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Antibiotic Heteroresistance and Treatment Failure: An Emerging Clinical Threat beyond Conventional Antimicrobial Resistance

Sumia Farid¹, Nusiba Albasheer², Sultan Hassan D. Almarwani³, Md Jamir Anwar^{4*}, Walid A. H. Eldaif⁴, Almutaz M. Idris⁴, Majed Faleh Mogbel Alharbi⁵, Wasi Uzzaman Khan⁶, Sundos Suleman Ismail Abdalla⁷, Mohd Rashid⁷, Khetam Aqeel N. Alrasheedi⁷, Ameenah Aqeel N. Alresheedi⁷, Mohammed Ghazi Bokhari⁸, Khulud Hamdan Albalawi⁹

¹Department of Pharmaceutics, Faculty of Pharmacy, Elrazi University, Khartoum 11115, Sudan

²Department of Pharmaceutical Analysis, Sudan International University, Khartoum, Sudan

³Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa 31982, Saudi Arabia

⁴College of Applied Medical Science, Buraydah Private Colleges, Buraydah, 51418, Saudi Arabia

⁵The Prince Faisal Cancer Center, King Fahd Specialist Hospital, King Abdullah Rd, An Naziyah-7505, Buraydah-52366, Saudi Arabia

⁶Department of Pharmacology, MM College of Pharmacy, Maharishi Markandeshwar (Deemed to be University), Mullana, Haryana 133207, India

⁷Department of Pharmacy, College of Dentistry and Pharmacy, Buraydah Private Colleges, Buraydah -51418, Al-Qassim-31717, Saudi Arabia

⁸Al-Haram Hospital in Madinah, Yusuf bin Ahmed Al-Shirazi, Al Fath District-6754, Madinah-42312, Saudi Arabia

⁹King Salman Armed Forces Hospital in the Northwestern Region, King Abdul Aziz Rd, Military Area, Tabuk 47512, Saudi Arabia

*Corresponding author: Md Jamir Anwar, Email: zameeranwar@gmail.com

ABSTRACT:

Background: Traditional resistance categorisation may not capture the complexity of the bacterial populations and antimicrobial resistance (AMR) is one of the biggest global health crises. With antibiotic heteroresistance a large percentage of the population is susceptible while a small percentage is much less susceptible. The resistant subpopulations cannot be identified in the normal antimicrobial sensitivity testing, but are continuously selected by the use of antibiotic drugs that lead to persistent infection, treatment failure and finally to the development of stable resistance.

Objective: This review will provide a general overview of the biological and molecular mechanisms of antibiotic heteroresistance, as well as identification and characterization, distribution in clinically relevant bacterial pathogens, relationship with treatment failure and new therapeutic and diagnostic strategies.

Method: The literature on Bacterial Heteroresistance, AMS, Resistance Evolution, Clinical Treatment Outcomes, Diagnosis, PK/PD Optimization, Antimicrobial Combinations, BART and Emerging Therapeutic Technologies was critically reviewed.

The microbiological, genomic, single-cell and clinical and translational data have been integrated to provide an overview of heteroresistance in the population level.

Results: Heteroresistance has been reported in various Gram-positive and Gram-negative pathogens and against a number of clinically relevant classes of antimicrobial agents, such as carbapenems, colistin, vancomycin, daptomycin and tigecycline. AHR can arise as a result of gene amplification, chromosomal mutation, copy number variation, regulation, increased/decreased membrane permeability, efflux activity, metabolic adaptation and/or horizontal gene transfer. Biofilm, gradients of antibiotics, nutrient limitation, stresses and host-specific factors may contribute to phenotypic heterogeneity. In addition to conventional susceptibility testing, which fails to detect low frequency resistant subpopulations, there are methods with a higher resolution like population analysis profiling, time-kill studies, single cell approaches, microfluidics and multi-omics. Therapeutically, there is a risk of subpopulation enrichment and emergence of resistant subpopulations which may be responsible for persistent infection, relapse, emergence and treatment failure. Other strategies to limit the clinical importance of heteroresistance involve rational combination therapy, dosing based on PK/PD, anti-biofilm agents and strategies, antimicrobial peptides, rapid molecular diagnostics, and AI-based predictions and host-directed therapy.

Conclusion: AHR represents a critical, underrecognized dimension of resistance that challenges standard diagnostic paradigms. By evading routine AST and serving as a reservoir for development of resistance. Future research aimed at defining and validating detection assays, at longitudinal monitoring of heteroresistance, at studying heteroresistance at the single-cell and multi-omics levels and at prospective studies, which investigate the link between heteroresistance and treatment outcomes, would be of great value. Incorporating the concept of susceptibility at a population level into the diagnostics and therapeutic process for antimicrobial treatment will be essential for enabling precision antimicrobial therapy and preventing treatment failure.

1. INTRODUCTION

The increased resistance of bacterial pathogens to antimicrobial drugs is increasingly undermining the effectiveness of treatment and eroding the current successes of antimicrobial chemoprophylaxis, a growing problem known as antimicrobial resistance (AMR). The issue is not only about the treatment of a single infection, but also about the broader issue of infectious diseases. Major surgery, transplantation, cancer chemotherapy and intensive-care interventions are procedures that rely on effective antibacterial prophylaxis, and are compromised by resistance. The scope, significance and scale of the problem is highlighted by global estimates of around 4.95 million deaths caused by bacterial antimicrobial resistance in 2019, which already make resistant bacterial infections a significant proportion of the global infectious disease burden (World Health Organization, WHO), (2024a). Worrying trends have been further witnessed in more recent surveillance. The WHO estimates that in 2023, one in six laboratory-confirmed bacterial infections were antibiotic-resistant and that, from 2018 to 2023, more than 40% of pathogen–antibiotic combinations were found to be resistant (WHO, 2025). The observations highlight that antimicrobial resistance is a dynamic microbiological phenomenon that is continually changing and influenced by exposure to antimicrobials, bacterial adaptation, transmission, healthcare practices, and ecological pressures.

Growing complexity of AMR is also evident in the evolving priorities of international public health organisations. The 2024 WHO Bacterial Priority Pathogens List includes 24 pathogen groups from 15 families, sorted by priority for research and development, surveillance and public health action. Some of these are of particular concern, such as carbapenem-resistant *Acinetobacter baumannii*, Enterobacterales that are also resistant to carbapenems and third generation cephalosporins, and rifampicin-resistant *Mycobacterium tuberculosis*, all of which are included in the critical-priority category. In the high-priority category are other clinically important organisms: vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, resistant *Salmonella*, *Shigella* and *Neisseria gonorrhoeae* (WHO, 2024b). The significance of these organisms is not just that they are capable of being resistant to specific

antibiotics, but the fact that they also can cause infections that are hard to treat, survive in the healthcare environment, spread the resistance determinants, and limit already-restricted treatment options.

In this general AMR scenario, the emergence of AHR is an interesting and clinically relevant type of bacterial phenotypic heterogeneity. The standard definition of antimicrobial resistance is based on the antimicrobial resistance of the bacterial population as a whole, and isolates are classified based on their known susceptibility breakpoints. However, heteroresistance undermines this population-level view, as an apparently susceptible isolate may harbor a small subpopulation that is more resistant than the majority population, and thus capable of growing at concentrations of antibiotics that are higher than those tolerated by the majority population. Therefore, the bacterial culture might seem to be sensitive based on standard testing, but actually a minority of the cells have a much more resistant phenotype. Heteroresistance was defined by El-Halfawy and Valvano (2015) as the resistance of subpopulations within a seemingly isogenic bacterial population to a given antibiotic. Importantly, this phenomenon has been known for decades, but the definition, detection criteria and interpretation of clinical significance of this phenomenon have not always been consistent. Heteroresistance is important because the fitter the bacteria, the more they are likely to survive and cause reinfection. A standard susceptibility test is most frequently the phenotype of the tested population, and will likely not detect rare resistant cells. Once antimicrobial treatment is initiated, however, the antibiotic establishes a strong selection pressure that inhibits or kills the majority of the susceptible cells, while the minority of more resistant cells are able to survive. These cells that survive can then multiply and dominate the population. Heteroresistance, in this context, could give bacteria an evolutionary edge when exposed to antimicrobials and could, given the right circumstances, aid in the transition to more robust resistance. Heteroresistance has thus been addressed in recent literature not only as a diagnostic challenge but also as a potential intermediate state between apparent susceptibility and complete resistance (El-Halfawy & Valvano, 2015; Xie et al., 2025).

One of the most interesting characteristics of this phenomenon is the fact that it is different from other types of bacterial survival. Bacteria that are considered antibiotic resistant can grow at levels of antibiotic that would not allow other susceptible bacteria to grow, and tolerance occurs when bacteria survive for a long period of time in the presence of an antibiotic, but does not necessarily lead to higher minimum inhibitory concentration (MIC) values. Persistence, on the other hand, often means that there are a few phenotypically tolerant cells that persist in a population of cells that are otherwise susceptible. Theoretically, heteroresistance can be explained by any of these phenomena as they all refer to a bacterial population heterogeneity, but differ in that they include subpopulations with significantly different levels of antimicrobial susceptibility. This separation is not a purely linguistic one. The similarity of heteroresistance to treatment failure by tolerance or persistence can make it difficult to interpret laboratory reports and to understand the actual mechanisms behind the failure. Recent research in the field of antibiotic tolerance also focused on therapeutic failure despite lack of resistance by the bacteria in conventional susceptibility testing, indicating a need to account for various bacterial survival phenotypes when assessing antibiotic treatment failure (Deventer et al., 2024).

The clinical issue is especially important because the heteroresistant subpopulations may escape detection during routine antimicrobial susceptibility testing. Traditional methods like broth microdilution, disk diffusion, and automated susceptibility testing primarily aim to determine the major mechanism of action of an isolate. They therefore will not necessarily be able to reliably detect a minority population that has a significantly higher resistance phenotype. The problem is exacerbated by the presence of low frequency heteroresistant subpopulations, which may not be stable when not exposed to the selecting antibiotic. Thus, a minority with resistance may be lost or not be seen when the organism is cultured in the absence of antimicrobials, but it may flourish rapidly when antimicrobials are used. This dynamic behavior introduces another paradox: the laboratory result may be a good reflection of the majority of the population at the time of sampling but fail to predict what may occur following antibiotic exposure. Population analysis profiling (PAP), which quantifies bacterial growth over a spectrum of antibiotic concentrations and yields data on the distribution of resistance in a population has been traditionally considered as the main method for investigating heteroresistance. However, PAP is tedious, time-consuming and not easily integrated into everyday clinical laboratory practice. Differences among studies and among laboratories have also been created because of a lack of universally accepted diagnostic criteria. The lack of standardization, speed and clinical utility of methods for detecting heteroresistance continues to be a significant challenge in current practice.

Although the clinical consequences failing to diagnose heteroresistance may be important, it may vary between organisms, antibiotics and kinds of infections. Heteroresistance occurs in Gram-positive and Gram-negative bacteria and among resistance to multiple clinically important classes of antimicrobials. Recently, a species known for multidrug resistance (MDR) and its significant role in healthcare-associated infections (HAIs). Resistant minority populations may make treatment more

complicated, and may help to select for higher-level resistance during treatment or lead to microbiological persistence (Bognar et al., 2024). These include the same issues as seen with *A. baumannii*, *P. aeruginosa*, *S. aureus* and other important bacterial pathogens. It is important to note however that not every heteroresistance is the reason for clinical treatment failure. The correlation between heteroresistance and clinical results was reported to be complex and sometimes even conflicting and was found to be influenced by the genotype of the bacteria, the antibiotic exposure, site of infection, host factors, stability and level of resistant subpopulation. This uncertainty highlights a critical aspect of antibiotic heteroresistance that warrants further exploration. The other side of the coin, however, is that it could potentially be an unrecognized mechanism that leads to seemingly susceptible infections that fail to respond to antimicrobial therapy. However, its prevalence and role in treatment failure is difficult to understand, due to the lack of uniformity in definition and clinically validated diagnostic approaches. The current evidence, therefore, suggests that the conventional susceptibility test alone cannot be used as an exclusion criterion for heteroresistance, which may not always be linked with therapeutic failure. It can lead to rapid spread of antibiotic-resistant cells in the presence of antibiotics, and it is a potential cause of low antibiotic cure rates and in some cases antibiotic resistance remaining stable.

From this perspective, antibiotic heteroresistance is a new type of AMR phenomenon that should be considered as a new episode of the AMR problem, and not as another form of AMR. The problem is that it is possible that a routine susceptibility test may provide a 'snapshot' of the major bacterial population, but miss the minority phenotypes that are significant only under the influence of antimicrobial exposure. It is important to be aware of this hidden population structure as it may help to better understand the unexplained treatment failure, recurrent infections, and apparent evolution of resistance during treatment. However, more appropriate terminology is required to facilitate recognition, and standardized laboratory procedures are necessary to facilitate epidemiological monitoring, more complete, and better clinical evidence demonstrating that specific heteroresistant phenotypes are associated with therapeutic outcome. This is already a challenge for resistance diagnostics with resistances that make time and accurate antimicrobial decision making difficult, and requires approaches that are capable of detecting clinically relevant bacterial heterogeneity (Hassall et al., 2024).

2. OBJECTIVE OF STUDY

Therefore, the present review will concentrate on the new clinical problem of antibiotic heteroresistance, which is the phenomenon wherein bacterial subpopulations exhibit resistance beyond the traditional binary classification of "susceptible" or "resistant". It is focused on the biological basis of heteroresistance, differences between heteroresistance and other survival phenotypes, the limitations of the currently used susceptibility testing methodology and the prevalence of heteroresistance among clinically important bacteria and its potential association with antimicrobial failure. Special emphasis is placed on translating this complex microbiological phenomenon to clinically relevant risk assessment. This review will show how heteroresistance is an important factor in the current debate on AMR and that detection and understanding of it in clinical settings is important to prevent the apparent susceptible infection from escalating into a difficult-to-treat clinical failure.

3. SELECTED METHODES FOR STUDY

Study Design: Specify whether observational cohorts, randomized controlled trials (RCTs), or *in vitro* surveillance studies are included. **Search Strategy & Databases:** Define explicit search queries (using MeSH terms and boolean operators) across standard electronic databases (e.g., PubMed, Scopus, Web of Science, Embase). **Population:** Specify exact bacterial strains or patient cohorts exhibiting antibiotic heteroresistance (AHR). **Intervention/Exposure:** Define diagnostic detection methods (e.g., Population Analysis Profiling/PAP, broth microdilution, automated systems) or specific antibiotic regimens. **Comparator:** Non-heteroresistant (monomorphic resistant or fully susceptible) strains or alternative treatment regimens. **Outcome:** Clinical failure rates, mortality, relapse, or diagnostic sensitivity/specificity.

4. BODY OF STUDY INCLUDING MECHANISMS AND DISCUSSION

4.1. Biological Basis and Molecular Mechanisms of Antibiotic Heteroresistance

Antibiotic heteroresistance is most readily understood as a population level phenomenon, whereby bacteria within an isolate which appear as a single follow a spectrum of susceptibility to an antimicrobial agent. Instead, a majority of cells is susceptible and a minority of cells is resistant and can survive and grow at significantly higher concentrations of antibiotics. The resistant fraction could represent a genetically different or a genetically the same, but physiologically different fraction of the cells or be affected by reversible changes in the expression of genes and cellular state. This diversity allows for a better understanding of the heteroresistance as a phenomenon that is not a stable characteristic of an entire bacterial population as is commonly

believed. Heteroresistance is also frequently dynamic, with some subpopulations becoming resistant to an antibiotic and others reverting to sensitivity when the antibiotic is no longer applied. This is especially true for amplification-mediated heteroresistance, where higher gene dosage results in a short-term increase in resistance (Andersson et al., 2019; Brameyer, 2026). Heteroresistance is then a multifactorial situation. Genetic changes can lead to changes in antibiotic targets, in dose of resistance genes, in membrane permeability and in the concentration of the antibiotic inside the cell; regulatory/physiological changes can cause phenotypic differences between cells of similar genotype. This heterogeneity is further increased by environmental conditions such as gradients of antibiotic concentration, nutrient limitations, oxidative stress and the development of biofilms. The resulting phenotype is the result of the interaction of bacterial genotype, cellular physiology and the microenvironment and does not reflect one resistance mechanism alone (El-Halfawy & Valvano, 2015; Xu et al., 2025: Figure 1).

4.2 Phenotypic and Genotypic Foundations

Population heterogeneity as the basis of heteroresistance

The bacterial culture does not have to be considered as a bunch of identical cells, acting in a similar way. Within a single clone, cells may exhibit variations in their growth rates, metabolic rates, patterns of gene expression, responses to stress, membrane composition and antibiotic susceptibility. This heterogeneity may result from random variation in transcription and translation, from the unequal distribution of cellular elements during cell division, from metabolic differences or from variation in the genetic makeup that can occur during the expansion of a population. The differences are especially significant when bacteria are subjected to antibiotic stress as cells in different physiological states will be subjected to different degrees of antimicrobial stress. Heteroresistance can occur when the majority and the minority phenotype are significantly different in terms of the degree of antibiotic resistance. The MIC determined using a standard susceptibility test can then be influenced by the dominant cells, while the MIC of the minority population is not easily detected. However, in the presence of antibiotics, the proportion of each subpopulation may be significantly altered. The resistant minority is selected for and increases in size, and susceptible cells are inhibited, but not killed. This dynamic behaviour is a plausible biological explanation for the occurrence of resistant growth within antimicrobial therapy of an isolate designated as susceptible in the laboratory (Andersson et al., 2019). There is an important difference between genotype and phenotype. There are a few heteroresistant populations (HAP) with detectable genetic alterations, like amplification of resistance genes, or mutations in antibiotic target and regulatory pathways. However, in other situations, cells capable of having nearly the same genome may exhibit a variety of resistance phenotypes due to a variation in the level of expression of the resistance determinant, or due to differences in the physiological state of the cells. Therefore, heteroresistance should not be only considered a genotype or phenotype phenomenon. Instead, both mechanisms can be responsible, jointly, for the appearance and perpetuation of resistant minority populations (El-Halfawy & Valvano, 2015; Xu et al., 2025; Elbehiry, A. 2026: Figure 1).

Adaptive and stable heteroresistance

Heteroresistance can be generally expressed on the basis of its stability in varying environmental conditions. In many of the documented cases the resistant phenotype is unstable. Once the antibiotic pressure is eliminated, the resistant subpopulation will slowly decline and the original susceptibility type may be restored. Mechanisms of these types include tandem gene amplification, changes in plasmid copy number or transient activation of regulatory elements, and are often associated with this reversibility. These states can impose a fitness cost in environments where antibiotics are not present, and cells with extra resistance determinants could be outcompeted when antibiotics are not present (Andersson et al., 2019; Pal & Andersson, 2024). In contrast, the resistance tends to be more stable when it is heteroresistant due to a more stable genetic change. Initial stabilization of a resistance phenotype may be by point mutations, chromosomal rearrangements, mobile genetic elements or compensatory mutations. This forms an evolutionary continuum, in which transient resistance can sometimes be an intermediate state that can lead to stable resistance. Relevance for the clinic is especially important because the exposure to antibiotics can result in the selection of subpopulations that initially survive through reversible mechanisms and later develop more permanent adaptations. Differences between adaptive and stable heteroresistance are, therefore, not absolute. Selection can drive a population from a majority of reversible to increasingly stable resistance components. On the other hand, the frequency of unstable resistant variants can be decreased by removing their selective pressure in the form of antibiotics. The results vary with the fitness cost of the resistance mechanism, the level and time of antibiotic exposure, the genetic make-up of the organism and the ecology of the environment (Andersson et al., 2019; Brameyer, 2026 and Band, V. I. et al., 2019: Figure 1).

Clonal diversity within bacterial populations

Bacterial populations can be sorted during replication, especially when bacteria are chronically infecting a host and/or experiencing mutational stress. Mutations that target antibiotic resistance can, therefore, occur in various subpopulations of an expanding bacterial population, whether in the antibiotic target, in regulatory proteins, in membrane components, in efflux proteins or in metabolic pathways. Then, based on selection, the variants are enriched. This process can result in a population of cells, which are closely related, but have varying susceptibility to antibiotics. It is important to note that the resistant population did not necessarily come from an unrelated strain because of the clonal diversity. Infecting clone to infecting clone variation can result in heteroresistance. Within-host evolution may be particularly relevant for chronic infections where the bacteria are challenged by variable concentrations of antimicrobials and host immune responses, nutrient depletion, and spatially variable microenvironments.

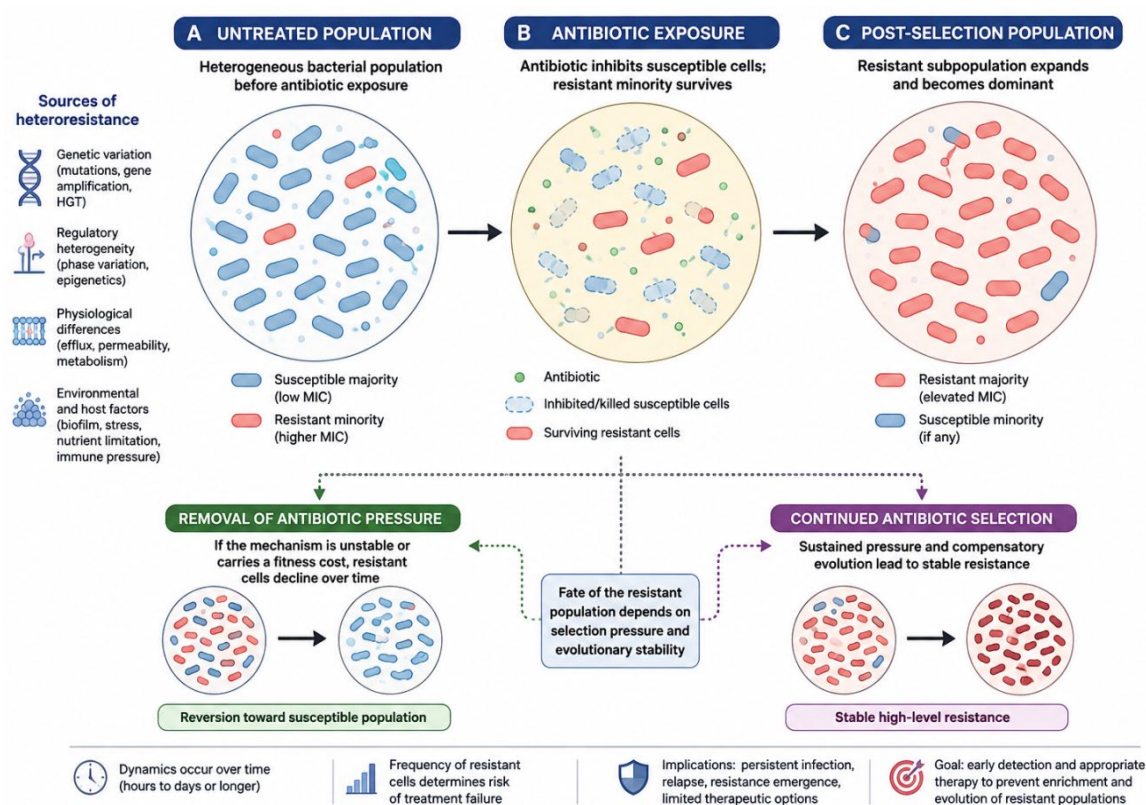


Figure 1. Conceptual origin and dynamics of antibiotic Heter resistance.

Genetic Mechanisms

Genetic mechanisms that cause measurable variation within subpopulations is one of the most well-known explanations for heteroresistance. These mechanisms include gene amplification, chromosomal mutation, copy number variation, structural rearrangements and acquisition of resistance determinants through horizontal gene transfer. The changes that can occur can result in an increased expression of resistance genes, changes in the genes targeted by antibiotics, reduced accumulation within the cell, or changes in the control of the antimicrobial susceptibility network.

Gene amplification

Gene amplification is one of the best characterised mechanisms that has been associated with unstable heteroresistance. Repeatedly, tandem duplication of a genomic region can lead to a temporary increase in the number of copies of a resistance associated gene. The resulting gene dosage increase can then result in increased production of an enzyme, target protein, transporter or any other determinant of resistance. The bacteria might only carry a fraction of the amplified region at the given moment leading to an mosaical distribution of antibiotic susceptibility (Andersson et al., 2019). The reason why amplification is important is that it's reversible. Homologous recombination can take place at tandem repeats, causing the

expanded segment to go away once the selection pressure is relaxed. Therefore, a population can rapidly shift from a susceptible to a resistant state, and from a resistant to a susceptible state when antibiotics are not available and available, respectively. This gives an evolutionary benefit as bacteria can temporarily gain resistance without having to permanently carry the fitness cost associated with a stable resistance mutation (Pal & Andersson, 2024). Gene amplification thus might serve as an evolutionary buffer. With the addition of antibiotics, these have the ability to create copies of resistance-associated genes, and survival increases. When the pressure is taken off, a reduced-cost cellular state can be restored by copy number reduction. Some heteroresistant phenotypes are not readily reproducible in normal laboratory assays, which is in part due to this flexibility.

Chromosomal mutations

Small chromosomal changes and point mutations might cause heteroresistance. Mutations that alter the antibiotic target, membrane proteins, the regulatory system/metabolic pathway can occur in just a fraction of the cells. Enrichment of these in the population can occur if they are selectively advantageous when the antimicrobial is used. Mutations in genes which affect the uptake or efflux of antibiotics are especially important as small changes in the concentration of the drug in the cell may have dramatic effects on susceptibility. Similarly, mutations in the target molecule can increase the resistance to antibiotics and mutations in control genes can increase the production of any already existing resistance mechanism. Importantly, not all mutations lead to clinically relevant resistance, the phenotype is dependent on the nature of the mutation, the genetic background of the organism and the concentration of the antibiotic and the growth state (Xu et al., 2025).

Copy-number variation

There is another type of CNV, called chromosomal amplification, that is not the one described above. Bacteria may have a cell population of various copy numbers of plasmids and other mobile genetic elements. The cells that carry more copies of plasmid may make more resistance protein than cells that carry fewer copies. Therefore, copy number variation of the plasmid can lead to variation in the resistance of different individuals within a population even though they all carry the same resistance gene. This process is especially critical when resistance genes are located on plasmids which have a measurable metabolic cost. Plasmid cells with high copy numbers may be more fit under antibiotic selection, but less fit in the absence of the antibiotics. This results in dynamic changes in the composition of the bacteria

Horizontal gene transfer

There is also the possibility of horizontal gene transfer, as an alternative source of the acquisition of resistance determinants by bacterial populations. Bacteria can get genetic material from other cells and/or from the environment by transformation, transduction and conjugation. Plasmids, transposons and integrative elements are all examples of mobile genetic elements that may contain genes for antibiotic-inactivating enzymes, altered targets, efflux systems and other resistance-associated functions. Mostly the horizontal gene transfer is seen as the transfer of resistance genes between lineages of bacteria but it can also cause intra-population heterogeneity. Subsets of cells that acquire or retain a resistance bearing element could produce genetically distinct subgroups of cells that produce genetically distinct antimicrobial phenotypes. This exchange can happen particularly in the biofilm, where bacterial cells are collected in a matrix and physically in contact for a long period of time with each other (Liu et al., 2024).

Table 1. Major genetic mechanisms contributing to antibiotic heteroresistance.

Genetic mechanism	Primary molecular consequence	Effect on antibiotic susceptibility	Typical characteristic
Tandem gene amplification	Increased resistance-gene dosage	Increased expression of resistance determinant	Often unstable and reversible
Point mutation	Altered target, regulator, transporter, or metabolic pathway	Variable increase in resistance	May be stable
Chromosomal rearrangement	Changes gene structure or expression	Altered resistance phenotype	Can be stable or unstable
Plasmid copy-number	Variable dosage of plasmid-borne	Unequal resistance-gene	Potentially reversible

variation	genes	expression	
Horizontal gene transfer	Acquisition of new resistance determinants	Introduction of new resistance phenotype	Can facilitate dissemination between cells
Compensatory mutation	Reduces fitness cost of resistance	Promotes persistence of resistant phenotype	May stabilize resistance

Adapted conceptually from the mechanistic frameworks described by Andersson et al. (2019), Xu et al. (2025), El-Halfawy et al. (2015), Band, V. I. et al. (2019), and Brameyer (2026).

4.3 Epigenetic and Regulatory Mechanisms

Other heteroresistance has nothing to do with a change in the DNA sequence. Bacteria are equipped with highly complex regulatory mechanisms which enable them to change their gene expression in response to changing environmental conditions very quickly. These mechanisms can result in various states within genetically identical cells, and may influence the resistance to antibiotics but do not cause permanent mutations.

Phase variation

Bacterial populations can switch from one state of expression of some genes to another by means of phase variation. These switches can happen by alteration of repetitive DNA segments, mispairing of repetitive DNA segments during replication, inversion of DNA segments, and regulation of methylation of DNA. This yields the creation of subpopulations with distinct expression of specific surface structures, metabolic activities, virulence-associated characteristics or resistance-associated pathways. Phase variation can be reversible, making it an ideal candidate to the changing environment. If the genetic modification of permanently altering every bacterium is too costly then there is no need to alter the cells permanently, but only enough for the population to maintain the phenotypic diversity. Phase variation does not necessarily mean heteroresistance, but it is a mechanism which may render antibiotic resistance heterogeneous.

DNA methylation and epigenetic control

Coordination of gene expression can be achieved by changes in methylation at multiple genomic locations that are caused by phase-variable DNA methyltransferases. These systems (also known as phasevarions) are able to regulate clusters of genes that are involved in host-specificity, stress response, and metabolism or resistance to antibiotics. The importance of methylation-based regulation is that phenotypic variation may be generated without a change in the actual structure of the nucleotide sequence of the DNA. A bacterial population can then be in a range of regulatory states, thus providing an adaptive response to environmental stresses. A mechanism to generate reversible bacterium phenotypes upon exposure to their different environmental stresses has been highlighted recently and this mechanism is known as epigenetic phase variation (Hama Soor et al., 2026).

Small regulatory RNAs

Small non-coding RNAs (sRNAs) are capable of altering the stability and/or translation of target mRNAs to have an impact on adaptation in bacteria. sRNAs are capable of interacting with the transcriptional and post-transcriptional regulatory networks and may impact stress response, membrane associated proteins, metabolic pathways and resistance determinants. The role of sRNAs in heteroresistance may vary with the context. These do not act as resistance determinants, but rather can alter the response of the cells to antibiotic exposure in terms of both the strength and timing of their responses. As a result, there is the potential for different levels of sRNA which can lead to phenotypic heterogeneity within a single cell.

Global stress-response regulators

A single molecular pathway is involved very rarely in the use of antibiotics. These regulatory networks are interconnected and comprise oxidative stress responses, envelope stress responses, stringent response pathways, two-component regulatory systems and other global regulators, all of which govern the responses of bacteria. These pathways may involve membrane permeability, metabolic pathway activity, growth rate, and gene expression involved with resistance, as well as DNA repair. Out as a result, cells under varying stresses can become in various physiological states. One set can continue to grow quickly and be sensitive to antibiotics, while another set can slow down, and then engage protective pathways. Different regulations may impact the spread of susceptibility among people.

Physiological Drivers

Ultimately, genetic and regulatory changes affect the susceptibility to antibiotics in terms of the physiology of cells. Several factors such as drug entry, efflux, modification or degradation, target accessibility and cellular metabolic activity are involved in the concentration of an antibiotic in its intracellular target. Any variation among single cells in any of these processes can lead to some cells being more resistant to antibiotics than others, which is called heterogeneity.

Efflux pumps

Efflux systems decrease the concentration of antibiotics within the bacterial cell by pumping them out of the cell. Major efflux systems consist of resistance-nodulation-cell division (RND), major facilitator superfamily (MFS), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE) and ATP-binding cassette (ABC) families. They can be expressed either constitutively or in response to other signals and stress in the environment (Sun et al., 2019). Efflux activity may not be the same in all cells of a heterogeneous population. These differences in expression of efflux genes can thus contribute to differences in accumulation of antibiotics in cells. The more active the efflux system the more resistant the cells and may survive concentrations that inhibit neighbouring cells. This is particularly relevant when there is exposure to antibiotics and the efflux related regulatory pathways are activated.

Changes in membrane permeability

Many antibiotics will face the bacterial envelope as their initial barrier. Changes in the expression of porins, in the composition of the membrane, in the cell envelope structure and in the transport, pathways can decrease the entry of antibiotics. Gram-negative bacteria may be more susceptible to the effects of changes in outer-membrane porins, as decreased permeability could lead to lower concentrations of β -lactam, fluoroquinolones, aminoglycosides, and other drugs inside the cell. Therefore, cell-to-cell variability of porins or envelope-stress responses can lead to cell-to-cell variability in the penetration of antibiotics. Such differences can occur simultaneously with efflux activity and result in an overall decrease in the amount of drug that is accumulated intracellularly.

Cell wall remodeling

The synthesis and remodeling of cell walls are highly dynamic and are regulated by the growth rate, environmental stresses and regulatory networks. Alterations in peptidoglycan architecture, in cell-envelope turnover and in regulation of these pathways may affect the susceptibility of cell-wall-synthesizing antibiotics. When bacterial cells are growing, the rate of synthesis of cell wall components is not necessarily the same for all cells. Thus, the sensitivity of cells in different physiological states might differ with antibiotics that are active against the cell-walls. Heteroresistance therefore depends on growth state and cellular physiology and this is one reason why it is important to take this into account when interpreting heteroresistance.

Metabolic adaptation

Antibiotic activity is strongly affected by the cellular metabolism. Cytotoxic agents which affect active cellular growth may not work as well on metabolically altered or slowly growing cells. But metabolic adaptation is not the same as conventional resistance, and should not be deemed to be a form of heteroresistance. Its significance is because metabolic heterogeneity might result in a continuum of susceptibilities of the cells in the same population. Respiratory activity, ATP availability, biosynthetic activity, redox state and nutrient utilization can vary between individual cells and impact their susceptibility to antibiotic exposure. This metabolic heterogeneity can be combined with genetic resistance mechanisms and thus affect the growth of subpopulations with heteroresistant gene expression.

4.4 Environmental and Host-Induced Factors

Heteroresistance does not come without its surroundings. The levels of antibiotics, nutrients, oxygen, host-derived molecules and metabolic products change continually for bacteria. These conditions provide selection pressures which can change the behavior and/or frequency of resistant subpopulations.

Antibiotic exposure gradients

The levels of antibiotic drugs in a patient's body are not necessarily equal throughout a patient's body. Levels of drugs may vary between tissues, abscesses, mucus layers and biofilms, intracellular compartments, and poorly perfused areas. Pharmacokinetic variability could therefore create a wide array of antimicrobial levels to which bacterial populations are exposed, instead of a consistent level. Nonsusceptible cells with slightly higher resistance can be important in the context of sub-inhibitory antibiotic exposure, as these cells are suppressed by the antibiotic, but not killed. Exposure to low concentrations or to varying concentrations can thus provide opportunities for selection and adaptation. Experimental studies have shown that antibiotic exposure, at low levels, can promote the evolution of higher-level resistance, highlighting the need to consider antibiotic concentration as an evolutionary variable, but not only a pharmacological parameter (Gifford et al., 2018).

Biofilm-associated heteroresistance

Bacterial heterogeneity is particularly favored in the case of biofilms. The cells inside a biofilm are affected by varying concentration of oxygen, nutrients, pH, metabolic activity, accumulation of wastes and penetration of antibiotic. A single microbial community generates these gradients, which determine the physiological niches at different locations. The extracellular polymeric substance matrix may also provide a barrier to antibiotic penetration, chelate certain antimicrobial agents and change the chemical environment at the local level that can affect the activity of the drug. Biofilms also have cells with varying metabolic states, such as slow-growing and dormant cells, and cells that are actively growing. All of these make them highly heterogeneous with respect to their antimicrobial susceptibility (Stewart & Franklin, 2008; Jo et al., 2022; Liu et al., 2024). Notably, the terms 'biofilm associated tolerance' and 'heteroresistance' aren't interchangeable. Physiological tolerance of biofilms can cause a higher tolerance without the presence of a significant resistant minority with a higher MIC. However, the spatial and physiological differences in biofilms might give rise to genetic and phenotypic heteroresistance developing, remaining or becoming clinically significant.

Host immune pressure

Infection results in heterogeneously distributed and time-varying immune-mediated stresses on bacteria. Alteration of bacterial physiology by phagocytic cells, antimicrobial peptides, reactive oxygen and/or nitrogen species, complement components and inflammatory mediators. The stress can impact gene expression, membrane properties, metabolism and stress-response pathways. The host environment can thus provide an extra selective environment. It is also possible that a bacterial subpopulation relatively more resistant to oxidative stress, or a nutrient limitation, may also be more resistant to antibiotics. The dynamics of host adaptation and antibiotic exposure can thus play a role in the persistence of resistant minorities in infection.

Nutrient limitation and oxidative stress

The availability of nutrients can differ significantly depending on the anatomical location and/or structured bacterial community. Carbon or nitrogen or iron (or other essential nutrients) limitation may affect the growth rate and metabolic activity of bacteria. For instance, in oxidative metabolism, virulence pathways and cellular stress responses could be affected by iron-limitation. Oxidative stress also triggers a series of other protection mechanisms that can also modify cell physiology. Stress can impact metabolic state and oxidative balance, which can affect antibiotic activity. But the relationship is complex, and should not be taken to mean a one-way street in all bacterial species.

Mechanistic Integration

All of the above mechanisms indicate that the development of antibiotic heteroresistance is not dependent on a single mechanism of molecular events. Instead, it is the result of interactions between diversity of populations and selective pressure. Resistant subpopulations can arise genetically, and regulatory and physiological mechanisms may control the survival and growth of these cells. This is how the strength and direction of selection will vary in different environments. It can be useful to consider this as a 'cycle'. Genetically or phenotypically, a bacterial population is variable, and this variability leads to an unequal susceptibility of bacteria. Then, with antibiotic exposure, it's a selection for the resistant cells, up to a certain degree. Resistant minority survive and in relative abundance. If the resistance is only temporary, then a population can once again become susceptible if antibiotics are removed from their environment. Transiently heteroresistant phenotype

however, may serve as a 'stepping stone' to a more stable resistance phenotype if there are stable mutations or compensating changes (Andersson et al., 2019; Pal & Andersson, 2024).

In biofilms and host associated environments, this process can be enhanced to create an environment with short-distance, spatially structured niches that exhibit significant variation in antibiotic concentrations, nutrient levels, oxygen tension, and immune pressure. So, the bacterial population that is met in the clinic will not be a homogeneous population of bacteria. Instead, it is living ecosystem, which is characterized by its genetic and physiological diversity and ability to interact with each other and with their environment (Jo et al., 2022; Liu et al., 2024). This integrated view is very important as it is the reason why traditional susceptibility testing has not been able to detect heteroresistance. Traditional assays will be representative of a complex bacterial population and have an average susceptibility, whereas heteroresistance is a distributional phenomenon. Clinically relevant information can be in the presence of minority populations with higher MICs as well as in the stability and frequency of the dominant population with lower MICs.

This is a growing opinion, which has recently been substantiated by mechanistic studies. Heteroresistance can result from a change in plasmid copy-number, point mutations, physiological adaptations, plasmid regulation, accumulation or variation in the dosage of the gene. Depending on the species and class of antibiotics, genetic background and environment, all of the above mechanisms can play a role in MDR (Xu et al., 2025; Brameyer, 2026). Further investigations of heteroresistance will then require single-cell or subpopulation level studies via techniques for measuring phenomena other than the average. To summarise: the biological mechanisms of antibiotic heteroresistance can be described as the continuum of variation, selection, survival, expansion and stabilisation. The first variation can be genetic or non-genetic, the selection done by the antibiotics and other environmental conditions; the few cells with a favourable phenotype survive; the proportion of these cells increases within the population; after a while, the genes coding for the phenotype of the cells with the new resistance will be genetically stabilised and the short-lived phenotype will be replaced by a more durable form of resistance. Knowing this continuum is essential to understand the unpredictability of what can happen with apparently susceptible bacterial populations in the treatment of AMR and the contribution of heteroresistance to the modern AMR problem.

5. BODY OF STUDY INCLUDING DIAGNOSTIC APPROACHES, METHODOLOGICAL BLIND SPOTS AND DISCUSSION

One of the most challenging issues in modern antimicrobial susceptibility testing is the detection of heteroresistance which is a population-distribution phenomenon and not a single MIC value. A standard susceptibility test usually represents the properties of the dominant population of bacteria, as heteroresistance can be due to a small proportion of bacteria that are very resistant to the antibiotic. Thus, it is conceivable that a resistant subpopulation exists within an isolate and survives selective pressure of antibiotics and grows in size, whereas the isolate is reported as susceptible. This diagnostic gap has become more widely recognised as an important limitation of routine antimicrobial susceptibility testing (AST) and is likely to be a constraint for the minority of the resistant or unstable phenotype if the prevalence of the resistant is low (Hassall et al., 2024; Guliaev et al., 2025). The methodological issue is complicated by a lack of universal standardized heteroresistance diagnostic process. Population Analysis Profiling (PAP) is an important reference approach, but too labor intensive to be used in routine use. However, new computational methods, gene and genomic analysis, microscopy, time-kill assays, and gradient diffusion assays are emerging as complementary approaches to study. This is a transition from population-average measurements to techniques that allow for the detection of subpopulation structure, individual cell response, genotype to phenotype relationships and dynamics as a function of antibiotic exposure.

5.1 Limitations of Conventional Antimicrobial Susceptibility Testing

The traditional format of AST was not ideal for detecting minority resistant populations; however, routine AST is useful for clinical decision. Both broth microdilution and disk diffusion methods classify an isolate to a specific phenotype by the most common phenotype identified under certain conditions. Both automated susceptibility platforms are similar in the way that the isolates are classified. This approach is OK when the bacteria community is fairly homogeneous, but can be a problem when a susceptible mass is present with a resistant mass.

Broth microdilution

A broth microdilution method is typically used to determine the MIC of an antimicrobial and is still a useful reference method for phenotypic susceptibility testing. This method is used to determine the growth of the bacteria at different levels of antibiotic concentration and to discover which level will cause the bacteria to stop growing according to a certain criterion.

However, the final MIC is not necessarily indicative of a small resistant population, since it is a measure of the response of the entire population. This is of particular interpretation with heteroresistant isolates. If the resistant subpopulation is a small percentage of inoculum, the endpoint chosen to measure the MIC might not be significantly affected by the growth of the resistant subpopulation. So a particular culture may be viewed as being sensitive, although made up of cells that grow at a much higher concentration. This restriction, especially applies in cases where the resistance is enriched in the minority, after a long time of antibiotic use. However, growth kinetics, extended incubation times or changes in susceptibility following exposure to antibiotics can be utilized to determine heteroresistance using broth microdilution. Such changes, however, would deviate from the standard format of the routine assay, and could further decrease interlaboratory comparability.

Disk diffusion

Disk diffusion is low cost, easy to perform and widely available and particularly useful in a resource constrained Microbiology laboratory. The diameter of inhibition around the antibiotic containing disk is compared to the established interpretive criteria. The method, however, yields a spatial average measurement and was not able to detect a low frequency resistant subpopulation. In a few cases, resistant colonies can be seen in a zone of incomplete inhibition. These colonies may be useful to indicate heterogeneous susceptibility, but the absence of these colonies does not rule out the possibility of heteroresistance. The recognition of these colonies may also depend on the density of the inoculum, the environment in which they are grown, the diffusion characteristics of the antibiotics, the morphology of the colonies and the experience of the observer.

Automated susceptibility platforms

Automated AST systems offer enormous advantages in the clinical laboratory for the standardisation of testing of many antimicrobials, speedy reporting, and the ability to connect these systems with laboratory information systems. Most of these platforms, however, still use population-level growth measures and existing susceptibility algorithms. If a minority resistant population does not make a significant change to the calculated susceptibility category of the system, then the minority resistant population may go undetected. This limitation is representative of a general problem with the current diagnosis of AMR: although the tests are quick, they are not necessarily sensitive for such a diverse AMR phenotype. Even a rapid response may be misinformed if the assay is only measuring the dominant population. This is the importance of speed, and the capability of future AST systems to give a higher resolution of clinically relevant AST resistance phenotypes, that is observed in current diagnostic reviews (Hassall et al., 2024; Reszetnik et al., 2024).

5.3 Gold Standard Detection Methods

Population Analysis Profiling

Population Analysis Profiling (PAP) is considered the main reference method to explore antibiotic heteroresistance. PAP does not just test one end-point of antibiotic action on bacteria; it tests bacterial survival over a range of concentrations. The population-level susceptibility distribution is obtained by measuring the number of bacteria at several different concentrations of the antibiotic and plotting the results against the antibiotic concentration. PAP has a good capacity to separate a majority of susceptible individuals from a minority of individuals who can resist higher concentrations of antibiotics. In this regard PAP is directly concerned with the characteristic of heteroresistance of the biological system. It can offer information regarding the frequency and magnitude of the resistant subpopulation as well. But there are considerable drawbacks to using PAP. Multiple concentrations of antibiotics, serial dilutions, plating, incubation, counting of colonies and careful interpretation. The process is therefore much more tedious than the normal AST. Also, variations in preparation of inocula, media, incubation conditions, and criteria for interpretation of the results can affect the outcome. Conventional PAP is difficult to implement routinely, as is evident from recent studies that are evaluating their simplified PAP formats. A recent clinical isolate-based PAP approach using a simplified agar system also demonstrates the continuing work to make detection more practical at the population level without sacrificing the key benefits of the reference method.

PAP-AUC methodology

PAP-AUC is an extension of the population analysis, which also determines the area under the population analysis curve (AUC). The method quantifies the entire profile of observed bacterial survival along the antibiotic gradient instead of just the "growth" or "no growth" data that is observed at a select point. This can help to compare isolates and can be a more quantitative measure of heteroresistance. The higher the AUC, the more bacteria will survive at higher concentrations of

antibiotic. However, PAP-AUC cannot be considered independent of the quality and design of the population analysis and is not a concept in itself.

Gradient diffusion methods

There are a few relatively simple gradient diffusion assays, such as the modified E-test assays, that allow for screening of heterogeneous resistance. A strip is laid on an inoculated agar surface which contains a continuous antimicrobial concentration gradient. In a heteroresistant population, the colonies may grow inside or outside the main inhibition ellipse, which indicates the presence of resistant minority colonies. One of the main features of gradient diffusion is its simplicity. May serve as a screening tool prior to confirmation by PAP or other reference test. But the technique alone might be insufficiently sensitive and specific. The colony morphology, preparatory procedure of inoculum, incubation conditions, and the antibiotic–organism relationship may also affect the interpretation. More recent assessments of modified gradients have thus focused on the use of these as screening tools and not on substituting population analysis.

Table 2. Comparison of major approaches for detecting antibiotic heteroresistance.

Method	Principal measurement	Major advantage	Major limitation	Potential role
Broth microdilution	Population-level MIC	Standardized and quantitative	Minority resistant cells may be masked	Routine AST; preliminary assessment
Disk diffusion	Inhibition-zone diameter	Simple and inexpensive	Low sensitivity for minority populations	Screening
Automated AST	Automated growth/susceptibility classification	Rapid and high throughput	Population averaging may obscure heteroresistance	Routine clinical testing
PAP	Survival across antibiotic gradient	Detailed population distribution	Laborious and time-consuming	Reference/confirmatory method
PAP-AUC	Quantitative PAP curve area	Comparative quantitative analysis	Dependent on PAP methodology	Research and confirmation
Gradient diffusion/E-test	Growth along concentration gradient	Practical and accessible	Variable sensitivity	Screening
Time-kill assay	Viability over time during antibiotic exposure	Dynamic characterization	Labor intensive	Mechanistic characterization
Flow cytometry	Single-cell physiological response	High cellular resolution	Requires specialized instrumentation	Research/advanced diagnostics
Microfluidics	Single-cell growth and drug response	High spatial and temporal resolution	Standardization and cost challenges	Emerging research/rapid AST

Adapted conceptually from the mechanistic frameworks described by *Jayathilaka, et al., 2024, Elbehiry, A. et al., 2026, Niño-Martínez et al., 2019 and Li, W et al., 2025.*

5.4 Advanced Phenotypic Approaches

Conventional AST has been set aside by the development of new techniques that describe the dynamics or single-cell behaviour of bacteria. These methods are especially beneficial because heteroresistance is not just a variation in the final growth, but a variation in the way individual cells grow over time and with varying concentrations of antibiotic.

Time-kill assays

Time-kill experiments track numbers of viable bacteria over a period of time after exposure to antibiotics. This gives information on the killing time of the bacteria as opposed to just showing if visible growth occurs at an endpoint. Time-kill assays can identify biphasic and/or heteroresistant killing patterns in heteroresistant populations. A high proportion of

susceptible individuals can coexist with a high proportion of individuals that either die off slowly or survive. If the exposure to antibiotics is prolonged, the survivors may be recovered and tested for change in susceptibility. The dynamic nature is then the greatest benefit of time-kill analysis. Can be used to separate rapid killing from delayed survival and can help give insight into the behaviour of minority populations when exposed to treatment. Drawbacks are its labor-intensive nature, the need for repeated sampling, using viable-count measurements, and the interpretation difficulties in establishing universal interpretation criteria.

Single-cell imaging

The advantage of microscopy-based methods is that they give a much more direct insight into the heterogeneity of bacteria. Time-lapse microscopy allows for the tracking of individual cells over time after exposure to antibiotics and for the growth, division, elongation, lysis, changes in morphology, or survival of the individual cells to be recorded. This view of a single cell is of special importance because the average of a cellular population may mask the rare but biologically significant behavior. Imaging can show that, in some cells, growth is arrested rapidly, in some the cells die and, in some, growth continues even in the presence of antibiotics. This information can be used to differentiate heteroresistance from tolerance and persistence and can be used to reveal cellular trajectories not seen in bulk assays. The recent developments in automated microscopy and image analysis have also made it easier to extract quantitative phenotypic information from a large number of individual bacterial cells.

Flow cytometry

Flow cytometry can be used to rapidly analyse the characteristics of individual bacterial cells (size, membrane integrity, nucleic-acid content, metabolic activity, and fluorescence). Together with antibiotic exposure and suitable fluorescent markers it is capable of detecting different subpopulations of cells. Throughput is one big benefit. Flow cytometry can analyse thousands of events in a brief time frame, whereas conventional microscopy could analyse relatively small numbers of cells. This means that it is appealing for minority physiological states. The major drawback is that it is difficult to interpret. A phenotypically distinct population, as identified by flow cytometry does not necessarily equate to a heteroresistant population. Thus, there is still a need for appropriate validation against susceptibility measurements and, if possible, population-based and/or genetic methods.

Microfluidics

Microfluidic systems enable the highly controlled environment where individual bacterial cells can be challenged with antibiotics, and growth and survival data is monitored. These platforms can create a gradient of antibiotics and allow for real-time monitoring of the response of the bacteria. Microfluidics is especially exciting for heteroresistance as it has the potential to connect between population level susceptibility testing and single cell biology. Individual cells can be tracked before, during and after antibiotic treatment, and cell survival can be correlated with stable resistance, antibiotic-induced physiological change or stochastic cellular responses. Microfluidics is becoming a more and more ubiquitous technology in the new field of rapid-AST, which has the potential to shorten testing time without sacrificing phenotypic data. Microfluidics and other miniaturized methods were revealed as key elements of the fast-evolving diagnostic field in a review of next-generation phenotypic AST technologies conducted in 2024 (Resznetnik et al., 2024).

5.5 Genomic and Multi-Omics Approaches

The phenotypic assays determine the actions that the bacteria take in the presence of the antibiotics while the genomic and multi-omics approach helps to explain the different behavior of the various subpopulations. These methods can be useful especially if there are mutations, gene amplification, changes in gene expression, changes in protein levels or changes in metabolism associated with heteroresistance.

Whole-genome sequencing

Whole-genome sequencing (WGS) can be used to detect mutations, resistance genes, mobile genetic elements, copy-number changes and genomic relationships between populations of bacteria. When resistant and susceptible fractions are isolated separately to be sequenced, WGS can be very informative in the context of heteroresistance. The sequencing of subpopulations can be used to compare them and identify changes that may not be noticeable in bulk sequencing. However, conventional WGS of a total isolate may not be able to detect variants that occur at low frequencies due to the fact that the sequence reads will mostly consist of the major population. Minority variants could be more easily resolved using deep

sequencing, long-read methods or sequencing of isolated subpopulations. Important, the genetic potential, not the phenotype is predicted. Resistance can be associated with a gene that is poorly expressed or resistance can take on multiple forms, not all of which can be guessed from the sequence data. Therefore, the most effective use of genomic information is in conjunction with phenotypic AST (Mustafa, 2024; Li et al., 2025).

Transcriptomics

As RNA expression level is measured in transcriptomic approaches, they can capture the change in gene expression in bacterial cells when exposed to antibiotics. Activation of efflux systems, stress responses, membrane remodeling pathways, metabolic adaptations, and resistance-associated genes may be detected by RNA sequencing. Transcriptomics is of special interest for heteroresistance, the comparison of resistant and susceptible subpopulations. Some differences in transcription profiles may be used to help determine if the resistant phenotype is driven by an increase in the expression of an existing resistance determinant or development of a new DNA sequence. One drawback is that the expression of genes is very dynamic and dependent on growth conditions, on the sampling time, the concentration of antibiotics, and on environmental factors. Thus, careful experimental design of the transcriptomic approach is required and where possible, validation of the findings with phenotypic or protein measurements.

Proteomics

Proteomics is the study of proteins found in actual bacterial cells, and it can thus establish an intermediate link between expression of genes and the phenotype. This is very useful in the case of resistance mechanism through efflux pumps, antimicrobial modifying enzymes, membrane proteins, stress response proteins and metabolism. For clinical microbiology, mass-spectrometry-based approaches are gaining considerable interest as they can characterize bacterial proteins on a rapid time scale and with more complex workflows, they can deliver information regarding the resistance-associated protein pattern. However, the identification and more in-depth protein characterization by conventional MALDI-TOF should not be regarded as an equivalent. The bottom up and top-down proteomics yields a much higher molecular resolution, but are also more technically challenging (Lavigne et al., 2024).

Metabolomics

Small molecule and metabolic states are provided by metabolomics. The relevance of this to heteroresistance is due to the tight link between bacterial metabolism and antibiotic susceptibility. Resistant minority cells can have other energy metabolic, redox, amino acid metabolic, membrane biosynthesis, or central metabolic pathways.

5.6 Emerging AI and Machine Learning-Based Diagnostics

Artificial intelligence (AI) and machine learning (ML) are beginning to influence AMR diagnostics have enabled the analysis of complex data sets which are not easily interpretable with regular statistical methods. This is especially important for heteroresistance as the phenotype can be described by multi-dimensional data such as growth curves, colony morphology, genome variations, single-cell behavior, and patterns of responses to antibiotics.

Predictive susceptibility models

Relationships between the bacterial genomic features and observed susceptibility phenotypes can be learned by machine-learning models. Various models such as random forests, gradient boosting algorithms, logistic regression, support vector machines and neural-network models have been explored for the prediction of AMR. One of the main features is the prospect of predicting resistance based on the genomic data before the phenotypic data is obtained. In 2024, a systematic review was conducted on WGS and ML approaches to predict AMR, which found high accuracy in various models and pathogens with significant differences between data sets and resistance types (Ardila et al., 2024). The field of heteroresistance has since advanced beyond theory and has become a reality. Guliaev et al. (2025) created ML models based on features developed from WGS to identify heteroresistance in *E. coli*. Their work is significant as it shows that computational models can be used to find a phenotype which is not detectable by standard susceptibility testing. But to achieve good performance from ML models, the training data must be high quality and representative. A model developed with a different population of a given species of bacteria, geographic location, antibiotic or laboratory procedure, may have different performance characteristics. Before broader implementation, external validation of the tool(s), standardized datasets, interpretability and clinically meaningful outcome measures will therefore be crucial.

Automated image analysis

Digital microscopy and automated image analysis provide another promising route. Computer-vision algorithms can measure the size of colonies, their shape, growth dynamics, number of divisions, elongation, lysis, and more, rather than having to manually examine each colony by a microbiologist. This method may prove to be useful for heteroresistance as these resistant minority colonies or atypical growth might harbor information which is hard to count manually. Integrating time-lapse microscopy and automated image analysis may be able to create high-dimensional phenotypic responses to antibiotics.

Clinical decision-support tools

The final goal of AMR diagnostics via a computational approach is not only to be able to classify bacterial phenotypes but also to give clinically relevant information. Decision-support systems might be able to integrate microbiological, genomic, patient, antimicrobial pharmacokinetic, and local antimicrobial resistance data to guide personalised choices of treatment. But there is a much greater level of evidence than simply laboratory prediction that is needed to make it clinically viable. Algorithms need to be externally tested, easily explainable to allow physician interpretation, stable to ensure that the laboratory workflows do not affect the algorithm's results and allow for incorporation into the current diagnostic systems. AI should, therefore, be considered as a complement to microbiological knowledge and skills, and not a substitute for laboratory confirmation.

Table 3. Emerging technologies for characterization of heteroresistance.

Technology	Information generated	Resolution	Major strength	Key challenge
WGS	Mutations, resistance genes, mobile elements	Population/genomic	Identifies genetic determinants	Low-frequency variants may be missed
Deep sequencing	Minority variants and copy-number differences	Subpopulation	Better detection of rare variants	Cost and bioinformatic complexity
Transcriptomics	Gene-expression profiles	Population/subpopulation	Reveals active regulatory pathways	Highly condition-dependent
Proteomics	Protein abundance and proteoforms	Molecular	Links expression to phenotype	Technical complexity
Metabolomics	Metabolic state	Molecular	Captures physiological adaptation	Difficult causal interpretation
Single-cell imaging	Growth, division, death, morphology	Single cell	Direct visualization of heterogeneity	Throughput and analysis
Flow cytometry	Cellular physiological states	Single cell	High throughput	Phenotype requires validation
Microfluidics	Dynamic single-cell antibiotic response	Single cell	Controlled exposure and real-time observation	Standardization and clinical translation
Machine learning	Predictive patterns	Dataset/model	Integrates complex variables	Generalizability and interpretability
Computer vision	Automated phenotypic features	Colony/single cell	Rapid quantitative analysis	Requires high-quality image datasets

Integrating Conventional, Phenotypic, Genomic, and Computational Diagnostics

None of the existing diagnostic techniques are ideal and can solve the heteroresistance issue entirely. Although conventional AST is still essential for the clinical susceptibility classification, it suffers from the limitation of failing to consider minority phenotypes in a population. PAP is sensitive for detecting resistant subpopulations, but is time consuming. The advanced phenotypic techniques offer better temporal and cellular resolution and the genomic and multi-omics approaches offers mechanistic information. With the help of AI and machine learning, these various data sources can be combined and patterns that are hard to detect with traditional analysis methods can be identified. Thus, a future diagnostic process might not be linear, but rather be composed of several tests depending on the context. The first susceptibility test might be a routine AST, with follow-up testing for heteroresistance based on certain clinical and/or microbiological characteristics. The phenotype might then be confirmed using PAP or an appropriately validated alternative and the underlying mechanism might be characterized using WGS and multi-omics. These measurements can be combined in computational models to generate a heteroresistance profile that will be a probability-based measure in the future. This aligns with the general trend of phenotypic diagnostics, molecular characterization and integration of data for AMR diagnostics, which all aim to provide rapid, accurate diagnosis and treatment. Recent reviews highlight the growing strength of the capability of rapid phenotypic AST and the increase in the use of genomic surveillance in clinical and public-health decision making (Resznetnik et al., 2024; Li et al., 2025). The big difficulty lies in the standardization. The definition of heteroresistance and the concentrations and size of the inoculum, the duration of incubation, the detection limit and the criteria for determining resistant subpopulations may differ in different studies. Such differences may result in seemingly conflicting prevalence estimates and comparison of prevalence across studies may be difficult. Standardization of diagnosis must thus go hand in hand with technology. The other important question is whether the percentage of resistant minority is found and whether there is a clinical significance.

A lab test can detect a small subpopulation resistant to the antibiotic, but this does not necessarily indicate that the phenotype will lead to failure to treat a patient. The clinical interpretation should take into account the concentration of the antibiotic at the site of the infection, the severity of the infection (bacterial load), immune system status, site of infection, duration of therapy, fitness of the resistant population and stability of phenotype. Therefore, the detection of antibiotic heteroresistance necessitates a change in the paradigm of conventional susceptibility testing to a more population-based approach to diagnosing antibiotic resistance. Although they are still very important in the clinic, both broth microdilution and disk diffusion and the automated systems are based on the assumption of a population average and may fail to detect rare resistant cells. Gradient diffusion can be used as a screening strategy and PAP and PAP-AUC can give more direct characterization of resistant subpopulations. The assays of time-kill and flow and microscopic and microfluidic technologies complement the other approaches in detection, and bring it into the dynamic and single-cell arena. Genomic and multi-omics technologies provide another layer of information by identifying genetic, transcriptional, proteomic and metabolic processes related to the degree of the response to the antibiotics and the variability of the response. With the rise of AI and ML, there is an opportunity to harness this confluence of complex data streams for use in predictive diagnostic systems. Important is the recent study showing that ML can be used to detect heteroresistance in clinical *E. coli* samples, indicating that computational methods might be used in the future to identify subpopulations that are resistant, but not detected in a routine WGS run (Guliaev et al., 2025). Finally, the best diagnostic approach will rarely be a one-size-fits-all solution to the problems of current AST. Rather, there are structured trends toward the field becoming integrated, multi-resolution diagnostics with population level susceptibility interpreted with the behavior of resistant-subpopulations, genomic variation, cellular physiology, and the prediction from computer models. This could make heteroresistance more of a clinical feature, as opposed to primarily a laboratory phenomenon, and help inform antimicrobial therapy decisions.

6. BODY OF STUDY INCLUDING PATHOGEN-SPECIFIC HETERORESISTANCE AND CLINICAL BURDEN, AND DISCUSSION

Heteroresistance to antibiotics is no longer just a microbiological curiosity, but a clinically relevant characteristic of several important bacterial pathogens. The clinical significance occurs when the susceptibility test report is simply but importantly different from what is actually in an infecting population. An isolate may be considered susceptible if most of the cells are inhibited by an antibiotic but there is also a minority of cells that resist the antibiotic at much higher concentrations. When the cells are exposed to antimicrobial therapy, these minority cells can increase in frequency, and in some situations, develop into a resistant population (Xu et al., 2025). Especially it is concerning that this phenomenon occurs among organisms which already have a considerable multidrug resistance. The pathogens that are considered to be international threats to antimicrobial resistance include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Vancomycin-resistant *E. faecium*, methicillin-resistant *S. aureus*, carbapenem-

resistant *P. aeruginosa*, carbapenem-resistant *A. baumannii*, and Enterobacterales resistant to multiple antibiotics are included in the high or critical priority category of the WHO 2024 Bacterial Priority Pathogens List, reflecting the clinical impact of resistance to these organisms (World Health Organization [WHO], 2024). Heteroresistance does not occur at an even rate for pathogens or antibiotic class. The extent of these variations, depending on bacterial lineage, antimicrobial exposure, geographical location, laboratory methodology, type of infection and the definition of heteroresistance, is given by the available studies. Therefore, the prevalence data should be interpreted with caution, and not be directly comparable without taking into account methodological differences (Xu et al., 2025).

6.1 Gram-Positive Pathogens

Staphylococcus aureus

S. aureus is one of the best-studied of Gram-positive pathogens in the context of heterogeneous antibiotic susceptibility. Heterogeneous vancomycin-intermediate *S. aureus* (hVISA) is a clinical identified phenotype that consists of a resistant subpopulation within a predominantly susceptible population. Of special interest in the treatment of serious infections caused by methicillin-resistant *S. aureus* (MRSA) are these populations, since vancomycin still has a place in many invasive infections caused by MRSA. The biological basis of vancomycin heterogeneity is complex and could involve changes in cell-wall architecture, regulatory pathways and several mutations in the chromosomal DNA. If cells' cell wall structure is altered, this can hinder the access of vancomycin to its target, and a minority population may withstand antibiotic exposure. However, recent studies have highlighted that vancomycin adaptation in *S. aureus* is driven by a wide range of mutations, not just a single resistance determinant (Fait et al., 2024). Heteroresistance also occurs in *S. aureus* other than vancomycin. A high prevalence of heteroresistance was observed in 2024 in a large genomic study, which revealed multiple mutations with a high frequency, across the core genes and not a single genetic pathway. What is significant about this observation is that it indicates that heteroresistance may be acquired repeatedly in various evolutionary pathways within the same species (Heidarian et al., 2024). On a clinical level, the biggest worry is that traditional MIC testing can overestimate the treatment resistance of an *S. aureus* population. This becomes a problem in particular if the infection is deep-seated, the bacterial load is high, or if the infection is subjected to extended antimicrobial treatment. However, heteroresistance shouldn't be taken as an indicator that treatment is not going to work. The clinical outcome is influenced by the antibiotic exposure, site of infection, bacterial density, host immunity, and stability and frequency of the resistant subpopulation.

Enterococci

The role of enterococci is of great importance due to their ability to acquire and spread resistances and survive in the healthcare setting. If vancomycin resistant, *Enterococcus faecium* is a high-priority resistant pathogen, according to WHO (2024). The case of vancomycin heteroresistance in *E. faecium* demonstrates the potential for significant phenotypic diversity from genetic amplification that is unstable. Instability of genetic amplification can produce ample phenotypic diversity as illustrated by vancomycin heteroresistance in *E. faecium*. In 2024, the vancomycin heteroresistance was reported to be associated with unstable tandem amplification of the *vanM* gene cluster on a conjugative plasmid. This amplification can lead to a higher concentration and expression of resistance genes in a group of cells, resulting in a population with both susceptible and highly resistant cells (Sun et al., 2024). The clinical significance is great since VRE or VRE-heteroresistant enterococci may restrict treatment options in the bloodstream, intra-abdominal, urinary, and device-associated infections. In addition, enterococci have the capacity for genetic exchange, and there is the possibility of the spread of heterogeneous resistance phenotypes, which would increase the spread of antimicrobial resistance genes.

Streptococci

Heteroresistance has also been shown for streptococci, but the evidence is not as extensive as for *S. aureus* and Gram-negative pathogens. *Streptococcus pneumoniae* is one such example that is informative. Previous population analysis studies showed that pneumococcal heteroresistance to penicillin occurred in several geographically widespread multidrug-resistant clones. The resistant subpopulations were able to grow at higher concentrations of penicillin than was observed for the dominant population based on the MIC, and were demonstrated to be reversible in antibiotic-free media (Hakenbeck et al., 2007). Later studies suggested that PBP2x is involved in the heteroresistant phenotype, and other PBP2x alterations have been confirmed. These observations fit the hypothesis that heteroresistance may enable pneumococci to evolve at intermediate levels of antibiotic resistance where subpopulations can test their ability to survive at higher drug concentrations prior to the selection of a stable high-level resistance phenotype (Engel et al., 2014). The clinical relevance of streptococcal

heteroresistance is less clear than that of several phenotypes of Gram-negative bacteria. However, its presence suggests that clinically relevant heteroresistance is not limited to multidrug resistant Gram-negative organisms.

6.2 Gram-Negative Pathogens

Genetic diversity in heteroresistance is particularly intriguing in gram-negative bacteria, which are already highly effective at expressing very efficient efflux pumps, low permeability outer membrane, mobile resistance determinants and are extraordinarily plastic in their genome. Such attributes can help with the quick adaptation under antibiotic pressure. *A. baumannii* and Enterobacterales that are resistant to carbapenems have been identified as critical priority bacteria and *P. aeruginosa* as high-priority bacteria by WHO (2024).

Klebsiella pneumoniae

K. pneumoniae is a classical bacterium in literature that is heteroresistant. CRKP frequently has complex resistance patterns and resistance has been found to the polymyxins, carbapenems, tigecycline, and ceftazidime-avibactam among other clinically important antibiotics (Xu et al., 2025). Currently only evidence for polymyxin heteroresistance exists. Polymyxin heteroresistance was detected in 118 of 154 patients with CRKP, in a retrospective genomic cohort of isolates from patients receiving polymyxin B treatment. Of these 33, 26 were rendered 100% resistant after being treated. Strikingly, resistant subpopulations were found before the full resistance was detected in the vast majority of patients that subsequently became fully resistant (Luo et al., 2024). This observation is important for an evolutionary interpretation as it might indicate that at least in some CRKP populations, heteroresistance can serve as a stepping stone on the way to stable high-level resistance. But it is not causal, though. Only some heteroresistant variants will develop into complete resistance and host factors and exposure to treatment will remain important. Other emerging problems are the heteroresistance to tigecycline. Characterization of recent CRKP isolates revealed resistant subclones that were able to grow in the presence of high concentrations of tigecycline, with some CRKP phenotypes being stable following multiple antibiotic-free passages. Genomic analysis indicated involvement of determinants linked to efflux and complex resistance backgrounds; detailed functional validation of the individual genes will be required (2025).

Acinetobacter baumannii

A. baumannii (AB) is a critical priority pathogen (WHO, 2024) and one of the most problematic pathogens in the world especially as a carbapenem-resistant *A. baumannii* (CRAB) (WHO, 2024). The colistin heteroresistance of *A. baumannii* has been of special interest owing to the fact that colistin has been a salvage or last line option for treatment of infections with highly resistant isolates. In a large clinical study of 173 isolates of CRAB, 67.1% of the bacteria were colistin heteroresistant. Most importantly, 80.2% of the heteroresistant isolates evolved to a fully resistant phenotype in the experimental environment used. More 14-day mortality was also seen among the bacteremic patients that were heteroresistant (Kon et al., 2023). Some of the most definitive evidence of the clinically relevant association between heteroresistance has been reported. Also, they present an example of why colistin heteroresistance is interesting: A population of cells that looks susceptible, can contain cells that will rapidly develop resistance to colistin. However, prevalence figures reported from studies are very variable. The results of a systematic review and meta-analysis put the overall prevalence of *Acinetobacter* heteroresistance to colistin at approximately 33%, although there was significant heterogeneity among the studies (Li et al., 2020). More recent clinical trials have continued to identify sub-populations that are resistant and have demonstrate that past antibiotic use can impact the emergence of resistant sub-populations (2025).

Pseudomonas aeruginosa

P. aeruginosa is a bacterium with a very high capacity for adaptive resistance via activation of efflux-pumps, porin modification, target modification, formation of *P. aeruginosa* biofilm and regulatory adaptation. The combination of these properties makes it a good organism to develop heterogenous antimicrobial phenotypes. Heteroresistance has been reported for carbapenems, colistin, newer β -lactam/ β -lactamase inhibitor combinations and fluoroquinolones. There have been significant reports of heteroresistance to imipenem and meropenem in available clinical studies, with some studies reporting high frequencies. Heteroresistance has been reported for both colistin and levofloxacin, but it remains unclear if these are clinically relevant (Bassetti et al., 2023). This is particularly worrisome for newer agents, especially for heteroresistance. For example, the cefiderocol heteroresistance has been investigated in clinical *P. aeruginosa* strains and mutations in tonB-dependent receptors have been proposed to play a role. The siderophore mediated uptake mechanism is unrelated to porin mediated uptake, therefore a decrease in susceptibility could occur because of changes in these receptors (2024). The clinical

evidence should however be used with caution. Even though heteroresistance is often identified in the laboratory, there are no consistent studies to show greater mortality or failure of treatment in all *P. aeruginosa* infections. This is significant as microbiological heterogeneity does not necessarily equate with a bad clinical outcome.

Escherichia coli

Heteroresistance in *E. coli* is less studied compared to *K. pneumoniae* and *A. baumannii*, and was only recognized as being as significant as recently. Multiple classes of antibiotics can be heteroresistant and it can be particularly difficult to identify heteroresistance in bloodstream infections, which often require prompt treatment decisions. The contemporary literature, based on recent studies, indicate that heteroresistance frequencies may be quite variable with respect to the antibiotic tested. The heteroresistance to colistin is apparently less prevalent in *E. coli* than in other Gram-negative organisms and there may be a lot more heteroresistance towards others (Xu et al., 2025). Especially, the increasing capacity to perform whole genome sequencing, and machine learning, are relevant to *E. coli*. Such tools might be helpful in identifying genomic patterns which are not easily identified in routine AST which are associated with heteroresistant phenotypes. It could also be beneficial when bacteria are found in the blood stream, and also when it is necessary to know, for example, the location of hidden subpopulations of resistance in a short time to guide the choice of antimicrobials.

6.3 Heteroresistance to Last-Line Antibiotics

Heteroresistance has probably the greatest clinical impact with antibiotics which are already in use due to limited traditional treatment options. Colistin, vancomycin, carbapenems, tigecycline, and daptomycin are therapeutics for different niches, but each case of heteroresistance demonstrates the same issue: populations susceptible on the surface may harbor cells with the capacity to survive when exposed at therapeutic concentrations.

Colistin

Colistin heteroresistance is well reported for *A. baumannii* and Enterobacterales. The phenotype is clinically of concern because infections from an organism that is resistant to numerous other antimicrobial drugs may be treated with colistin. In CRAB, it has been shown that heteroresistance can evolve to full resistance in many experimental evolution experiments, and is suggested to be associated with poor clinical outcomes in bacteremia. (Kon et al. 2023) Polymyxin heteroresistance has also been linked to emergence of complete resistance in treatment in CRKP. These results suggest that resistant minority populations can serve as reservoirs for the development of high-level resistance under the drug selection of therapy (Luo et al., 2024).

Vancomycin

Heterogeneity of vancomycin is of special significance in *S. aureus* and enterococci. Heterogeneous VIRSA phenotypes in *S. aureus* may make MRSA infection difficult to manage. This is because in *E. faecium*, cells with relatively high or low van susceptibilities can coexist through the amplification of van gene clusters, which is unstable. (Sun et al., 2024) In the case of vancomycin treatment, which is often long-term for severe infections, the selective environment can lead to the survival and growth of less-sensitive subpopulations.

Carbapenems

Carbapenem heteroresistance is a significant feature of *K. pneumoniae* and *P. aeruginosa*. The worry is not only for the short-term survival of a minority population, but the prospect that they might gain or develop further resistance mechanisms. The clinical significance of carbapenem heteroresistance is compounded by the fact that the WHO has designated carbapenem-resistant Enterobacterales, carbapenem-resistant *A. baumannii* as critical priority pathogens and carbapenem-resistant *P. aeruginosa* as high-priority pathogens. (WHO, 2024).

Tigecycline

Tigecycline continues to be a useful drug in certain MDR infections, especially where multiple traditional drugs have failed. The emergence of heteroresistance to tigecycline, however, has been reported increasingly in CRKP. Efflux-pump regulation and plasmid-associated determinants of resistance may be factors in the generation of resistant subpopulation (2025). Of particular concern is that with antibiotic removal, tigecycline heteroresistance could persist and that some resistant subpopulations might not only not disappear, but continue to survive.

Daptomycin

Daptomycin is a valuable drug alternative for the treatment of refractory infections caused by Gram-positive bacteria, especially resistant enterococci and *S. aureus*. Logically, there is a biological basis for heterogeneous susceptibility to daptomycin, which can occur by changes in membrane charge, phospholipid metabolism, cell-envelope homeostasis, and regulatory pathways. In comparison to vancomycin heteroresistance, however, the clinical literature specifically describing daptomycin heteroresistance is less extensive. Therefore, it would be more accurate to state that daptomycin is an emerging area of concern rather than to assume that a uniform clinical heteroresistance phenotype has been defined for all of the pathogens of interest. This is crucial for clinical advice when extrapolating lab observations to the clinic.

6.4 Biofilm-Associated and Chronic Infections

It is worth noting also that the links between heteroresistance and biofilms are especially pertinent as both chronic infections and biofilms create conditions conducive to bacterial heterogeneity and extended periods of exposure to antimicrobials. Biofilms contain cells with different metabolic states and in different nutrient/oxygen/antibiotics/host molecule concentrations. Such gradients establish highly structured environments in which susceptible and less susceptible subpopulations can coexist. Biofilm associated resistance does not always equal heteroresistance. There are three different types of resistance: conventional genetic resistance, physiological adaptation, and tolerance associated with biofilms. However, the biofilm environment can enable the development and maintenance of variation among phenotypes, due to spatial refuges and extended periods of selection (Liu et al., 2024).

Device-associated infections

Foreign surfaces, such as indwelling catheters, prosthetic joints, vascular devices, cardiac implants, and others can provide surfaces for bacterial biofilm to form. Structured communities of organisms, including *S. aureus*, enterococci, *P. aeruginosa* and Enterobacterales, can form on these surfaces. The removal of established biofilms is difficult as antibiotics may not reach them sufficiently, metabolism may be heterogeneous between bacteria in the biofilm, and immune clearance may be hampered. Such properties can lead to situations where resistant minority populations are able to adapt to repeated exposure to antimicrobial agents. The formation of device-associated biofilms has been highlighted in recent reviews, as it can lead to chronic infection and even necessitate the removal of the device in addition to antimicrobial therapy (Liu et al., 2022).

Respiratory infections

Bacterial heterogeneity is another setting where it can become clinically relevant, for chronic respiratory infections. *P. aeruginosa* plays a significant role in chronic lung disease such as CF, where it lives in mucus surfaces with high oxygen and nutrient gradients. Some of these structured communities contain actively growing cells and others in slower growing metabolic states. Further exposure to antibiotics may then result in the selection of antibiotic resistant or tolerant subpopulations. Thus, the clinical relevance of heteroresistance and other survival phenotypes in biofilm-associated *P. aeruginosa* is a clinically relevant scenario in which these mechanisms may interact (Liu et al., 2024).

Urinary tract infections

Another environment in which repeated bacterial exposure to antibiotics has been shown to occur is in the urinary tract in women with recurrent UTIs. Uropathogenic *Escherichia coli* is a major contributor to community-acquired UTI, whereas other pathogens, such as *Klebsiella pneumoniae* and enterococci, are a growing problem in HAIs. Recurrent UTIs (RUTIs) generate repeated selection events because of frequent exposures to antibiotics. Furthermore, urinary catheters can harbor bacterial surfaces where bacteria can aggregate and thrive despite antibiotic treatment. With the increasing antimicrobial resistance in uropathogenic bacteria, treatment and outcomes are already linked, and hidden resistant subpopulations are a promising field to investigate (Simoni et al., 2024).

Wound infections

Chronic wounds create a unique ecological niche where there is tissue damage, inflammation, a nutrient gradient, oxygen deprivation, polymicrobial flora and frequent exposure to antimicrobial agents. Biofilm-forming *S. aureus*, *P. aeruginosa*, Enterobacterales and other organisms could remain in wounds and be responsible for delayed healing. The clinical implications are not just on the issue of antibiotic resistance. Biofilms of bacteria in chronic wounds can lead to chronic inflammation and hinder tissue healing, and multiple species of bacteria can make microbiological diagnosis and treatment

more difficult. Biofilm formation, impaired healing and chronic infections are strongly linked with the wound microbiota, which has been highlighted recently (Uberoi et al., 2024).

6.5 Epidemiology and Global Distribution

The epidemiology of heteroresistant infections is complicated by the fact that there are several different definitions of resistant subpopulations as well as antibiotic quality varying, and different population analysis methods and criteria for identification of resistant subpopulations. Hence, it is important to note that reported prevalence is not an exact estimate but rather a spectrum. Evidence available, however, indicates that there is geographical fitness and widespread prevalence of heteroresistance. It has been reported from Asian, European, Middle Eastern and other regions of the world of North and South American. Commonly reported frequencies include those of highly resistant Gram-negative organisms, including *K. pneumoniae* and *A. baumannii* that are most often polymyxin-resistant or other last-line antibiotics (Xu et al., 2025).

Hospital settings

There are risk factors at the hospital level that can contribute to development and persistence of heteroresistance, such as high rates of antimicrobial use, sicker patients, longer courses of antimicrobials, intensive-care units, invasive devices, frequent and close transmission networks and multi-class exposure. The ESCAPE pathogen *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp. are especially relevant in this scenario as they are responsible for substantial burden of disease within and outside of healthcare settings and have many resistance mechanisms and high-risk clones (Miller & Arias, 2024).

Community-acquired infections

The primary focus on heteroresistance has been on hospital-acquired organisms (HAI), but should not be considered as the only event produced in the hospital. There are, of course, resistant subpopulations of community pathogens as well, because the exposure to antibiotics in the community is common, and antibiotic resistance determinants are within the population of community pathogens. Resistant clones and mobile genetic elements are likely to cross over between reservoirs in the hospital, community, animal and environment, making the distinction between hospital versus community epidemiology more and more ill-defined. Surveillance, therefore, should be expanded beyond hospital antibiograms, to encompass a broader perspective of One Health.

Geographic trends and surveillance

The differences between regions are expected as a result of differences in antibiotic use, infection control, frequency of antibiotic resistance in bacteria and antibiotic sensitivity testing between countries, or bacterial clones. WHO has hence called for a regionalised global priority-pathogen framework (in accordance with the local context and not as a global ranking) (WHO, 2024). This is particularly true in the case of heteroresistance. A high prevalence in one area does not necessarily correlate with a high biological susceptibility to heteroresistance, but might reflect either over-representation of a particular clone in the area or previous exposure to a particular antibiotic or sensitivity of detection method used. In the future, the presence of heteroresistance should be included as one of the variables to monitor along with the bacterial lineage, history of antibiotics, type of infection, frequency of the resistant subpopulation, the way the resistance was detected, and clinical outcome of the infection.

Clinical Interpretation and Overall Significance

Clinical interpretation and overall significance are the terms used to describe the overall significance of a clinical interpretation. Heteroresistance is clinically significant in a variety of different ways in different pathogens. The more evidence that is available the more likely it is that a resistant minority will exist prior to treatment and that it can be demonstrated when the patient starts antibiotic treatment and when microbiological or clinical deterioration has been observed while the patient is on antibiotic treatment. Resistant subpopulations have been reported before treatment in the two cases of colistin-resistant bacteria from China (CRAB) and polymyxin B-resistant bacteria from Pakistan (CRKP), followed by the emergence of resistant bacteria after treatment (Kon et al., 2023; Luo et al., 2024). Heteroresistance does not necessarily signify treatment failure, although, of course, it is an indication of treatment failure. There is still a lot of variation in the clinical literature and some studies have shown no significant difference between outcomes of heteroresistant and non-heteroresistant infections. The variations may be due to differences in the number of bacteria, exposure to antibiotics, host's immune system, site of infection, or the way the bacteria is detected. The differences may be due to the number of bacteria present, exposure to

antibiotics, immune system of the host, nature of the infection and detection methods for the presence of the bacteria. It is therefore important to highlight the clinical message, i.e. the presence of heterogeneity in a population may result in an apparent susceptibility but will in all cases yield a different treatment. This holds true particularly with infections caused by multidrug resistant organisms, with limited treatment options, already available. Heteroresistance can also be an unnoticed source of resistance from an epidemiological point of view. A resistant minority can remain unnoticed until they come into contact with antimicrobials and then get "enriched". Resistant populations can then pick up compensatory mutations or stable resistance mechanisms and account for the development of resistant strains. This phenomenon in turn links the person to the wider evolution and public health processes (Shepherd et al., 2024; Xu et al., 2025). Overall, evidence indicates that heteroresistance is most clinically relevant for last-line antimicrobials, pathogens in the hospital with high clinical risk, chronic infections, biofilm-associated infections, and long-term exposures to antimicrobials. It is, therefore, increasingly important to recognise this phenotype as being part of AMR surveillance and research. Future research is needed to go beyond prevalence estimates, and to create standardised links between heteroresistance, patterns of exposure to antibiotics, dynamics of resistant subpopulations, response to treatment, and the outcome of patients.

7. CLINICAL IMPACT, PK/PD CONSIDERATIONS, AND TREATMENT FAILURE WITH DISCUSSION

The standard thinking on antimicrobial resistance to an infection that does not clear up with antibiotics is the following: "The organism that caused the infection is already resistant to the antibiotic," "The antibiotic was not present at the site of the infection at high enough levels," or "The organism became resistant during antibiotic therapy. The case of heteroresistance is more complex. In this case the laboratory may test the isolate as sensitive, and most bacteria might be inhibited but a few survive and multiply in the presence of the antibiotic. The clinical course can therefore be paradoxical: therapy is based on a 'susceptible' result, and bacteria do not die out during therapy nor do they become susceptible. The correlation between heteroresistance and treatment failure is logical, but not necessarily convincing. Although there has been experimental work that suggests that there is a relationship, and the pharmacodynamic models support this, animal studies and a handful of retrospective clinical studies seem to support this, prospective human data are relatively scarce. The infection site, the number of bacteria and the exposure to the antimicrobial agents, but also the immunity of the host and the fitness of the subpopulation of bacteria which has become resistant, all have an impact on treatment outcome. Thus, heteroresistance, and not just the absence of treatment failure, would be considered a risk factor.

7.1 Mechanisms Linking Heteroresistance to Clinical Failure

Expansion of resistant subpopulations during therapy

Heteroresistance is linked to treatment failure via a central mechanism selective enrichment. The population can be particularly susceptible with a subpopulation of cells growing at much higher levels of antibiotics. During the beginning of the therapy, there are more susceptible cells killed and the resistant ones are left. The proportion of cells that are resistant increases as treatment progresses. In some instances, such as when the resistant phenotype is the result of the amplification of genes or variation in the copy number of a plasmid, it can be especially rapid. Such mechanisms can give rise to large variation in the dosage of resistance genes of individual cells in a closely related population. After a period of antibiotic exposure, cells carrying more copies of the resistance gene will have a selective advantage and cells in the absence of selective pressure may revert to the pre-antibiotic state. This would provide a plausible explanation for the emergence of a clinically susceptible isolate that is predominantly resistant during therapy. This process was shown to be clinically relevant especially in the case of carbapenem-resistant *Klebsiella pneumoniae*. Among 154 polymyxin B-treated patients in a retrospective cohort, 118 patients were found to have clinically undetected polymyxin heteroresistance prior to treatment and 33 (28%) of these heteroresistant infections went on to develop resistance to polymyxin B during treatment. The same evolutionary trajectory has been seen in patients that are critically ill with polymyxin-heteroresistant CRKP. Of the patients who had never received polymyxin before, 69.7% were heteroresistant, and 38.1% of the heteroresistant isolates later proved to be also polymyxin resistant and responsible for therapeutic failure. These observations indicate that there is a possibility of heteroresistance being an evolutionary step between susceptibility and stable resistance, but these are not certain.

Antibiotic selection pressure

This pressure can be intense or mild, and can persist for a short or long period of time, depending on which subpopulation of bacteria will be retained. The population may rapidly restructure if the minority is resistant but the majority is wiped out. The same goes for treatment with "last line" antibiotics, particularly. The antibiotics used in the treatment of MDR Gram-negative

bacteria have a narrow therapeutic window and can generate high selective pressure. Such heteroresistant populations can thus be a clinically significant survival niche where exposure to antibiotics controls the dominant population but not completely eliminate the resistant subpopulation. The evolution of colistin resistance and heteroresistance in *K. pneumoniae* has been the subject of experiments that showed that resistance is extremely sensitive to the pattern of exposure and not just to the initial MIC.

Relapse and recurrent infections

Inadequate eradication can lead to persistence of the population of the infecting organism and reinfections. It is important to note that heteroresistance is different from tolerance or persistence, however. Tolerance is defined as resistance without any increase in MIC, heteroresistance is defined as resistance by a minority that grows at high concentration of the drug. The cells that are phenotypically tolerant are those that survive from the antibiotic and re-grow, and persistence typically constitutes a small percentage of this. These phenotypes may be observed in a clinical context. Recurrent infection can therefore be due to several interrelated mechanisms, including: Antimicrobial resistance due to the presence of a stable genetic resistance, Heteroresistant subpopulations, Persister cells, Biofilm formation, and Defective penetration of antimicrobials. If not performed, this complicates it to simply attribute the failure to recur to heteroresistance.

Treatment failure as an evolutionary process

It can be easily understood that the progression is:

Sensitive majority and resistant minority → antibiotic exposure → preferential killing of sensitive cells, enrichment of resistant cells, loss of efficacy of antibiotic treatment, stable resistance, persistence/relapse. This is consistent with the wider evidence of the emergence of antimicrobial resistance in patients by selection of pre-existing resistant variants, de novo mutations, horizontal gene transfer, or by the introduction of resistant lineages. The other phenomenon is another one: Heteroresistance a small number of resistant individuals may be present from the beginning of treatment and not be detectable with the normal AST.

7.2 Evidence from Clinical Studies

Clinical evidence of the association of heteroresistance to failure is growing but is significantly variable for different pathogens, antibiotics, and infection types.

Bloodstream infections

Some of the best clinical evidence can be obtained for bloodstream infections, in which one can obtain a relatively direct specimen of the infecting organism prior to and during therapy. A noteworthy recent study explored heteroresistance to cefotaxime, gentamicin and piperacillin–tazobactam in 255 patients who had *Escherichia coli* bloodstream infections. Heteroresistance was found in 125 isolates, but 96% of these heteroresistant isolates were classified as susceptible by routine susceptibility testing. Gentamicin heteroresistance predicted higher risk of admission to the intensive-care unit and death (around 7 times greater in the reported analysis) among patients treated with gentamicin, and among those treated with gentamicin, gentamicin heteroresistance was associated with higher risk of admission to the intensive-care unit and death. Also, patients who received Piperacillin–tazobactam had an increased risk for admission to intermediate care when used as part of heteroresistance. These results are important as they show three clinically relevant points in one go: relatively common heteroresistance; routine AST can greatly underestimate its presence; and The chosen heteroresistant phenotypes may be correlated with adverse clinical outcomes. However, these findings were obtained in a retrospective cohort, and are not sufficient to prove that the negative outcome was due to heteroresistance. May be due to severity of illness, selection of treatment, comorbidities and other confounders.

Pneumonia

Pneumonia poses a unique problem as the concentration of antibiotics in the lungs is much higher than the plasma concentration. Biofilm-like structures, necrotic tissue, abscesses, and epithelial lining fluid (ELF) influence local antimicrobial exposure. Heteroresistance can thus be clinically significant if the level of the infection at part of the infection area is within the zone of suppression of susceptible cells and resistant minority cells survive. This is notable in the case of respiratory infection caused by *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. This notion has been supported by experimental pharmacodynamic studies. In a murine lung infection model, the colistin-resistant subpopulation of *A. baumannii* was shown to differ significantly in response to colistin treatment when compared to a colistin-susceptible strain lacking the colistin-

resistant subpopulation, in the same model used to assess colistin susceptibility by MIC testing. There was no further reduction of the bacteria with increasing exposure to colistin as compared to the non-heteroresistant strain. Although animal models cannot be directly compared to outcome in human patients, these findings may be relevant to understanding why, in some instances, MIC-guided therapy may be over-optimistic with regards to the ability to eradicate bacteria.

Sepsis

The impact of treatment failure is worsened by the effects of sepsis as bacterial growth can be speedy and delayed successful antimicrobial treatment is correlated with poor outcomes. If a resistant subpopulation does exist, it is likely to be particularly harmful here since much of the initial therapy choice is determined by standard susceptibility or from clinical trial experience. Blood cultures may be positive at first and then continue to grow for a few days while the antibiotic is being used, but then, with further improvement in the clinical condition, a blood culture becomes positive again, but with resistance to that antibiotic. This could potentially result in the misconception that the organism has become resistant to the drug, as long as the original isolate is post hoc tested for resistant subpopulations. Specifically, the setting of sepsis is of interest with regard to the *E. coli* bloodstream-infection cohort, which showed an association with higher rates of intensive-care admission and death in those who received the associated antibiotic treatment regimen.

Urinary tract infections

Recently the evidence base for treatment failure associated with heteroresistance has been growing to include complicated urinalysis infections. The post hoc analysis of 291 index isolates from the ALLIUM phase 3 clinical trial piperacillin–tazobactam arm showed that 11.5% of patients had infections with piperacillin–tazobactam-heteroresistant isolates, although all isolates were found to be resistant at or below the traditional resistance breakpoint. Treatment failure rates were greater for patients with heteroresistant as compared to those with non-heteroresistant isolates. The unadjusted odds ratio was 2.13 but the adjusted odds ratio was not as well defined and did not meet the usual criteria for statistical significance (1.74, 0.82–3.71). Patients not wearing a removable urinary catheter seemed to be at especially high risk. This study is of special interest because it was performed with isolates from a prospective clinical trial as opposed to only isolates from a laboratory setting. Thus, it is a more valuable connection between the microbiological phenotype and clinical efficacy.

Table 4. Clinical evidence linking heteroresistance with treatment outcomes.

Infection/pathogen	Antibiotic phenotype	Major observation	Clinical implication	Evidence limitation
<i>E. coli</i> bloodstream infection	Gentamicin heteroresistance	Associated with increased ICU admission and mortality among treated patients	Potentially clinically important hidden resistance	Retrospective cohort
<i>E. coli</i> bloodstream infection	Piperacillin–tazobactam heteroresistance	Associated with increased intermediate-care admission	Possible treatment-risk marker	Observational design
Complicated UTI	Piperacillin–tazobactam heteroresistance	Treatment failure occurred more frequently	Supports clinical relevance of undetected HR	Post hoc analysis
CRKP infection	Polymyxin heteroresistance ^B	Resistant subpopulations frequently evolved toward full resistance	Strong evidence for within-host evolution	Retrospective study
Critically ill CRKP patients	Polymyxin heteroresistance	Resistant subpopulations expanded during exposure; therapeutic failure observed	Important risk during last-line therapy	Observational cohort
<i>A. baumannii</i> pneumonia model	Colistin heteroresistance	Poor bacterial response despite apparently susceptible baseline MIC	Demonstrates PK/PD implications	Animal model

<i>S. aureus</i> infection	hVISA	Associated in previous studies with persistent bacteremia and poorer outcomes	Supports relevance in Gram-positive disease	Definitions and studies vary
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7.3 Pharmacokinetic and Pharmacodynamic Considerations

Heteroresistance is a phenomenon that cannot be explained on a microbiological level only. Antibacterial activity is related to the time-dependency of drug exposure to bacterial susceptibility. This can become complex, for example when the cells in the infecting population have very different susceptibility profiles.

Antibiotic exposure variability

Pharmacokinetics is the relationship between an antibiotic concentration and the host, while pharmacodynamics is the effect of the concentration of the antibiotic on the bacterial population. The plasma level can be very different from the level of exposure at the site of infection. Augmented renal clearance, renal function, hepatic function, fluid distribution, obesity, extracorporeal therapies, critical illness, and the penetration of antimicrobials into certain tissues may all influence antimicrobial exposure. With a homogeneous, susceptible population, traditional PK/PD parameters (e.g., fAUC/MIC, C_{max}/MIC, %fT>MIC) can be helpful for estimating antimicrobial activity. The relationship is complicated by heteroresistance, in which the MIC of the dominant population may not reflect the susceptibility of the resistant minority. Thus, an exposure that may seem sufficient based on the reported MIC can be insufficient against the resistant subpopulation. This is one of the reasons why MIC does not fully describe the activity of an antibiotic.

The inoculum effect

Antimicrobial activity may be modified by changes in the density of bacteria. Even if the frequency of the resistant subpopulation is the same, the number of resistant cells at the baseline is higher at a high inoculum. For instance, a 10-5 resistant minority in a population of 10⁶ organisms would be ~10 resistant cells while in a population of 10¹⁰ organisms it would be ~10⁵ resistant cells. Therefore, a high number of bacteria can significantly boost the number of cells that can be selected. This is especially relevant for high burden infections such as abscess, endocarditis, severe pneumonia, deep wound infections and more. Population biology may thus play a role in the relationship between inoculum size and treatment outcome as well, in that the larger the number of bacteria present at the time of treatment, the more likely the presence of a clinically important resistant minority.

Sub-MIC antibiotic concentrations

Concentrations of antibiotics vary little from place to place inside an infected tissue. Gradients can arise around biofilms, necrotic tissue, abscesses, poorly perfused areas or respiratory secretions. In these areas, the concentration of the drug could be less than the MIC of the predominant population. Because sub-MIC exposure can help to inhibit susceptible bacteria while not killing resistant bacteria, it is important. Such exposure does not result in immediate sterilisations, but may establish a selective environment, favoring a relative advantage for resistant subpopulations. The results of pharmacodynamic studies of colistin-resistant and heteroresistant *K. pneumoniae* provide evidence that resistance can develop based on the exposure history and intensity of exposure to a drug rather than on baseline MIC less than a drug susceptibility breakpoint. A semimechanistic pharmacodynamic study of colistin in *E. coli* also showed that despite having similar EC₅₀ values, minor heteroresistant cells could have significantly different maximal killing characteristics compared to the dominant cells. This emphasises the need for population-level parameters to be considered as being unsuitable for describing the behavior of resistant minorities.

PK/PD interpretation in heteroresistant populations

Useful conceptual model:

Exposure to antibiotics → control of the susceptible majority population → survival of the resistant minority population → relative increase in the resistant minority population → regrowth of the population with stabilization of the resistant population.

This model indicates that, beyond reaching an exposure sufficient to achieve the dominant population, successful therapy would be facilitated by creating a robust and enduring pharmacodynamic effect that would result in the eradication of

clinically relevant subpopulations of clinical resistance. However, it is not always a good idea to increase the dose of the antibiotic. Progression to higher doses may be restricted by the toxicity of the kidneys, brain, or liver. Therefore, the therapeutic approach should take into account the need for adequate exposure, toxicity, and/or combination therapy or source control.

7.4 Host Factors Influencing Treatment Outcomes

Heteroresistance is not a stand-alone. Different outcomes of the same bacterial phenotype can be observed in different patients, depending on the context of the biological system (host immunity, tissue architecture, microbiota, bacterial density, and comorbid diseases) in which antimicrobial treatment takes place.

Immune status

After antimicrobial treatment, the bacterial load has been lowered, and patients with intact immune responses might be able to clear the remaining minorities of bacteria. However, immunocompromised persons can continue to be at risk from relatively small survivors. Neutropenia, hematological malignancy, transplantation, immunosuppressive therapy, advanced chronic disease and other factors may make the host more susceptible to bacteria which have survived the antibiotic treatment. If this is the case, a recalcitrant minority that would have been driven out might get a chance to grow. One of the reasons for being careful when interpreting associations between heteroresistance and mortality is that there are variations in which people live. Variations in who lives are one of the reasons why associations between heteroresistance and mortality should be interpreted with caution. Severely ill patients are more likely to be infected with resistant organisms and to have an inherent risk of death. Recent reviews thus stress the need to better understand the independent contribution of heteroresistance to clinical outcomes.

Comorbidities

Dysfunction of the kidneys may affect antibiotic exposure, potentially resulting in the over- or under-exposure of the antibiotic, depending on the particular antibiotic and dosing plan. Diabetes, malignancy, chronic lung disease, vascular disease, and other comorbidities may affect tissue perfusion, immune function, the severity of infection, and bacterial persistence. Also, chronic exposure to antibiotics can create the selective pressure for the emergence or growth of antibiotic-resistant minorities. It can, therefore, be as crucial to a patient's medical record as the susceptibility profile obtained at the time of diagnosis.

Infection site

The success of treatment depends largely on the site of infection. Concentrations easily obtained in plasma may not be attainable in specific body compartments such as bone, lung secretions, abscess cavities, necrotic tissue or biofilms. This spatial heterogeneity creates the possibility for a bacterial population to be exposed to multiple different concentrations of an antimicrobial at any given time. A vulnerable majority can be killed off in high-perfused regions, while resistant cells remain in the poorly penetrated regions. This spatial selection can help to maintain the persistent infection even if the plasma PK/PD seems to be adequate.

Microbiome interactions

Microbiome may affect treatment outcome by competing, horizontal gene transfer and metabolic interactions, as well as ecological recovery after antibiotic treatment. The normal microbiota may be altered by broad spectrum antibiotics (dysbiosis) thus diminishing colonization resistance and increasing the growth of resistant organisms. Enterobacterales and enterococci are especially significant in the gut. A suppressed heteroresistant organism can then grow in an antibiotic-disrupted microflora. The gut can thus serve as a reservoir of resistant subpopulations and as a site from which bacteria can spread to more normally sterile sites.

7.5 Clinical Case Reports and Meta-Analyses

Representative clinical evidence

The first clues about the possibility of clinical implications of heteroresistance were in case reports. A typical case was a patient developing *A. baumannii* meningitis who received colistin treatment and then received a fully resistant population as a result of a population initially being heteroresistant. This observation helped to develop the biological plausibility of treatment associated enriched resistant minorities. Recent clinical studies have progressed from case studies. Luo et al. characterised an

undocumented heteroresistant group of CRKP patients before treatment and a group of patients who developed complete resistance during treatment with polymyxin. Likewise, the 2025 complicated UTI analysis also offers some data that heteroresistance could be linked to treatment failure even in cases where traditional AST shows that the organism is susceptible. The results are particularly meaningful as the isolates tested were not just high-level resistance, but also taken from a clinical trial population.

Meta-analytic evidence

It is important to note that each individual study may have small populations, and definitions and detection methods may vary within the different studies. Heteroresistance however is an unusually challenging meta-analytical problem, as there is not full methodological uniformity in the phenotype itself. This year, a systematic review and meta-analysis of colistin heteroresistance in *K. pneumoniae* revealed prevalence of approximately 31.5% in 18 studies. But the statistical heterogeneity was very high, around 94.9% (I^2). This finding not only provides an example of its prevalence, but also the challenges of direct comparison of prevalence estimates across studies. In contrast, clinical outcome meta-analyses are more developed in the case of vancomycin-intermediate *S. aureus* than heteroresistance when it comes to Gram-negative pathogens. This could be attributed to the advanced hVISA research in comparison to other VISAs and the availability of clinical studies for hVISA. For other pathogen-antibiotics, though, laboratory studies still outnumber animal studies, case reports and retrospective cohorts.

Why mortality should be interpreted cautiously

A link between heteroresistance and mortality is biologically plausible, but is hard to separate out. If bacterial infection is severe, death is dependent upon several factors including: sex and race; severity of sepsis; immune status; organ dysfunction; source control; the time it takes until the influence of the treatment is observed; antimicrobial exposure; infection site; bacterial burden; co-infections; and availability of alternative antibiotics. These comparisons (direct comparisons) of the mortality rate of heteroresistant vs. non-heteroresistant infections can overestimate the role of the bacterial phenotype. The most convincing evidence comes from research that measures the level of heteroresistance before therapy is started, treats the appropriate antibiotic and shows that the resistant fraction of the bacteria increases during treatment, leading to a clinical decline or microbiological failure upon completion of treatment. However, caution must be used when making decisions about causation when controlling for confounding factors.

Overall Interpretation: When Does Heteroresistance Become Clinically Dangerous?

The clinical significance of heteroresistance is more likely to be important if more than one condition is present at the same time. The resistant minority can become clinically dominant because of high concentrations of bacteria, exposure to less than the normal doses of antibiotics, long use of antibiotics in the community, a small minority of resistant bacteria that grow rapidly or are large in number, poor penetration of tissue by the antibiotics and decreased host immunity. However, if the subpopulation of the population with resistance is very small, or the fitness cost is high, or the exposure to the antimicrobial agent has effectively removed the resistant subpopulation, or the resistant subpopulation has been rapidly eliminated by the host immune system, then heteroresistance can be largely unmeasured. That's why, sometimes, it's not easy to see the connection between heteroresistance and treatment failure. If a person has a positive heteroresistance test it does not necessarily indicate that treatment will fail. Instead, it specifies a susceptibility of the population which under certain selective and host conditions may become clinically significant. Existing evaluations also call for careful consideration of the overall clinical impact, a topic that remains controversial, but increased by the number of experimental and observational studies. Selected last-line therapies and high-risk Gram-negative infections are the most convincing, at this time. The case of polymyxin heteroresistant CRKP is a particularly good example as resistant subpopulations can be identified prior to treatment, and can then grow to become resistant during treatment. The phenomenon does not occur only in laboratory models, or with organisms that are extensively drug-resistant, as evidenced by the recent studies of clinical UTI and *E. coli* bloodstream infection. All of these observations are indicative of a change of perspective on treatment failure. The MIC should no longer be considered a "typical" description of the bacterial population from which the isolate was cultured. Instead, the susceptibility should be interpreted with the knowledge of the bacterial burden, infection site, antibiotic exposure, host factors, clinical response, and, if a bacterial subpopulation is suspected (when no clinical failure is evident), the presence of a bacterial subpopulation.

8. THERAPEUTIC STRATEGIES AND FUTURE INTERVENTIONS WITH DETAILS DISCUSSION

The challenge of heteroresistance with antibiotics is a very similar, but not identical, problem to that of classical antimicrobial resistance. With conventional resistance, the susceptibility profile is typically representative of the behavior of the majority of bacterial cells. However, in heteroresistance, a susceptible majority can exist with a smaller subpopulation that can withstand much higher levels of antibiotics. This forms a therapeutic blind spot: it may seem like the right course of action, but antibiotic use can result in the mobilisation of the minority resistant population, leading to microbiological persistence or stable resistance. In recent years, however, it has been suggested that clinical and experimental efforts to manage heteroresistant infections should focus on optimizing antimicrobial exposure, early detection of clinically relevant resistant subpopulations, and source control measures that can decrease the advantage that resistant cells have over susceptible cells (Luo et al., 2024; Xu et al., 2025). For pathogens already of concern due to a limited number of treatment options, the therapeutic challenge is particularly important. carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant or third-generation-cephalosporin-resistant Enterobacterales are listed as critical priority pathogens in the 2024 WHO Bacterial Priority Pathogens List, and carbapenem-resistant *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* are included in the high-priority organisms (World Health Organization [WHO], 2024). Therapies thus should not just try to add more antibiotics to heteroresistance. They should seek to kill the minority or resistant bacteria, not allow the minority to grow, keep the antibiotic effect on the bacteria, and decrease the ecological and evolutionary conditions that make resistant bacteria thrive.

8.1 Optimizing Antimicrobial Therapy

Combination antibiotic therapy

When heteroresistance is a potential problem for the efficacy of a single agent in an infection, combination therapy is one of the most common recommended regimens. The principle is simple: If you have a resistant subpopulation that is able to survive with particular mechanism with one antibiotic, another antibiotic with a different target or resistance pathway could suppress that population. Combination therapy should not be considered treatment of choice per se, however. The effect of a drug combination may be synergistic, additive, indifferent or antagonistic, so that there will be no significant protection of the combination from resistance, but the toxicity will be greater. In recent years, studies on combinations of antibiotics have highlighted the importance of considering the evolutionary effects of drug interactions in addition to more classical indicators of anti-bacterial synergy (Bognár et al., 2024). The best combinations for heteroresistant infections are those where the second drug is effective against the minority of resistant organisms and not just a repetition of the selective pressure from the first drug. This would ideally involve considering both the PK/PD properties of the combination and the resistance mechanism, population structure, and infection site, in practice. One other benefit of rational use of combination therapy is the potential to decrease the chance that one microbe will be resistant to both. However, this principle is highly dependent on the biological connections between the resistance mechanisms. These may reduce the expected benefit as they can be either cross-resistance or collateral resistance.

Sequential therapy

Sequential therapy can be a useful approach when given long term, the single antibiotic may give rise to the emergence of a resistant subpopulation. The premise is to shift antimicrobial pressure in response to microbiological response, evolution of antimicrobial susceptibility, or clinical improvement. One benefit is that the bacteria do not experience the same selective conditions over an extended period of time. Sequential therapy is not to be confused with empiric antibiotic cycling, however. Switching regimens without microbiological grounds without destroying the population of the pathogen may generate many selective pressures. Sequential therapy may then only be useful in heteroresistant infections with repeated microbiological monitoring and knowledge of the changing phenotype of the bacterium.

High-dose optimization

When bacteria are exposed to more antibiotics, sometimes it can be more effective at killing the bacteria and make it less likely that resistant cells survive. However, the dose should not be interpreted as “more drug equals more benefit. Whether increased exposure will be beneficial depends on the toxicity of the compound, ability of the compound to penetrate tissues, patient-specific pharmacokinetics and susceptibility distribution of the population of bacteria. The desired objective should be to give an exposure which is sufficient to kill the bacteria but not so great that the safety margin is exceeded. This is especially true for antibiotics that have a narrow therapeutic index including polymyxins. If a subpopulation MIC is higher than the

MIC of the dominant population, the highest subpopulation MIC should be considered. The susceptibility of the dominant population and the susceptibility of the resistant subpopulation can differ widely in experimental systems and conventional dose optimization may result in a lower exposure level needed to eliminate the resistant cells.

PK/PD-guided dosing

Fixed dosing is not a rational dosing strategy as it does not consider drug exposure at the individual patient level, which is what PK/PD-guided therapy does. Therapeutic drug monitoring, population PK, Bayesian dosing and individualized renal-function assessment can assist in keeping antimicrobial levels in an effective range. In the case of heteroresistance, the conventional MIC should be used in addition to the population distribution of susceptibility, for PK/PD optimization. This is particularly important if resistant subpopulations can survive at levels close to the clinically achievable concentrations. In critically ill patients, however, the importance of PK/PD optimization increases because of the potential, even in these patients, for significant variability in antimicrobial exposure due to changes in volume of distribution, increased renal clearance, organ dysfunction, extracorporeal therapy, and significant physiological changes.

Table 5. Therapeutic approaches for managing antibiotic heteroresistance.

Strategy	Primary objective	Potential benefit against heteroresistance	Major limitation
Combination therapy	Attack different bacterial targets	May suppress resistant minorities and reduce selection of single-drug resistance	Toxicity, antagonism, cross-resistance
Sequential therapy	Reduce prolonged selective pressure	May prevent continuous enrichment of one resistant phenotype	Requires reliable repeat susceptibility testing
High-dose optimization	Increase antimicrobial exposure	May improve killing of resistant subpopulations	Toxicity and tissue penetration constraints
PK/PD-guided dosing	Individualize drug exposure	Reduces underexposure and maximizes pharmacodynamic effect	Requires PK/PD expertise and monitoring
Therapeutic drug monitoring	Measure actual exposure	Particularly useful in critically ill patients	Not available for every antibiotic
Source control	Reduce bacterial burden	Decreases population size and probability of resistant-cell survival	Depends on infection type and anatomical accessibility

Overall, the optimal antimicrobial strategy should be individualized rather than based on a universal “combination versus monotherapy” rule. The current literature supports rational combination design and exposure optimization, but the specific regimen should depend on the pathogen, resistance mechanism, antibiotic, infection site, and patient characteristics (Bognár et al., 2024).

8.2 Novel Therapeutic Approaches

Traditional antibiotics are mainly pressure on the populations of bacteria by targeting the essential cellular processes. Novel strategies aim either to minimize this selective pressure, overcome existing resistance mechanisms or to sensitize resistant bacteria to existing antibiotics.

Anti-biofilm agents

Bacteria residing in biofilms have altered metabolism, limited penetration of antibiotics, extracellular polymeric substances, and significant physiological heterogeneity, making biofilms an important therapeutic challenge. These characteristics can occur along with genetically resistant subpopulations and provide a setting for increased selection for persistence and resistance. There have been recent reviews that highlight the challenge of biofilm associated infections due to the fact that cells in the biofilm are physiologically different from planktonic bacteria cells (Liu et al., 2024). Anti-biofilm approaches include: Disintegration of extracellular polymeric substances; low or no toxicity; and enzymatic breakdown of matrix components of biofilms; quorum-sensing interference; biological processes that take place at the surface; and dispersal agents;

and antibiotics that can infiltrate the structure of biofilms. One possible example is liposomal antibiotic formulations. The ability of nanocarrier systems to enhance antibiotic penetration into biofilms and deliver higher drug concentrations in regions of bacterial aggregation (Panthi et al., 2024) can benefit the treatment of these infections. In the case of heteroresistance, the key benefit of biofilm-directed therapy is indirect but significant: Decreasing biofilm biomass and/or spatial protection can increase the availability of resistant minority bacteria for antimicrobial killing.

Efflux pump inhibitors

Multidrug resistance can be caused by efflux pumps eliminating the antimicrobial agent from the cell. The differential expression of efflux systems in heterogeneous populations may also play a role in phenotypic heterogeneity of susceptibility. These are thus being looked at as antibiotic adjuvants: efflux pump inhibitors. The concept is appealing because the inhibitor does not have to have good intrinsic antibacterial activity; in fact, it could be an inhibitor of another antibiotic that will restore the intracellular activity of that antibiotic. Nevertheless, it is difficult to translate into clinic. Candidates for inhibitors should have adequate specificity, favorable pharmacokinetics, low toxicity and compatibility with the protected antibiotic.

Antimicrobial peptides

Another therapeutic class that is seen as promising is the class of antimicrobial peptides (AMPs). Most of the AMPs interact with the cellular membranes or other conserved cell structures, and some have mechanisms of action that are very different from traditional antibiotics. A significant variety of naturally occurring and engineered AMPs are described in recent reviews and new candidates for peptides are being identified by genome mining or computational methods (Kumar et al., 2024). Of special interest are their potential role in the development of heteroresistance, as agents that act by several or quite fast mechanisms could have different evolutionary constraints as compared to traditional single-target agents. Peptide stability, toxicity, proteolytic degradation, and pharmacokinetics, however, are important challenges, as is manufacturing costs and delivery.

Nanotechnology-based antibiotics

There are two significant ways in which nanotechnology could help manage heteroresistance. Firstly, nanoparticles can have direct antibacterial activity. Secondly, nanoparticles can serve as carriers that selectively carry antibiotics into bacterial cells or bacterial biofilms. The use of metallic, polymeric, lipid-based and hybrid systems have been explored as antimicrobial nanoparticles. Hypothetical mechanisms involve disruption of the bacterial membrane, generation of oxidative stress, the delivery of the compound inside the cell, and disruption of essential bacterial processes (Mondal et al., 2024). Combination nanotherapy, where a nanoparticle at the same time enhances drug delivery and provides an independent antibacterial effect, could possibly prove to be the most promising application in the end. But this doesn't mean that the laboratory is ready for the clinic. A detailed evaluation of biodistribution, toxicity, immunological effects, long term accumulation, manufacturing reproducibility, and regulatory safety is required for nanomaterials.

8.3 Precision Medicine and Personalized Therapy

Heteroresistance is a key problem in standard antimicrobial treatment, because the susceptibility of a population of bacteria from which the susceptibility value is taken may not be uniform. Precision antimicrobial therapy is trying to overcome that limitation by combining all of the pieces of information related to microbiology, genomics, pharmacology and clinical.

Rapid molecular diagnostics

RMDs can detect resistance genes or mutations much sooner than culture-based susceptibility testing. They may be most useful in combination with molecular assays or quantitative or phenotypic assays that can detect minority populations. A resistance gene found in low frequency could be an early sign of the presence of a minority of bacteria that are resistant. In contrast, the lack of a known resistance determinant does not rule out heteroresistance as some phenotypes may occur as the result of amplification, regulation changes, physiological adaptation or other resistance mechanisms not tested by the assay. Therefore, molecular diagnostics should be used in addition to, not instead of, phenotypic diagnosis.

Biomarker-guided therapy

Candidates for potential biomarkers are resistant-subpopulation frequency; percent of total DNA that is occupied by the resistance genes; and population-level MIC distributions; bacterial burden; circulating microbial DNA; inflammatory biomarkers; pharmacokinetic exposure; and Susceptibility changes that vary from one time period to another throughout the

treatment. It could be possible to combine these variables into a heteroresistance risk score for use in a future clinical workflow to select patients for enhanced microbiological monitoring or alternative treatment.

AI-assisted antimicrobial selection

AI and machine-learning technologies may have the potential to combine microbiological and clinical factors that are hard to interpret together. Possible applications include: Prediction of heteroresistance on the basis of genomics; Automated detection of resistant colonies in population analysis assays; and understanding of time-kill curves; prognosis; Determining the best antibiotic combinations; and recommendations for an individualized PK/PD dose.

The biggest challenge is that machine learning models need high-quality, well-categorized datasets. Various definitions, detection thresholds and experimental conditions are commonly employed in heteroresistance studies. If AI models are not harmonized, they risk detecting laboratory-specific patterns instead of clinically relevant biological signals. Ideally, future machine learning systems should be trained on multi-center datasets that include paired genomic, phenotypical, pharmacological and clinical outcome data.

8.4 Surveillance and Antimicrobial Stewardship

Heteroresistance is not just a therapeutic challenge, it's a diagnostic and surveillance challenge. Normal surveillance programs could underestimate the burden of resistance if resistant subpopulations are not identified.

Laboratory reporting strategies

The primary method in use in clinical laboratories is to use standardized susceptible, intermediate, and resistant categories. Although these categories are still important for clinical decision, they may not be able to identify clinically relevant subpopulation behavior. A possible future lab model would be: Routine testing: susceptible. Susceptible by routine testing, heteroresistance suspected/confirmed. Before it can be widely used, there would need to be standardisation of terms and validated thresholds. Standardizing heteroresistance to each isolate is not likely to be feasible. A targeted approach might be more achievable, targeting: last-line antibiotics; high-risk Gram-negative pathogens; persistent bacteremia; unexplained treatment failure; recurrent infection; isolates with distinct colony forms; and Prolonged antimicrobial therapy patients.

Clinical antimicrobial stewardship

Aimed at minimizing selection pressure that allows the expansion of resistant subpopulation, antimicrobial stewardship programs can decrease the selection pressure. Core interventions include: Avoiding unnecessary antibiotics; choosing the most effective narrow spectrum; using the right dose at the right time; when microbiological results are obtained, de-escalating; minimising unwanted exposure for a long period; and incorporating PK/PD principles; and reassessment of therapy if patient is not improving. Stewardship is more relevant for heteroresistance, as suboptimal or prolonged antimicrobial exposure can offer exactly the environment in which the resistant minority can grow.

Infection prevention and control

The prevention of transmission is still critical as heteroresistant bacteria may spread from patient to patient, in a similar way to other resistant bacteria. Strategies for the hospital should include: Hand hygiene; environmental cleaning; appropriate isolation precautions; seeking out high-risk populations as needed; device-care bundles; rapid identification of resistance; and – surveillance during outbreaks, either genomic or phenotypic. The WHO underscores the importance of AMR control, going beyond the development of new antibiotics, with integrated surveillance, infection prevention and control, access to diagnostics, and research into new treatment options (WHO, 2024).

8.5 Drug Discovery Targeting Heteroresistance

Developing antimicrobials that take bacterial population heterogeneity into account from the beginning of the discovery process will be the long-term solution for heteroresistance. In the past, when screening for the determination of antibiotic candidates, one MIC value has been used for a relatively homogenous bacterial population. In pathogens that are heteroresistance, this approach could overlook clinically significant minorities of resistant microorganisms. The following should be assessed in future drug discovery: Resistant-subpopulation frequency; mutant prevention concentration; population analysis profiles; emergence of resistance over serial exposures; antibiotic combination effects; benefits from fitness; and intracellular and biofilm activity; and the direction of evolution of resistance within therapeutic concentrations.

New antimicrobial targets

Setting new targets that are likely to be conserved and hard to avoid may lower the chance of the emergence of rapid resistance. Of special interest are bacterial processes essential during the infection, but not so essential during laboratory growth. The recent AMR antibacterial pipeline assessments by WHO highlight the inadequacy of the current development pipeline and the need for increased innovation in the development of antibiotics, especially against critical priority pathogens (WHO, 2024). Most importantly, drugs being considered for the future should be tested for not only eliminating the dominant bacteria, but also for reducing the presence of the opportunists. The ability to "contain" resistant minorities should be made a specific aspect of preclinical development.

Adjunctive therapies

This is meant to be used as an alternative to finding new antibiotics. Potential approaches include: β -lactamase inhibitors; efflux pump inhibitors; anti-biofilm compounds; membrane-active agents; quorum-sensing inhibitors; bacteriophages; phage-derived enzymes; antimicrobial peptides; immune modulators; and drug-delivery systems. The advantages of adjunctive methods are their ability to enhance existing antibiotic therapy without a need for a totally new class of antimicrobial drugs.

Host-directed therapeutics

Host-directed therapy aims to augment the patient's own capacity to clear bacterial populations. Potential strategies include modulation of innate immune responses, enhancing phagocytic function, restoring epithelial barriers, and modifying inflammatory pathways. This approach is especially intriguing for heteroresistance as the exposure to an antibiotic does not have to achieve full bacterial clearance. When the population of bacteria is reduced by antimicrobial therapy and the immune system kills the minority that survives, the chance of the resistant-subpopulation proliferating can be lowered. Caution must be exercised with host directed therapy, however. Over-activation of immune systems can lead to damage of tissues or aggravate general inflammation, especially when it comes to sepsis.

Future Therapeutic Framework

So, the new therapeutic approach to heteroresistance may be considered as a four-layer approach: Rapid detection optimized exposure to antibiotic therapy suppression of resistant sub-populations prevention of recurrence and transmission. This framework is not a simple "Which antibiotic is active against this isolate?" but rather "Which antibiotic do you recommend for this isolate?". What treatment strategy will kill all of the bacteria and cause no selection of resistant subpopulations?

The present development landscape backs the need for such diversification. As of December 2023, WHO has reported that 97 antibacterial products are in clinical development, of which 57 are antibiotics and 40 are non-traditional antibacterial agents. However, WHO declared that overall pipeline is still inadequate to combat the rising occurrence and dissemination of AMR (WHO, 2024). This gap is especially germane with regard to heteroresistance, as new antimicrobials should be developed that include resistance testing at the level of the population, and not just the conventional MIC-based approach. A paradigm shift is needed in how antimicrobial therapy is thought about to manage heteroresistance. The infecting organism should not necessarily be considered a uniform population with a single susceptibility phenotype. Instead, it may have a small, but clinically significant resistant subpopulation that is enriched by antibiotic selection. The most urgent therapeutic concerns are thus correct diagnosis, appropriate antimicrobial exposure, timely combination therapy (when appropriate), intensive source control and ongoing clinical evaluation. Some novel strategies like the use of anti-biofilm compounds, anti-microbial peptides, phage therapy, efflux pump inhibitors, nanotechnology, and host-directed interventions can be effective in preventing resistant minorities from becoming dominant. From a research perspective, heteroresistance should be a clear endpoint in the development of antibiotics. WHO's ongoing priority framework underscores the need for novel antibiotics, alternative antibiotics, diagnostics, and equitable R&D for high-risk resistant pathogens.

9. FUTURE PERSPECTIVES, RESEARCH GAPS, AND CONCLUSIONS

Heteroresistance a new dimension of antimicrobial resistance is increasingly recognised as an important but relatively under recognised dimension. One of the main problems is the absence of a common set of diagnostic and epidemiological criteria. Many laboratories use population analysis profiling, while the different definitions and detection limits, the bacterial inoculations, and the different antibiotic concentrations and interpretation of results, make it difficult to compare between studies. The incidence of heteroresistance may also be under-estimated as small subpopulations of resistant organisms may not be detected by routine susceptibility testing as shown by more recent studies. One key research challenge is thus to create

standardized, fast and clinically applicable diagnostic tools. Future studies should incorporate traditional phenotypic testing with single-cell microbiology, genomic analysis and quantitative methods that can detect rare resistant cells. In addition, single-cell and multi-omics technologies are highly attractive as they have the potential to capture biological heterogeneity which may be masked by measurements at the level of the population, but are currently too expensive, technically challenging and require complex data analysis, and not standardized in their workflows for routine clinical use (Lim et al., 2024).

Another important gap is the lack of knowledge about the evolution of heteroresistant populations during treatment. Longitudinal studies from the point of initial isolation of a heteroresistant population, through antibiotic exposure, microbiological response and eventually possible relapse should be performed. The integration of the whole genome sequence, transcriptomics, proteomics, metabolomics and assays of single cells could aid understanding of the reasons for the minority groups that are resistant and those that do not have any clinical consequences. This type of study can also identify potential biomarkers that might be associated with which heteroresistant populations have the potential to become stable. Heteroresistance needs to be taken into account in the clinical and public health context to make it a primary consideration in the study and monitoring of AMR. WHO's 2025 global surveillance report illustrates the expansion of global standardized AMR surveillance and the recent diagnostic landscape analysis shows that there remains a global need for better access to and availability of rapid, robust diagnostics for AMR. Heteroresistance could be added to the set of population-level attributes that are monitored in future surveillance systems—especially for last-line antibiotics and priority bacterial pathogens. Implementation should be based upon valid diagnostic criteria and not upon new and not standardised testing of all patients.

For the future of heteroresistance studies, a combination of several disciplines including microbiology, evolutionary biology, pharmacology, genomics, single-cell analysis, artificial intelligence and clinical medicine will be needed. The ability of single-cell technologies to detect rare states of bacteria that are not detected in bulk measurements, as well as multi-omics technologies that are potentially able to link those with the underlying molecular mechanisms. To make this shift to practice, however, there is a need for reliable assays, multi-centre validation, clinically relevant thresholds and a demonstration that the identification of clinically relevant heteroresistance will improve decisions made in clinical practice and patients' outcomes. Lastly, heteroresistance is not to be considered as another type of a lab induced antimicrobial resistance. It is an intraspecific susceptibility diversity which cannot manifest its clinical effects until it is detected when selecting antibiotics. In the context of infection with multidrug-resistant and last-line antibiotic resistant pathogens, it is of particular relevance as it may play a role in failed treatment, resistance and persistence of the infection. It remains a biological and clinical phenomenon, but there is great uncertainty regarding prevalence and standardisation of detection, and the extent to which it represents a unique contribution to patients' outcomes. The next step in future research should be to understand the dynamics of these populations of heteroresistant organisms and create strategies that can detect and eradicate the heteroresistant populations before they gain clinical dominance.

Conflict of Interest

The authors declare they don't have any conflict of interest.

Author contributions

The initial draft of the manuscript was prepared by the first author, who led the background synthesis, methodology layout, and primary textual development. The corresponding author oversaw the entire drafting process, providing critical structural guidance, technical validation, and project oversight. Every co-author actively participated throughout the preparation phases: identifying relevant data sources, extracting and validating evidence, formulating structured comparison tables, and performing thorough line-by-line editorial revisions. Following a rigorous internal review to ensure academic integrity and methodological rigor, all listed authors gave their unanimous approval for the manuscript to be submitted for journal publication.

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