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Exposure to sub-inhibitory concentrations of gentamicin, ciprofloxacin and cefotaxime induces multidrug resistance and reactive oxygen species generation in meticillin-sensitive *Staphylococcus aureus*

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Abstract

Purpose. The role of antibiotics below their MIC in the development of bacterial drug resistance is becoming increasingly important. We investigated the effect of sub-MICs of bactericidal antibiotics on the susceptibility pattern of *Staphylococcus aureus* and evaluated the role of free radicals.

Methodology. A total of 12 *S. aureus* strains were recovered from pus samples and their antibiograms determined. The test isolates were treated with sub-MIC levels of tetracycline, gentamicin, ciprofloxacin and cefotaxime. Alterations in their respective breakpoints were observed along with measurements of free radical generation by nitro blue tetrazolium test.

Results/Key findings. Gentamicin, ciprofloxacin and cefotaxime exposure significantly altered the breakpoints of exposed isolates against several tested antibiotics and higher levels of free radicals were generated after antibiotic exposure.

Conclusions. Our study demonstrates that sub-MIC levels of antimicrobials can lead to resistance and cross-resistance across several classes of antibiotics in wild strains of *S. aureus*, possibly by free radical production. The molecular mechanisms behind the acquisition of drug resistance at low antibiotic concentrations and the specific target genes of reactive oxygen speciesneed to be explored further.

INTRODUCTION

Antibiotics are lifesaving drugs and one of the most precious discoveries in the field of medicine. Unfortunately, today we stand at the brink of the antibiotic era due to multidrug-resistant bacteria harbouring several antibiotic resistance genes. The principal causes of this catastrophic situation are transferable drug resistance, inadvertent antibiotic exposure, selection pressure and mutagenesis. Therefore, understanding the dynamics of development of antibiotic resistance is indispensable. Although the selection of drug-resistant mutants at high antibiotic concentrations is a well-known fact, the role of antibiotics below their MIC has only recently gained importance in this context. Sub-MICs may be defined as concentrations that allow susceptible strains to grow, but at a slower rate as compared to the growth in drug-free media [1]. Interestingly, antibiotics in sub-MICs are commonly found in livestock, the environment and the food industry, through which they can easily gain access to humans [2]. In addition, inappropriate dosing, poor compliance and use of low quality drugs often result in the presence of low concentrations of antibiotics in our system. Generation of reactive oxygen species (ROS) by bacteria exposed to sub-inhibitory antibiotic doses has been demonstrated in a few studies as the underlying mechanism for acquisition of multidrug resistance [3–5]. In the present study, we have aimed to investigate the effect of sub-MICs of bactericidal antibiotics, i.e. tetracycline, gentamicin, ciprofloxacin and cefotaxime, on the susceptibility pattern of meticillin-sensitive *Staphylococcus aureus* (MSSA) and its correlation with free radical generation in bacteria.

METHODS

Selection of MSSA isolates

Pus samples were collected from patients with wound infections without any history of recent antibiotic intake. Pus was cultured aerobically for 48 h, on 5 % sheep blood agar (SBA),

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Keywords: meticillin-sensitive *Staphylococcus aureus*; sub-inhibitory antibiotics; antibiotic resistance; reactive oxygen species.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MAR, multiple antibiotic resistance; MSSA, meticillin-sensitive *Staphylococcus aureus*; NBT, nitro blue tetrazolium; ROS, reactive oxygen species.

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nutrient agar and MacConkey's agar media (HiMedia). *S. aureus* isolates were identified by Gram-staining, catalase and coagulase tests. Further strain confirmation was carried out by using a Vitek2 Compact system (bioMériuex). The colony morphology and Gram-stain character of the identified strains were noted. The isolates were tested for *mecA*-mediated oxacillin resistance by the disc diffusion method using a cefoxitin disc (30 µg) on Mueller–Hinton agar (MHA) plates [6] and 12 MSSA strains were selected for the study.

MIC determination of the antibiotics under study

Four antibiotics, namely tetracycline, gentamicin, ciprofloxacin and cefotaxime (HiMedia), belonging to distinct classes and having different mechanisms of action were selected for investigation. MIC values, defined as the lowest concentrations at which no growth was visible, were determined for the aforesaid drugs against each of the 12 *S. aureus* isolates and an ATCC strain (*S. aureus* ATCC 29213) by using the broth microdilution method [7]. Stock solutions of the four antibiotics (2.048 mg ml^{-1} in water) were prepared from the respective pure compounds obtained from HiMedia. The first dilution for the measurement of MIC was achieved by diluting the stock solutions four times in Mueller–Hinton broth (MHB; HiMedia), attaining a value of 256 µg ml^{-1}. Serial dilutions were further made in MHB in 96-well micro-titre plates to obtain 12 concentrations in the range of 0.125–256 µg ml^{-1}. Broth culture of the test strain containing 0.5 McFarland (~1×10^8 c.f.u. ml^{-1}) inoculum density was inoculated in each well at a 1 : 10 ratio to maintain a final density of ~1×10^7 c.f.u. ml^{-1}, except for a 'contamination control row'. After incubation for 18 h at 37 °C, the optical density (OD) was measured at 630 nm by using an ELISA reader (ErbaLisaScan II; Transasia) to determine the MIC values [6, 8]. The OD_{630} of the 'blank' row showing no growth served as a 'contamination control' for the procedure. The tests, as well as the readings, were performed in duplicate to check reproducibility of results.

Exposure of the strains to sub-inhibitory antibiotic concentrations

Each of the 12 *S. aureus* isolates was exposed to its respective sub-inhibitory concentrations (0.25×MIC) of the four antibiotics, i.e. tetracycline, gentamicin, ciprofloxacin and cefotaxime [9]. A set of the same test isolates incubated in drug-free media was employed as a control. The stock solutions of these antibiotics were diluted in peptone water to obtain the strain-specific sub-MICs of the drugs. A single colony of each isolate from the agar plates was suspended in the antibiotic solutions and incubated aerobically at 37 °C for 4 h. The tests were performed in duplicate and the results compared to avoid any bias due to selection of a resistant mutant while choosing single colonies from the culture plates. The OD_{630} was adjusted to slightly higher than 0.5 McFarland to counterbalance for any cell loss. Following incubation, the solution was washed and the supernatant discarded to free the bacterial cells of any residual antibiotic. Finally, the cell deposit was suspended in peptone water at a concentration of 0.5 McFarland units.

Antibiogram of the tested strains with and without antibiotic exposure

The antibiogram for the MSSA test strains with and without drug exposure was determined under similar conditions by Kirby–Bauer disc diffusion method. The antibiotic susceptibility pattern was determined in duplicate against azithromycin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, cotrimoxazole, gentamicin, linezolid and tetracycline (Becton Dickinson). The results were interpreted following Clinical and Laboratory Standards Institute (CLSI) guidelines [6] and *S. aureus* ATCC 29213 was used as standard for the entire experiment. This provided the susceptibility patterns of the test isolates following sub-MIC antibiotic treatment as well as those of the untreated cells (controls). The zones of inhibition of the test strains were compared with those of the controls and alterations in breakpoints were noted.

The multiple antibiotic resistance (MAR) indices for both antibiotic-treated cells and controls were separately calculated using the formula [10]:

$$\text{MAR index for an antibiotic} = \frac{\text{No. of antibiotic–resistant isolates}}{\text{No. of antibiotics tested} \times \text{No. of isolates}}$$

Estimation of free radicals in *S. aureus* cell suspensions

Modified colorimetric nitro blue tetrazolium (NBT) assay was performed to evaluate the extent of ROS generation by the test and control groups of *S. aureus* [11]. Briefly, bacterial suspensions of 0.5 McFarland standard from test and control groups were centrifuged at 500 ***g*** for 10 min and washed. The bacterial pellet was then suspended in PBS (200 µl) and freshly prepared NBT (0.01 %) was added to this solution. The mixture was incubated for 45 min at 37 °C, following which it was washed again (500 ***g*** for 10 min). As a result, blue water-insoluble intracellular formazan crystals were produced from the yellow water-soluble tetrazolium salt in the presence of bacterial superoxide anions. The quantity of formazan crystals within each cell was proportional to the production of free radicals [12]. In order to evaluate the formazan product, the bacterial cell pellet was dissolved in KOH solution (2 M, 60 µl in DMSO). The resultant colour reaction was measured spectrophotometrically on a microplate reader at 630 nm. The tests and readings were performed in duplicate to exclude technical errors.

Statistical analysis of free radical generation

To determine the statistical significance of the difference in free radical generation in antibiotic-treated and -untreated *S. aureus* cell suspensions, we performed Student's *t*-test, taking $P\leq0.05$ as significant.

RESULTS

Characteristics of tested bacteria

Twelve strains of *S. aureus* were isolated from pus collected from six male and six female patients with stitch infections (n=5), ulcers (n=2), wound infections (n=4) and umbilical stump infection (n=1). The patients were aged between

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2 days and 55 years. The colonies on solid media were round, opaque, 1–2 mm, with entire edges, and cream to golden yellow in colour, producing β haemolysis on 5 % SBA. Catalase and coagulase tests were positive. The antibiogram patterns of the isolates were obtained by using the Kirby–Bauer disc diffusion test, as depicted in Table 1. The MICs of the four bactericidal antibiotics under investigation (tetracycline, gentamicin, ciprofloxacin and cefotaxime) were obtained in the range of 0.125–128 μg ml^{-1} (Table 1).

Effect of sub-MIC antibiotic exposure on the susceptibility pattern of bacteria

The isolates expressed notable shifts in breakpoints after 4 h of exposure to sub-MIC antibiotic concentrations. For instance, on exposure to a sub-inhibitory concentration of cefotaxime, a ciprofloxacin-sensitive isolate (SA1) was found to exhibit resistance to the same drug when tested under similar conditions using the Kirby–Bauer method. Treatment with a sub-inhibitory concentration of cefotaxime demonstrated a remarkable effect on the gentamicin susceptibility of *S. aureus*, where 7 out of 12 test strains were observed with an altered breakpoint, consequently developing resistance. Cefotaxime treatment also induced resistance towards azithromycin in susceptible wild strains. Similarly, sub-MICs of ciprofloxacin and gentamicin exposure led to acquisition of gentamicin, ciprofloxacin, clindamycin and azithromycin resistance in several tested isolates. No breakpoint change was observed against tetracycline, cefoxitin, linezolid, chloramphenicol and cotrimoxazole in any of the 12 isolates after pretreatment with any of the antibiotics. The results are demonstrated in Table 2, Fig. 1. Thus, different classes of bactericidal antibiotics at their respective sub-inhibitory concentrations exercised distinct effects on the susceptibility of a particular isolate towards the tested drugs. The overall MAR indices were higher in antibiotic-treated cells in comparison to untreated *S. aureus* strains, and maximum MAR index was obtained in gentamicin- exposed strains (Fig. 2).

Effect of sub-MIC antibiotic exposure on ROS generation in bacteria

In an NBT assay for semi-quantitative ROS detection, higher optical density values were obtained in the *S. aureus* cells treated with sub-MIC levels of antibiotic exposure in comparison to their untreated counterparts (Fig. 3). Significantly (P<0.05) greater numbers of free radicals were generated in cells treated with cefotaxime, gentamicin and ciprofloxacin than the untreated controls. The bacterial cells did not display any significant variation in the amount of ROS generation after treatment with tetracycline (Fig. 3).

DISCUSSION

Sub-MIC antibiotic concentration and its relation with acquired drug resistance

S. aureus is a major human and animal pathogen responsible for a myriad of superficial and deep suppurative infections as well as various toxin-mediated diseases [7]. It is also a component of normal human microbiota inhabiting anterior nares and skin of people working in healthcare settings [13]. This convenient colonization in healthcare workers aids the spread of the organism, often leading to nosocomial outbreaks [14, 15]. Of greater importance is the ability of the bacteria to develop antibiotic resistance and tolerance, subsequently ending in treatment failures. Sporadic and epidemic cases of infection with meticillin-resistant *S. aureus*,

Table 1. Antibiograms and MICs of *S. aureus* isolates without sub-inhibitory antibiotic exposure

Isolate number	Antibiogram*									MIC (μg ml^{-1})†			
	Tet	Gen	Cip	Cef	Lin	Clin	Chl	Cot	Azi	Tet	Gen	Cip	Cex
SA1	S	S	S	S	S	S	S	S	S	0.5	0.25	0.5	1
SA2	S	S	R	S	S	S	S	R	R	0.5	4	8	2
SA3	S	S	R	S	S	S	S	S	R	0.25	0.25	16	2
SA4	S	S	R	S	S	S	S	S	S	0.25	0.25	8	1
SA5	S	S	S	S	S	S	S	R	S	1	0.5	1	2
SA6	S	S	S	S	S	S	S	S	S	1	0.5	0.25	0.5
SA7	S	S	S	S	S	S	S	S	S	1	0.25	1	0.5
SA8	S	S	R	S	S	S	S	R	R	1	1	128	2
SA9	S	S	R	S	S	S	S	R	R	1	0.125	32	4
SA10	S	S	R	S	S	S	S	R	S	0.25	0.25	128	2
SA11	S	R	S	S	S	S	S	R	R	4	64	1	4
SA12	S	S	R	S	S	S	S	S	R	4	0.25	128	2

*Antibiograms of test isolates were determined by using the Kirby–Bauer disc diffusion method against tetracycline (Tet), gentamicin (Gen), ciprofloxacin (Cip), cefoxitin (Cef), linezolid (Lin), clindamycin (Clin), chloramphenicol (Chl), cotrimoxazole (Cot) and azithromycin (Azi). 'S' represents sensitive and 'R' resistant against the individual antibiotics.

†MICs of *S. aureus* strains against pure compounds of four bactericidal antibiotics; tetracycline (Tet), gentamicin (Gen), ciprofloxacin (Cip), cefotaxime (Cex) were tested by using the broth microdilution method.

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Table 2. Breakpoint change in *S. aureus* isolates after antibiotic exposure

The column headings show the sub-MIC antibiotics to which the *S. aureus* isolates (denoted by SA1–12) were exposed and the first column denotes the antibiotics tested by using the Kirby–Bauer method. The table demonstrates the changes in breakpoint observed in individual isolates after treatment with antibiotics (shaded cells). For explanation of the antibiotic abbreviations, see footnotes Table 1.

Breakpoint change (S→R)	Antibiotic (0.25×MIC)			
	Tetracycline	Gentamicin	Ciprofloxacin	Cefotaxime
Tet	Nil	Nil	Nil	Nil
Gen	SA4 (*n*=1)	SA4, SA5, SA10 (*n*=3)	SA5, SA9, SA10, SA12 (*n*=4)	SA2, SA3, SA4, SA5, SA6, SA9, SA10 (*n*=7)
Cip	SA1 (*n*=1)	SA5 (*n*=1)	SA1, SA5, SA7 (*n*=3)	SA1, SA5, SA7 (*n*=3)
Cef	Nil	Nil	Nil	Nil
Lin	Nil	Nil	Nil	Nil
Clin	Nil	SA4, SA5, SA7, SA8, SA10 (*n*=5)	SA3 (*n*=1)	Nil
Chl	Nil	Nil	Nil	Nil
Cot	Nil	Nil	Nil	Nil
Azi	SA1, SA4 (*n*=2)	SA1, SA4, SA5, SA7 (*n*=4)	SA1, SA5, SA7 (*n*=3)	SA1 (*n*=1)

vancomycin-resistant *S. aureus* and even linezolid-resistant *S. aureus* have been reported worldwide [16–20].

In this study, we selected 12 MSSA isolates obtained from pyogenic infections. The strains were susceptible to most of the tested antibiotics with the exception of ciprofloxacin, cotrimoxazole and azithromycin (Table 1).

Selection of resistant mutants in an environment of antibiotic excess has been cited as the most important cause of the current crisis that we are going through. Thus, the mutant selective window hypothesis (antibiotic concentrations between the MIC of the susceptible wild-type population and the MIC of the resistant population) has received attention as the primary reason behind the rise of drug-resistant mutants. However, the impact of antibiotics below MIC levels in the evolution of resistant strains is yet to be entirely delineated. Some previous studies have demonstrated preferential expansion of the resistant subpopulations of *S. aureus* when grown in sub-MICs of antibiotics [3, 21–23]. The role of sub-MIC drug exposure is indeed more alarming because low concentrations of antibacterial drugs are abundant in the environment, livestock, food industry, sewage and humans. The source of the drugs is varied, ranging from naturally occurring antibiotics produced by fungi and bacteria to synthetic drugs manufactured by the pharmaceutical industry [1]. Accidental and deliberate release of these drugs into soil and water bodies provides a conduit to human and animal ecological systems. In addition, use of antibiotics in agriculture, pisciculture, animal husbandry, veterinary and human therapeutics leads to a persistence of low levels of antibiotics in the environment. Therefore, there is at hand an optimal atmosphere of sub-MIC drugs which effectively selects and enriches the population of mutant bacteria that gradually gain resistance to commonly used antibiotics. In our study, the strains acquired novel resistance for clindamycin, azithromycin, gentamicin and ciprofloxacin after treatment with sub-inhibitory bactericidal antibiotics of four distinct classes.

Effect of sub-MIC of ciprofloxacin

Sub-MIC treatment with ciprofloxacin, a second-generation flouroquinolone, was associated with development of gentamicin (*n*=4), azithromycin (*n*=3), ciprofloxacin (*n*=3) and clindamycin (*n*=1) resistance (Table 2, Fig. 1). A recent study by Yim *et al.* [24] demonstrates that flouroquinolones at sub-MIC levels are potent activators of stress responses in *Escherichia coli* and *Salmonella* Typhi, resulting in up-regulation of several SOS and virulence genes, altered metabolism, membrane permeability, mutagenesis and antibiotic resistance [24]. Flouroquinolone exposure can lead to favourable *gyrA* mutations and activate several multidrug efflux pumps in clinical isolates of bacteria including *S. aureus*. Furthermore, non-quinolone antibiotics can promote persistence of resistance-bearing plasmid-mediated quinolone resistance genes and decreased susceptibility to fluoroquinolones [4]. As all these outcomes occur at non-inhibitory drug concentrations, it can be anticipated that a significant array of unexpected effects may occur during the course of fluoroquinolone therapy.

Effect of sub-MIC of cefotaxime

On sub-MIC exposure to cefotaxime, a beta lactam antibiotic, 7 out of the 12 isolates developed resistance to gentamicin, in addition to ciprofloxacin (*n*=3) and azithromycin (*n*=1; Table 2). Although use of gentamicin as a solo therapeutic agent in infections with Gram-positive cocci is not recommended, it is often employed in combination with beta lactam antibiotics [6]. Accordingly, induction of gentamicin resistance by cefotaxime will be an unforeseen complication in patient management by physicians employing this combination. Kohanski *et al.* have illustrated that the use of sub-inhibitory beta lactams also induces the SOS response via RecA-mediated processes, whence on

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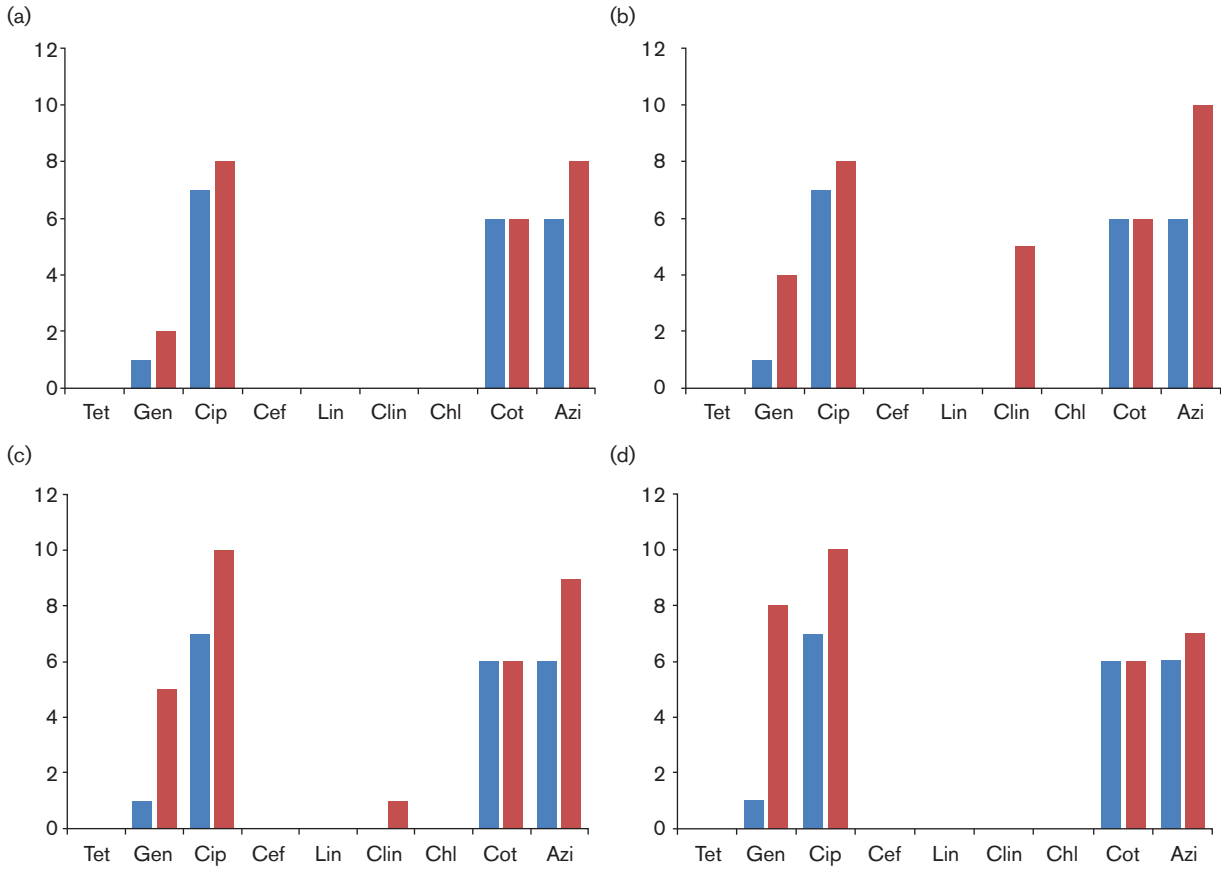

Fig. 1. Comparative picture of the susceptibility pattern of tested isolates without any antibiotic exposure (blue) and that of the same strains tested after treatment with sub-inhibitory levels of antibiotics (red) viz. tetracycline (a), gentamicin (b), ciprofloxacin (c) and cefotaxime (d). The x-axis shows the drugs comprising the antibiogram and the y-axis denotes the number of resistant isolates. The breakpoint changes were observed for gentamicin, ciprofloxacin, clindamycin and azithromycin, while no alteration was obtained for tetracycline, cefoxitin, linezolid, chloramphenicol and cotrimoxazole.

treatment with low levels of ampicillin, wild-type *E. coli* acquired resistance to ampicillin, norfloxacin, kanamycin, tetracycline and chloramphenicol [3, 25]. An *in vivo* study performed by McVicker *et al.* in zebra fish embryos illustrated that pre-existing resistant strains were preferentially selected on treatment with sub-curative doses of tetracycline and oxacillin [23]. Additionally, *de novo* generation of resistance mutations in susceptible isolates has also been reported to expand the resistant bacterial population [26].

Effect of sub-MIC of gentamicin

Gentamicin is an aminoglycoside that inhibits protein synthesis by binding to the 30S subunit of bacterial ribosome. In this investigation, sub-inhibitory concentrations of gentamicin led to the development of clindamycin (n=5), azithromycin (n=4), gentamicin (n=3) and ciprofloxacin (n=1) resistance in the tested MSSA isolates (Table 2, Fig. 1). Emergence of small colony variants of *S. aureus* exhibiting aminoglycoside resistance after gentamicin exposure has been demonstrated previously [27]. They are auxotrophic with altered growth, metabolism and virulence [13]. Sub-lethal exposure to kanamycin, another aminoglycoside, has been implicated in inducing cross-resistance to other aminoglycosides as well as resistance to tetracycline and ampicillin [28].

Effect of sub-MIC of tetracycline

Effect of sub-MIC tetracycline exposure was not especially noteworthy (Table 2, Fig. 1). Although previous reports have depicted low-level tetracycline as a potential inducer of resistance, our study could not significantly corroborate the earlier findings [23].

The MAR index of the isolates was also higher after antibiotic treatment (Fig. 2), which can be used to predict the possibility of acquiring multidrug resistance upon low levels of drug exposure. The increase of MAR index was principally due to azithromycin, ciprofloxacin, clindamycin and gentamicin. Azithromycin and clindamycin are the primary drugs (group A CLSI) used for treatment of staphylococcal infections; hence development of resistance to these drugs is detrimental to therapeutic recovery. Gentamicin is used only in combination with a beta lactam agent; hence, as already explained above,

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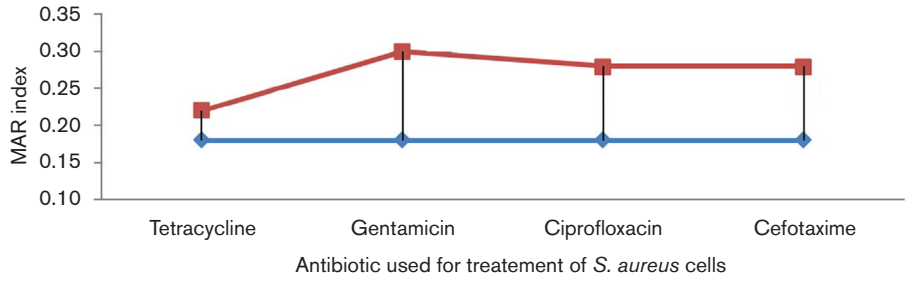

Fig. 2. Multiple antibiotic resistance (MAR) indices of *S. aureus* isolates pre (blue) and post (red) sub-MIC antibiotic exposure. The MAR index is given by the formula: (No. of antibiotic-resistant isolates)/(No. of antibiotics tested×No. of isolates). The numerator for the untreated isolates is 20 and after exposure due to increase in the number of resistant isolates, the numerator increased accordingly (tetracycline, 24; gentamicin, 33; ciprofloxacin, 31; cefotaxime, 31). Nine antibiotics and 12 isolates were tested.

the rise in resistance after antibiotic exposure will adversely affect patient disease outcome. Although ciprofloxacin is included as a supplemental drug (group C CLSI) in *S. aureus* infections, its importance cannot be overlooked. Most skin and soft tissue infections are mixed, with more than one bacterium isolated, and it is very difficult to determine the primary pathogen. As flouroquinolones have a broad spectrum of activity, they are often used to target both Gram-negative and -positive organisms, which make them liable to acquire resistance. Also, fluoroquinolones are the most common over-the-counter drugs that people consume without any knowledge of the aetiological organism (if any) causing their symptoms. Hence, the rates of resistance to fluoroquinolones are very high in both nosocomial and community settings, which makes testing these drugs preferable in both Gram-negative and Gram-positive infections [6].

Mechanisms inducing drug resistance in low antibiotic concentrations

The present investigation revealed that breakpoint changes were primarily observed in antibiotics acting at the level of DNA replication and translation (azithromycin, clindamycin, gentamicin and ciprofloxacin; Table 2, Fig. 1). Thus, intervention in bacterial replication and protein synthesis appears to be the major site of altered gene expression and resistance acquisition. Previous studies have stated that altered protein synthesis is a major mechanism by which low levels of antibiotics induce resistance in *S. aureus*, either directly or by inducing virulence, via biofilm formation and quorum sensing [29–31]. Similarly, DNA damage due to SOS response or altered gene expression as well as biofilm formation can be attributed to flouroquinolone resistance in *S. aureus* exposed to sub-MIC levels of drugs [29, 32].

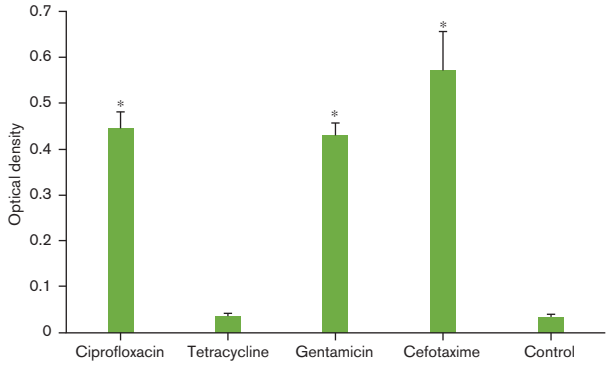

Fig. 3. Generation of free radicals (measured in terms of optical density) in *S. aureus* isolates after treatment with sub-inhibitory concentrations of antibiotics. Data presented as mean±SD. *P<0.05 is considered as significant difference compared to the untreated control group (confidence interval, CI 95 %).

Mechanisms that induce drug resistance in low antibiotic concentrations are still not completely understood, mainly due to the diverse types of mutations produced in bacteria at such concentrations. Selection of pre-existing mutants, which is the chief mechanism of drug resistance in strains exposed to high antibiotic levels, is one of the major mechanisms. An exhaustive review by Anderson and Hughes [1] detailed the various effects of exposure to low levels of antibiotics on bacteria. Sub-MIC levels of antibiotics provoke RecA- or LexA-mediated SOS responses in bacteria, giving rise to horizontal gene transfer and recombination via prophages, integron incorporation and transposition. In addition, stress-induced, sigma factor-based (RpoS, SigB) mutagenesis is also observed in exposed cells [1].

Antibiotic exposure and ROS generation

Free radical generation is a common mechanism through which bactericidal antibiotics kill target bacteria. Recent studies have illustrated a positive correlation between high ROS generation following low levels of antibiotic exposure and development of antibiotic resistance [3, 5, 25, 33]. In this investigation, we have observed significantly higher levels of free radical generation by bacterial cells after sub-inhibitory cefotaxime, gentamicin and ciprofloxacin exposure as compared to their untreated counterparts (Fig. 3). This substantiates the role of ROS generation after treatment with the aforementioned antibiotics in bestowing drug resistance among bacteria. Although the sample size is insufficient to make a strong conclusive statement, the

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observations from this investigation point towards a significant hypothesis that should alarm health professionals and warrants communication to fellow researchers. Pertaining to the current antibiotic crisis and constant streaming of these drugs into the environment, this study highlights the disastrous effects of sub-MIC levels of antibiotics on the susceptibility patterns of common pathogens. Kohanski *et al.* [3] illustrated an increase in MIC after exposure of bacteria to sub-MIC antibiotic levels via free radical-mediated mechanisms while observing no such alteration in anaerobic environment or in the presence of reducing substances. A study by Li *et al.* [5] demonstrated that sub-lethal vancomycin treatment may induce protective ROS generation in heterogeneous vancomycin-resistant *S. aureus*. Thus, the free radicals generated in low antibiotic levels induce various degrees of oxidative damage and the ensuing mutations in bacterial cells give rise to drug resistance. Kohanski *et al.* [3] found point mutations in the genes responsible for flouroquinolone (*gyrA*) and aminoglycoside (*rspL*) resistance in bacterial cells pre-treated with sub-MIC antibiotics. Additionally, activation of common efflux pumps via radical-induced mutagenesis may be responsible for the evolution of multidrug-resistant strains [3].

Conclusion

Our study demonstrates that sub-MIC levels of bactericidal antimicrobials can lead to resistance and cross-resistance across several classes of antibiotics in wild strains of *S. aureus*. We have also found that generation of free radicals is probably one of the chief mechanisms responsible for the development of resistance. Thus, low levels of drugs in our ecological and biological system can enhance multidrug-resistant strains which may also disseminate resistance genes to wild strains. However, the molecular mechanisms behind the acquisition of drug resistance at low antibiotic concentrations and the specific target genes of ROS need to be explored further. Importantly, antibiotic stewardship must not only be confined to healthcare systems but also extend to veterinary, food and pharmaceutical industries to combat the global drug resistance crisis.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was conducted in accordance with institutional ethical guidelines and ethical clearance was obtained. Informed consent was obtained from all participating subjects with assurance of confidentiality.

References

1. **Andersson DI, Hughes D.** Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014;12:465–478.
2. **Nikolić N, Mirecki S, Blagojević M.** Presence of inhibitory substances in raw milk in the area of Montenegro. *Mljekarstvo* 2011;61:182.
3. **Kohanski MA, Depristo MA, Collins JJ.** Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* 2010;37:311–320.
4. **Redgrave LS, Sutton SB, Webber MA, Piddock LJ.** Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol* 2014;22:438–445.
5. **Li GQ, Quan F, Qu T, Lu J, Chen SL *et al.*** Sublethal vancomycin-induced ROS mediating antibiotic resistance in *Staphylococcus aureus*. *Biosci Rep* 2015;35:e00279.
6. **CLSI.** *Performance Standards for Antimicrobial Susceptibility Testing;* Twenty-Fifth Informational Supplement. CLSI Document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
7. **Collee JM, Fraser AG, Marmion BP, Simmons A (editors).** *Mackie & McCartney Practical Medical Microbiology*, 14th ed. India: Elsevier; 1996.
8. **Dey D, Ray R, Hazra B.** Antitubercular and antibacterial activity of quinonoid natural products against multi-drug resistant clinical isolates. *Phytother Res* 2014;28:1014–1021.
9. **Maioli E, Marchese A, Roveta S, Cagnacci S, Cavallini F *et al.*** *In vitro* activity of ceftibuten at sub-inhibitory concentrations in comparison with other antibiotics against respiratory and urinary tract pathogens. *J Chemother* 2007;19:152–160.
10. **Kawane RS.** Studies on antibiotics and heavy metal resistance profiling of *Escherichia coli* from drinking water and clinical specimens. *Biosci Discovery* 2012;3:292–295.
11. **Tunc O, Tremellen K.** Oxidative DNA damage impairs global sperm DNA methylation in infertile men. *J Assist Reprod Genet* 2009;26:537–544.
12. **Esfandiari N, Sharma RK, Saleh RA, Thomas AJ, Agarwal A.** Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. *J Androl* 2003;24:862–870.
13. **Peacock SJ.** *Staphylococcus*. In: Borriello PS, Murray PR and Funke G (editors). *Topley and Wilson's Microbiology and Microbial Infections*, 10th ed. Washington, DC: ASM Press; 2005.
14. **von Eiff C, Becker K, Machka K, Stammer H, Peters G.** Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. *N Engl J Med* 2001;344:11–16.
15. **Davis KA, Stewart JJ, Crouch HK, Florez CE, Hospenthal DR *et al.*** Methicillin-resistant *Staphylococcus aureus* (MRSA) nares colonization at hospital admission and its effect on subsequent MRSA infection. *Clin Infect Dis* 2004;39:776–782.
16. **McDonald M.** The epidemiology of methicillin-resistant *Staphylococcus aureus*: surgical relevance 20 years on. *Aust N Z J Surg* 1997;67:682–685.
17. **Tsiodras S, Gold HS, Sakoulas G, Eliopoulos GM, Wennersten C *et al.*** Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet* 2001;358:207–208.
18. **Conly JM, Johnston BL.** VISA, hetero-VISA and VRSA: the end of the vancomycin era? *Can J Infect Dis* 2002;13:282–284.
19. **Appelbaum PC.** The emergence of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 2006;12:16–23.
20. **Iguchi S, Mizutani T, Hiramatsu K, Kikuchi K.** Rapid acquisition of linezolid resistance in methicillin-resistant *Staphylococcus aureus*: role of hypermutation and homologous recombination. *PLoS One* 2016;11:e0155512.
21. **Trzcinski K, Cooper BS, Hryniewicz W, Dowson CG.** Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2000;45:763–770.
22. **Liu A, Fong A, Becket E, Yuan J, Tamae C *et al.*** Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. *Antimicrob Agents Chemother* 2011;55:1204–1210.

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23. **Mcvicker G, Prajsnar TK, Williams A, Wagner NL, Boots M *et al.*** Clonal expansion during *Staphylococcus aureus* infection dynamics reveals the effect of antibiotic intervention. *PLoS Pathog* 2014;10: e1003959.
24. **Yim G, Mcclure J, Surette MG, Davies JE.** Modulation of *Salmonella* gene expression by subinhibitory concentrations of quinolones. *J Antibiot* 2011;64:73–78.
25. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007;130:797–810.
26. **Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L *et al.*** Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* 2011;7:e1002158.
27. **Massey RC, Buckling A, Peacock SJ.** Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. *Curr Biol* 2001;11:1810–1814.
28. **Chen LX, He S, Li C, Ryu J.** Sublethal kanamycin induced cross resistance to functionally and structurally unrelated antibiotics. *J Exp Microbiol Immunol* 2009;13:53–57.
29. **Li D, Renzoni A, Estoppey T, Bisognano C, Francois P *et al.*** Induction of fibronectin adhesins in quinolone-resistant *Staphylococcus aureus* by subinhibitory levels of ciprofloxacin or by sigma B transcription factor activity is mediated by two separate pathways. *Antimicrob Agents Chemother* 2005;49:916–924.
30. **Goerke C, Köller J, Wolz C.** Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006;50:171–177.
31. **Otto MP, Martin E, Badiou C, Lebrun S, Bes M *et al.*** Effects of subinhibitory concentrations of antibiotics on virulence factor expression by community-acquired methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2013;68:1524–1532.
32. **Ubeda C, Maiques E, Knecht E, Lasa I, Novick RP *et al.*** Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol* 2005;56:836–844.
33. **Dwyer DJ, Kohanski MA, Collins JJ.** Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* 2009; 12:482–489.

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