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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.

Objectives: 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: A 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, epidemiology and clinical impact of heteroresistance in ESKAPE pathogens are discussed here.

Content: Heteroresistance is only clearly defined for heterogeneous vancomycin intermediate *Staphylococcus aureus*. 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, which is the reference 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-analyses in the case of heterogeneous vancomycin intermediate *S. aureus* 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 treatment failure, heteroresistance should be tested by reference laboratories. Additional studies using standardized methods are needed to improve our understanding of heteroresistance and further assess its clinical impact. **Mélanie Roch, Clin Microbiol Infect 2023;29:320**

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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 classifies 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

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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 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 the 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. [2], Dewatcher et al. [5] and Anderson et al. [3], 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, the definition of heteroresistance 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, Fig. 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 with the 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} and 10^{-6}, able to grow in the presence of an antibiotic concentration of at least two-fold the breakpoint [6,10].

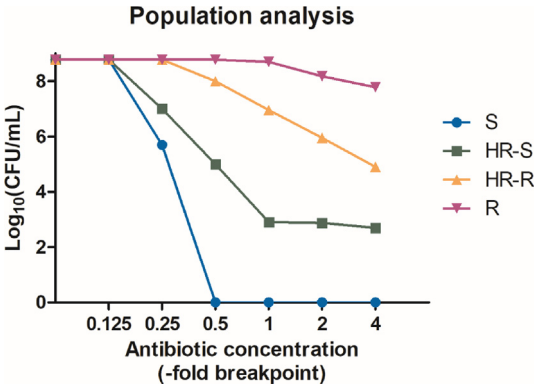

Fig. 1. Examples of population analysis curves of isolates displaying various antimicrobial susceptibility testing phenotypes: susceptible (S), resistant (R) and heteroresistant classified as susceptible or resistant by classical antimicrobial susceptibility testing methods. Number of bacteria growing from the 2McF inoculum (CFU/mL, y axis) is plotted for each antibiotic concentration (two-fold increase displayed on x axis).

The only type of heteroresistance with a clear consensus definition is the heterogeneous vancomycin intermediate *Staphylococcus aureus* (hVISA). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines hVISA as a *S. aureus* isolate susceptible to vancomycin (MIC $\leq$2 mg/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 because the frequency of the resistant subpopulation is too low to be detected from a standard inoculum.

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

- Inoculum: While some laboratories use overnight broth culture [6,10], a standardized 2 McFarland (2McF) inoculum (approximately 6.10^8 CFU/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, Fig. 1).
- Media: In the absence of guidelines, except for hVISA detection that has to be performed on Brain Heart Infusion (BHI) agar, most studies have used Mueller-Hinton agar for PAP. The standardized composition of Mueller-Hinton agar, 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 24-hour incubation time can be extended to 48 hours to aid the detection of slower-growing subpopulations [6,12]. For detection of hVISA, EUCAST recommends a 48-hour incubation and recent publications suggested that 72 hours 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 10-µL 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 the absence of antibiotic pressure [7,15]. Therefore, it is critical to minimize the number of subculture steps as the proportion of the 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 [3] and fungal [17] species. 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 among studies. The true prevalence of heteroresistance in ESKAPE pathogens remains unclear: on one hand, it is likely underestimated due to its difficult detection [12]; on the other hand, most studies reuse existing multidrug-resistant isolate collections, creating a sampling bias. The clinical impact of heteroresistance remains poorly evaluated. However, a 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 Table S1.

Heteroresistance in Gram-positive ESKAPE pathogens

Heteroresistance was first described for methicillin-resistant *S. aureus* in the 1960s [18], although the most studied and clearly defined heteroresistance type is for hVISA, which has been extensively reviewed [19]. The prevalence of hVISA varies depending on the population studied; a meta-analysis conducted by Zhang et al. [20] estimated that approximately 6% of methicillin-resistant *S. aureus* isolates are hVISA. Several clinical studies and case reports have described the association among hVISA and a worse clinical outcome, including persistent bacteraemia 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 subpopulations expressing the resistance and leading to breakthrough bacteraemia [23]. Recently, heteroresistance was also reported for the newly released omadacycline both in *S. aureus* and *Enterococcus faecalis* [24,25].

Heteroresistance in Gram-negative ESKAPE pathogens

Polymyxin heteroresistance

In a large carbapenem-resistant Enterobacterales retrospective study of 408 isolates (United States), 10% were found to be 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]. These data are in sharp contrast with *E. coli* data, in which 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 was a single case of a neutropenic patient with *K. pneumoniae* bloodstream infection, in which 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%) in 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.

β-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 a *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 reported in a large retrospective cohort in China, where imipenem and meropenem heteroresistance was 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, their 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 carbapenem-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 Study of Cefiderocol (S-649266) or Best Available Therapy for the Treatment of Severe Infections Caused by Carbapenem-resistant Gram-negative Pathogens (CREDIBLE - CR) 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, United Sates: 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].

Tigecyclin heteroresistance

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 are no data available on the clinical impact of tigecycline heteroresistance.

Fosfomycin heteroresistance

Fosfomycin heteroresistance is frequently observed in Enterobacterales, with prevalence estimated at approximately 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 with 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 are scarce data on aminoglycoside heteroresistance. In *K. pneumonia* isolates, amikacin heteroresistance was reported at a rate of 8.4% in China [59]. In a 104 carbapenem-resistant Enterobacterales collection in the United States, 24%, 5% and 29% of the isolates showed heteroresistance to amikacin, gentamicin and tobramycin, respectively [6]. Heteroresistance to tobramycin and gentamicin was 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 because 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 a 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 to the presence of heteroresistance, although further confirmation by the PAP reference method is necessary. However, only isolates with a high frequency of resistant subpopulations will display these phenotypes. In case of treatment failure, isolates before and after treatment should be analysed 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 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 favour heteroresistance [22,63]. In addition, particularly for hVISA, high bacterial load infection, chronic (osteoarticular) infections and persistent bacteraemia 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, Fig. 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. [6] proved heteroresistance to be an important mechanism underlying effective combination treatments against multidrug-resistant Enterobacterales. 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 subpopulations to each antibiotic are independent. Therefore, using antibiotic combinations, we expect that one antibiotic eradicates the subpopulation resistant to the other and vice versa. For example, aminoglycoside/β-lactam combination was bactericidal in an *in vitro* time-kill experiment with Enterobacterales heteroresistant to both molecules, and in contrast single-molecule treatment 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 appears to be 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 confirm 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: (1) the undetected resistant subpopulations in isolates otherwise considered susceptible that might lead to clinical failure, and (2) the identification of susceptible subpopulations in pan-drug resistant gram-negative bacteria 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.

Authors contributions

M.R. and D.O.A. contributed to the conception, literature review and writing of the manuscript. R.S. contributed to the critical review of the manuscript.

Transparency declaration

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2022.10.018.

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