ± 0.000	0.042 ± 0.000	0.132 ± 0.049	0.160 ± 0.000
Fold change in susceptibility			-2	-2	-2	-2	2	2
Oxy BAC (0.001%)								
<i>S aureus</i>	0.0026 ± 0.0000	0.0052 ± 0.0000	0.0026 ± 0.0000	0.0052 ± 0.0000	0.0026 ± 0.0000	0.0026 ± 0.0000	0.0026 ± 0.0000	0.014 ± 0.0000
Fold change in susceptibility			0	0	0	-2	0	3
<i>E coli</i>	0.0104 ± 0.0000	0.0104 ± 0.0000	0.0104 ± 0.0000	0.0104 ± 0.0000	0.0052 ± 0.0000	0.0104 ± 0.0000	0.0208 ± 0.0000	0.0208 ± 0.0000
Fold change in susceptibility			0	0	-2	0	-2	2
Oxy BAC (1%)								
<i>S aureus</i>	0.0052 ± 0.0000	0.0052 ± 0.0000	0.0026 ± 0.0000	0.0026 ± 0.0000	—	—	NT	NT
Fold change in susceptibility			-2	-2				
<i>E coli</i>	0.0416 ± 0.0000	0.0416 ± 0.0000	0.1040 ± 0.0000	0.1040 ± 0.0000	—	—	NT	NT
Fold change in susceptibility			2.5	2.5				

NOTE. Values are mean ± SD or as otherwise indicated. A negative value denotes an increase in susceptibility.

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; NT, not tested; —, no survivor.

Table 3
Fold changes in MIC and MBC (compared with baseline data) after 1, 5, and 10 passages in biocide- and biocidal-free product or biocide and biocidal product

		Folds change in MIC and MBC							
		At 24 h		Passage					
		MIC	MBC	1		5		10	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Triclosan-free	<i>Staphylococcus aureus</i>	9	2	9	37	2	2	3	2
	<i>Escherichia coli</i>	32	4	39	37	16	2	16	2
Triclosan (0.0004%)	<i>S aureus</i>	9	2	9	18	2	2	2	4
	<i>E coli</i>	32	4	163	9	32	2	16	4
CHX-free	<i>S aureus</i>	5	-4	5	-4	5	-2	5	-2
	<i>E coli</i>	4	-2	3	-4	3	-4	3	-2
CHX (0.00005%)	<i>S aureus</i>	5	-4	5	-4	5	-2	5	-4
	<i>E coli</i>	4	-2	3	-2	3	-2	3	-2
H ₂ O ₂ -free	<i>S aureus</i>	>2	>2	>2	>2	>2	>2	>2	>2
	<i>E coli</i>	2	2	2	2	2	2	2	2
H ₂ O ₂ (0.001%)	<i>S aureus</i>	>2	>2	>2	>2	>2	>2	>2	>2
	<i>E coli</i>	2	2	2	4	4	4	2	4
Oxy BAC-free	<i>S aureus</i>	0	3	2	4	2	4	16	8
	<i>E coli</i>	2	2	0	8	0	8	4	8
Oxy BAC (0.001%)	<i>S aureus</i>	0	3	2	4	2	4	16	8
	<i>E coli</i>	2	2	0	4	2	4	8	8

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

in the presence of the biocide. Hence the risk associated with exposure to H₂O₂ (0.001%) is associated with the permanent exposure to this oxidizing agent at that concentration (Fig 1). The use of this decision tree (Fig 1) based on the susceptibility profile results provides the information necessary for manufacturers to make a case for the safety of their products in terms of development and selection for antimicrobial resistance. It also provides the regulators with an easy tool to assess the risk imparted to bacteria after biocidal product exposure.

DISCUSSION

The objective of this work was to make use of a predictive protocol⁷ to determine the effect of bacterial exposure to TRI, CHG, H₂O₂, and OxyBAC F31 RO 1331, which active is H₂O₂. The protocol

used is designed to expose bacteria to product under during use conditions, which reflect the worse-case scenario of product usage (eg, dilution, prolonged exposure during application). This was the case for Oxy BAC, which was tested at a concentration of 1% for 30 seconds. Because of the bactericidal activity of Oxy BAC, it was also decided to test lower concentrations and longer contact time, which did not reflect product usage in practice but would allow bacterial survival and exposure to long contact time. Likewise, with TRI, CHG, and H₂O₂, the concentrations tested allowed enough bacterial survival (data not shown) after exposure so that a change in the antimicrobial susceptibility profile could be measured after additional testing.

Evaluation of biocidal products, rather than just active ingredients, is important to consider because the formulation will impact on the overall efficacy of the product, but this has often been

Table 4

Changes in antibiotic susceptibility profile after exposure to biocide and biocidal product and 1, 5, and 10 passaging in biocide- and biocidal-free product or biocide and biocidal product

		Exposure			Passage with biocide			Passage without biocide		
		30 s	5 min	24 h	1	5	10	1	5	10
Triclosan (0.0004%)	<i>Staphylococcus aureus</i>	CIP	CIP	—	—	AMP	AMP	AMP	AMP	AMP
	<i>Escherichia coli</i>	—	—	—	AMP	AMP	AMP	AMP	—	—
CHG (0.0004%)	<i>S aureus</i>	—	—	—	—	—	—	—	—	—
	<i>E coli</i>	—	—	TOB	TIM	—	AMP	TIM	—	—
H ₂ O ₂ (0.001%)	<i>S aureus</i>	—	—	CIP	CIP, AMP	CIP	CIP	CIP	—	—
	<i>E coli</i>	—	—	AMP	—	—	—	—	—	—
Oxy BAC 0.001%	<i>S aureus</i>	—	—	—	—	—	—	TIM	—	—
	<i>E coli</i>	—	—	—	—	AMP	AMP	—	—	—

NOTE. Where the antibiotic is named, the bacterium became clinically resistant to that antibiotic according to European Committee on Antimicrobial Susceptibility Testing breakpoints.²⁰

Abbreviations: AMP, ampicillin; CHG, chlorhexidine gluconate; CIP, ciprofloxacin; TIM, ticarcillin-clavulanic acid; TOB, tobramycin; —, no change in susceptibility.

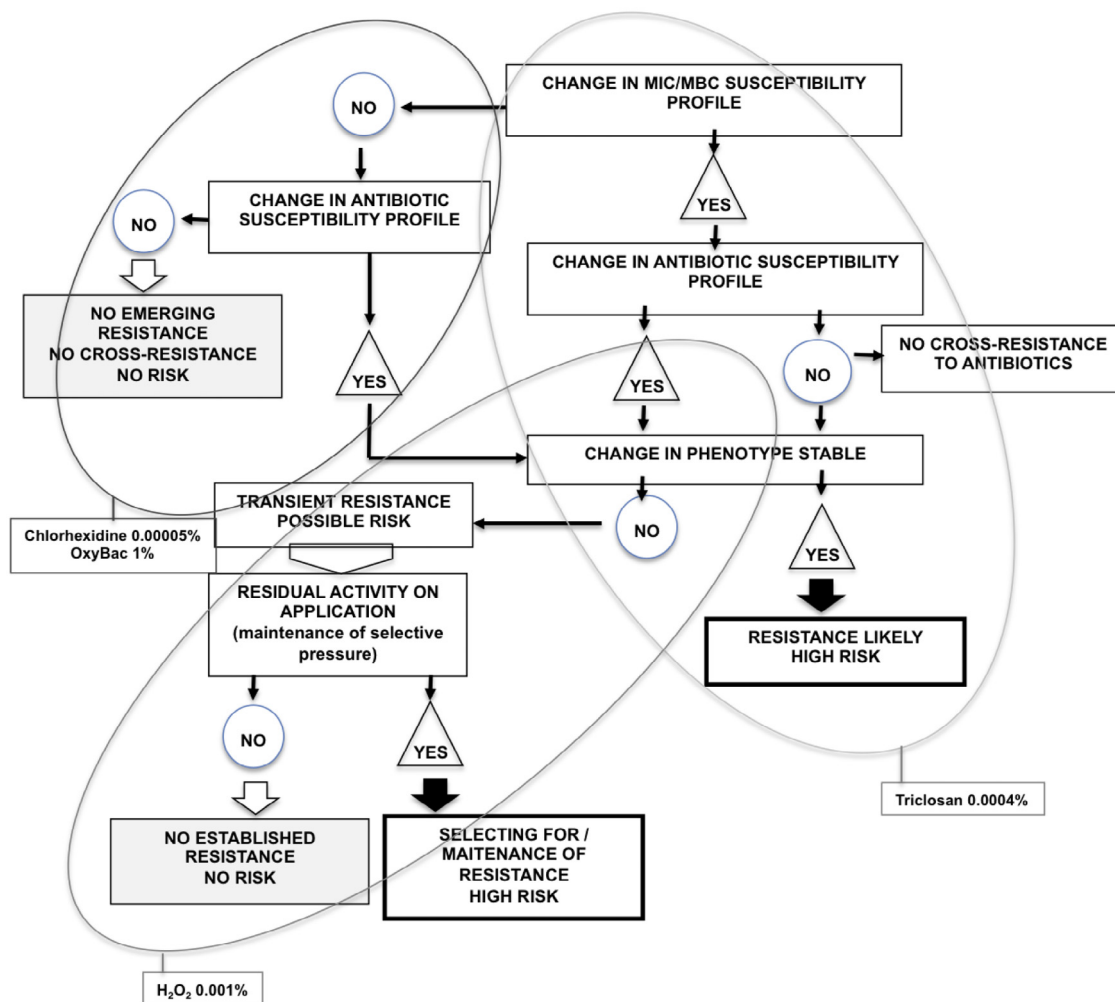


Fig 1. Bacterial resistance to biocides: decision tree.

overlooked.⁷ Knapp et al evaluated the effect of the exposure to 3 biocidal products at their during use concentrations and found a significant increase in MBC; however, the concentrations attained were still below or equal to the concentration of active in these products during use.¹¹ Kurenbach et al reported a decreased antimicrobial susceptibility of *E coli* and *S enterica* Typhimurium after exposure to 3 commercially available herbicides.²¹ This contrasts with the study from Condell et al, who did not observe any correlation between reduced susceptibility to 8 food industry biocide formulations and

resistance to clinically relevant antimicrobial compounds in a panel of 189 *Salmonella* isolates. The use of unformulated TRI, chlorhexidine, and benzalkonium chloride was associated with an increased tolerance to antimicrobials.²²

TRI and CHG were chosen because both biocides have been implicated in a change in antimicrobial susceptibility profile, notably a documented rise in MIC and MBC associated or not with a change in antibiotic susceptibility profile. TRI in particular has been shown to produce increased antimicrobial insusceptibility in a wide range

of bacteria.²³⁻²⁸ In some studies, TRI insusceptibility correlates with multiple drug resistance^{13,29}; however, a recent report minimized the impact of TRI exposure on the development of antibiotic resistance in *S aureus*.³⁰ Furthermore, it has been observed that bacterial-resistant subpopulations to TRI, benzalkonium chloride, and chlorhexidine in clinical isolates may be uncommon.³¹ Several mechanisms have been implicated in bacterial resistance to biocides.^{7,15,32,33} Efflux has been implicated in producing resistance to both TRI and antibiotics,³⁴ including ampicillin^{13,22} and ciprofloxacin.^{35,36} Our study was essentially an observational one, and at this stage we did not investigate the mechanisms of resistance implicated in the change of susceptibility profiles to antimicrobials.

In our study, short and long exposure to TRI (0.0004%) elicited a significant and stable increase in MIC in *E coli*, but it did not elicit a clinical change in antibiotic susceptibility. Prolonged exposure (subculturing) in the presence of TRI however produced a stable clinical resistance change in ampicillin. A prolonged treatment to TRI has been associated with stable changes in antimicrobial susceptibility profiles in *S enterica* serovar Typhimurium.¹³ Here, the response of *S aureus* to TRI was somewhat different from that of *E coli*, with a significant increase in MBC after short contact time with TRI together with a clinical resistance to ciprofloxacin. Subculturing *S aureus* in the presence of TRI or not resulted in stable ampicillin resistance. Shorter *S aureus* exposure to TRI yielded larger changes in MIC-MBC. Although we do not have a direct mechanistic explanation for these observations, we can speculate that cumulative damage may have occurred. TRI is a phenolic compound and will affect the bacterial membrane somewhat. Longer exposure in TRI may cause sufficient membrane damage to the bacteria, negating any bacterial adaptation. Cumulative damage could also explain the lack of stability in antimicrobial tolerance observed by Knapp et al after *S enterica* Typhimurium was exposed to chlorhexidine and benzalkonium chloride.¹¹ Exposure of both *S aureus* and *E coli* to TRI presented a risk for the development of resistance in both bacteria. These results confirm conclusions from other studies^{13,22,32} and establish the use of TRI as an acceptable positive control.

Bacterial resistance to CHG has been reported in staphylococci.^{14,26,27,30,37} Here we did not observe a change in the chlorhexidine susceptibility profile after short and long exposures to the biguanide. Subculturing bacteria in CHG containing broth did not alter the antimicrobial susceptibility profile. Some changes in antibiotic susceptibility profile were observed in *E coli* after 24-hour exposure to chlorhexidine (0.00005%), but these changes were not stable. Changes in the antibiotic susceptibility profile after chlorhexidine (>0.0002%) exposure have been reported in *P stutzeri*³⁸ and *S enterica*.¹² A recent study observed a positive moderate correlation between CHG and antibiotic resistance in *S aureus*.³⁰

We also investigated exposure to a low concentration of H₂O₂ (0.001%). Although the bacterial susceptibility profile to H₂O₂ did not change after short or long (24 h) exposure to (Table 2), or repeated subculturing in (Table 3), the oxidizing agent, a stable change in ciprofloxacin resistance was observed when *S aureus* was subcultured in the presence of H₂O₂. This clinical resistance to ciprofloxacin was unstable without H₂O₂ selective pressure (Table 3). It is conceivable that prolonged exposure to H₂O₂ induced the expression of the SoxRS system, which itself regulates the expression of efflux pumps among a number of defense mechanisms.³⁹ The induction of the OxyR regulon after H₂O₂ exposure could also lead to the production of scavengers, notably the regulation of katG catalase.³⁹ The level of expression of catalase genes, such as *katG*, was not investigated here. As a contrast, only an unstable clinical resistant to ampicillin was observed in *E coli* exposed to H₂O₂ for 24 hours. Wang et al⁴⁰ found that several regulatory genes responsive to oxidative stress and antibiotic resistance (*marRAB*, among

others) were upregulated after *E coli* exposure to H₂O₂. Such a bacterial response was not observed when *S aureus* was exposed to the biocidal product (Table 3). Instead, clinical resistance to ampicillin was observed in *E coli* after the fifth subculturing in the presence to Oxy BAC (0.001%). Other clinical resistance to antibiotics was reported, but these were not stable; notably, these were resistance to tobramycin in *E coli* after 24-hour exposure to CHG (0.00005%) and ticarcillin-clavulanic acid after subculturing *E coli* and *S aureus* to CHG (0.00005%) and Oxy BAC (0.001%), respectively (Table 3). From the literature it is likely that the mechanisms eliciting cross-resistance are multifactorial.^{32,33,41} It is interesting to note that many of the clinical resistances to antibiotics were not stable. Without a better understanding of the mechanisms involved, it is difficult to ascertain whether these unstable changes are caused by efflux expression driven by the selective pressure or detrimental mutations that only confer an advantage in the presence of the biocide. Except for the clinical resistance to ampicillin in *S aureus* as a result of TRI exposure, all other clinical resistances to antibiotics were lost after >1 subculturing in biocide-free media (Table 3).

This study looked at combining standard efficacy protocols to determine the propensity of biocide and biocidal products to cause antimicrobial resistance in bacteria. Standard MIC-MBC microdilution broth test protocols¹⁹ combined with standard antibiotic susceptibility testing²⁰ were used to ensure result reproducibility. Although our study was not repeated over time, Knapp et al¹¹ reported on experimental reproducibility of antimicrobial susceptibility testing using these protocols, where experiments were performed over a 6-month period. A small variation in MIC-MBC was observed, but this was deemed not to be practically significant because the use of microdilution broth means that a change in just one dilution will impinge negatively on the SD, especially when high concentrations are concerned.¹¹

We have brought forward the concept of during use concentration, which reflects the worst-case scenario of concentration and contact time for a biocidal product during application. This also acknowledges that a biocidal product may be diluted during use or remain present for an extended length of time. This is quite different from the in use concentration of a biocide reported on packaging, which is the concentration that is used to make a product claim after for example British Standards Institute and Food and Drug Administration standard efficacy testing. By acknowledging the actual conditions of use of a biocidal product (ie, any dilution resulting from use, prolonged contact time), this test provides a realistic assessment of the selective pressure exerted by the product on application. It is informative to note that high MICs to cationic biocides, but not to TRI, have been positively correlated with the prediction of multidrug resistance in staphylococci.³⁷

Our study made a number of interesting observations, notably where a stable clinical resistance to antibiotics was measured. Unfortunately, the aim was not to investigate the mechanisms of resistance involved at this stage, and such observations warrant additional studies.^{5,7} Instead we presented a decision tree to help manufacturers understand the risks associated with their products (Fig 1). The use of such a decision tree should favorably address the request from the U.S. Food and Drug Administration and European Union Biocidal Products Regulations (<http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm378542.htm>)³ for manufacturers to provide information on antimicrobial resistance and cross-resistance in bacteria after the use of their products.

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