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Narrative review

Antibiotic heteroresistance in ESKAPE pathogens, from bench to bedside

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Abstract

BACKGROUND. Heteroresistance refers to subpopulation-mediated differential antimicrobial susceptibility within a clonal bacterial population. Usually, it designates a resistant subpopulation identified within an isolate considered susceptible by classical antimicrobial susceptibility testing. Heteroresistance lacks a uniform microbiological definition for diagnostic laboratories and its clinical impact remains unclear for most bacterial species.

OBJECTIVE. This narrative review aims to provide a practical overview on the latest developments in the field of heteroresistance for both clinical microbiologists and physicians, with a particular focus on ESKAPE pathogens.

SOURCES. Literature search was performed on Pubmed and Google with the key words: heteroresistance; (heterogeneity OR heterogeneous) AND "antibiotic resistance". Among the 836 publications selected based on their abstracts, the most relevant for the detection, the epidemiology and the clinical impact of heteroresistance in ESKAPE pathogens are discussed here.

CONTENT. Heteroresistance is only clearly defined for vancomycin in *Staphylococcus aureus* (hVISA). We compiled a larger microbiological definition to be applicable to other bacterial species and antibiotics in the clinical context. We highlighted the key technical points of population analysis profile, the gold standard for detecting heteroresistance. Heteroresistance to polymyxins, β-lactams (carbapenems, cefiderocol), fosfomycin, tigecycline, and aminoglycosides is frequently reported in multidrug-resistant Gram-negative pathogens. Treatment failure due to heteroresistance has been described in case reports or retrospective studies, so far confirmed by meta-analysis in the case of hVISA only. Finally, to treat pandrug resistant bacterial infections, the option of targeting susceptible subpopulations of resistant isolates using tailored antibiotic combinations, is also discussed.

IMPLICATIONS. Systematic heteroresistance screening by clinical laboratories is not currently recommended. Nevertheless, we should be aware of this phenomenon, and in specific cases, such as

41 treatment failure, heteroresistance should be tested by reference laboratories. Additional studies
42 using standardized methods are needed to improve our understanding of heteroresistance and further
43 assess its clinical impact.

44

Introduction

Antimicrobial resistance is a major threat to modern medicine and public health [1]. Phenotypic antimicrobial susceptibility testing (AST) remains the cornerstone of tailored–directed anti-infective therapies. AST classify the isolates as susceptible or resistant assuming the dogma that a bacterial isolate is a uniform entity. The possibility that one isolate can be formed of subpopulations displaying different phenotypic properties, such as differential antimicrobial susceptibility patterns, would represent a challenge for both microbiology laboratories and clinicians.

Heteroresistance is defined as a variability of antibiotic-susceptibility within an isogenic clonal population. Usually, heteroresistance refers to bacteria with a resistant subpopulation within an overall susceptible isolate. The resistant subpopulation replicates in presence of the antibiotic potentially leading to treatment failure [2-4], which differs from the growth arrest phenotype in persistence and tolerance. While heteroresistance has been previously reviewed by El-Halfawy et al., Dewatcher et al. and Anderson et al. [2, 3, 5], the present narrative review focuses on essential concepts of heteroresistance for laboratories and clinicians: its microbiological definition, its detection, and the latest developments about its clinical significance in ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.).

More recently, heteroresistance definition was expanded to the presence of a susceptible subpopulation within a strain classified as resistant by standard AST [6]. While previously neglected, this aspect of heteroresistance could offer interesting treatment perspectives for pan-resistant bacteria and will also be discussed here.

Microbiological definition

Heteroresistance refers to the presence of a resistant subpopulation in an overall susceptible strain (HR-S profile, Figure 1). There is no clear consensus on the frequency and the level of resistance that

the resistant subpopulation should display to be classified as heteroresistant. Some publications define heteroresistance compared to the minimum inhibitory concentration (MIC) of the main population [2, 7], from a clinical perspective it is reasonable to think that heteroresistance should overlap the breakpoint concentration to be significant [6, 8, 9] meaning that the isolate contains both susceptible and resistant subpopulations that are expected to respond differently to the antimicrobial treatment. Isolates with all their subpopulations remaining either susceptible or resistant should respond to the antibiotic treatment as expected from their AST category. The frequency of the resistant subpopulation should exceed the intrinsic spontaneous mutation rate [3]. **Therefore, heteroresistance could be defined as the detection of a resistant subpopulation from an overall susceptible isolate by standard MIC assay, at a minimum frequency between 10^{-8} to 10^{-6}, able to grow in the presence of an antibiotic concentration of at least two-fold the breakpoint [6, 10].**

The only type of heteroresistance with a clear consensus definition is the heterogenous vancomycin-intermediate *Staphylococcus aureus* (hVISA). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines hVISA as a *S. aureus* isolate susceptible to vancomycin (MIC≤2mg/L) but with minority populations (>10^{-6} cells) growing on vancomycin >2 mg/L, by population analysis profile (PAP) investigation [11] . This definition matches the more general definition established above. Beyond hVISA, a consensus definition of heteroresistance, for clinical microbiological laboratories remains to be established by leading societies in the field.

Detection method

The molecular mechanisms of heteroresistance remain poorly understood and its detection relies only on phenotypic assays [3]. By definition reference AST and MIC determination from EUCAST and CLSI generally fail to identify heteroresistance, since the frequency of resistant subpopulation is too low to be detected from a standard inoculum.

The gold standard method for the detection of heteroresistance is the PAP [2, 3]. In this technique, a higher inoculum is spread onto agar plates containing 2-fold antibiotic increment. After incubation, colonies are counted, and log10 CFU/mL are plotted versus antibiotic concentrations (Figure 1). However, a consensus method should be clearly defined to ensure better comparability between studies:

- **Inoculum**: While some laboratories use overnight broth culture [6, 10], a standardized 2 McFarland (2McF) inoculum (approximately 6.10^8CFU/mL), which can be easily prepared in clinical laboratories, would improve reproducibility ensuring that $>10^8$ CFU/mL are used for detection of low frequency subpopulations.
- **Antibiotic concentrations**: Based on the definition, antibiotic concentration tested should match multiple of breakpoint (0-, 0.125-, 0.25-, 0.5-, 1-, 2-, 4-fold, **Figure 1**)
- **Media**: In the absence of guidelines, except for hVISA detection that has to be performed on BHI agar, most studies have used Mueller-Hinton agar (MHA) for PAP. The standardized composition of MHA, already used as a reference media for standard AST, should allow good reproducibility of PAP. Nevertheless further validation of the optimal media across species should be performed.
- **Incubation:** Standard 24h incubation time can be extended to 48h to aid the detection of slower-growing subpopulations [6, 12]. For detection of hVISA, EUCAST recommends a 48h incubation and recent publications suggested that 72h could improve the detection of slow-hVISA isolates [11, 13].
- **Spreading the inoculum** onto a full plate is a critical point for heteroresistance confirmation because a high density of cells could lead to the so-called inoculum effect, artificially increasing the resistance [3]. Spot-PAP, performed by spotting 10µl drops on each antibiotic concentration, presents the advantage to allow simultaneous testing of multiple strains on the same plate but would need to be validated before its implementation in clinical laboratories.

hVISA is the only type of heteroresistance with a standardized detection assay recommended by EUCAST [11] following the protocol described by Wootton et al. [14]. The area under the curve (AUC) obtained from the PAP graph is compared to the AUC of the reference strain Mu3: a ratio of ≥0.9 confirms the hVISA phenotype. This PAP-AUC method cannot be extrapolated to other species as it requires a control strain displaying stable heteroresistance to the antibiotic of interest.

Importantly, heteroresistance reverts in absence of antibiotic pressure [7, 15]. Therefore, it is critical to minimize the number of subculture steps as the proportion of resistant subpopulation will progressively decrease leading to false negative results. Similarly, long-term storage was shown to alter heteroresistance phenotype [16]. This instability might partly explain the variable rates obtained from retrospective studies. The conditions and duration of storage should be disclosed and discussed in future studies.

PAP analysis is a highly time- and workforce-consuming method, usually not implemented in clinical laboratories but performed by reference laboratories to retrospectively detect heteroresistance in case of treatment failure or by research laboratories for epidemiological or mechanistic studies. For hVISA, EUCAST recommends three rapid screening methods [11], based on high-inoculum gradient strip or spot plating, and positive isolates should be sent to a reference laboratory for PAP-AUC. For other types of heteroresistance, methods applicable for routine detection should be developed and validated.

Epidemiology and clinical implications

Heteroresistance has been described in many bacterial species [3] and fungi [17]. Here, we address heteroresistance epidemiology and clinical impact in ESKAPE pathogens particularly for Gram-negative bacteria where novel data are available. Still, the lack of uniform definition and the variability of methods used to detect heteroresistance hampers comparison between studies. Heteroresistance

true prevalence in ESKPAPE pathogens remains unclear: on one hand, it is likely underestimated due to its difficult detection [12]; on the other hand, most studies reuse existing multi-drug resistant isolate collections creating a sampling bias. Clinical impact of heteroresistance remain poorly evaluated. However, small proportion of surviving bacteria could lead to treatment failure in particular clinical situations such as high-inoculum infections or immunocompromised patients. Here, we compiled the types of heteroresistance described in ESKAPE pathogens together with the data available on their prevalence and clinical impact. Details of the studies presented are available in **Supplementary Table 1**.

Gram-positive ESKAPE pathogens

Heteroresistance was first described for methicillin resistant *S. aureus* (MRSA) in the 1960s [18] although the most studied and clearly defined heteroresistance type is for hVISA, which has been extensively reviewed [19]. Prevalence of hVISA varies depending on the population studied, a meta-analysis conducted by Zhang and colleagues estimated that around 6% of the MRSA isolates are hVISA [20]. Several clinical studies and case reports have described the association between hVISA and a worse clinical outcome including persistent bacteremia and treatment failure but with little significant impact on mortality [19, 21, 22]. Vancomycin-variable Enterococci, susceptible isolates but carrying silent *vanA* genes, can be considered as heteroresistant as they produce minor subpopulation expressing the resistance and leading to breakthrough bacteremia [23]. Recently, heteroresistance was also reported for the newly released omadacycline both in *S. aureus* and *E. faecalis* [24, 25].

Gram-negative ESKAPE pathogens

- **Polymyxin heteroresistance**

In a large carbapenem-resistant Enterobacterales retrospective study of 408 isolates (USA), 10% were found heteroresistant to colistin [12]. In *Enterobacter* spp. the proportion of polymyxin

heteroresistance varies between 15% and 57% [12, 26]. In *K. pneumoniae* heteroresistance appears to be prevalent. In carbapenem-resistant isolates prevalence has been reported ranging from 8.4% in a large US study to 60% locally in Greece [12, 27]. Heteroresistance rates in carbapenem-susceptible isolates were lower, at 1.3% in a South-Korean study of 252 isolates [28]. This data is in sharp contrast with *E. coli* data where polymyxin heteroresistance seems rare (<2%) [12] .

Polymyxin heteroresistance in Enterobacterales has been suggested to cause treatment failures and impact clinical outcome [12]. In animal infection models, heteroresistant *Enterobacter cloacae* and *K. pneumoniae* isolates treated with colistin, led to overgrowth of the resistant population and treatment failure [29, 30]. In clinical practice, reports of colistin treatment failure due to polymyxin heteroresistant Enterobacterales remain scarce. There is a single case of a neutropenic patient suffering from *K. pneumoniae* bloodstream infection, where initial heteroresistance led to a fully resistant isolate under polymyxin-B treatment [31].

Polymyxin heteroresistance has rarely been described in *P. aeruginosa* [32]. In contrast, *A. baumannii* polymyxin heteroresistance is more established with a prevalence estimated at 33% (95% CI 16-53%) from a recent meta-analysis [8]. Two case-reports have described clinical treatment failure associated with post-surgical meningitis due to heteroresistant carbapenem-resistant *A. baumannii* [33-35]. In both cases, after colistin treatment of the heteroresistant isolate, a fully resistant isolate was recovered from cerebrospinal fluid. In one case, addition of rifampicin allowed for a successful outcome.

Its frequent detection in *Enterobacter* spp. and *K. pneumoniae* probably warrants caution with the use of colistin monotherapy against these pathogens. For carbapenem-resistant *A. baumannii,* combination therapies, as recommended for moderate to severe (or high-risk) infections [36, 37], should mitigate that risk.

Beta-lactam heteroresistance

Heteroresistance to carbapenem is well established [6] and has been reported in *E. coli* [38], *E. cloacae* [39] and *K. pneumoniae* in both carbapenemase-negative and carbapenemase-producers with low carbapenem MIC [40, 41]. Carbapenem regimen failed to be bactericidal against *K. pneumoniae* heteroresistant isolate in time-kill experiments and led to treatment failure in infected mice [42]. In *P. aeruginosa*, heteroresistance has been reported in several studies [43, 44] and its role in treatment failure was pointed in a large retrospective cohort in China, where imipenem and meropenem heteroresistance were detected in 54% and 73% of the 451 isolates, respectively [44]. Heteroresistance to carbapenems in *A. baumannii* has been described with up to 20% prevalence in a nationwide Spanish cohort [45, 46], and one clinical case of treatment failure in Brazil [47].

Regarding cephalosporins, while cefepim and ceftazidime heteroresistance have been reported in Enterobacterales, its prevalence and clinical impact remain uncertain [6]. In contrast, high heteroresistance rates are reported for cefiderocol, a recently marketed siderophore-cephalosporin antibiotic [48]. Clinical trials confirmed its effectiveness against carbapenemem resistant Enterobacterales but raised concerns on its effectiveness against carbapenem-resistant *A. baumannii* infections despite *in vitro* susceptibility [49], leading to usage restriction in this indication [36]. Although isolates from the CREDIBLE-CR study were not directly assessed for heteroresistance, Weiss's group drew attention to the high rates of cefiderocol heteroresistance in a collection of carbapenem resistant isolates from Georgia (USA): 30% in *K. pneumoniae* (30%), 9% in *P. aeruginosa* and 59% in *A. baumannii* [50] suggesting heteroresistance as a possible cause of poor clinical outcome. This association should be further studied to better understand the role of cefiderocol heteroresistance in treatment failure [51].

- **<u>Tigecyclin heteroresistance</u>**

Heteroresistance to tigecycline has been reported at variable rates in small epidemiological studies of hundreds of isolates: 7.8% in *K. pneumoniae* (China), 20% in *E. cloacae* (China) and up to 56% in

A. baumannii (South Korea) [52-54]. There is no data available on the clinical impact of tigecycline heteroresistance.

- **Fosfomycin heteroresistance**

Fosfomycin heteroresistance is frequently observed in Enterobacterales, with prevalence estimated around 10% [55]. Some studies have suggested that universal heteroresistance in *K. pneumoniae* could be the cause of oral fosfomycin treatment failure [56, 57], possibly leading to fosfomycin inferiority compared to nitrofurantoin for uncomplicated urinary tract infection [58]. Recently, EUCAST guidelines discontinued interpretation of oral fosfomycin for Enterobacterales other than *E. coli* [56, 57].

- **Aminoglycosides**

Overall, there is scarce data on aminoglycoside heteroresistance. In *K. pneumonia* isolates , amikacin heteroresistance was reported at 8.4% rate in China [59]. In a 104 carbapenem-resistant Enterobacterales collection (USA), 24%, 5% and 29% of the isolates showed heteroresistance to amikacin, gentamicin and tobramycin, respectively [6]. Heteroresistance to tobramycin and gentamicin were also reported in *A baumannii* [60].

Heteroresistance risk factors and when to look for it

One of the key questions is when to look for heteroresistance since systematic testing is not feasible in a clinical microbiology laboratory. Indications of heteroresistance can be microbiological, epidemiological and/or clinical. During standard AST, the presence of sporadic colonies growing in the inhibition area of gradient strip or disc diffusion assay [2], or the skip-well phenotype observed in colistin broth microdilution MIC assay for *Enterobacter* spp. [61] might hint for the presence of heteroresistance, although further confirmation by PAP reference method is necessary. However, only isolates with a high frequency of resistant subpopulation will display these phenotypes. In case of treatment failure, isolates before and after treatment should be analyzed for heteroresistance to increase our understanding of the clinical impact of heteroresistance.

Ideally, a priori targeted investigation could be performed based on heteroresistance prevalence and clinical risk factors. The main risk factor defined for heteroresistance is a prior exposure to this antibiotic, or to antibiotics that could induce cross-resistance [44, 62]. Underdosed antibiotic regimens of vancomycin or colistin have been shown to favor heteroresistance [22, 63]. In addition, particularly for hVISA, high bacterial load infection, chronic (osteo-articular) infections, and persistent bacteremia were associated to heteroresistance. Complicated central nervous system infections, particularly due to *A. baumannii* are likely situations at risk [33-35].

Taking advantage of heteroresistance to treat infections due to pan-resistant bacteria

A neglected facet of heteroresistance is the presence of a susceptible subpopulation within an overall resistant population (HR-R profile, Figure 1) [6]. With the increase of multidrug resistance, finding antimicrobials with at least partial activity might be a necessary therapeutic strategy.

A recent breakthrough study by Band et al. proved heteroresistance to be an important mechanism underlying effective combination treatments against multidrug-resistant Enterobacterales [6]. Heteroresistance to multiple antibiotics was found in 86.5% of their collection of 104 carbapenem-resistant Enterobacterales isolates [6]. In these cases of heteroresistance to multiple drugs, resistant sub-populations to each antibiotic are independent. Therefore, using antibiotic combinations we expect that one antibiotic eradicates the sub-population resistant to the other and vice versa. For example, aminoglycoside/beta-lactam combination was bactericidal in in-vitro time-kill experiment with Enterobacterales heteroresistant to both molecules, while single molecule failed to inhibit growth. The same strategy was also effective against a pan-drug resistant *K. pneumoniae* in a mouse infection model using the appropriate combination of antibiotics for which the strain was in fact heteroresistant. Only drug combinations targeting multiple heteroresistance displayed effective killing

[6]. These observations could explain the controversial and conflicting results observed across in vitro studies and clinical practice using combination therapies. Assessing resistance profiles beyond classical susceptibility testing by searching for heteroresistance coupled with combination testing by time-kill (reference method), gradient strip or checkerboard synergy assay, might offer novel solutions to clinicians. Further studies are needed to determine the feasibility, efficiency, and clinical impact of such strategies.

Conclusions

Heteroresistance is still “an emerging field in need of clarity” [2]. While heteroresistance seems a prevalent phenomenon, at least among multidrug-resistant isolates, its clinical significance remains understudied, mostly relying on case reports. Beyond the bench, prospective studies are needed to confirmed the true scale and clinical impact of heteroresistance at the bedside. Clear definitions and uniformization of detection methods, in particular for Gram-negative pathogens, are urgently needed to allow comparison between laboratories and studies. Two facets of heteroresistance merit further investigation: i) the undetected resistant subpopulations in isolates otherwise considered susceptible that might lead to clinical failure, and ii) the identification of susceptible subpopulations in pan-drug resistant Gram-negative to tailor combination treatments. If further studies confirm the clinical impact of heteroresistance, rapid screening methods should be developed to implement testing in clinical laboratories.

Transparency declaration

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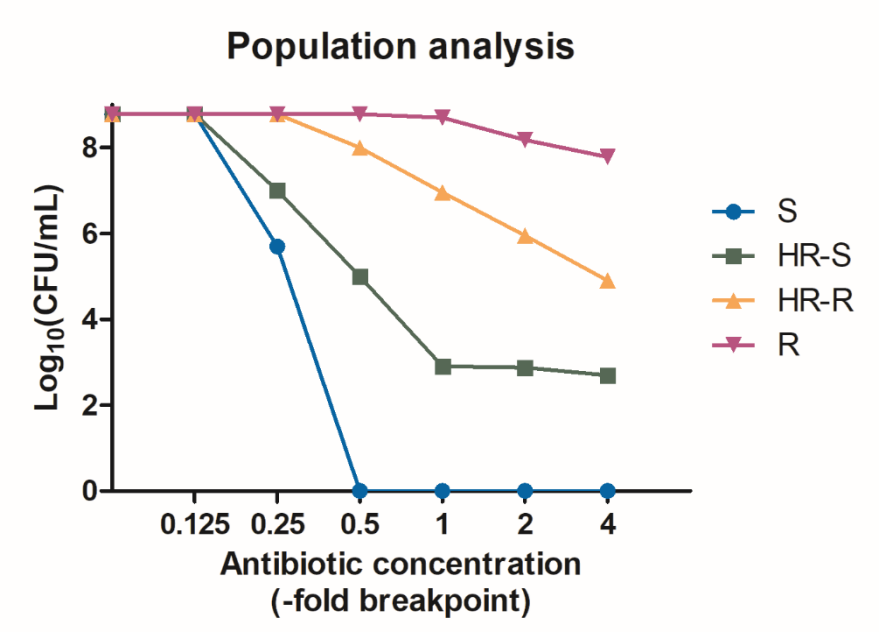
Contribution

MR and DOA contributed to the conception, literature review and writing of the manuscript. RS contributed to the critical review of the manuscript.



301 **Figure 1**: Examples of population analysis curves of isolates displaying various AST phenotypes:
302 susceptible (S), resistant (R) and heteroresistant classified as susceptible (HR-S) or resistant (HR-R) by
303 classical AST methods. Number of bacteria growing from the 2McF inoculum (colony forming unit/mL,
304 (CFU/mL), y axis) is plotted for each antibiotic concentration (2-fold increase displayed on x axis).

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