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Heteroresistance: A Gray Side of Antimicrobial Susceptibility Testing

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ABSTRACT

Antibiotic resistance is a universal warning to human health; by 2050, it is expected that the mortality rate due to antimicrobial resistance (AMR) will exceed 10 million. Heteroresistance (HR) is a phenomenon in which subpopulations of cells exhibit lower levels of antibiotic susceptibility compared to the main population. There are no standard methods to detect HR leading to inappropriate use of this expression. HR has been distinguished since 1947 and reported in Gram-negative and Gram-positive bacteria. Recently, HR is so prevalent in various bacterial species against the plethora of antibiotic classes. HR which has an unstable phenotypic character, having different mechanisms with non-standard methods to be determined, prevents recognition of the degree to which this phenomenon is precarious and its consequences. In 2009, World Health Organization (WHO) has defined antibiotic resistance (AbR) as a critical public health threat causing death rates more than that caused by cancer and such serious diseases. Consequently, understanding the novel and often under-recognized mechanisms of resistance that represent barriers to antibiotic efficacy is vital so as to combat resistance with new therapeutic approaches. Eventually, a fundamental issue is whether we can predict why some resistant clones have the ability to survive despite the perishing of the main population. In this review, we will assess the available literature on bacterial HR suggesting recommendations for the definition and determination criteria for antibiotic HR to help assess the treatment failure caused by heteroresistant bacteria.

Keywords: *Heteroresistant cells; Antimicrobial resistance; Phenotype; Genotype; Population Analysis Profile; Treatment failure*

INTRODUCTION

It is important to recognize novel and often unknown mechanisms of resistance that represent an impediment to the efficacy of antibiotic [1], as antibiotic resistance is a universal menace to human health [2]. According to WHO, infection with highly resistant bacteria such as multidrug-resistant (MDR) microorganisms causes a serious load on economies due to increased morbidity and mortality worldwide [3]. Over 2 million infections every year in the United States are caused by antibiotic-resistant bacteria, according to the Centers for Disease Control and Prevention (CDC), resulting in 23,000 deaths at least and increasing healthcare costs by US$55 billion [4]. Recently, resistance mechanisms to antibiotic classes are well illustrated, showing how bacterial species develop different levels of resistance and are categorized as both (resistance mutations (RsMt) and resistance genes (RsGn)) in a bacterial cell [5]. These well-illustrated

mechanisms generally provide predictability and correlation between bacterial phenotype and genotype; however, exceptions exist [6]. Heteroresistance is an example of an anomaly from the correlation rule [3, 5]; making it difficult to classify a clinical isolate as resistant or susceptible [2].

Heteroresistance definition

The broadest definition of HR is the presence of a heterogeneous resistance behavior of bacteria with subpopulations that reveal reduced levels of antibiotic susceptibility compared to the main population[3]. HR means that there are population-wide variable responses to antibiotics [6]. Bacterial species can respond to an antibiotic not only homogeneously to be sensitive, intermediate or resistant but also response shows heterogeneity sometimes, as shown below in (**Figure 1**) [7].

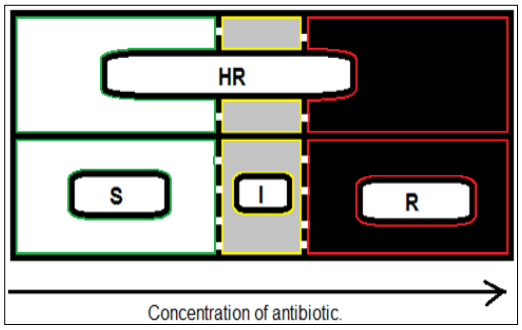

Figure 1. Heterogeneous response of bacteria to an antibiotic[7].Dotted lines illustrate the breakpoints of an antibiotic. S: sensitive homogeneous response. R: resistant homogeneous response. I: intermediate homogeneous response.HR: heterogeneous response.

However, the use of the term "Heteroresistance" began in 1970s[8], it was first described in 1940s for *Haemophilus influenzae*[9] and for Gram-positive staphylococci 20 years later [10].

Another definition of HR depends on egression out from breakpoints of an antibiotic [11] as defined by the Clinical and Laboratory Standards Institute (CLSI), the British Society of Antimicrobial Chemotherapy (BSAC), and other international bodies, relies on single cutoff concentrations, which doesn't depict the diversity in resistance among members of a bacterial population [3].

Factors to be considered when defining heteroresistance

Several factors should be considered when defining heteroresistance[2], such as clonality of heteroresistance, where heteroresistance can be detected in populations (polyclonal heteroresistance) that result from mixed infections or from rare resistant mutants that slowly increase in proportion during antibiotic treatment in a population of susceptible bacteria. In these two cases, HR will not be detected in cultures from purified clones. Instead, antimicrobial susceptibility tests (AST) performed on pure clones would result in detection of a fully susceptible or fully resistant phenotype depending on which of the two populations (resistant or susceptible) the purified clone originated from. Heterogeneity in resistance could also be generated from a single clone that differentiates into two populations (sensitive and resistant) at high frequency in the absence of antibiotic pressure (monoclonal heteroresistance) [12]. In this case, a pure culture from a purified clone displays an HR phenotype (for example, a disc diffusion antimicrobial susceptibility test performed on a purified clone reveals the growth of resistant colonies within the inhibition zone[13]. Other factors that should also be considered include the level of resistance of the subpopulation compared with that of the main population, the frequency of the resistant subpopulation and the stability of the HR phenotype[12](Heteroresistance is stable if the resistance of the subpopulation does not decrease or revert to susceptibility following growth in the absence of antibiotics)[3].

Mechanisms of heteroresistance

- Polyclonal Heteroresistance

A sample population from a patient would show polyclonal HR either due to the infection might consist of a mix of two genetically totally different populations (susceptible and resistant) [14] or the development of uncommon spontaneous resistant mutants that are increasing proportionally in the population within antimicrobial treatment, which leads to two distinct populations (susceptible and resistant).

AST methods used directly on multiple samples, without a single-cell colony purification step [15], usually lead to problems in resistance detection due to several co-existing subpopulations [12]. So, it is noteworthy that the analysis of isolated clones adequately reflects the characteristics of the clinical samples.

- Monoclonal Heteroresistance

Pure colonies (pure colony is that originated from a single isolate) may show monoclonal HR due to genetic heterogeneity or indigenous heterogeneity [2]. Apart from the study of nalidixic acid and kanamycin resistance in *Salmonella enterica*[16], no experimental evidence supports the existence of a non-genetic mechanism that leads to the generation of HR.

However, non-genetic mechanisms lead to the formation of so-called persister cells (Bacterial populations produce **persister cells** that neither die nor

grow in the presence of bactericidal antibiotics, i.e. having the same frequency before and after exposure to antibiotics) against several antibiotic classes [17]. The persistent subpopulation cannot grow in the drug's presence although it tolerates exposure to its bactericidal concentration [13]. On the other side, the resistant subpopulation of heteroresistant cells exhibits an outright resistance phenotype that allows for growth at antibiotic concentrations that inhibit cell growth of the main population [11].

Genetic modulation of heteroresistance expression

Most HR cases are clarified to be unstable; in 88% of 34 confirmed HR cases, the resistance was transient and MIC reverted to the susceptibility level of the main parental isolate [12]. Unstable genetic amplifications and other mutational events (for example, small deletions or point mutations) are the causes of unstable antibiotic HR[13] and increasing the expression levels of amplified resistant genes enhance the increased resistance of the subpopulations[12].

Unstable Heteroresistance Related to Unstable Tandem Amplifications.

Amplification of resistance genes is responsible for HR phenotypes confirmed by two different tests:(a) Deletion of resistance genes amplified in the resistant subpopulations resulted in the loss of heteroresistant phenotypes and reduction of MIC of the resistant population. (b) Overexpression of resistance genes by cloning resulted in increased resistance of the subpopulations to antibiotics and converted them into a fully resistant population [12]. This is the most common mechanism for HR and also the most difficult to detect because of its high instability and transient nature.

Unstable Heteroresistance Linked to Point Mutations and Small Deletions.

Point mutations mean the alteration of only one nucleotide in a particular gene's sequence, leading to a minor effect on the produced protein. So, point mutations in the resistance gene lead to the conversion of a heteroresistant population into a sensitive one[2]. Small deletions have the same effect; small deletions in the sensor gene lead to loss of function mutation. The mutations involved in unstable HR were investigated for 18 cases. No amplifications were detected in 7 out of 18 clones with unstable resistance. Instead, point mutations, insertion sequences (IS) insertions or small deletions were found. These mutations did not affect known horizontally-transferred resistance genes and were all chromosomally located[12].

Methods of heteroresistance detection

BSAC, CLSI and other international bodies develop clinical laboratory standards and recommendations for practices concerning antimicrobial resistance [18]. So, AST methods, such as MIC determination, disc diffusion techniques, and standard criteria to define isolates as susceptible, intermediately resistant or resistant to any antibiotic, are generally agreed upon worldwide. On the other side, HR is poorly characterized, and consensus-based standards to define it are very scarce. As shown below, there are several methods to measure HR to antibiotics in clinical isolates but nearly all of them have a possibility of error to an extent [3].

1. Population analysis profile method (PAP)

PAP method depends on quantitative measurement of bacterial growth at different antibiotic concentration gradients (on nutrient broth or agar) using spread plate technique for CFU counting[3]. HR phenotypes are detected when the inhibitory concentration of the antibiotic to affect the resistant subpopulation is > 8 times the highest non-inhibitory concentration [6] giving good information on frequency and MIC of the resistant subpopulation[2].

Although PAP is considered as the most accurate method to be used for HR detection and known as the standard gold method [3], it is infrequently used in clinical laboratories due to a factor of sample size and its high cost [6, 20].

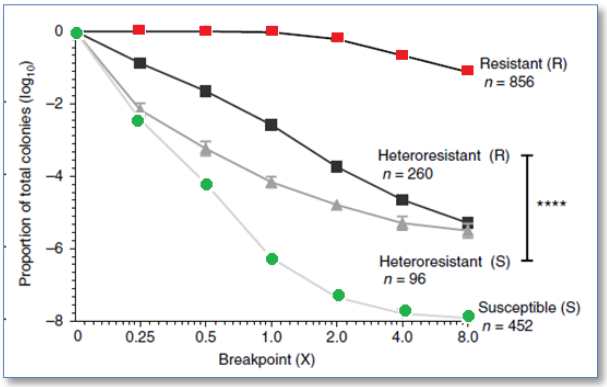

Figure 2. Representation of the PAPs of 104 isolates on 16 antibiotics [23]. (Red squares, 'Resistant (R)'), resistant by clinical testing and heteroresistant by PAP (Black squares, 'Heteroresistant (R)'), susceptible by clinical testing and heteroresistant by PAP (Grey triangles, 'Heteroresistant (S)') and susceptible by clinical testing and PAP (Green circles, 'Susceptible (S)'). Data are presented as mean ± s.d. ****$P < 1 \times 10^{-17}$ using two-tailed Welch's *t*-test of average logs killing at 1× breakpoint concentration for Heteroresistant (R) versus Heteroresistant (S) ($t = 9.01$).

A modified PAP assay is developed by comparing the area under the curve (PAP-AUC) of a given strain to that of a reference heteroresistant strain[3], which represents an important drawback as any change

in the response of the control strain to the effect of the antibiotic will lead to significant changes in the result[21]. This method was used to detect *S.aureus* HR to vancomycin (hVISA) [7].Reference ratios between the test and control strains of the PAP-AUC method, classified into three categories <0.9, 0.9 to 1.3, and >1.3, were considered indicative of vancomycin-susceptible *S.aureus*, hVISA, and vancomycin-intermediate *S.aureus*(VISA), respectively[22]. The following Figure 2 shows an illustration of the detection of heteroresistant bacterial isolates against several antibiotics as an example of some studies on HR.

2. Disc diffusion and E-test assays

Disc diffusion tests and E-test strips have been used to measure HR as recommended for classical *in vitro* susceptibility testing [24, 25]. The lack of standard guidelines leads to disserving detection of HR using E-test and disc diffusion assays, the same with PAP method [6]. Simply, an apparent indication of HR is the presence of distinct colonies growing within the clear zone of inhibition in the antibiotic disc (**Figure 3**) or E-test strip. Still, the wide behavior of the population cannot adequately be described by the inhibition zone diameters, or that is called cutoff concentrations. The disc diffusion test and the E-test show both poor specificity and sensitivity, as indicated by high frequencies of false negative and false positive samples, respectively [2, 12, 26].

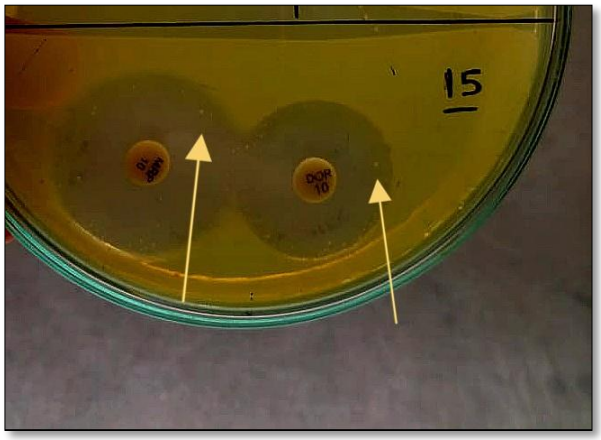

Figure 3.Heteroresistant subpopulations in disc diffusion method [27]. Heteroresistant colonies detected on carbapenems discs MRP; Meropenem DOR; Doripenem

3. Other methods to detect heteroresistance

Mostly, these methods depend on increasing the proliferation of the few members of the population [28], by prolonging the incubation time; increased MIC values of the same strain are determined after bacterial regrowth at later time points in time-kill assays after an initial significant growth reduction [29]. Flow cytometry using a fluorescent penicillin derivative is another approach employed to assess heteroresistance in methicillin-resistant *S.aureus* (MRSA) compared to isolates with known HR [30]. There are other under-development methods used for HR measurement [21, 31] such as, droplet digital PCR, a powerful technique that permits the detection of point mutations or whole genes involved in resistance [12] even if they are only in such a low level of bacteria in the main population, had promising results, particularly when used on stool samples containing *H. pylori*[32].Whole genome sequencing is a cost-effective and promising technique; however, the detection of subpopulations presents at low frequencies, less than 1%, is a stumbling block [31-33]. On the other side of advantages, it is more accurate than traditional phenotypic tests as shown for *M. tuberculosis*[34]. Hopefully, fewer error rates of sequencing and lower costs will be available to identify resistant populations present in < 1% frequency to avoid false positive and negative results detected by traditional methods. Such genotypic tests cannot be applied to all antibiotics and pathogens yet due to relying on a comprehensive understanding of mutations and genes involved in resistance [19]. Moreover, some conflicts between genotypic and phenotypic analysis are present, which could tangle genotypic results analysis [5, 13]. All used methods for the characterization of HR need to be confirmed using the golden standard PAP to eliminate a high percentage of error possibility as there are no available accurate, practical methods, and the development of new clinical AST is a must. Automated broth systems (for example, VITEK 2, MicroScan, WalkAway) may be used for the detection of HR phenotypes[3,] but they are rarely used due to their poor result because of the small inoculum sizes of resistant subpopulations [2, 35]. Significant errors are observed when one bacterial isolate is characterized as susceptible to an antibiotic when tested by a type of AST but is characterized as resistant to the same antibiotic when tested by another one [2]; this assures that underdeveloped mechanisms of resistance are still unrecognized so, improvement of AST and development of novel methods are urgently needed.

Heteroresistance and Treatment Failure

The effect of heteroresistance on antibiotic treatment failure has been a matter of extensive contest. However, increasing data strongly suggests that HR may be responsible for poor outcomes and recurrent infections with pathogens classified misguidedly as susceptible. The statistical modeling of the pharmacodynamics of HR in *K. pneumoniae* and other species supports this hypothesis [12].

HR detection is a precondition to selecting the appropriate antibiotic to reach an effective treatment outcome. Although some studies found no correlation

between phenotype and treatment failure, HR can lead to treatment failure in case it is not measured [1, 3]. Wrong treatment protocols can lead to severe prognosis in many cases, such as bacteremia resulting from using vancomycin as an inappropriate antibiotic in the treatment of heterogeneous vancomycin-intermediate *S. aureus* (hVISA) [35] not only persistent bacteremia but also, increased mortality, prolonged hospital stays which is financially expensive, in addition to other complications [22, 35, 36]. Treatment failure has been linked to vancomycin heteroresistance in *Staphylococcus epidermidis* and *S. aureus* in some retrospective studies [7, 35, 37]. Carbapenems and colistin HR in *Acinetobacter baumannii* also lead to treatment failure [38-40]. Such studies proved that there is no correlation between HR and treatment failure [3, 41], for example, vancomycin-treated hVISA [38, 42] and studies of colistin heteroresistant *A. Baumannii* isolates [43], it is crucial to clarify which parameter of HR can lead to treatment failure, the resistance level of heteroresistant subpopulations and their frequencies can influence the treatment protocol. Also, it should be kept in mind that methods of HR detection and their related factors may have a hand in this correlation and explain some of the differences found between some studies[38]. Recurrent heteroresistant infections treated repeatedly with the same antibiotic may allow the evolution of drug-resistant bacteria [44].

Studies for HR have been done to correlate the relationship between the frequency of resistant subpopulations, their resistance level and treatment failure. Two of these studies reported that the presence of even small resistant subpopulations (10^{-6} to10^{-2}) resulted in treatment failure in colistin heteroresistance[45]. It has also been reported that there was a higher frequency of colistin HR subpopulations *in vivo* than detected *in vitro* on the culture medium in the absence of antibiotic usage meaning that false results can be obtained due to underestimation of the frequency of HR in standard laboratory conditions [46] similar to *S. aureus* subpopulations resistance to glycopeptides [47] a model of rabbit endocarditis also showed vancomycin treatment failure due to presence of two different hVISA[48].

In another study, 37 MDR ESBL/AmpC-producing clinical isolates of *K. pneumoniae* were particularly resistant to conventional antimicrobials other than carbapenems. Among the isolates, three strains exhibited HR to IPM and carried several ESBL and/or AmpC genes. Mice infected with a lethal dose of any of the three heteroresistant isolates could not survive in the presence of IPM treatment, as the frequency of the IPM heteroresistant strains was increased in the peritoneum of mice at 24 h after infection. And these heteroresistant strains failed IPM therapy in experimentally infected mice [19].

It is notable to consider that most experiments focused on heteroresistance have been performed *in vitro* or using animal models. So, these results may not indicate the mechanisms in the host organism. Many further issues should be kept in mind, for example, the relationship between the pathogen and the host immune response, besides the co-existence of several heterogeneous subpopulations, including persister and viable but non-culturable (VBNC) bacteria, which are complicated to detect [49].

It has been lately demonstrated that human contact restrictions and other strategies to avoid SARS-CoV-2 transmission during the COVID-19 pandemic have limited the spread of *K. pneumoniae* and other pathogens [50]. However, the extensive use of disinfectants and increased administration of antibiotics to prevent bacterial co-infections during the COVID-19 pandemic may enhance the emergence and spread of drug resistance [51]. Several studies have indicated that the prevalence of carbapenems-resistant *K. pneumoniae* co-infection in COVID-19 patients may reach more than 50% of cases [52]. Moreover, *K. pneumoniae* heteroresistance toward chlorhexidine, the most widely used disinfectant in hospitals, has been described previously with high prevalence [53].

Therapeutic strategies against heteroresistant isolates

It is recommended, in some studies, to use antibiotic combinations to combat treatment failure caused by this phenomenon[23]. Recent findings suggest that combinations of clinically approved antibiotics can be used as therapeutic strategies against heteroresistant *K. pneumoniae* (**Table 1**) [54-58].

Heteroresistance and other scientific terms perplexity

It should be noted that the term "heteroresistance" has been used to describe mixed populations of bacteria with stable genetic differences, including closely related bacteria that developed co-infections with two unrelated strains [59] or mutations[12,38]So, it is important to distinguish HR from other forms of subpopulation-mediated resistance such as **persistence** and **tolerance**[1, 13]. Resistance or Tolerance? Persistence or Heterogeneity? Both are important questions to answer.

Resistance: an isolate is considered resistant to certain antibiotic when a higher concentration of the antibiotic is essential to produce the same effect in a resistant strain as is produced in a susceptible strain [60]. A bacterial strain with a higher MIC than another strain will be considered more resistant, and MIC is the minimum concentration of an antibiotic that is required to prevent net growth of the microorganism [61] (**Figure 4**).

Table 1. Drug combinations effective against heteroresistant *K. pneumonia*e isolates.

Drug Combination	Susceptible isolates
Colistin + Meropenem	MDR, colistin-heteroresistant.
Polymyxin B + tigecycline	Carbapenem-resistant, heteroresistant to Polymyxin B, and resistant, heteroresistant or susceptible to tigecycline
Colistin + Fosfomycin	Carbapenem-resistant, heteroresistant to colistin and Fosfomycin.
Fosfomycin + Ceftazidime	Carbapenem-resistant, heteroresistant to Fosfomycin and Ceftazidime
Fosfomycin + Sulfamethoxazole/Trimethoprim	Pandrug-resistant, heteroresistant to Fosfomycin and Sulfamethoxazole/Trimethoprim
Amikacin + Piperacillin/Tazobactam	Pandrug-resistant, heteroresistant to Amikacin and Piperacillin/Tazobactam
Polymyxin B + Ceftazidime/Avibactam	Carbapenem-resistant, heteroresistant to Polymyxin B

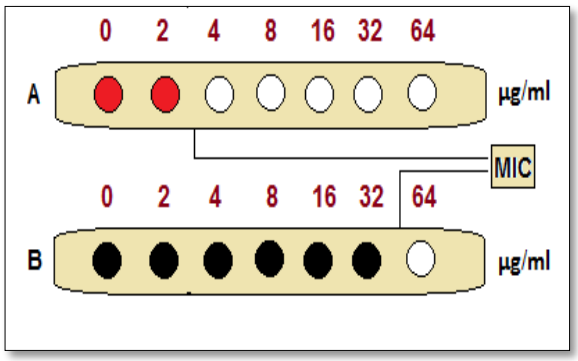

Figure 4. Susceptible versus Resistant bacterial strains [13].A: Susceptible bacterial strain. B: Resistant bacterial strain.

Tolerance: the ability of a bacterial population to survive a temporary exposure to antibiotics, even at concentrations that distance surpass MIC [62].Tolerant strains can have the same MIC as non-tolerant; MIC is not instructive as a metric to detect tolerance [63, 64]. Notably, more prolonged exposure to an antibiotic is required to produce the same level of killing in a tolerant strain as is produced in a susceptible strain rather than a higher concentration of an antibiotic. Detection of minimum duration of killing (MDK) using Time-kill curves at different antibiotic concentrations is an excellent approach for assessing tolerance (**Figure 5**). In tolerance, a whole population of bacteria can survive transient exposure to high antibiotic concentrations, and there may be no preexisting resistant cells before antibiotic exposure[3, 13].

In persistence, small subpopulations of bacteria are temporarily quiescent with a very slow growth rate leading to increased resistance to antibiotics, HR represents the subpopulations that can not only survive but also replicate even under antibiotic stress in contrast to persister bacteria that can lead to relapse of the infection after therapy cutoff, but it is not able to cause the acute failure of treatment [3]. A persistent strain of bacteria has a similar MIC and a similar MDK_{99} to a susceptible one, but the minimum duration of therapy essential to kill 99.99% of a bacterial population ($MDK_{99.99}$) is significantly higher for a persistent strain (**Figure 6**) [13].

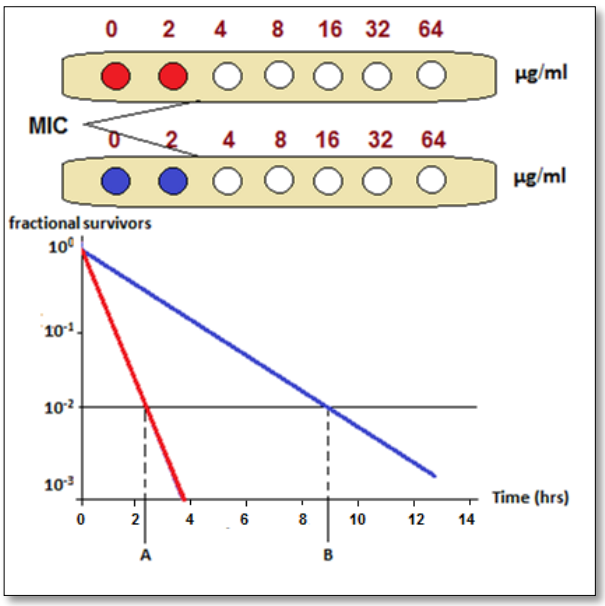

Figure 5. Susceptible versus tolerant bacterial strains [13]. Red color indicates a susceptible bacterial strain. Blue color indicates a tolerant bacterial strain. A: MDK_{99} for a susceptible strain. B: MDK_{99} for a tolerant strain. MIC for a tolerant bacterial strain is similar to that of a susceptible one; however, the minimum duration for killing, for example, MDK_{99} (minimum duration of treatment which is essential to kill 99% of a bacterial population) of a tolerant strain is substantially higher than the MDK_{99} of a susceptible strain. So, it is a matter of exposure time rather than the antibiotic concentration.

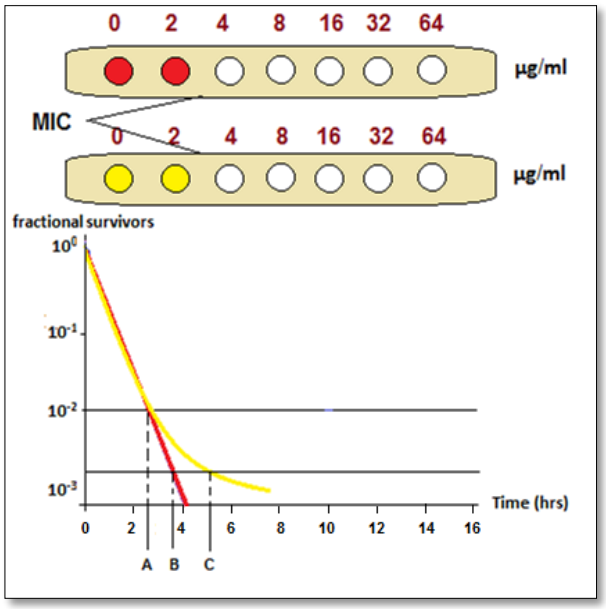

Figure 6. Susceptible versus persistent bacterial strain [13]. Red color indicates a susceptible bacterial strain. Yellow color indicates a persistent bacterial strain. A: MDK_{99} for a susceptible and a persistent strain. B: $MDK_{99.99}$ for susceptible strain. C: $MDK_{99.99}$ for a persistent strain.

CONCLUSION

For those antibiotic treatments that efficiently inhibit the growth of bacteria, subpopulations that are not killed by the antibiotic can nevertheless emerge. Identical bacterial cells in a population can exhibit phenotypic heterogeneity in terms of antibiotic susceptibility, which leads to difficulties in unambiguously classifying bacteria as susceptible or resistant.HR can lead to treatment failure due to inappropriate selection of an antibiotic depending on the sensitivity of the main population regardless of the susceptibility manner of tiny colonies of the subpopulation. The absence of an accurate method to define HR is an extra factor in this problem, so understanding the mechanisms of antimicrobial resistance and finding new approaches to detect HR are necessary.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Contributions

All authors read and approved the final version of the manuscript.

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