

Antibiotic Resistance in Environmental Bacteria

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Abstract

Antibiotics can enter the environment through sources such as wastewater, agricultural runoff, and human and animal waste. Especially when present at low concentrations that do not kill bacteria, they can affect bacterial growth and may contribute to the development and spread of antibiotic resistance. This review observes how prolonged exposure to sub-lethal or sub-MIC antibiotic concentrations affects bacterial growth and resistance development. Findings from previous studies were compared, focusing on growth curves, changes in lag phase, growth rate and final density, changes in MIC, resistance mutations, and horizontal gene transfer (HGT). The studies showed that the response to sub-lethal antibiotic exposure was not always the same. Some antibiotics delayed bacterial growth, mainly by increasing the lag phase, while certain low concentrations also produced higher growth rates or final densities than the antibiotic-free control, suggesting a hormetic effect. Resistance was found to develop through the selection and accumulation of mutations in some studies, while others showed that sub-MIC exposure increased the transfer of existing resistance genes through HGT. Overall, the findings suggest that low antibiotic concentrations can put enough pressure on bacteria to favour resistance while still allowing them to survive and reproduce. This makes environmental antibiotic pollution a concern because resistance can develop or spread outside clinical settings. Future research should use longer exposure periods, more antibiotics and bacterial strains, and more realistic environmental conditions.

I. Introduction

Antibiotic resistance is the ability of bacteria to survive and grow even when exposed to antibiotics that would usually inhibit their growth or mitigate them. Some of the ways this resistance develops in bacteria are: genetic mutations, horizontal gene transfer, and adaptive physiological changes.

Sub-lethal concentrations of antibiotics are those concentrations which are too low to outright kill the bacteria, and hence can lead to the development of antibiotic resistance. The development of antibiotic resistance can make it harder to treat or stop the growth of bacteria using antibiotics. Sub-lethal concentrations or sub-MIC concentration matter and are of importance as they can greatly affect the bacterial physiology, horizontal gene transfer, biofilm formation, and influence mutation and adaptation in bacteria.

There are three ways in which such low concentrations of antibiotics arise in the environment: wastewater, runoff, and agricultural use.

- 1) Wastewater : Antibiotics are consumed in large volumes across the world among both humans and animals. A large amount of these drugs pass through human and animal bodies unmetabolised and enter the wastewater as such through urine and excreta. Wastewater treatments can reduce the concentrations of these antibiotics and dilute them after which these low concentrations of drugs enter water bodies like lakes, ponds etc.
- 2) Agricultural use: Livestock bred and raised on farms also require large amounts of antibiotics for health or growth. Through wastes and manure, when these wastes are used as natural fertilizer for agricultural use, these drugs seep into the soil and enter the environment.
- 3) Run-off: When rainfall or irrigation passes over the fields with antibiotic laden fertilizers and manure, it causes agricultural run off.

Over the years it has become evident that low concentrations of antibiotics can directly affect the rate of emergence of antibiotic resistance in a bacterial population. These sub-inhibitory concentrations of antibiotics can trigger multiple biological responses in bacteria such as mutation rate changes, horizontal gene transfer and biofilm formation.

- 1) Biofilm Formation : Bacteria in biofilms are embedded in a protective matrix which is extracellular. Within these biofilms, bacteria may enter dormant states or even activate stress responses which makes them less prone to antibiotics. This leads to persistent infections that are difficult to treat.

- 2) Mutation rate changes: Lethal doses of antibiotics can select for pre-existing resistant enzymes. While sub-lethal doses can do that, they can also favor the emergence of new resistant determinants by increasing the mutation rate and their spread through the stimulation of the horizontal gene transfer. Sub-lethal levels of many antibiotics damage DNA or stall replication, activating bacterial stress networks like the SOS response. This network switches on error-prone DNA polymerases (such as Pol IV and Pol V) that intentionally lower replication fidelity to help the cell survive, inadvertently generating high rates of random mutations.
- 3) Horizontal Gene Transfer: Unlike vertical transfer of genetic information where the information passes down through inheritance i.e from parents to offspring, Horizontal gene transfer can lead to a bacterium acquiring antibiotic gene resistance from another bacterium where it already exists, without requiring the bacterium to develop the resistance mechanism. While mutation requires the bacterium to undergo a genetic change to develop resistance, in HGT the mechanism may already exist in another bacterium and can be transferred.

II. Existing Research

Existing research shows that bacteria evolve resistance under sub-lethal exposure via two main routes: de novo mutation during prolonged low-level exposure and horizontal gene transfer (conjugation) promoted by sub-MIC antibiotics.

- 1) de novo Mutation: When a genetic mutation takes place for the very first time in an organism after any alteration, it is called de novo mutation. It is not inherited by the organism from the parent, however it can be passed down to future generations i.e to offsprings.
In a recent study, researchers exposed *Klebsiella pneumoniae* to progressively increasing sub-inhibitory concentrations of Cephalothin for over 14 days. The concentration at the beginning was about 0.5 µg/mL and was gradually increased to 7.5 µg/mL.
It was observed that the bacteria population gradually developed high resistance to Cephalothin, despite not acquiring a conventional β-lactamase resistance gene.
When whole genome sequencing was done, mutations in several genes were identified. This leads to the fact that low-level exposure of *K. pneumoniae* to Cephalothin for a prolonged period of time gave rise to mutations and genetic variations, which proved to be an advantage as it gave rise to the progressive development of resistance in the bacteria against Cephalothin.
- 2) Horizontal gene transfer (conjugation): When a resistance gene is transferred from a bacterium with the gene existing in it to a bacterium without that gene, it is known as Horizontal Gene Transfer. When the transfer is done through a plasmid i.e when a plasmid containing the resistance gene is transferred from one bacterium to another, it is called conjugation.
A strain of *Klebsiella pneumoniae*, a drug with multiple resistance genes, was studied in 2022 along with *E. coli* bacteria while both were exposed to sub- MIC concentrations of meropenem, ciprofloxacin, cefotaxime and amikacin to observe the effects of sub-MIC exposure of these antibiotics on the conjugation transfer and frequency from *Klebsiella pneumoniae* to *E. coli*. The results showed that the exposure to sub-MIC concentrations lead to an increase of the conjugation frequency. For instance it was observed that ciprofloxacin increased the conjugation frequency significantly with one increase observed being nine times the original frequency. It is imperative to note that the results weren't the same for all concentrations and antibiotics. Post the sub-MIC exposure, there was an observed increase in the expression of genes which were involved in machinery and facilitation of conjugative transfer.

These observations and findings lead to the below possibilities about the effect of sub-lethal exposure of antibiotics:

- It affects the bacterial physiology and its gene expression.
- It can lead to the generation of resistance genes in a bacteria.
- It can increase the conjugative frequency of a bacteria.

III. Research Gap

The studies till now have contributed significantly and are of immense importance to the research going on. They have shown how sub-MIC concentrations of antibiotics affect bacterial physiology, lead to HGT techniques and affect the gene expression of bacteria. However, there are some limitations to these studies that need to be addressed as they can impact further insights regarding the topic. Most of these experiments and studies use bacterial strains that have antibiotic-resistance genes or even are clinically resistant, making it harder to

understand how resistance might develop in a previously susceptible population. Also, a large number of studies examine exposure over relatively shorter periods i.e usually only 24–48 hours. While these experiments might give clarity on how the resistance initially develops, they do not usually reflect the gradual changes that can take place when bacteria is exposed to low levels of antibiotics over longer periods, as can happen in our environment. Differences in how sub-MIC concentrations and bacterial responses are measured also make it difficult to compare the findings of different studies.

This shows a gap in research about what happens when a non-resistant environmental bacterium is exposed to low levels of antibiotics for a longer period of time. Although resistance can develop through both de novo mutations and horizontal gene transfer (HGT), relatively few studies have looked at both of these mechanisms together while also tracking how bacterial growth changes over time. Following the growth of the bacteria along with changes in antibiotic resistance could help show not only whether resistance develops, but also how it develops. Studying these changes over a longer period could give a better understanding of how continuous, low-level antibiotic exposure in the environment may contribute to the development and spread of antibiotic resistance.

All this leads to a very important question- how does prolonged exposure to sub-MIC concentrations of a commonly used antibiotic influence the bacterial growth (which can be understood with the help of growth curves) or lead to the development of resistance (through either HGT or de novo mutation) in an environmental bacteria isolate? The expected pattern would be that bacterial growth is initially reduced when first exposed to the antibiotic, as the concentration places stress on the bacterial population. On continued exposure and repeated growth, however, the population may gradually adapt, leading to improved growth compared to the initial response. At the same time, repeated exposure is expected to increase the likelihood that antibiotic resistant variants become more common through natural selection acting on newly arising mutations. If transferable resistance determinants are available in the surrounding bacterial population, HGT may also contribute to the emergence or spread of resistance.

IV. Methodology

Design/Approach

A progressive exposure approach was implemented by the authors for the experiments in order to observe resistance development and its mechanisms. Exposure was performed by gradually raising the concentration of an antibiotic in the subsequent passages during the experiment with simultaneous maintenance of an antibiotic-free control population under the same conditions. This allowed the researchers to differentiate between changes due to passages and sub-culturing and those resulting from exposure. For example, bacterial cells underwent progressive exposure to cephalothin. For this, bacterial cells were cultured using a continuous exposure method with increased cephalothin concentrations during the 14-day experiment. Cells transferred to a new culture medium with the increased concentration during exposure, while an antibiotic-free control population was maintained separately under the same conditions. By doing this, researchers could observe resistance development and changes in bacterial characteristics with continuous exposure.

Exposure was prolonged to observe the gradual changes in the bacterial population rather than focusing on changes at the beginning of the experiment. Moreover, the starting concentration and increases in concentration were selected based on the susceptibility of the tested strain. Therefore, the continuous exposure allowed resistance development to occur as a result of prolonged exposure to gradually increased antibiotic concentrations. Finally, bacterial growth was assessed during the exposure to observe the changes occurring in the population as it becomes more resistant to the antibiotic under prolonged exposure. Continuous exposure was a better option to observe resistance development and its mechanisms since it realistically simulated a prolonged treatment scenario. In addition, a control population without antibiotic exposure allowed the researchers to assess changes that occurred specifically due to exposure. The researchers could compare the control population to the exposed one and observe how exposure affected bacterial resistance and characteristics.

Data Collection

Bacterial growth was estimated by measuring the optical-density (OD) values at certain time points during the experiment. By performing measurements on a regular schedule, the researchers could obtain growth curves reflecting changes in the bacterial population over time. Growth curves, along with similar curves for the control population, enabled the researchers to compare growth characteristics, such as lag phase, growth rate and cell density at the stationary phase. Theophel et al. used an online kinetic assay to estimate changes in the 96-well plate containing bacteria under study. The researchers measured cell density in the wells at specified time points during the 48-hour measurement period. As a result, the study obtained growth curves that helped identify the effect of antibiotic exposure on bacterial growth. The authors observed that the lag phase was a sensitive indicator of exposure since changes in the lag phase duration were more correlated with exposure concentrations for certain antibiotics than other parameters, such as maximum growth rate and cell density.

In addition to growth measurements reflected in growth curves, resistance mechanism observations included MIC measurements at various stages of the experiment. Estimation of the MIC for each stage of the experiment allowed the researchers to observe the trends in resistance development during prolonged exposure. Therefore, the changes in MIC, as well as bacterial growth curves, could be used to determine the effect of prolonged exposure on bacterial resistance.

V. Data Analysis

Growth curve analysis consisted of evaluating the characteristics of growth, such as the lag phase, growth rate, and cell density at the stationary phase, and comparing them to the control population. The parameters were selected based on the analysis of growth curves reflecting bacterial growth at different exposure concentrations. In addition to visual analysis, growth curve data can be used to calculate lag time, growth rate, carrying capacity, and other parameters using mathematical models. These parameters can then be compared between the studied populations using statistical analysis, such as ANOVA. Such analysis allows determining whether the observed differences are statistically significant.

Analysis of changes in resistance mechanisms was performed by comparing the MIC of the bacterial population at different stages of the experiment. An increase in MIC indicates resistance development. In addition, when gene transfer was studied, the frequency of gene transfer in the populations before and after exposure could be compared using statistical analysis to determine whether exposure affected gene transfer frequency.

VI. Results

Primary Findings

The growth trend of the bacteria varied depending on the antibiotic, with sub-lethal exposure causing a delay in the start of bacterial growth in some cases with the lag phase being longer than the antibiotic-free control. However, the bacteria didn't necessarily show reduced growth; in some instances (fosfomycin, daptomycin, fusidic acid, cefoxitin, and gentamicin), the bacteria displayed higher maximum growth rates and/or final optical density than the control. This indicated that for some antibiotics, at sub-lethal concentrations, the bacteria could show a growth-stimulation or hormetic effect despite the delay in growth.

Amongst the different growth parameters, the lag phase appeared to be the most informative for indicating the effect of increasing antibiotic concentrations. Unlike the maximum growth rate and final cell density, the lag phase increased more distinctly for some of the antibiotics tested. This highlighted the importance of observing the complete growth curve in addition to the final optical density in order to assess the effects of sub-lethal antibiotic exposure.

However, it should be noted that, whilst the study looked at changes in the growth and susceptibility patterns of the bacteria, the emergence of resistance was not necessarily followed for several generations. Therefore, the study cannot conclude that resistance emerged solely through mutation, horizontal gene transfer (HGT) or a combination of both. As this study looked primarily at the effects of sub-lethal antibiotic exposure on changes in bacterial growth and susceptibility patterns, but not the emergence of resistance, its relevance to the review is the demonstration that sub-lethal antibiotic exposure can cause various changes in the growth pattern of bacteria, amongst them the delayed growth as well as the possibility of growth stimulation at low concentrations.

Group/Category Comparisons

When comparing the different concentration groups, it was evident that the development of resistance followed different trends for the lower and higher sub-lethal concentrations. Less costly resistant mutants became fixed earlier in populations exposed to the lower sub-lethal concentration, whereas more costly resistant mutants that caused a greater reduction in growth were retained longer at the higher sub-lethal concentration. This demonstrated antibiotic stress influenced which resistance mutations were favoured, and how quickly they dominated the bacterial population.

The resistance mutations also differed in the extent to which they impacted the growth of the bacteria in the absence of the antibiotic; some provided resistance with little impact on growth, which indicated their lower fitness cost. However, a different mutation was observed predominantly in the higher-concentration group. This mutation caused a significant growth defect in the absence of the antibiotic. This indicated that resistance did not necessarily result in an equal fitness cost for the bacteria, and the concentration of antibiotic could influence whether less costly or more costly resistant mutants dominated the population.

Overall, the comparison demonstrated that sub-lethal exposure to a lower concentration of antibiotics favoured earlier fixation of resistance mutations of lesser impact on bacterial growth, whereas higher concentrations of antibiotics allowed more costly resistant mutants to persist. The antibiotic-free populations were

a useful comparison for growth in the absence of sub-lethal antibiotic exposure and allowed the identification of resistance mutations that caused a fitness cost to the bacteria.

Secondary Comparisons (transfer/mechanism)

Comparing the antibiotic-exposed groups with the antibiotic-free control demonstrated that sub-MIC exposure resulted in an increased frequency of plasmid transfer between the bacterial strains. This was the case for all four antibiotics tested, but the increase varied; meropenem increased conjugation frequency by ~2.3 – 4.1 times, whereas ciprofloxacin increased conjugation frequency by ~3.4 – 9.0 times, compared to the antibiotic-free control. Cefotaxime increased transfer by ~3.3 – 4.8 times, and amikacin by ~2.4 – 3.5 times. All increases were statistically significant at the concentrations tested, and demonstrated that even sub-lethal antibiotic exposure could promote transfer of a plasmid harbouring resistance.

The increase in conjugation did not follow a linear trend for each sub-MIC antibiotic, but instead different sub-MIC concentrations of antibiotics caused different increases in conjugation frequency, with ciprofloxacin demonstrating the highest increase. The study also demonstrated increased expression of genes associated with conjugation and the type IV secretion system after antibiotic exposure, which could provide an explanation for increased plasmid transfer between bacteria following exposure to sub-lethal concentrations of antibiotics. Overall, the findings indicated that sub-MIC exposure can create conditions conducive to horizontal transfer of plasmids, such that resistance can be transferred between bacteria by sub-lethal antibiotic exposure, rather than resistance emerging solely through mutation and natural selection.

Key/Unexpected Findings

An important finding that arose from the studies reviewed was that resistance did not appear to have a singular mechanism by which it developed. In some studies, prolonged sub-lethal antibiotic exposure resulted in genetic changes within the bacterial population, indicating resistance was emerging through mutation and natural selection. However, in other studies, sub-MIC exposure was found to increase the frequency of horizontal gene transfer (HGT) through conjugation, thereby allowing resistance genes to be transferred between the bacteria. This demonstrated that prolonged sub-lethal antibiotic exposure could result in resistance to antibiotics that develops through multiple mechanisms: increased mutation and natural selection, as well as increased horizontal transfer of resistance genes.

An unexpected finding was the observation that the use of low concentrations of certain antibiotics did not reduce bacterial growth. Whilst sub-lethal exposure caused a delay in the start of bacterial growth in some cases (by increasing the lag phase), some bacteria demonstrated higher growth rates or final densities than antibiotic-free control at certain low concentrations. This indicated a possible hormetic/growth stimulation effect, whereby very low levels of an antibiotic could yield differing results to higher concentrations.

VII. Discussion and Conclusion

Interpretation

The observed pattern reflects the fact that sub-lethal antibiotic exposure does not halt bacterial growth completely, thereby allowing more opportunities for differential resistance mutations to occur and be selected for than lethal exposure allows. Wistrand-Yuen et al.'s high level resistance arose via a step-wise accumulation of several resistance-conferring mutations, with only individually minor impacts on resistance levels. These mutations occurred through distinct mechanisms, including alteration of antibiotic target, lowering of antibiotic uptake, and increased antibiotic modification, whereas lethal-level exposure mostly led to resistance being conferred by a single, large-effect mutation.

According to the reviewed studies, mutation has indeed been shown to be the primarily demonstrated mechanism of new resistance emergence, whereas HGT provides an additional mechanism of conferring pre-existing resistance. The sub-MIC conditions are of particular importance, as they allow susceptible bacteria to remain viable and grow while subject to selection pressures from the antibiotic. These conditions are conducive to step-wise emergence of small-effect resistance mutations, as opposed to selection for those already carrying a mutation that would allow survival at high antibiotic concentrations. Thus, sub-lethal exposures can lead to the emergence of high-level resistance, with the mechanisms of resistance being distinct from those selected for by lethal concentrations.

Comparison with previous data

The findings from the current study align well with those of the previous analyses of resistance emergence under sub-lethal exposure. The *Klebsiella pneumoniae* study demonstrated that progressive exposure leads to step-wise emergence of resistance with specific mutations being fixed at higher MICs. Similarly, the *E. coli* study

demonstrated that lower sub-lethal concentrations resulted in the earlier outgrowth of less-costly resistance mutants, whereas more costly mutants persisted longer at higher sub-lethal concentrations. Thus, the current study's findings align well with the reviewed data to demonstrate that the levels of sub-lethal exposure affect which resistance mechanisms are selected for.

The findings from the *Salmonella* study align with the current findings in that all three demonstrate that high-level resistance can emerge under weak sub-MIC selection pressures. Notably, the selection for resistance at sub-lethal concentrations of antibiotics did not follow the pattern predicted by the predominance of large-effect mutations at lethal concentrations. Rather, at sub-lethal concentrations, resistance was conferred by multiple small-effect mutations, leading to high levels of resistance despite individually minor changes. In fact, the findings align well with those of the *Klebsiella pneumoniae* study, as the sequential exposure led to step-wise resistance development without the acquisition of a known resistance gene.

Overall, the current findings therefore build upon the reviewed studies to demonstrate that sub-lethal antibiotic exposure can allow bacteria to continue growing while resistance mechanisms are gradually accumulated, with the pattern of resistance emergence being different compared to exposure at lethal concentrations. Moreover, the studies reviewed demonstrate that resistance to antibiotics does not necessarily arise from the horizontal gene transfer of pre-existing resistance genes; rather, the step-wise emergence of resistance mutations can also account for increased resistance levels. Meanwhile, the differences between the reviewed studies' findings highlight the fact that sub-lethal exposure does not necessarily have a uniform impact on resistance emergence, with exposure concentrations, duration, bacterial species, and fitness cost all playing a role.

Practical Implications

The findings from the reviewed studies have significant implications for antibiotic pollution in the environment, as bacteria can be exposed to low concentrations of antibiotics from numerous sources. Antibiotic residues from human use, livestock, agriculture, and aquaculture can enter the environment through human and animal waste, wastewater, agricultural runoff, and the discharge of treated or untreated sewage. Moreover, antibiotic residues can enter the soil and the groundwater through the irrigation of crops and the discharge of pharmaceutical production waste. Notably, the presence of these multiple sources of sub-lethal antibiotic exposure can mean that bacteria are exposed to sub-lethal concentrations of antibiotics repeatedly and chronically.

This is of particular importance due to the fact that environmental bacteria do not necessarily need to cause direct harm to be clinically relevant. The presence of sub-lethal concentrations of antibiotics can exert selective pressure to maintain resistance genes vertically and increase rates at which resistance genes are horizontally transferred between bacteria. The reviewed study demonstrated that environmentally-relevant concentrations of antibiotics increased both vertical persistence of resistance and horizontal transfer of resistance genes, including between disparate bacterial species, and inhibited such processes at higher concentrations of antibiotics. Thus, antibiotic pollution can lead to environmental conditions conducive to the maintenance and dissemination of antibiotic resistance genes between bacterial populations, rather than a direct selection for resistant strains.

The public health concern raised by the findings is that environmental bacteria can serve as a reservoir for resistance genes, which can then be disseminated throughout bacterial populations, including those more directly related to human, animal, or clinical populations. Thus, antibiotic pollution not only poses an environmental health risk, but represents a One Health threat due to the possible increased dissemination of antibiotic resistance genes between environmental, animal, and human bacterial populations. As such, the findings support the need to consider environmentally relevant concentrations of antibiotics and the prolonged exposure when considering the risk posed by antibiotic resistance.

Limitations & Future Research

There are several significant limitations that should be noted in regards to the reviewed studies' findings. One of the primary limitations is the fact that the exposure durations used in most studies are much shorter than the exposure periods that bacteria can be subjected to in the environment. Moreover, most studies focus on a single strain of bacteria and a single class of antibiotics, making it difficult to extrapolate the findings to other bacterial species or different classes of antibiotics. Another limitation is that laboratory settings are much more controlled than the conditions that bacteria can be exposed to in the environment. For example, bacterial growth in nutrient-rich laboratory broth is significantly different from bacteria growing in soil, wastewater, or agricultural runoff, where they would interact with other bacteria and environmental factors.

Another key limitation is the fact that there is no fully standardized method of studying sub-lethal antibiotic exposure, meaning that the findings from different studies can be difficult to compare. Reviewing the findings from the studies included in this review, it becomes evident that there are several factors that can be varied: determining the MIC, selecting sub-MIC concentrations of antibiotics, culturing conditions, and methods of determining bacterial response and resistance. Thus, studies can report differing findings even when examining

similar questions, which highlights the issue identified by Thurner and Alatraktchi: the difficulty of comparing different findings from various studies on sub-lethal exposure.

Future research efforts should therefore be focused on examining the impacts of prolonged exposure to sub-lethal concentrations of antibiotics. Moreover, future studies should seek to use multiple bacterial species and different classes of antibiotics when examining the effects of sub-lethal exposure. Additionally, studies should aim to use varying environmental conditions when examining the responses of bacteria to sub-lethal antibiotic exposure, as opposed to controlled laboratory conditions. Overall, following the recommendations of Thurner and Alatraktchi, future research can employ standardized methods when examining the effects of sub-lethal exposure on bacterial resistance to better compare the results across different studies.

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