

## ORIGINAL ARTICLE

# Understanding the risk of emerging bacterial resistance to over the counter antibiotics in topical sore throat medicines

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antibiotic resistance, bacitracin and tyrothricin, gramicidin, neomycin, over-the-counter, sore throat.

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## Abstract

**Aims:** The aims of this study were to explore the development of bacterial resistance and cross-resistance in four common human pathogens following realistic exposure to antibiotics found in over-the-counter (OTC) sore throat medicines: gramicidin, neomycin, bacitracin and tyrothricin.

**Methods and Results:** Bacterial exposure to in-use (concentration in the product before use) and diluted concentration (i.e. *during use*) of antibiotic where conducted in broth for 24 h or until growth was visible. The changes in bacterial susceptibility profile before and after exposure was determined using standardized ISO microdilution broth. Antibiotic testing was performed according to EUCAST guidelines. We demonstrated that test bacteria were able to survive exposure to the in-use concentrations of some antibiotics used in OTC medicines. Exposure to *during use* concentrations of bacitracin resulted in stable increase in minimal inhibitory concentration (MIC) (>8-fold) in *Staphylococcus aureus* and *Acinetobacter baumannii*. Exposure to tyrothricin resulted in a stable increase in MIC (2-4-fold) in *Klebsiella pneumoniae*, and exposure to neomycin resulted in a stable increase MIC (5000-fold higher than the baseline) in *Streptococcus pyogenes*. Clinical cross-resistance to other antibiotics (ciprofloxacin, fusidic acid, gentamicin, cefpodoxime, amoxicillin/clavulanic acid and cefotaxime) was also demonstrated following exposure to bacitracin or tyrothricin. Bacitracin exposure lead to a stable bacterial resistance after 10 passages.

**Conclusions:** Our results indicate that OTC antibiotic medicines have the potential to drive resistance and cross-resistance *in vitro*.

**Significance and Impact of the Study:** Tackling antibiotic resistance is a high worldwide priority. It is widely accepted that the overuse and misuse of antibiotics increase the risk of the development and spread of antibiotic resistance within communities. A number of OTC sore throat products, widely available across the world for topical use in respiratory indications, contain locally delivered antibiotics. Our findings showed that these antibiotics in OTC medicines present a risk for emerging cross-resistance in a number of bacterial respiratory pathogens.

## Introduction

The chemotherapeutic treatment of pathogens is increasingly arduous as antibiotic resistance becomes more prevalent. Currently, 700 000 deaths per year are attributed to antibiotic resistant diseases and this is expected to rise to 10 million by 2050 (IACG 2019). Alongside alarming figures of attributed deaths, antibiotic resistance has a wider bearing on our economy. It is predicted that by 2030, antimicrobial resistance could force up to 24 million people into extreme poverty due to its heavy impact on the economy (IACG 2019). In part, the misuse and over-use of antibiotics are driving factors in emerging resistance (Wright 2014). Around 80% of sore throat cases have a viral aetiology (Mäkelä *et al.* 1998; Ebell *et al.* 2000; Worrall 2007; Worrall 2008) and, even when the infection is bacterial, most cases resolve without antibiotics (Bisno 2001; Pelucchi *et al.* 2012; Spinks *et al.* 2013). The National Institute for Health and Care Excellence (NICE) recommends that no antibiotics be provided for upper and lower respiratory tract infection if the patient is not systemically unwell or at higher risk of complications (NICE 2019). The imprudent use of antibiotics can also decrease human immunity to a level that poses a risk of and increased susceptibility to subsequent disease (Keeney *et al.* 2014). A review of six studies of the common cold and five studies of acute purulent rhinitis suggested that antibiotics do not work for either the common cold or for acute purulent rhinitis and many people are affected by antibiotic side effects (Kenealy and Arroll 2013). Around 75% of all antibiotic prescribing is estimated to take place in the community setting of primary care (Zuckerman *et al.* 2007), of this, respiratory tract infections account for 60% (Tan *et al.* 2008). Nonetheless, the over-prescription of antibiotics still occurs with an estimated 20% of all primary care antibiotic prescriptions being deemed inappropriate (PHE 2017). Additionally, in 2016 it was estimated that 7% of antibiotic consumed in the EU are done so without a prescription (Johnson *et al.* 2018). Greece, Romania and Cyprus are the EU member states with the highest use of antibiotics without prescription, mainly driven by over the counter (OTC) retailing with 80–100% being obtained in a pharmacy (Paget *et al.* 2017). Antibiotic resistance is reported to be higher in countries that have a greater use of antibiotics (EARS 2017). While there is a significant body of evidence on resistance development to systemic antibiotics which are primarily restricted to dispensing with a prescription, very little data exist on topical antibiotics dispensed OTC for conditions like sore throat (Essack *et al.* 2019).

This study aims to (i) understand the development of bacterial resistance in nine common human pathogens to four locally delivered antibiotics (gramicidin, neomycin, bacitracin and tyrothricin) found in some OTC sore

throat medicines, and (ii) measure possible emerging cross-resistance to different classes of antibiotics following during use exposition to these OTC antibiotics.

## Methods

### Test antibiotics

Gramicidin and tyrothricin were purchased in powder form (Xellia Pharmaceuticals ApS, Copenhagen, Denmark), and neomycin and bacitracin were kindly provided by Reckitt Benckiser (Slough, UK). Gramicidin and tyrothricin were initially solubilized in 100% DMSO and then diluted into 50% DMSO; the final test concentration of DMSO was no more than 5% which was shown not to affect bacterial viability (ISO 2006).

### Micro-organisms

Bacteria investigated in this study were as follows: *Staphylococcus aureus* ATCC 6538, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* ATCC 19615, *Acinetobacter baumannii* ATCC 19568, *Enterobacter cloacae* 1781, *Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 10211, *Klebsiella pneumoniae* ATCC 13883 and *Pseudomonas aeruginosa* ATCC 27853. Bacterial cultures were inoculated onto Mueller-Hinton agar (MHA) for 24 h (37°C) and then sub-cultured into Mueller-Hinton broth (MHB) and incubated for another 24 h at 37°C. *Streptococcus pneumoniae* was incubated in 5% CO<sub>2</sub>. All working cultures were centrifuged at 5000 g and re-suspended in tryptone-sodium chloride diluent (TSC; 1 g l<sup>-1</sup> tryptone and 8.5 g l<sup>-1</sup> sodium chloride) and an optical density (OD<sub>600nm</sub>) equivalent to 10<sup>6</sup> CFU per ml was obtained following appropriate dilution in TSC.

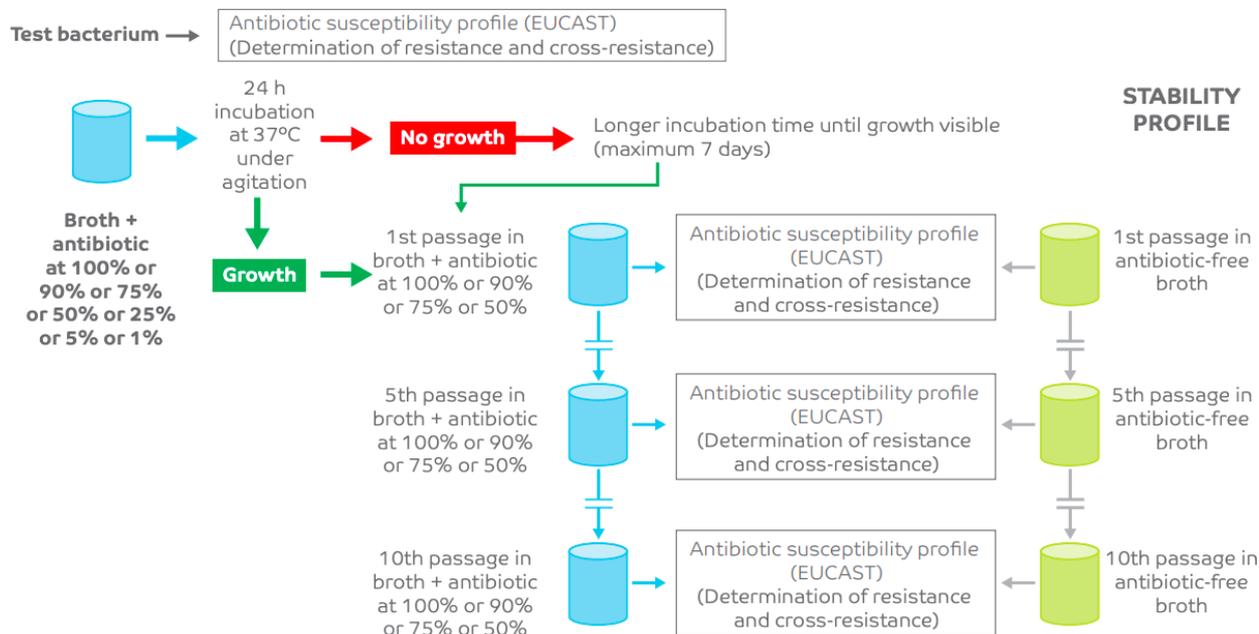
All cultures obtained after antibiotic exposure were frozen and stored at -80°C for further testing. Purity of suspensions and homogeneity were checked using the Gram-staining technique and selective media.

### Test protocol

A simple protocol based on the in-use antibiotic concentrations of the four commercially available OTC antibiotics was used (Figure 1). The in-use concentration of antibiotic is subjected to dilution during use of the product; here a decrease in concentration resulting from the dilution of product in saliva.

### Antibiotic susceptibility testing

Initially, baseline minimal inhibitory concentration (MIC) data were obtained using the ISO [20776-1]



**Figure 1** Protocol to study the impact of antibiotic exposure on emerging resistance following the use of the OTC products.

microdilution broth standard (ISO 2006). Briefly, antibiotics were diluted in MHB in halving concentrations along a 96-well plate. Standardized overnight bacterial cultures ( $10^6$  CFU per ml) were added to each well and incubated for 24 h at 37°C. Despite the inclusion of DMSO, tyrothricin and gramicidin produced a cloudy solution, for this reason, following incubation, visible growth was recorded and 20  $\mu$ l of each well suspension was plated onto an MHA plate, which was incubated for 24 h at 37°C. The MIC was considered the highest concentration in which no visible growth was observed.

#### Antibiotic cross-susceptibility testing

Antibiotic susceptibility was tested with the disc diffusion method in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2019) protocol. Briefly, bacterial suspensions (0.5 McFarland standard) were applied to an MHA plate and spread evenly using a sterile cotton swab (Fisher Scientific, Loughborough, UK). The antibiotic discs (Beckton Dickinson, Wokingham, UK) were placed onto the agar surface and plates were incubated (24 h; 37°C). Following incubation, zone diameters of growth inhibition were recorded and compared to EUCAST breakpoints (EUCAST 2019) to confirm clinical susceptibility. To investigate for antibiotic cross-resistance following exposure to the test

antibiotics, the bacteria were tested against a panel of 6–8 EUCAST recommended antibiotics from different classes (Table 1). Baseline antibiotic susceptibility profiles and clinical resistance status of tested isolates are presented in Table S1.

#### Investigating the ability for bacteria to grow in during-use antibiotic concentration

A number of different concentrations were tested to represent dilutions of antibiotic concentrations when OTC products are used. Examples of brand named medicines containing a range of in-use antibiotic concentrations are provided in Table S2. The selected 'in-use' concentration used in this study represented a compromised concentration used in these products and did not intend to represent each of the product listed. The original in-use concentrations that were subsequently diluted were as follows: gramicidin 3 mg ml<sup>-1</sup>, neomycin 2.5 mg ml<sup>-1</sup>, bacitracin 100 units, tyrothricin 4 mg ml<sup>-1</sup>. Test bacteria were exposed to the antibiotics for an initial exposure time of 24 h at 37°C under constant agitation. If no microbial growth was observed after 24 h, incubation was continued for a maximum of 7 days and the time taken until visible growth was observed as the 'initial exposure time'. If no growth occurred after 7 day exposure, lower concentrations were investigated successively until growth was observed: 90, 75, 50, 25, 10, 5 and 1%.

**Table 1** EUCAST recommended antibiotic discs tested for each strain

Strain	Antibiotics used in EUCAST test
<i>Staphylococcus aureus</i>	Benzylpenicillin, ceftazidime, ciprofloxacin, gentamicin, erythromycin, tetracycline, chloramphenicol, fusidic acid
<i>Streptococcus pneumoniae</i>	Benzylpenicillin, levofloxacin, vancomycin, erythromycin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole
<i>Streptococcus pyogenes</i>	Benzylpenicillin, levofloxacin, vancomycin, erythromycin, tetracycline, chloramphenicol, rifampicin
<i>Acinetobacter baumannii</i>	Trimethoprim, levofloxacin, ciprofloxacin, gentamicin, tobramycin
<i>Enterobacter cloacae</i>	Co-amoxiclav, piperacillin/tazobactam, cefotaxime, imipenem, ciprofloxacin, fusidic acid
<i>Escherichia coli</i>	Co-amoxiclav, piperacillin/tazobactam, cefotaxime, imipenem, ciprofloxacin, fusidic acid
<i>Haemophilus influenzae</i>	Ampicillin, cefotaxime, imipenem, ciprofloxacin, erythromycin, tetracycline, colistin
<i>Klebsiella pneumoniae</i>	Cefpodoxime, ceftazidime, co-amoxiclav, cefotaxime, ciprofloxacin, gentamicin, piperacillin/tazobactam, piperacillin
<i>Pseudomonas aeruginosa</i>	Ciprofloxacin, cefepime, ceftazidime, gentamicin, imipenem, piperacillin, piperacillin/tazobactam, tobramycin

### Phenotype stability for bacteria surviving in-use antibiotic exposure

Following 24 h exposure to the in-use concentration of an antibiotic, surviving bacteria were passaged into tubes containing broth and the antibiotic at the in-use concentration. Briefly, the exposure suspension was centrifuged (5000 g; 10 min) and re-suspended in TSC diluent and an OD<sub>600nm</sub> equivalent to 10<sup>6</sup> CFU per ml was obtained through appropriate dilution. One hundred microlitre of the washed suspension was added to a fresh tube of MHB containing the appropriate concentration of the relevant antibiotic and incubated for 24 h at 37°C. A maximum of 10 passages were performed. MIC and EUCAST tests were conducted after the 1st, 5th and 10th exposures.

## Results

### Antibiotic susceptibility testing

Baseline MIC values were predominantly below the level covered by the in-use concentration of the antibiotics. The exceptions were *S. aureus*, *A. baumannii* and *E. coli* with gramicidin and *K. pneumoniae* with neomycin

(Table 2). Despite the MIC value being lower than the in-use exposure concentration, there were instances of bacterial growth at the in-use concentration. *Pseudomonas aeruginosa* grew at 4 mg ml<sup>-1</sup> (100% of selected in-use concentration) tyrothricin after 24 h incubation. This exposure resulted in a shift in MIC value from 2.5 mg ml<sup>-1</sup> (baseline value) to >5 mg ml<sup>-1</sup> (Table 2). This twofold increase (Table 3) was however no longer observable after five passages both in the presence of 4 mg ml<sup>-1</sup> tyrothricin and broth only. *Klebsiella pneumoniae* and *A. baumannii* grew in the presence of tyrothricin at 4 mg ml<sup>-1</sup> (100% of selected in-use concentration) and 0.4 mg ml<sup>-1</sup> (10% of selected in-use concentration) respectively. These exposures did not result in a change to MIC value after the initial exposure or after 10 passages with or without antibiotic. *Staphylococcus aureus* grew in the presence of 100 U bacitracin (100% of proposed in-use concentration) after 144 h incubation and became less susceptible to the antibiotic with a fourfold increase (Table 3) in MIC from 3.18 U (baseline value) to 125 U (Table 4). *Streptococcus pyogenes* grew in the presence of 0.125 mg ml<sup>-1</sup> (5% of selected in-use concentration) neomycin after 144 h. This exposure resulted in a 125-fold increase of the baseline MIC; from 0.005 mg ml<sup>-1</sup> to 0.63 mg ml<sup>-1</sup> (Table 4). Furthermore, after one passage in the presence of neomycin (0.125 mg ml<sup>-1</sup>), the MIC increased to 25 mg ml<sup>-1</sup>, which was sustained over 10 passages (Table 4). When neomycin was removed and *S. pyogenes* was passaged in broth only, the MIC decreased to 0.16 mg ml<sup>-1</sup> (Table 4), which was still 36-fold higher than the baseline MIC (Table 3). The MIC of 0.16 mg ml<sup>-1</sup> was stable over 10 passages.

### Antibiotic cross-resistance

There were instances where exposure to the in-use concentration of an antibiotic affected the clinical susceptibility profile of different antibiotics from sensitive to resistant (Table 5). *Staphylococcus aureus* became resistant to ciprofloxacin after one passage in bacitracin, this change was not observed at passage 5–10. *Staphylococcus aureus* was resistant to ciprofloxacin, fusidic acid and gentamicin after one passage in 100 U bacitracin, this change was also not observed at passage 5–10. *Klebsiella pneumoniae* became clinically resistant to cefpodoxime, amoxicillin-clavulanic acid and cefotaxime after one passage in 4 mg ml<sup>-1</sup> tyrothricin. This change in susceptibility profile was not observed in passages 5 and 10 when passaged in broth only. However, with the consistent presence of 4 mg ml<sup>-1</sup> tyrothricin *K. pneumoniae* demonstrated stable cross-resistance over 10 passages.

**Table 2** MIC values before (baseline) and after exposure to antibiotics. Refer to the text for selected in-use concentration and percentage exposure time

	Tyrothricin (mg ml <sup>-1</sup> ) (in-use concentration percentage/exposure time)		Gramicidin (mg ml <sup>-1</sup> ) (in-use concentration percentage/exposure time)		Bacitracin (units) (in-use concentration percentage/exposure time)		Neomycin (mg ml <sup>-1</sup> ) (in-use concentration percentage/exposure time)	
	Baseline	Exposure	Baseline	Exposure	Baseline	Exposure	Baseline	Exposure
	<i>Staphylococcus aureus</i>	0.008	NG (1%/168 h)	> <b>30</b>	> <b>30 (100%/144 h)</b>	31.25	<b>125 (100/144 h)</b>	0.002
<i>Streptococcus pneumoniae</i>	<0.001	NG (1%/168 h)	<0.04	NG (1%/168 h)	<0.06	NG (1%/168 h)	<0.003	NG (1%/168 h)
<i>Streptococcus pyogenes</i>	0.002	NG (1%/168 h)	0.000006	NG (1%/168 h)	0.004	NG (1%/168 h)	0.005	0.63 (5%/144 h)
<i>Acinetobacter baumannii</i>	2	2 (10&5%/24 h) *	> <b>30</b>	> <b>30 (100%/24 h)</b>	31.25	<b>125 (5%/24 h)<sup>†</sup></b>	<2.5	<2.5 (5%/24 h) <sup>‡</sup>
<i>Enterobacter cloacae</i>	1	NG (1%/168 h)	15	NG (1%/168 h)	62.5	NG (1%/168 h)	0.006	NG (1%/168 h)
<i>Escherichia coli</i>	1.67	NG (1%/168 h)	> <b>30</b>	NG (1%/168 h)	1.95	NG (1%/168 h)	0.006	NG (1%/168 h)
<i>Haemophilus influenzae</i>	0.03	NG (1%/168 h)	0.004	NG (1%/168 h)	0.98	NG (1%/168 h)	0.003	NG (1%/168 h)
<i>Klebsiellapneumoniae</i>	1.04	0.02 (100%/24 h)	62.5	NG (1%/168 h)	3.91	NG (1%/168 h)	<b>3.12</b>	NG (1%/168 h)
<i>Pseudomonas aeruginosa</i>	2.5	> <b>5 (100%/24 h)</b>	30	<b>30 (100%/24 h)</b>	7.81	NG (1%/168 h)	0.12	NG (1%/168 h)

Bold text represents MIC that are higher than the 'in-use' concentration; text not in bold represents MIC phenotype that was lower than or equal to the 'in-use' concentration.

NG, no recoverable growth after initial exposure after 7 days incubation in 1% of in-use concentration.

\*After growth of *A. baumannii* at 10 and 5% in 24 h, no further growth was observed.

<sup>†</sup>The change in MIC resulting from 24 h exposure of 5% bacitracin occurred in only one of three repeats.

<sup>‡</sup>The change in MIC resulting from 24 h exposure of 5% neomycin occurred in only one of three repeats.

## Discussion

This study established the ability for five common human pathogens (*S. aureus* ATCC 6538, *A. baumannii* ATCC 19568 and *S. pyogenes* ATCC 19615, *K. pneumoniae*, *P. aeruginosa*) to survive a range of concentrations of several antibiotics used in topical OTC sore throat products, and to become less susceptible to these antibiotics and clinically resistant to unrelated ones. Clinical resistance to other antibiotics was demonstrated following exposure to tyrothricin and bacitracin, with resistance sustained after 10 passages following tyrothricin exposure. *Streptococcus pyogenes* is the most common bacterial cause of sore throat illness and is known to be broadly sensitive to penicillin, which is used as a first-line treatment for confirmed infection. The observed development of *S. pyogenes* resistance to neomycin is concerning as this antibiotic is widely used systemically, that is, for intestinal disease (Basseri *et al.* 2011).

The in-use concentration can be defined as the concentration of an active that is applied through disinfection.

The in-use concentration may be diluted throughout the disinfection process leading to the term 'during-use' concentration as discussed in previous works (Knapp *et al.* 2015; Weggate *et al.* 2016). The probability for low concentrations of antibiotic to affect a decrease in susceptibility is a concern as OTC antibiotic medicines commonly encounter a dilution effect due to the amount of saliva and mucus naturally produced in the area of intended application. The uncontrolled and indiscriminate use of locally applied antibiotics like tyrothricin for sore throat is cited to not be of clinical concern due to the absence of resistance (Strauss-Grabo *et al.* 2014); however, the study confirms that as with systemic antibiotics, locally applied antibiotics do lead to the development of antibiotic resistance and more concerningly, cross-resistance to those antibiotics used systemically to treat life-threatening infections. The protocol used in this study cannot reproduce exactly the *in situ* fate of antibiotics in OTC products when taken. Even where lower concentrations were used to mimic product dilution upon usage, contact time between a pathogen and the

**Table 3** Fold changes in MIC after exposure and passage in antibiotics or broth only. Values in bold represents an MIC higher than the baseline MIC

	Fold changes							
	Tyrothricin		Gramicidin		Neomycin		Bacitracin	
	Antibiotic	Broth only	Antibiotic	Broth only	Antibiotic	Broth only	Antibiotic	Broth only
<i>Staphylococcus aureus</i>								
144 h	NT	NT	No change		NT	NT	<b>4</b>	
P1							<b>8</b>	<b>4</b>
P5							<b>&gt;8</b>	<b>2</b>
P10							<b>&gt;8</b>	<b>2</b>
<i>Streptococcus pyogenes</i>								
144 h	NT	NT	NT	NT	<b>125</b>		NT	NT
P1					<b>5000</b>	<b>32</b>		
P5					<b>5000</b>	<b>32</b>		
P10					<b>5000</b>	<b>32</b>		
<i>Acinetobacter baumannii</i>								
24 h	No change		No change		No change		<b>4</b>	
P1					No growth		<b>8</b>	<b>8</b>
P5							<b>8</b>	<b>8</b>
P10							<b>8</b>	<b>8</b>
<i>Klebsiella pneumoniae</i>								
24 h	-52*		NT	NT	NT	NT	NT	NT
P1	<b>2</b>	<b>2.4</b>						
P5	<b>2</b>	<b>2.4</b>						
P10	<b>2</b>	<b>2.4</b>						
<i>Pseudomonas aeruginosa</i>								
24 h	<b>2</b>		No change		NT	NT	NT	NT
P1	No growth							
P5								
P10								

144 h, 144 hour initial exposure; 24 h, 24 hour initial exposure; P1, passage 1; P5, passage 5; P10, passage 10; NT, not tested.

\*Represents a MIC fold lower than the baseline value.

antibiotic *in situ* would be difficult to predict. *In vitro* study relies on setting and controlling set test parameters to improve result reproducibility, and ultimately provide reliable information on a potential risk associated with, in this case, antibiotic usage.

There is a direct link between antibiotic consumption and antimicrobial resistance (Bell *et al.* 2014). At very high concentrations, antibiotics apply a selective pressure that is fatal to susceptible micro-organisms therefore allowing resistant organisms to survive and replicate to form a dominant population (Wistrand-Yuen *et al.* 2018). Even very low (sub-lethal) concentrations of an antibiotic can produce a selective pressure that favours adaption (Gullberg *et al.* 2011, 2014; Liu *et al.* 2011; Sandegren 2014). Transient changes in antibiotic susceptibility, as seen in the case of *S. aureus* resistance to CIP, FA and CN after bacitracin exposure, suggest metabolic regulations that govern defence mechanisms such as efflux mechanisms and changes in the structure of the cell membrane to alter permeability (Tezel and Pavlostathis

2015). Stable changes in susceptibility, however, suggest genetic mutations that confer to newly acquired resistance that may be inherited by future generations (Meyer and Cookson 2010; Tezel and Pavlostathis 2015). The probability for low concentrations of an antibiotic to affect a stable decrease in susceptibility is a concern as OTC antibiotic medicines commonly encounter a dilution effect due to the amount of saliva and mucus naturally produced in the area of intended application.

The lag phase is when micro-organisms react to the environment around them and adapt to stressful influences (Rolfe *et al.* 2012). Here, the expression or resistance mechanisms that aim to decrease the detrimental concentration of an antimicrobial to a level that does not interfere with bacterial growth may be associated with an extended lag phase. The lag phase has been associated with primary metabolic changes that lead to increased drug tolerance (Fridman *et al.* 2014). There were instances where growth while in the presence of antibiotic was observed after just 24 h incubation. It has been

**Table 4** MIC changes after exposure and passage in antibiotics or broth only. Values in bold represents an MIC higher than the baseline MIC

		MIC							
		Tyrothricin (mg ml <sup>-1</sup> )		Gramicidin (mg ml <sup>-1</sup> )		Neomycin (mg ml <sup>-1</sup> )		Bacitracin (U)	
		Antibiotic	Broth only	Antibiotic	Broth only	Antibiotic	Broth only	Antibiotic	Broth only
<i>Staphylococcus aureus</i>									
144 h	NT	NT		No change		NT	NT	<b>125 ± 0</b>	
P1								<b>250 ± 0</b>	<b>125 ± 0</b>
P5				MIC remained >30 ± 0				<b>&gt;250 ± 0</b>	<b>65 ± 0</b>
P10								<b>&gt;250 ± 0</b>	<b>65 ± 0</b>
<i>Streptococcus pyogenes</i>									
144 h	NT	NT		NT	NT	<b>0.63 ± 0.00</b>		NT	NT
P1						<b>25 ± 0</b>	<b>0.16 ± 0.00</b>		
P5						<b>25 ± 0</b>	<b>0.16 ± 0.00</b>		
P10						<b>25 ± 0</b>	<b>0.16 ± 0.00</b>		
<i>Acinetobacter baumannii</i>									
24 h	No change			No change		No change		<b>125 ± 0</b>	
P1						No growth		<b>250 ± 0</b>	<b>250 ± 0</b>
P5								<b>250 ± 0</b>	<b>250 ± 0</b>
P10								<b>250 ± 0</b>	<b>250 ± 0</b>
<i>Klebsiella pneumoniae</i>									
24 h	0.02 ± 0.00			NT	NT	NT	NT	NT	NT
P1	<b>2.08 ± 0.72</b>	<b>2.5 ± 0.0</b>							
P5	<b>2.5 ± 0.0</b>	<b>2.5 ± 0.0</b>							
P10	<b>2.5 ± 0.0</b>	<b>2.5 ± 0.0</b>							
<i>Pseudomonas aeruginosa</i>									
24 h	>5 ± 0			No change		NT	NT	NT	NT
P1	No growth								
P5									
P10									

144 h, 144 hour initial exposure; 24 h, 24 hour initial exposure; P1, passage 1; P5, passage 5; P10, passage 10; NT, not tested.

suggested that shorter lag phases provide a selective advantage (Sleight and Lenski 2007). There were instances where growth whilst in the presence of antibiotic was delayed (i.e. bacitracin and *S. aureus*; neomycin and *S. pyogenes*). It has been inferred that an elongated lag phase provides adaptive survival advantages and promotes regrowth upon the removal of an antibiotic (Li *et al.* 2016).

Our findings question the validity of continued use (inclusion in topical OTC products) of neomycin, due to the extreme stable increase in MIC observed, tyrothricin and bacitracin for their potential to cause stable changes in MIC and cross-resistance to un-related antibiotics. Gramicidin as a topical antibiotic is used for its activity against Gram-positive micro-organisms (Swierstra *et al.* 2016; Berditsch *et al.* 2019), for this reason, *S. aureus* growth and change in antibiotic cross-susceptibility at the concentration of gramicidin used in common practice is concerning. *Staphylococcus aureus* resistance to Gramicidin S has been previously reported and attributed to consolidation and thickening of the cell wall (Orlova *et al.* 2007). These findings compliment the conclusions

of a recent review that found inconclusive evidence that the use of these four antibiotics (bacitracin, gramicidin, neomycin and tyrothricin) for sore throat was beneficial (Essack *et al.* 2019). Furthermore, the review concluded that it was not possible to predict the impact of use on antimicrobial resistance.

The outcome of our study warrants further investigation into the difference between the in-use and during-use antibiotic exposure concentration and their effect on the susceptibility and cross-susceptibility of common pathogens. Furthermore, our findings reinforce concern about the over-use and misuse of antibiotics and provide evidence to support enforcement of the WHO action plan (WHO 2013, 2015, 2018) and the global drive to tackle antibiotic resistance. O'Neill (2016) estimated that in the US and Europe alone, resistant infections cause at least 50 000 deaths per year. In accordance with the WHO action plan (WHO 2015) it is vital that the prescribing of antibiotics is regulated prudently and the public be educated and informed on the risks of self-medication. The consumption of antibiotics needs to be better controlled. Further restriction on the use of these

**Table 5** Clinically relevant antibiotic susceptibility phenotype changes after exposure survival and passage in antibiotics

Passage in	Tyrothricin— <i>Klebsiella pneumoniae</i>		Bacitracin— <i>Staphylococcus aureus</i>	
	Antibiotic	Broth only	Antibiotic	Broth only
Passage at which change was observed*	P1–P10	P1	P1	P1
Antibiotic—Zone diameter (mm) after exposure	Amoxicillin/clavulanate —0 Cefotaxime—0 Cefpodoxime—0	Amoxicillin/clavulanate —0 Cefotaxime—0 Cefpodoxime—0	Ciprofloxacin— 20	Ciprofloxacin— 20 Fusidic acid—23 Gentamicin—17
Antibiotic/Baseline Zone diameter (mm) before exposure	Amoxicillin/clavulanate/25 Cefotaxime/32 Cefpodoxime/31		Ciprofloxacin/26 Fusidic acid/31 Gentamicin/24	
Sensitive breakpoint <sup>†</sup> (mm)	Amoxicillin/clavulanic acid ≥ 19 Cefotaxime ≥ 20 Cefpodoxime ≥ 21		Ciprofloxacin ≥ 21 Fusidic acid ≥ 24 Gentamicin ≥ 18	
Resistant breakpoint <sup>‡</sup> (mm)	Amoxicillin/clavulanic acid < 19 Cefotaxime < 17 Cefpodoxime < 21		Ciprofloxacin < 21 Fusidic acid < 24 Gentamicin < 18	

\*P1 = change first observed at passage 1 and not observed after that; P1–P10 = change first observed at passage 10 and still present at passage 10.

<sup>†</sup>Breakpoints based on EUCAST (2019).

antibiotics in OTC medicine for sore throat seem necessary to manage antimicrobial resistance and to reduce it as much as possible.

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## Conflict of interest and authors' contributions

Evangelista, R. Atkinson, A. Shepard and O. Adegoke are employees of Reckitt Benckiser. All authors have significantly contributed to the protocol design and redaction of this manuscript.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Baseline antibiotic susceptibility profiles (zone of inhibition and clinical resistance status) of tested isolates according to EUCAST (2019) breakpoints ( $n = 3$ ).

**Table S2.** Examples of OTC antibiotic in-use concentrations.