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Development of disinfectant tolerance in *Klebsiella pneumoniae*

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SUMMARY

Background: Disinfectants are a critical infection control measure that are relied upon globally in a range of settings including healthcare, food production, and domestic environments. However, bacteria have been shown to survive disinfectant treatments when harboured in dry surface biofilms or when disinfectants are used ineffectively. This provides an opportunity for organisms to develop low-level tolerance to various disinfectants. The capability of bacteria to develop adaptations to non-antibiotic antimicrobial agents is often overlooked.

Aim: To report on the capability and readiness of clinically relevant *K. pneumoniae* to adapt to common disinfectants that are relied upon every day across the world, delivering much-needed insights into an often-overlooked aspect of antimicrobial resistance.

Methods: This study investigated the ability of *Klebsiella pneumoniae* NDM-1 strain NCTC 13443 to adapt to a range of common chemical disinfectants (benzalkonium chloride, didecyldimethylammonium chloride, polyhexamethylene biguanide, chlorocresol and bronopol) via serial passage exposure method.

Findings: After long-term adaptation, *K. pneumoniae* developed tolerance to all tested disinfectants, exhibiting a minimum inhibitory concentration increase of between 30 and 413% compared with the untreated parent samples. Characterization of disinfectant cross-tolerance showed that while cross-tolerance can occur, most adapted samples became more susceptible to the second disinfectant treatment, probably because of the fitness cost of adaptation. Observed cross–tolerance/collateral sensitivity was not always reciprocated between disinfectant-tolerant samples.

Conclusions: Results suggest the order of disinfectant exposure is important during tolerance development. This has significant implications for disinfectant cleaning routines, and is probably due to variations in the underpinning tolerance mechanisms, even when the disinfectants display similar mechanisms of action.

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Introduction

The widespread use of chemical disinfectants during the COVID-19 pandemic has highlighted our dependence on these agents for infection control, which is only likely to be reinforced with the rising prevalence of antimicrobial resistance (AMR). While AMR is typically associated with antibiotics, research has repeatedly found bacteria to be able to develop adaptations that reduce their susceptibility to the action of disinfectants [1], including species such as *Klebsiella pneumoniae* [2], *Staphylococcus aureus* [3,4] and *Pseudomonas aeruginosa* [5].

For the purpose of this study, tolerance is defined as the ability for a bacterial population to survive transient exposure of an otherwise lethal concentration of disinfectant, while resistance is an inherited property that is unaffected by the duration of exposure [6]. Typically, the development of tolerance or resistance through adaptation can only occur if bacteria survive long enough to mount a response. As the 'at use' concentration of disinfectants is typically many orders of magnitude above any given minimum inhibitory concentration (MIC), exposure of healthcare-associated infections (HCAIs) to sub-MIC concentrations of disinfectant is assumed to be improbable, and tolerance is unlikely to develop.

However, the efficacy of chemical disinfectants relies upon their correct usage, with external factors such as the presence of organic load [7,8], dilution factor [9,10] and exposure time [9] having a significant impact. Recent studies have highlighted the risk of dry surface biofilms within medical environments providing a reservoir of HCAIs [11], even after cleaning with disinfectants [8,12]. Bacteria harboured in this state can survive disinfectant exposure, providing further opportunities for adaptation development, facilitating further biofilm buildup. In addition, research has been exploring the efficacy of disinfectants and surface coatings with residual or persistent antimicrobial activity [13,14]. While the results of these studies are encouraging, any surviving bacteria will probably have undergone prolonged exposure to non-lethal concentrations of disinfectant, providing a significant opportunity for adaptations to develop.

These examples suggest that bacteria may be routinely unintentionally exposed to non-lethal concentrations of disinfectants, and are therefore being given an opportunity to survive, adapt, and develop tolerance or resistance. Such concerns have led to calls to introduce stewardship of disinfectants [15,16], alongside contributing to regulatory changes in the USA. As chemical disinfectants are critical to our current approach to infection control, the risk of bacteria being able to mitigate the efficacy of these agents is a serious issue.

In this study we elucidate the ability for a clinically relevant strain of *K. pneumoniae* to develop tolerance to a range of common chemical disinfectants. The characteristics and mechanisms of action of the disinfectants used in this study are overviewed in Supplementary Table A1. The disinfectant adaptation experiments were performed with no fixed time limit to investigate the theoretical adaptation limit and simulate a 'worst case' scenario. *K. pneumoniae* tolerance to disinfectants has been established in clinical and environmental isolates, with decreased susceptibilities to chlorhexidine [17], iodophor [18] and benzalkonium chloride (BAC) [19] reported. Short-term *in vitro* experiments have demonstrated *K. pneumoniae* tolerance to chlorhexidine [20], BAC [19], and polyhexamethylene biguanide (PHMB) in combination with betaine [4]. However, the ability for *K. pneumoniae* to adapt to didecyldimethylammonium chloride (DDAC), chlorocresol, bronopol and PHMB in isolation has not previously been evaluated. As disinfectant products and cleaning procedures utilized in healthcare environments often utilize a variety of disinfectants [21], cross-tolerance profiles of adapted samples are also quantified.

Methods

Bacterial strains and growth media

K. pneumoniae NCTC 13443 was selected due to its clinical relevance. Samples were cultured in 10 mL Mueller–Hinton Broth (MHB) (Thermo Scientific) overnight at 37 °C. Initial bacterial stocks were standardized to 5×10^5 cfu/mL.

Stock solutions of antimicrobial compounds

Antimicrobial compounds were selected based on their widespread use. Their characteristics are summarized in supplementary Table A1. BAC (Thor Specialities Limited), DDAC (Thor Specialities Limited), PHMB (Thor Specialities Limited) and bronopol (Thor Specialities Limited) were made up to a stock concentration of 10,000 μg/mL in ddH_2O immediately before testing. Chlorocresol (Lanxess Limited) was made up to a stock concentration of 10,000 μg/mL in dimethylsulphoxide (Corning) immediately before testing.

Disinfectant adaptation via serial passage

Bacterial samples were serially passaged at increasing concentrations of disinfectant in 200 μL volumes. Each volume consisted of 160 μL MHB, 20 μL disinfectant and 20 μL bacterial stock. The disinfectant concentrations used in passage one were selected to be below the respective MICs [21]. All disinfectants used an initial starting concentration of 1 μg/mL, except chlorocresol which began at 20 μg/mL. The concentration of each respective disinfectant increased in increments of 1 μg/mL on each subsequent passage, except chlorocresol which increased in increments of 20 μg/mL.

Samples were incubated for 24 h at 37 °C before visual inspection for growth. As the growth outcome of each passage was unknown, four concentrations of disinfectant were inoculated in parallel as follows: the same concentration of disinfectant as the previous passage, one increment higher, one increment lower, and no disinfectant. To inoculate the following passage, only the well containing the highest concentration of disinfectant that demonstrated growth was used. All other wells were not used further. Any leftover wells in the 96-well plate were used as sterility controls.

The daily passages continued at increasing disinfectant concentrations until samples were unable to demonstrate growth in the well containing the highest concentration of disinfectant. Passages were then continued for 15 days to allow further tolerance to develop and validate the stability of the existing tolerance, before the experiment was concluded. If samples developed the ability to grow at the next highest

increment of disinfectant during this time, the experiment was continued until the next breakpoint was reached. This process was repeated until the samples could no longer develop further tolerance over 15 subsequent passages. Adaptation to each disinfectant treatment was conducted on the same 10 biologically independent replicates.

Contamination checks were performed weekly by plating aliquots of each sample on to Mueller–Hinton agar and CHROMagar™ Orientation chromogenic agar to confirm that the samples were *K. pneumoniae*. Any contaminated samples were disposed of, and the experiment was repeated from the beginning.

Disinfectant cross-tolerance

Disinfectant cross-tolerance profiles of three disinfectant-tolerant *K. pneumoniae* biological replicates were identified via the determination of BAC, DDAC, PHMB, chlorocresol and bronopol MICs through the microdilution method as previously described [21]. The percentage increase in MIC for each sample was calculated and arranged into a heatmap using GraphPad Prism 9.4.1.

Results

Disinfectant adaptation via serial passage

Adapted *K. pneumoniae* samples were able to survive prolonged exposure to otherwise lethal concentrations of disinfectant, showing a 30–413% higher MIC than the parent samples (Table I). For a visualization of the progression of tolerance development over time, see Supplementary Figure A1. Small, temporary variations in the rate of tolerance development were seen between biological replicates but no variation was seen in the final tolerance across all 10 biological replicates in all conditions. *K. pneumoniae* tolerance to BAC, DDAC, PHMB and bronopol developed rapidly, with the parent sample MIC being exceeded on passages 23, 9, 9 and 11, respectively. Disinfectant tolerance occasionally collapsed in various samples, with growth only visible in the well containing no disinfectant. However, in all cases the tolerance was recovered in subsequent passages.

Disinfectant cross-tolerance

The percentage change in MIC between non-tolerant parent samples and tolerant samples (after tolerance development) are displayed as a heatmap in Figure 1. Raw MIC values are available in Supplementary Table A2.

The cross-tolerance profiles of disinfectant-tolerant *K. pneumoniae* samples varied, with MIC percentage change values ranging from -91.7% to 233.3%. Of the 20 possible disinfectant combinations, five demonstrated cross-tolerance, 12 showed reduced tolerance (collateral sensitivity), and three showed no change. PHMB-tolerant sample 3 and bronopol-tolerant sample 3 displayed cross-tolerance profiles that contrasted the other biological replicates.

The highest level of cross-tolerance was displayed by BAC-tolerant samples exposed to DDAC, showing a 150–233.3%

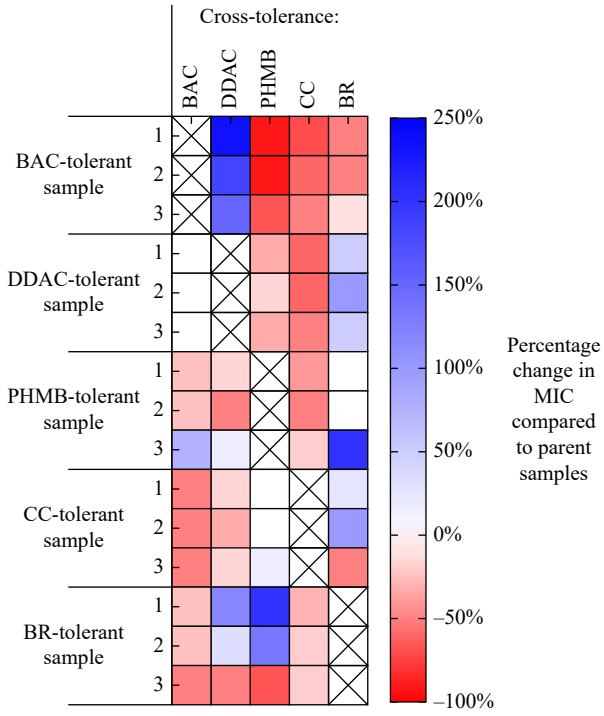

Figure 1. Cross-tolerance of disinfectant-tolerant samples of *Klebsiella pneumoniae* NCTC 13443 to other common disinfectants. Colour gradient represents the percentage change in minimum inhibitory concentration of the tolerant samples compared with the untreated parent samples, with blue and red indicating an increase or decrease in minimum inhibitory concentration, respectively. BAC, benzalkonium chloride; BR, bronopol; CC, chlorocresol; DDAC, didecyldimethylammonium chloride; PHMB, polyhexamethylene biguanide.

Table I
Minimum inhibitory concentration (MIC) values of common disinfectants against *Klebsiella pneumoniae* NCTC 13343 before and after disinfectant tolerance development

Disinfectant	Disinfectant class	MIC (μg/mL)		MIC increase (%)
		Parent samples	Tolerant samples	
Benzalkonium chloride	Cationic surfactant (QAC)	20	56	180
Didecyldimethylammonium chloride	Cationic surfactant (QAC)	6	14	133
Polyhexamethylene biguanide	Cationic polymeric biguanide	6	9	50
Chlorocresol	Phenol-derivative	200	260	30
Bronopol	Halogen-nitro	8	41	413

QAC, quaternary ammonium compound. $N = 10$.

increase in DDAC MIC after BAC adaptation. In contrast, the most significant increase in susceptibility was shown by BAC-tolerant samples exposed to PHMB, demonstrating a ~91.7% decrease in PHMB MIC. Interestingly, DDAC-tolerant samples were only ~33.3% more susceptible to PHMB after adaptation, despite the near-identical mechanism of action (MOA) to BAC.

PHMB tolerance led to a ~25% increase in susceptibility to BAC and DDAC in two out of the three biological replicates tested. The third biological replicate contrasted this, instead showing low-level cross-tolerance to the membrane active quaternary ammonium compounds (QACs), with a 75% and 16.7% reduced susceptibility to BAC and DDAC respectively.

Chlorocresol-tolerant *K. pneumoniae* samples displayed a 50% and ~15% increased susceptibility to BAC and DDAC respectively, but a 0%–13.3% decrease in susceptibility to PHMB. BAC, DDAC and PHMB-tolerant *K. pneumoniae* samples all demonstrated an increase in susceptibility to chlorocresol of between 20%-70%. Bronopol-tolerant samples displayed a ~25% increase in susceptibility to chlorocresol. However, chlorocresol-tolerant samples demonstrated a wide range of lower susceptibilities to bronopol, ranging from 25% to 200%.

Discussion

Upon sequential exposure to increasing concentrations of disinfectants over long periods of time, *K. pneumoniae* samples were readily able to develop adaptations to all tested disinfectants (Table I). The lack of variability in the final adapted MICs across biological replicates in all respective conditions indicates that the samples reached the limit of adaptation within the confines and parameters of the methodology. It also suggests similarity in the underpinning adaptations that were developed across biological replicates.

All adaptations were maintained for 15 passages, suggesting that the populations were resistant rather than demonstrating transient tolerance. As further genetic characterization of the samples will be required to confirm if adaptations are genetic resistance or sustained phenotypical adaptations, the samples are provisionally classified as 'tolerant'.

Known bacterial adaptations to QACs include modification of membrane lipid composition [22,23], up-regulation of broad-spectrum efflux pumps [19,22] and down-regulation of porins [22]. Despite near-identical MOAs, the final limit of sustainable tolerance varied between the two QACs (Table I), suggesting variations in the underpinning tolerance mechanisms resulting from differences in the chemical properties of the compounds.

BAC-tolerant samples showed high cross-tolerance to DDAC, as expected (Figure 1). The lower MIC increase after DDAC adaptation and lack of cross-tolerance to BAC indicates that the requirements for DDAC tolerance are more stringent and unique compared with BAC tolerance. This probably results from the compounds having different chemical properties, resulting in variability in the efficacy of adaptations. More generally, the varying cross-tolerance profiles show that cross-tolerance relationships between disinfectants are not automatically reciprocated, even when the MOAs are similar. Therefore, the order in which disinfectants are applied has a significant impact on potential tolerance development.

The mechanism of action of PHMB relies upon sequestering anionic lipids within biological membranes, disrupting bilayer organization and membrane function, leading to bacterial cell death [24]. *K. pneumoniae* was able to sustain a 50% increase in MIC after a total of 92 days of PHMB acclimatization (Table I). Higher concentrations of PHMB (a 133% increase in MIC) could be repeatedly tolerated on individual passages but were not sustainable for 15 consecutive passages (Supplementary Figure A1). *K. pneumoniae* can therefore repeatedly adopt a high PHMB-tolerant phenotype transiently, such as during cleaning routines.

Small-scale changes in *Escherichia coli* PHMB tolerance have been attributed to a reduction in anionic phospholipids in the outer leaflet [25]. More recently, López-rojas *et al.* demonstrated that two *K. pneumoniae* strains were unable to develop tolerance to PHMB when used in combination with betaine [4]. Our contrasting results suggest that *K. pneumoniae* is able to develop tolerance to PHMB in the absence of betaine, provisionally suggesting that multiple disinfectants in combination may impede tolerance development.

Despite MOA similarities between PHMB and the QACs, the adaptations that underpin BAC and DDAC tolerance in *K. pneumoniae* cause sensitivity to the MOA of PHMB, and vice versa (Figure 1). We hypothesize that this increased susceptibility is a result of the fitness cost of the adaptations developed by *K. pneumoniae* samples leaving them more vulnerable to the activity of the second disinfectant. Further molecular characterization of any developed adaptations will be required to investigate this further.

Recently, PHMB has been suggested to act via the binding and condensing of nucleic acids instead of membrane perturbation [26], unlike QACs. The variability in the cross-tolerance profiles of PHMB-tolerant biological replicates suggests variability in the possible mechanisms of PHMB tolerance, indicating multiple varying MOAs. Significant differences in the MOA of these disinfectants would also explain the increase in susceptibility observed in PHMB-adapted samples exposed to QACs and vice versa.

Of the disinfectants tested, chlorocresol provided the most significant challenge for *K. pneumoniae* tolerance development with a final MIC percentage increase limited to 30% (Table I). Chlorocresol is a phenol-derivative, operating via the disruption of the permeability barrier and inducing the leakage of low-molecular-weight material [27,28]. Tolerance to chlorocresol has not been reported previously, but tolerance to other phenolic disinfectants has been documented in various species *in vitro*. Underlying mechanisms remain poorly characterized, but suggested adaptations include removal of disinfectant via increased efflux pump activity [29] and limiting penetration of phenolic disinfectants via changes in lipid [30] and lipopolysaccharide (LPS) content [31].

The cross-tolerance profiles of chlorocresol-tolerant samples displayed an increased susceptibility to QACs (Figure 1). Similarly, QAC and PHMB-adapted samples were more susceptible to chlorocresol, suggesting variation in underpinning tolerance mechanisms despite all compounds acting on bacterial membranes. This collateral sensitivity reflects the fitness cost of adaptation. However, chlorocresol-tolerant samples demonstrated a 0%–13.3% decrease in susceptibility to PHMB. Similarly, bronopol-tolerant samples demonstrated a ~25% increased susceptibility to chlorocresol, while chlorocresol-tolerant samples demonstrated a decreased susceptibility to bronopol. These data further highlight that cross-tolerance relationships are not automatically reciprocated and vary depending on the order of exposure.

K. pneumoniae was readily able to develop bronopol tolerance, with a MIC 413% higher after adaptation (Table I). Previous experiments have reported an inability of bacteria to develop tolerance to bronopol after 12 and 20 passages, respectively [32,33], although the conflicting findings can be accounted for by variations in the methodology and test organisms.

The degree to which *K. pneumoniae* was readily able to adapt to bronopol was unexpected, as the disinfectant is known to operate via two distinct mechanisms: the cross-linking of primary amines in protein structures [34], and the generation of reactive oxygen species [35]. The two mechanisms together provide a harsh selection pressure, thus *K. pneumoniae* samples must have developed mechanisms to deal with both aspects of the antimicrobial activity. Additional investigations will be required to characterize this further.

The disparity in cross-tolerance profiles between the bronopol-tolerant biological replicates indicate significant variations in the possible underpinning tolerance mechanisms (Figure 1). This discrepancy in cross-tolerance profiles between biological replicates was also seen in PHMB-tolerant samples, which has also been suggested to operate via multiple MOAs. Collectively, these data indicate that having multiple MOAs provides bacteria with a greater number of potential routes to mitigate activity and develop tolerance.

In conclusion, we hereby demonstrated that *K. pneumoniae* can readily develop tolerance to common disinfectants. The developed adaptations can then confer cross-tolerance to other disinfectants used for infection control. While adapted *K. pneumoniae* did not demonstrate tolerance sufficient for survival at disinfectant concentrations at the point of use, this is still alarming. Previous studies have shown that bacteria can survive cleaning routines in healthcare settings when they are sheltered in dry surface biofilms [8,12] or when disinfectants are used ineffectively [7—10]. In practice, this provides an opportunity for tolerance or resistance to develop, facilitating increased bacterial survival, further biofilm development, reduced cleaning efficacy and ultimately higher risk of HCAI spread.

Interestingly, our results also show that adaptation to disinfectants often causes collateral sensitivity to other disinfectants. This is probably a result of the fitness cost of disinfectant adaptation, with bacteria needing to invest significant resources to adapt to a given antimicrobial compound. This limits the ability of cells to respond to subsequent treatments, leaving them more susceptible.

The nature of the cross-tolerance/susceptibility profiles varies depending on the disinfectants used and the order in which they were applied. Cross-tolerance variation even occurred between disinfectants that operate via the same MOA such as BAC and DDAC, indicating that the underlying tolerance mechanisms are probably significantly different. The cross-tolerance profiles of bronopol and PHMB-tolerant samples also varied between biological replicates, probably due to the multiple MOAs enabling multiple possible approaches for bacteria to develop tolerance. These data have significant implications regarding cleaning routines in healthcare environments, as different combinations of disinfectants can either promote or mitigate the development of tolerance, and ultimately the efficacy of disinfectant use.

This study raises new research questions, including the need to identify the underlying mechanisms that allow *K. pneumoniae* to readily develop tolerance so consistently. In future studies, the permanence of the adapted tolerance can be elucidated through passaging the samples in the absence of disinfectant, while specific tolerance mechanisms can be identified through detailed molecular characterization. In addition, while the disinfectants herein are among the most commonly used in healthcare settings, future research should be performed to investigate a broader range of clinically relevant disinfectants, alongside other HCAI-causing bacteria.

Collectively, this information provides a critical contribution to our understanding of the disinfectants we depend on for infection control every day across the world. With the ever-increasing prevalence of AMR, our reliance on existing infection control measures will only be reinforced. This study reports quantifiable data regarding the capability and readiness of clinically relevant *K. pneumoniae* to develop tolerance to common disinfectants, delivering much-needed novel insights into an often-overlooked aspect of AMR.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2024.11.006.

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