Priming with biocides: a pathway to antibiotic resistance?

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Title:

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Priming with biocides: a pathway to antibiotic resistance?

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Abstract

Aims

To investigate the priming effects of sub-inhibitory concentrations of biocides on antibiotic resistance in bacteria.

Methods and results

Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were exposed to sub-inhibitory concentrations of biocides via a gradient plate method. Minimum inhibitory concentration (MIC) and antibiotic susceptibility were determined, and efflux pump inhibitors (thioridazine and chlorpromazine) were used to investigate antibiotic resistance mechanism(s).

Escherichia coli displayed a 2-fold increase in MIC (32 to 64 mg l-1) to H_2O_2 which was stable after 15 passages, but lost after 6 weeks, and *P. aeruginosa* displayed a 2-fold increase in MIC (64 to 128 mg l-1) to BZK which was also stable for 15 passages. There were no other tolerances observed to biocides in *E. coli*, *P. aeruginosa* or *S. aureus*, however stable cross-resistance to antibiotics was observed in the absence of a stable increased tolerance to biocides. Six-fold increases in MIC to cephalothin and four-fold to ceftriaxone and ampicillin were observed in hydrogen peroxide primed *E. coli*. Chlorhexidine primed *S. aureus* showed a four-fold increase in MIC to oxacillin, and glutaraldehyde-primed *P. aeruginosa* showed four-fold (sulphatriad) and eight-fold (ciprofloxacin) increases in MIC. Thioridazine increased the susceptibility of *E. coli* to cephalothin and cefoxitin by four and two-fold respectively, and both thioridazine and chlorpromazine increased the susceptibility *S. aureus* to oxacillin by eight and four-fold respectively.

Conclusions

These findings demonstrate that sub-inhibitory concentrations of biocides can prime bacteria to become resistant to antibiotics even in the absence of stable biocide tolerance and suggests activation of efflux mechanisms may be a contributory factor.

Significance and impact of the study

This study demonstrates the effects of low-level exposure of biocides (priming) on antibiotic resistance even in the absence of obvious increased biocidal tolerance.

Introduction

The rise in multi-drug resistant bacteria means that some conventional antimicrobials have become ineffective in the treatment of infections (Walsh and Toleman 2011; Roca Subirà *et al.* 2012; Tuon *et al.* 2012;). Biocides have been used for centuries to control infectious agents (Maillard 2002; Russell 2002; Morente *et al.* 2013). They are either applied as or added to formulated products that are used as disinfectants, preservatives, pesticides, antiseptics and even cosmetics (Maillard 2002; Gilbert and McBain 2003; Knapp *et al.* 2015). Public awareness to infection control has caused a rise in the use of biocide and biocidal products in the home environment. As a result of increased use of biocides, especially at sub-inhibitory concentrations, there are concerns over how their selective pressure might potentially favour the development of less susceptible bacterial strains, as well as encourage the expression and dissemination of resistance mechanisms to biocides and other antimicrobial agents (McBain and Gilbert 2001; Fraise 2002; SCENIHR 2010; Maillard *et al.* 2013 Knapp *et al.* 2015; Pereira *et al.* 2021). There are several laboratory studies demonstrating a link between exposure of bacteria to sub-inhibitory concentrations and increased tolerance and resistance to biocides (Walsh *et al.* 2003; Escalada *et al.* 2005; Christensen *et al.* 2011; Knapp *et al.* 2013; Bock *et al.* 2016). Some studies show both increased tolerance to biocides plus antibiotic cross-resistance in bacteria after exposure to sub-inhibitory concentrations of biocides (Wand *et al.* 2016; Kurenbach *et al.* 2015). While exposure to biocides has been linked to reduced susceptibility and cross-resistance to antibiotics (Chuanchuen *et al.* 2001; Braoudaki and Hilton 2004; Tkachenko *et al.* 2007; Soumet *et al.* 2012; Slayden *et al.* 2000; Wand 2017), it is paramount that we understand what happens in the absence of obvious increased biocidal tolerance after low level exposure; will there be a change in bacterial resistance to antibiotics in that instance?

Since biocides have several target sites within bacteria (Maillard 2002; Russell 2002), the most common mechanisms for cross-resistance are via non-specific processes such as efflux-pumps (Costa *et al.* 2013; Bogomolnaya *et al.* 2013) and changes in properties of their cell wall, for example reduced permeability due to porin down regulation (Jaffe *et al.* 1982; Manzoor *et al.* 1999).

A review by Maillard *et al.* (2013) and a report from the scientific committee for emerging and newly identified health risks (SCENIHR, 2010) identified biocides as a risk due to selective pressure for less susceptible strains. Their findings highlighted a key gap in current knowledge in understanding the effect of low concentrations of biocides on bacterial cells, as well as the mechanisms involved in the development of resistance and cross-resistance (Jaffe *et al.* 1982; Maillard 2002; SCENIHR 2010; Knapp *et al.* 2015). Because of these concerns, the European Union and the United States have proposed regulatory changes requiring manufacturers of biocidal products to provide data on the risks of resistance development in organisms targeted by biocidal products (SCENIHR 2010; Knapp *et al.* 2015).

As previously demonstrated by several studies, prolonged exposure of bacteria to biocides under laboratory conditions can generate less susceptible mutants which can often display reduced susceptibility to various antibiotics (Karatzas *et al.* 2007; Randall *et al.* 2007; Karatzas *et al.* 2008; Whitehead *et al.* 2011; Fernández Márquez *et al.* 2017; Hardy *et al.* 2017).

The present study investigates the effect of biocide priming on bacterial resistance to antibiotics after continuous exposure of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* to low concentrations of hydrogen peroxide (H_2O_2), chlorhexidine (CHG), benzalkonium chloride (BZK) and glutaraldehyde (GTA). The three organisms used are all commonly associated with hospital acquired infections (HAIs) and the strains selected are those listed in the BS EN 1276:2009 disinfection test method. *Escherichia coli* is a Gram-

negative bacteria commonly transmitted to humans via the consumption of contaminated food and water (Kaper *et al*. 2004) and known to cause infections such as diarrhoea and haemorrhagic colitis to severe complications such as haemolytic anaemia and acute renal failure (Brzuszkiewicz *et al*. 2011). Outer membrane protein modification like the down regulation of porins and up regulation of efflux pumps such as AcrAB have been linked with increased antimicrobial resistance in *E. coli* (Ma *et al*. 1993; 1994). *Staphylococcus aureus* is a Gram-positive, opportunistic pathogen responsible for both hospital and community acquired infections (Boucher *et al*. 2010; Latimer *et al*. 2012). Upregulation of QacA-D, E and H (Kazama *et al*.1998; Heir *et al*.1998; Rouch *et al*. 1990) and NorA, NorB and NorC efflux pumps (Truong-Bolduc *et al*. 2006) have been linked to antimicrobial resistance in *S. aureus*. *Pseudomonas aeruginosa* is an opportunistic, intrinsically resistant, Gram-negative bacteria that is responsible for a range of infections to the eyes and ears to serious complications in cystic fibrosis and bronchiectasis patients (Lambert 2002; Soothill 2013). Upregulation of efflux pumps such as MexAB, MexCD and MexEF and outer membrane protein modification have been linked to antimicrobial resistance in *P. aeruginosa* (Schweizer 1998; Poole 2001). This study aims to understand the impact of prolonged exposure of bacteria to low concentrations of biocide, and the possible cross-resistance to antibiotics.

Materials and Methods

Bacterial strains and storage

Escherichia coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538 strains were grown in tryptone soya broth (TSB) (OXOID, Basingstoke, Hampshire, England) at 37°C with shaking at 100 rpm for 24 hours and stored on protect beads (Scientific Laboratory Supplies Limited, Nottingham, United Kingdom) at -80°C.

Preparation of inoculum

Bacterial strains were grown on Mueller Hinton agar (MHA) (OXOID, Basingstoke Hampshire, England) at 37°C for 24 h and a single colony was transferred to 10 ml sterile saline. The turbidity of the suspension was adjusted to the equivalence 0.5 McFarland (absorbance range between 0.08-0.13) at 625 nm line with the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2015) and of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000).

Preparation of biocide working solutions

A two-fold dilution series of biocides (mg l^{-1}) was prepared in Mueller-Hinton broth following the BS EN ISO 20776-6 guidelines/protocol (BSI, 2006). Hydrogen peroxide (H_2O_2) concentration range 1-512 mg l^{-1} (Fisher Scientific Belgium), formulated chlorhexidine gluconate (CHG) concentration range 0.976-500 mg l^{-1} (Molnlycke Health Care Ltd Manchester UK), benzalkonium chloride (BZK) concentration range 1-512 mg l^{-1} and glutaraldehyde (GTA) concentration range 8-4096 mg l^{-1} (Sigma-Aldrich).

Preparation of antibiotic working solutions

Antibiotics discs Mastring-S (M13 and M14) were obtained from Mast Diagnostics UK. Antibiotics and efflux pump inhibitors were purchased from Sigma Aldrich and used at the following concentrations: oxacillin sodium salt concentration 0.0020-8 mg l^{-1}, cefoxitin sodium salt 0.25-32 mg l^{-1}, cephalothin sodium salt 0.25-32 mg l^{-1}, ceftriaxone disodium salt hemi (heptahydrate) 0.016-2 mg l^{-1}, ampicillin 0.25-32 mg l^{-1}, sulfathiazole 37 % v/v, sulfadiazine 37% v/v, sulfamerazine 26 % v/v, ciprofloxacin sodium salt 0.125-2 mg l^{-1}, thioridazine hydrochloride (TZ) 1.9–500 mg l^{-1} and chlorpromazine hydrochloride (CPZ) 4-256 mg l^{-1}. Stock solutions were prepared by dissolving salts in sterile distilled water and working solutions were prepared in Mueller-Hinton broth following the BS EN ISO 20776-6 guidelines/protocol (BSI, 2006).

Adaptation of bacterial strains to biocides using gradient plate

A two-fold dilution series of biocides were prepared in sterile distilled water following the EUCAST guidelines of ESCMID (EUCAST 2015; ESCMID 2000). The required biocide (1 ml) was added to 19 ml sterile molten nutrient agar to give specific final concentrations (from a concentration just below the MIC of the biocide against tested bacteria strain). The molten agar and biocide mixture was poured into sterile Petri dishes and set at an angle, after which plates were placed on a flat surface and 20 ml sterile molten agar were poured over the first layer and allowed to set. Plates were left at 4°C for 24 h to allow diffusion of biocide. The 24 h bacterial cultures were streaked along the concentration gradient starting at the point of the gradient plate containing the lowest concentration of biocide. These streaked plates were incubated at 37 ° C for 24 h. Bacterial colonies that grew the furthest along the gradient towards the high concentration were used to streak a new gradient plate containing the next highest concentration, until no further increases in tolerance were observed, at which point the furthest-growing colonies were selected and stored on protect beads at -80 ° C. For example, the H_2O_2 MIC against *E. coli* was 32 mg l^{-1}, therefore the bacteria was first streaked on a plate containing 16 mg l^{-1} H_2O_2 (concentration 2-fold below the MIC), then moved to 32, 64, 128 and 256 mg l^{-1} until no growth were observed on the 512 mg l^{-1} plate.

Stability of adaptive resistance

To confirm the stability of the primed-strains produced, the gradient plate isolates were sub-cultured 15 times in 10 ml of tryptone soya broth without biocide. MIC between parent and primed strains were compared after one, two, ten and fifteen subcultures, following EUCAST guidelines ESCMID (EUCAST 2015; ESCMID 2000).

Determination of minimum inhibitory concentrations (MICs)

The MICs were determined for each strain before and after exposure to the biocides. This was carried out using the colony suspension method of testing in accordance with BS EN ISO 20776-1 guidelines/protocol (BSI, 2006). Briefly, 50 μl of the standardised inoculum was dispensed into each well of a 96-well plate containing 50 μl of the appropriate concentration of biocides or antimicrobial agents to give a final inoculum concentration of 5×10^5 CFU/ml. The inoculated 96-well plate was incubated at 37°C for 24 hours. The lowest concentration of compound that prevented bacterial growth after 24 h of incubation was used to establish the MIC. Bacterial growth was determined in two ways: by visual observation of growth on the 96-well plate and by measuring absorbance at 625 nm in a Spectramax plus plate reader. All experiments were carried out as biological and technical triplicates.

Antibiotic susceptibility testing using disc diffusion test

The antibiotic susceptibility of each bacterial strain was determined before and after biocide treatment with biocides using the disc diffusion method of antimicrobial susceptibility testing. This was done in accordance with the guidelines of European committee on antimicrobial susceptibility testing (EUCAST 2015). Briefly, antibiotic discs were applied to surfaces of the inoculated plates within 15 minutes of inoculation. After 24 h of incubation at 37 °C, zones of inhibition were measured with a digital Vernier calliper.

The effect of efflux pump inhibitors

Standardised bacterial suspensions of primed strains were prepared from 24 h culture as previously described. Suspensions were grown in 96 well plates for 24 h at 37 °C in concentrations of antibiotics they were resistant to, plus an efflux pump inhibitor (EPI) at half its MIC (a concentration that does not inhibit growth of strain). H_2O_2 primed *E. coli* (EcH2O), GTA primed *P. aeruginosa* (PaGTA) and BZK primed *P. aeruginosa* (PaBZK) were tested with TZ, and CHG primed *S. aureus* (SaCHG) was tested with CPZ and TZ. EcH2O, 40 mg l⁻

[1] TZ was tested with cephalothin and cefoxitin, PaGTA, 250 mg l^{-1} TZ was tested with ciprofloxacin and sulphatriad, PaBZK 250 mg l^{-1} TZ was tested with BZK, and SaCHG was tested with oxacillin with either 32 mg l^{-1} CPZ or 15.625 mg l^{-1} TZ. The lowest concentration of antibiotic plus EPI that prevented bacteria growth after 24 h of incubation was used to establish MIC.

Confirmation of strain identity

16S rRNA genes were amplified from genomic DNA of parent and biocide-treated strains using Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs) and primer pairs 16S rRNA For 1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S rRNA Rev 1 (5′-ACGGCTACCTTGTTACGACTT-3′) for *S. aureus*, 16S rRNA For 2 (5′-AGAGTTTGATCATGGCTCAG-3′) and 16S rRNA Rev 2 (5′-ACGGTTACCTTGTTACGACTT-3′) for *E. coli*, and 16S rRNA For 2 and 16S rRNA Rev 1 for *P. aeruginosa*. PCR products were analysed on 0.8% (w/v) agarose gels and sequenced by automated DNA sequencing (Eurofins).

Fitness of primed strains

Overnight cultures of parent and primed bacterial strains were inoculated in antibiotic-free Mueller Hinton broth (MHB) and absorbance at 600 nm was recorded every 10 minutes for 16 hours. Resulting data and standard deviations were plotted. Each experiment was conducted in biological triplicates.

Results

Effect of sub inhibitory concentrations of biocides

The MIC of four biocides against pre and post primed strains of bacteria are summarised in Table 1. There was an initial increase in MIC to H$_2$O$_2$ from 32 mg l^{-1} for the *E. coli* parent strain

to 64 mg l^{-1} after H$_2$O$_2$ priming (EcH$_2$O$_2$), which was stable after 15 passages but lost after storage for six weeks. The MIC of *E. coli* did not change after exposure to other biocides used in this study. Apart from a two-fold increased MIC from 64 mg l^{-1} for the parent strain of *P. aeruginosa* to 128 mg l^{-1} (stable after 15 passages) after BZK priming (PaBZK), there was no other obvious increased tolerance observed to biocides tested in *P. aeruginosa*. No biocide tolerance was observed with *S. aureus*, but bacteria exposed to CHG (SaCHG) were included in further testing to determine whether there was any observed antibiotic cross-resistance.

The results of antibiotic susceptibility tests for parent and primed strains of bacteria are summarised in Table 2. Two sample t-tests assuming unequal variances (p=0.05) were used to compared the data and statistically significant changes were observed in the following five cases. Changes in zones of inhibition were observed in EcH$_2$O$_2$, where there was no zone of inhibition around cephalothin or ampicillin discs compared to the parent strain *E. coli* strain, where 10.43 mm (cephalothin) and 18.88 mm (ampicillin) zones of inhibition where observed. The EUCAST (EUCAST 2020) zone diameter breakpoint for ampicillin and *Enterobacterales* reports resistance at <14 mm, indicating a change in clinical susceptibility; the corresponding data for cephalothin is not available. For SaCHG there was no zone of inhibition around oxacillin discs compared to 22.28 mm for the parent strain (EUCAST breakpoint data not available) and for PaGTA and PaBZK there was no zone of inhibition around sulphatriad discs compared to 15.11 mm for the parent strain (EUCAST breakpoint data not available).

To investigate the resistance more closely, the MIC of selected antibiotics against biocide primed strains of bacteria were determined (summarised in Table 3); the strain and antibiotic combinations tested were based on the results obtained from antibiotic disc diffusion tests. Due to the increased resistance of EcH$_2$O$_2$ to cephalothin (a first-generation cephalosporin), both cefoxitin and ceftriaxone, second and third generation cephalosporins respectively, were included in the MIC testing to see if the cross resistance extended to newer antibiotics. There

was an eight-fold increased MIC for EcH$_2$O$_2$ to cephalothin from 4 mg l^{-1} for the parent strain to 32 mg l^{-1} for EcH$_2$O$_2$, a four-fold increase in MIC for EcH$_2$O$_2$ to cefoxitin to 16 mg l^{-1} compared to 4 mg l^{-1} in the parent strain, and a two-fold increase to ceftriaxone (parent 0.0625 mg l^{-1} and primed strain 0.125 mg l^{-1}) and ampicillin (parent 2 mg l^{-1} and primed 4 mg l^{-1}). The MIC breakpoint (EUCAST 2020) for *Enterobacterales* reports resistance at >2 mg l^{-1} with ceftriaxone indicating no change in clinical susceptibility, data for cephalothin and cefoxitin are not available. A four-fold increased MIC for oxacillin was observed in SaCHG (2mg l^{-1} compared to parent strain *S. aureus* 0.5 mg l^{-1}; breakpoint data not available). In PaGTA an eight-fold increased MIC to ciprofloxacin was observed (1 mg l^{-1} compared to 0.125 mg l^{-1} for the parent *P. aeruginosa* strain) and a four-fold increased MIC to sulphatriad was also recorded. The MIC breakpoint (EUCAST 2020) for *Pseudomonas* spp. and ciprofloxacin reports resistance at >0.5 mg l^{-1} indicating clinically significant resistance in PaGTA (data for sulphatriad is not available).

The effect of efflux pump inhibitors

The MICs of cephalothin and cefoxitin against EcH$_2$O$_2$ were reduced in the presence of the efflux pump inhibitor TZ (from 32 mg l^{-1} to 8 mg l^{-1} for cephalothin and from 16 mg l^{-1} to 8 mg l^{-1} for cefoxitin; Table 4). The MIC of oxacillin against SaCHG was reduced from 2 mg l^{-1} to 0.25 mg l^{-1} in the presence of TZ and to 0.5 mg l^{-1} in the presence of CPZ (Table 5). The effect of TZ on the MIC of ciprofloxacin and sulphatriad against PaGTA could not be determined due to the turbidity, caused by insolubility, observed when 250 mg l^{-1} of TZ (a concentration that does not inhibit growth) was combined with either ciprofloxacin or sulphatriad. To further determine the effect of TZ in the presence of either antibiotics, contents of the previously observed turbid wells were spread onto agar and further incubated at 37°C for 24 hours. Reduced growth on agar of PaGTA was observed from wells containing ciprofloxacin + TZ and sulphatriad + TZ, compared to wells with only ciprofloxacin, TZ or

sulphatriad (data not shown). The same observation was seen in the case of PaBZK, where no growth on agar was observed from wells containing 128 mg l-1 BZK + TZ, compared to significant growth from wells with only BZK or TZ (data not shown).

Confirmation of strain identity

16S rRNA sequencing confirmed all parent (wild type) and biocide primed strains used/ generated in this study were at least 99.9% identical to the type strains; *E. coli* ATCC 8739, *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442 (data not shown).

Fitness of primed strains

Results of 16-hour growth curves of parent and primed strains are shown in Figure 1. There was no difference in the length of the lag phase between the *E. coli* parent strain and primed isolate, but growth was decreased from 4 h onwards (Fig. 1A). For *S. aureus*, the CHG primed isolate had an extended lag phase compared to the parent strain, but overall growth was the same from 6 h onwards (Fig. 1B). Interestingly, the *P. aeruginosa* BZK and GTA isolates both had an extended lag phase and decreased overall growth when compared to the parent strain (Fig. 1C). This effect was more pronounced with the BZK isolate.

Discussion

The goal of the present study was to test whether biocide priming has an effect on bacterial resistance to antibiotics. Low concentrations of H_2O_2 have previously been shown to promote tolerance to the biocide (Imlay and Linn 1987; Bogomolnaya *et al.* 2013) as was initially seen in this study with *E. coli*. In contrast to previous studies, here the increased tolerance to H_2O_2 was short lived. Stable cross resistance of EcH_2O_2 to cephalothin, cefoxitin, ceftriaxone and ampicillin antibiotics was observed, similar to the findings of Pereira *et al.* (2021). Previous studies show H_2O_2 activates the SoxRS and the OxyR regulons (Aslund *et al.* 1999; Manchado

et al. 2000) both of which control the expression of over 100 oxidative stress response genes (Demple 1991; Blanchard *et al.* 2007; 2012), including *acrAB*, which encode a multidrug efflux pump (Dukan *et al.* 1996; Storz and Imlay 1999). Although this might explain the cross tolerance to antibiotics after H_2O_2 priming observed here, further work is required to see if either the SoxRS or OxyR regulons are activated in the present study, by comparing the expression levels of genes involved in both parent and primed strains. This could help to establish the mechanism(s) behind the differences seen between this study and others. Specifically, the ability of biocides to prime for antibiotic resistance without the usual observation of stable biocidal tolerance. Exposure of *S. aureus* to CHG did not reduce its susceptibility to the biocide, contrasting the findings of Hardy *et al.* where prolonged exposure of *S. aureus* isolates to CHG led to reduced susceptibility (Hardy *et al.* 2017). Here, cross-resistance to oxacillin was observed with no corresponding tolerance to the biocide.

The results from the present study shows an increased MIC to BZK in PaBZK in comparison to the parent *P. aeruginosa* strain. Activation of multidrug efflux systems such as the RND-type MexCD-OprJ was previously linked to the use of sub-inhibitory concentrations of membrane damaging biocides including BZK and CHG (Morita *et al.* 2003; Fraud *et al.* 2008). Previous adaptation of *P. aeruginosa* to sub-inhibitory concentrations of BZK was attributed to increased efflux (Loughlin *et al.* 2002; Mc Cay *et al.* 2010). In their work, Mc Cay *et al.* observed cross-resistance with ciprofloxacin in BZK primed *P. aeruginosa* grown in continuous culture (2010), in contrast to this study whereby no cross-resistance to antibiotics, including ciprofloxacin, was seen. This could be attributed to the different methodology used when priming strains as here a serial batch method, gradient plating, was used in comparison to the continuous culture method employed by Mc Cay *et al.* (2010). Our findings are consistent with those of Loughlin *et al.,* where cross resistance was observed with BZK and other quaternary ammonium compounds after serial batch culture (Loughlin *et al.* 2002). The varied

outcome in these studies shows how different growth conditions may influence adaptation and selection of bacteria to antimicrobials.

There are several reports of bacterial resistance to GTA (Kirschke *et al.* 2003; Simoes *et al.* 2006; Svetlikova *et al.* 2009; Tschudin-Sutter *et al.* 2011; Simões *et al.* 2011; Kampf *et al.* 2013), which was not observed in this study. Cross-resistance after priming with GTA to two unrelated antibiotics, ciprofloxacin and sulphatriad, was however observed. A study using *Pseudomonas fluorescens* biofilms showed that exposure of the bacteria to GTA significantly induced expression of two genes encoding multidrug efflux pumps, PFLU2929 and PFLU3876, which appear to be orthologs of OprN and PA5159 in *P. aeruginosa* (Vikram *et al.* 2015). The cross-resistance observed with unrelated antibiotics in this study might suggest efflux is involved, as seen previously (Poole 2002; Sanchez *et al.* 2005; Maseda *et al.* 2009; Ferreira *et al.* 2011).

Phenothiazines such a TZ and CPZ have been shown to potentiate the effect of antimicrobials against bacteria (Wainwright *et al.* 1998; Viveiros and Amaral 2001), eliminate antibiotic resistant plasmids (Evdokimova *et al.* 1997; Radhakrishnan *et al.* 1999) and inhibit bacterial efflux pumps (Viveiros and Amaral 2001; Ordway *et al.* 2003; Costa *et al.* 2013; Machado *et al.* 2018). The most well studied efflux pump inhibitor is CPZ, but both CPZ and TZ have the same antimicrobial properties against efflux and phagocytosed bacteria (Ordway *et al.* 2002a; 2002b; Machado *et al.* 2018). Here we tested the effect of TZ at sub-inhibitory concentration on EcH$_2$O$_2$ in the presence of varying concentrations of cephalothin and cefoxitin, to which the primed-strain had previously shown cross-resistance. TZ greatly increased the susceptibility of EcH$_2$O$_2$ to cephalothin and cefoxitin, suggesting that efflux mechanisms may be involved in the observed cross-resistance. Our results corroborate the findings of Amaral *et al.* where CPZ reduced the MIC of ceftazidime and ceftriaxone against *E. coli* from 1.0 mg l^{-1} to 0.08 and 0.07

mg l^{-1} respectively (Amaral *et al.* 1992). These findings suggest that increased efflux might be a contributory factor in the cross-resistance observed with EcH$_2$O$_2$.

When the effect of both TZ and CPZ on MIC levels of oxacillin on SaCHG was evaluated, the results revealed a significant reduction of MIC in the presence of CPZ and TZ. These results suggest that efflux mechanisms may be contributing to the cross-resistance observed. Furthermore, this agrees with the findings of Kristiansen *et al.* (2006) and Costa *et al.* (2013).

Although efflux contributes highly to antimicrobial resistance in *P. aeruginosa*, reduced influx/impermeability has also been shown to contribute (Li *et al.*, 2000), for example mutation in or loss of the transmembrane porin OprD is significant in resistance to carbapenems (Bradford *et al.* 1999). Thus, a combination of increased efflux and decreased influx may contribute to antimicrobial resistance in *P. aeruginosa* resistance, even though it was not fully demonstrated with the methods used here.

The increased susceptibility seen with some biocide primed strains to antibiotics in the presence of EPIs suggests efflux as a contributory mechanism to the cross resistance observed in this study. The next step will be to employ the use of q qPCR to compare gene expression in parent and primed strains of bacteria.

The growth curve results comparing both parent and primed strains indicated that the resistance phenotype did have an impact on overall growth in the case of *E. coli*, the length of the lag phase with *S. aureus* and both overall growth and lag phase with *P. aeruginosa*. This suggests that although there may be potential fitness cost associated with these phenotypic adaptations in terms of initial or overall speed of replication, this is not impacting on the conservation of the resistance after repeated sub-culture under laboratory conditions. This agrees with other findings, where generally most mutations had an impact on fitness (Melnyk *et al.* 2015). It is

not unexpected to observe this with biocide induced cross-resistance, as biocides generally have broader modes of action in terms of cellular targets.

Results from this study clearly demonstrate that continuous exposure to sub-inhibitory concentrations of biocides far below the recommended in-use concentration can, under laboratory conditions, prime bacteria to become resistant to antibiotics even in the absence of increased tolerance to the biocides. This raises the important question of whether this phenomenon is occurring in clinical settings and contributing to dissemination to antimicrobial resistance.

Conflict of interest

No conflict of interest.

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Conflict of Interest, none declared.

Authors contribution

Pat Adkin[1], Andrew Hitchcock[2], Laura J. Smith[1], Susannah E. Walsh[1,3*]

Pat Adkin and Andrew Hitchcock carried out the experiments. Pat Adkin wrote the manuscript with support from Susannah E. Walsh and Laura J. Smith. Susannah E. Walsh and Laura J. Smith supervised the project and designed the experiments.

Tables

Table 1. MIC of biocides against pre and post primed strains of bacteria

EcH$_2$O$_2$, H$_2$O$_2$ primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa;* and PaBZK, BZK primed *P. aeruginosa*. [*]EcH$_2$O$_2$ tolerance to H$_2$O$_2$ was unstable. Only in [†]PaBZK was there a stable two-fold increase to tolerance BZK.

Table 2. Antibiotic susceptibility profiles of pre and post primed bacteria strains

Bold *, changes in zones of inhibition where observed compared to parent strain. NZ, no zone (6mm disc diameter). Standard error shown in parentheses (n = 3). EcH_2O_2, H_2O_2 primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa;* and PaBZK, BZK primed *P. aeruginosa.*

Table 2. MICs of selected antibiotics against parent and primed strains of bacteria

Bold [*], increase in MIC compared to parent strain. EcH_2O_2, H_2O_2 primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa*. Standard error shown in parentheses (n = 3).

Table 3. The effect of TZ on the tolerance of cephalothin and cefoxitin in EcH$_2$O$_2$

EcH$_2$O$_2$, hydrogen peroxide primed *E. coli*. TZ, thioridazine. Reduced MIC for [*]cephalothin and [†]cefoxitin were observed in the presence of 40mg l^{-1} thioridazine.

Table 4. The effects of TZ and CPZ on oxacillin in SaCHG

Reduced MIC to oxacillin was observed in the presence of TZ and CPZ. SaCHG, CHG primed *S.* aureus; TZ, thioridazine; CPZ, chlorpromazine. Standard error shown in parentheses (n = 3).

Tables

Table 1. MIC of biocides against pre and post primed strains of bacteria

Strain	MIC (mg l^{-1}), n=3			
	hydrogen peroxide	glutaraldehyde	benzalkonium chloride	chlorhexidine
E. coli	32	1024	16	15.6
EcH$_2$O$_2$	*64	1024	16	15.6
S. aureus	4	512	4	7.8
SaCHG	4	512	4	7.8
P. aeruginosa	32	1024	64	31.3
PaGTA	32	1024	-	31.3
PaBZK	-	-	†128	-

EcH$_2$O$_2$, H$_2$O$_2$ primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa;* and PaBZK, BZK primed *P. aeruginosa*.

*EcH$_2$O$_2$ tolerance to H$_2$O$_2$ was unstable. Only in †PaBZK was there a stable two-fold increase to tolerance BZK.

Table 2. Antibiotic susceptibility profiles of pre and post primed bacteria strains

Antibiotics (µg)	Zone of inhibition (mm) (S.E.)						
	EcATCC	EcH2O2	SaATCC	SaCHG	PaATCC	PaGTA	PaBZK
Ampicillin (10)	*$**18.88**$(1.50)	*$**NZ**$	31.90(0.05)	31.81(0.14)	NZ	NZ	NZ
Cephalothin (5)	*$**10.43**$(0.24)	*$**NZ**$	31.67(0.21)	31.84(0.16)	NZ	NZ	NZ
Colistin (25)	14.41(0.24)	14.37(0.23)	NZ	NZ	15.92(0.59)	15.87(0.59)	13.86 (0.39)
Gentamicin (10)	21.67(0.22)	21.90(0.46)	22.82(0.084)	22.76(0.12)	15.96(0.82)	15.62(0.58)	13.23 (3.29)
Streptomycin (10)	18.18(0.94)	17.98(0.83)	19.11(0.06)	18.78(0.21)	10.00(0.18)	9.75(0.37)	7.20 (3.60)
Sulphatriad (200)	31.26(0.62)	31.20(0.56)	26.13(0.85)	26.14(0.83)	*$**15.11**$(0.58)	*$**NZ**$	*$**NZ**$
Tetracycline (25)	21.99(1.27)	21.93(1.27)	23.16(0.37)	23.09(0.37)	8.779(0.05)	8.78(0.07)	10.00 (0.34)
Cotrimoxazole (25)	27.46(1.02)	28.11(0.77)	20.35(0.19)	20.57(0.13)	NZ	NZ	NZ
Chloramphenicol (25)	20.42(0.33)	21.37(0.33)	20.03(0.21)	19.97(0.22)	NZ	NZ	NZ
Erythromycin (5)	NZ	NZ	17.12(0.42)	17.08(0.38)	NZ	NZ	NZ
Fusidic Acid (10)	NZ	NZ	26.71(0.12)	26.50(0.22)	NZ	NZ	NZ
Oxacillin (5)	NZ	NZ	*$**22.28**$(0.44)	*$**NZ**$	NZ	NZ	NZ
Novabiocin (5)	NZ	NZ	22.39(0.07)	22.35(0.02)	NZ	NZ	NZ
Penicillin G 1 unit	NZ	NZ	21.26(0.24)	21.23(0.22)	NZ	NZ	NZ
Streptomycin (10)	18.55(0.99)	19.04(1.21)	16.13(0.67)	16.09(0.61)	8.96(1.26)	8.96(1.25)	10.87 (0.34)
Tetracycline (25)	20.77(0.32)	20.94(0.15)	21.06(0.26)	20.94(0.21)	NZ	NZ	7.82 (0.27)

Bold *, changes in zones of inhibition where observed compared to parent strain. NZ, no zone (6mm disc diameter). Standard error shown in parentheses (n = 3). EcH$_2$O$_2$, H$_2$O$_2$ primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa;* and PaBZK, BZK primed *P. aeruginosa.*

Table 3. MICs of selected antibiotics against parent and primed strains of bacteria

Strains	MIC (mg l^{-1})						
	cephalothin	cefoxitin	ceftriaxone	ampicillin	oxacillin	sulphatriad	ciprofloxacin
E. coli	4	4	0.0625	2	-	-	-
EcH$_2$O$_2$	*32	*16	*0.125	*4	-	-	-
S. aureus	-	-	-	-	0.5	-	-
SaCHG	-	-	-	-	*2	-	-
P. aeruginosa	-	-	-	-	-	256	0.125
PaGTA	-	-	-	-	-	*1024	*1

Bold *, increase in MIC compared to parent strain. EcH$_2$O$_2$, H$_2$O$_2$ primed *E. coli*; SaCHG, CHG primed *S. aureus*; PaGTA, GTA primed *P. aeruginosa*. Standard error shown in parentheses (n = 3).

Table 4. The effect of TZ on the tolerance of cephalothin and cefoxitin in EcH$_2$O$_2$

Strain	MIC (mg l^{-1})				
	TZ	Cephalothin	+ TZ	Cefoxitin	+ TZ
EcH$_2$O$_2$	128	32	*8	16	†8

EcH$_2$O$_2$, hydrogen peroxide primed *E. coli*. TZ, thioridazine. Reduced MIC for *cephalothin and †cefoxitin were observed in the presence of 40mg l^{-1} thioridazine.

Table 5. The effects of TZ and CPZ on oxacillin in SaCHG

Strain	MIC (mg l^{-1}), mode n=3				
	TZ	CPZ	Ox	+ ½ TZ	+ ½ CPZ
SaCHG	31.25	64	2	0.25	0.5

Reduced MIC to oxacillin was observed in the presence of TZ and CPZ. SaCHG, CHG primed *S.* aureus; TZ, thioridazine; CPZ, chlorpromazine. Standard error shown in parentheses (n = 3).