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Antimicrobial Heteroresistance: an emerging field in need of clarity

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SUMMARY

'Heteroresistance' describes a phenomenon where subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic. Unfortunately, lack of standard methods to determine heteroresistance has led to inappropriate use of this term. Heteroresistance has been recognized since at least 1947 and occurs in Gram-positive and Gram-negative bacteria. Its clinical relevance could be considerable, since more resistant subpopulations may be selected during antimicrobial therapy. However, using non-standard methods to define heteroresistance, which are costly and involve considerable labor and resources, precludes evaluating the clinical magnitude and severity of this phenomenon. We review the available literature on antibiotic heteroresistance and propose recommendations for definitions and determination criteria for heteroresistant bacteria. This will help assessing the global clinical impact of heteroresistance and developing uniform guidelines for improved therapeutic outcomes.

INTRODUCTION

Infections by multidrug-resistant bacteria impose a serious encumbrance worldwide on society and economy and account for increasing global morbidity and mortality (1). Variable responses to antibiotics from bacterial cells within the same population, known as heteroresistance, is a poorly characterized phenomenon that further complicates the study of antibiotic resistance and its clinical relevance is uncertain. Heterogeneous antibiotic resistance was first described in 1947 for the Gram-negative bacterium *Haemophilus influenzae* (2), and almost 20 years later for Gram-positive staphylococci (3), but the first reported use of the term ‘heteroresistance’ occurred in 1970 (4). Heterogeneous resistance, population-wide variation of resistance, and heterogeneity

of response to antibiotics are also used to describe this phenomenon. The Clinical and Laboratory Standards Institute (CLSI), the British Society of Antimicrobial Chemotherapy (BSAC), and other international bodies develop clinical laboratory standards and recommendations for practices concerning antimicrobial resistance (5). Therefore, antimicrobial susceptibility testing methods such as MIC and disc-diffusion techniques, and standard criteria to define isolates as susceptible, resistant or intermediately resistant to any antibiotic are generally agreed upon worldwide. In contrast, heteroresistance is poorly characterized and consensus-based standards to define it are lacking.

In the literature, the term 'heteroresistance' has been indiscriminately applied to describe not only population-wide variation in antibiotic resistance but also other observations, and methods to determine heteroresistance vary significantly among laboratories. Confusion regarding this phenomenon precludes establishing its clinical significance and implementing proper therapeutic interventions and guidelines. Therefore in this review, we critically assess the published literature on heteroresistance, expose contradictions and variations in its definition, and recommend an operational definition and uniform criteria to assess heteroresistant bacteria.

MULTIPLE DEFINITIONS OF HETERORESISTANCE

Heteroresistance means population-wide, variable response to antibiotics (6). Several reports including the earliest studies describing the phenomenon applied this definition without specifying a particular antibiotic concentration range (3, 4, 7, 8). In contrast, concentration ranges were indicated for heteroresistance in *Acinetobacter baumannii* where subpopulations grew in 3 to 10 µg/ml colistin while the culture's MIC ranged from 0.25 to 2 µg/ml (9). Others described heteroresistance when a subset of the microbial population was resistant to an

antibiotic while the rest of the population was susceptible based on the concentration breakpoints of traditional *in vitro* susceptibility testing (10). This definition excludes cases where the bacterial culture comprises subpopulations with varying levels of resistance, but the entire population is either sensitive (Fig. 1D) or resistant (Fig. 1F) to the antibiotic.

Other definitions of heteroresistance contributed to the misconception about the nature of the phenomenon. Some of them were based on single cut-off concentrations, which did not describe the variation in resistance among members of a bacterial population. For example, heteroresistance was defined by growth of *A. baumannii* colonies on plates containing 8 μg/ml of colistin, with confirmation of MIC of 8 μg/ml by subsequent broth microdilution method (11). Similarly, heterogeneously resistant staphylococci were defined as any culture containing subpopulations at a frequency of 1 in 10^6 CFU/ml or higher with MIC > 4 μg/ml for vancomycin or ≥ 16 μg/ml for teicoplanin (12) or simply with MIC above these specified in CLSI guidelines for breakpoints of vancomycin or teicoplanin (13). A similar definition was adopted by setting a cut-off diameter of 10 mm in disc diffusion assays below which the strain was considered heteroresistant rather than merely resistant (14). Another approach defined heteroresistance as high MIC of *Enterococcus faecium* against vancomycin (>256 μg/ml) by broth dilution, but low MIC (=1.8 μg/ml) by Etest (15).

Other forms of heterogeneous bacterial behaviour against antibiotics were reported as heteroresistance. Certain *S. aureus* strains displayed methicillin resistance at high antibiotic concentrations (64 to 512 μg/ml) and susceptibility at low concentrations (2 to 16 μg/ml) (16). This phenomenon, termed "Eagle-type" resistance, was similar to the Eagle killing by penicillin described earlier, in which the bactericidal action of penicillin paradoxically decreased at high antibiotic concentrations (17). Similar patterns of bimodal growth in population analysis profiles

were observed in *A. baumannii* with cefepime, where growth inhibition after an initial peak of growth at low antibiotic concentration was followed by another peak of growth at higher concentration (18). Certain *S. aureus* strains displayed 'thermosensitive' heteroresistance where cultures growing in high methicillin concentrations at 30°C lost this ability within 30 minutes after shifting the growth temperature to 37°C (19). A temperature shift in the reverse direction caused equally rapid expression of methicillin resistance (19).

Adding to the confusion, 'heteroresistance' was applied to describe infections with bacterial strains having different levels of resistance to an antibiotic. Amoxicillin-resistant and -susceptible *Helicobacter pylori* isolates (MICs of 2 μg/ml and 0.06 μg/ml, respectively) were observed in different biopsies from one patient, a case described as 'inter-niche' heteroresistance (20). More recently, pairs of *H. pylori* isolated from the same patients had different levels of resistance to levofloxacin, metronidazole and in only one case to clarithromycin; the antibiotic resistant strains were mostly derived from a pre-existing sensitive strain rather than from infection with different strains of *H. pylori* having different levels of antibiotic resistance (21). Similarly, heteroresistance in *Mycobacterium tuberculosis* was defined as coexistence of anti-tuberculosis drug-susceptible and -resistant bacteria in the same patient (22, 23). More recently, heteroresistance in *M. tuberculosis* was redefined as coexistence of populations with different mutations in a drug resistance locus within a sample of organisms (24). Therefore, heteroresistance does not have a uniformly consistent definition, making retrospective comparisons to assess its true clinical significance impossible.

MEASURING HETERORESISTANCE

Population analysis profiling (PAP)

The PAP method is considered the gold standard for determining heteroresistance. In this method, the bacterial population is subjected to a gradient of antibiotic concentrations (either on plates or in liquid medium) and bacterial growth at each of these concentrations is quantified. The PAP is typically performed using the format of standard MIC determination with 2-fold antibiotic increments and by spread-plate techniques for CFU counting (3, 4, 6, 8, 14, 16, 18, 19, 25-41). Counting CFU by dropping smaller aliquots was as efficient as spread-plate (6, 42). Turbidimetric PAP assays are also performed using 2-fold antibiotic increments (6, 43), and antibiotic increments wider than 2-fold steps (2, 44).

Recently, heteroresistance was considered if the antibiotic concentration exhibiting the highest inhibitory effects was at least 8-fold higher than the highest non-inhibitory concentration (6), which allows comparisons of the isolate's behaviour against different antibiotics. However, most studies lacked criteria to define homogeneous vs. heterogeneous resistance. Lack of a standardized method to perform PAP, in particular the selection of antibiotic concentration increments led to confounding observations. For example, several studies investigated the response to glycopeptide antibiotics using PAP assays with narrow increments in antibiotic concentrations, such as 1 µg/ml steps (9, 13, 45-65) and even as low as 0.1 µg/ml steps (66). In these cases, a homogeneous strain could be inaccurately considered heteroresistant, and sometimes the same strain appeared as homogenous in one curve and heterogeneous in another (12).

A modified PAP assay comparing the area under the curve (PAP-AUC) of a given strain to that of a reference heteroresistant strain was used to determine *S. aureus* heteroresistance to vancomycin (67-81). The PAP-AUC ratios between test and control strain of <0.9, 0.9 to 1.3,

and >1.3 were considered indicative of vancomycin susceptible *S. aureus*, heterogeneous vancomycin intermediate *S. aureus* (hVISA), and vancomycin intermediate *S. aureus* (VISA), respectively (67, 72, 74, 76). Because this method relies on the vancomycin response of the *S. aureus* control strain, any instability in the antibiotic resistance of the control would cause significant changes in the results. The typical PAP method is time-consuming and labor intensive, and may not be suitable for clinical laboratories that screen hundreds of isolates for heteroresistance. A variation of PAP to screen clinical isolates for heteroresistance against glycopeptides used plates containing a single concentration of either vancomycin or teicoplanin (56, 68-70, 75, 82-84). However, comparative studies indicated that this method is not reliable for detecting heteroresistance (83, 85).

Disc diffusion and Etest assays

Disc diffusion (3, 14, 18, 55, 86-92) and Etest strips were used to detect heteroresistance as recommended for traditional *in vitro* susceptibility testing (6, 15, 18, 50, 63, 64, 66, 68, 71, 73, 76, 80, 86, 88-91, 93-101). Special Etest strips were developed for glycopeptides resistance detection (GRD Etest) (69, 74, 75, 81, 102). These are double-sided strips where one side contains vancomycin and the other teicoplanin. As with PAP, lack of standard guidelines hampers detection of heteroresistance using Etest and disc diffusion assays. An obvious indication of heteroresistance is the appearance of distinct colonies growing within the clear zone of inhibition in the disc diffusion or Etest assays. However, many reports set cut-off concentrations or inhibition zone diameters to decide on the heterogeneity of the response of the bacterial population to antibiotics as discussed before, but such cut-off values cannot sufficiently describe the population-wide behaviour.

184

185 **Additional methods to characterize heteroresistance**

186 Agar plates containing a linear gradient of antibiotic concentrations were used to determine the
187 antibiotic susceptibility of clinical isolates and identify antibiotic-resistant cells within bacterial
188 populations (103). Flow cytometry using a fluorescent penicillin derivative is another approach
189 employed to assess heteroresistance in methicillin-resistant *S. aureus* (MRSA) compared to
190 isolates with known heteroresistance (104). Other methods to characterize heteroresistant
191 bacteria have included bacterial re-growth at later time points in time-kill assays after an initial
192 significant growth reduction (9, 40), and increased MIC values of the same strain on prolonging
193 the incubation time (27). Both methods allow time for proliferation of less abundant and more
194 resistant members of the population. Also, uninterpretable and irreproducible MIC results in the
195 form of 'skipwells' (wells exhibiting no growth although growth still occurs at higher
196 concentrations of the antibiotic) could suggest heteroresistance, which was further confirmed by
197 PAP in isolates of *Enterobacter cloacae* and *Enterobacter aerogenes* against polymyxin B (105).

198

199 **HETERORESISTANCE IN DIFFERENT BACTERIAL SPECIES**

200 Heteroresistance denotes the presence of subpopulations of bacterial cells in the same culture
201 with higher levels of antibiotic resistance. Individual subpopulations of more resistant bacteria
202 were often isolated, but their stability differed. Typically, after five to ten serial passages in
203 antibiotic-free medium some highly resistant subpopulations reverted to the heterogeneous
204 resistance phenotype displayed by their original population (3, 30, 40), whereas others retained
205 their high-level resistance (6, 28). Most of the reported incidences of heteroresistance involve
206 bactericidal antibiotics including β-lactams, glycopeptides, antimicrobial peptides,

fluoroquinolones, aminoglycosides, and the nitroimidazole antibiotic metronidazole that acts on anaerobic bacteria (Tables 1 and 2). No systematic comparisons of the response of heteroresistant bacteria to bacteriostatic versus bactericidal antibiotics have been reported, except for one study in *Burkholderia cenocepacia* (6) showing heteroresistance to different classes of bactericidal antibiotics and homogenous responses to bacteriostatic antibiotics. Two studies reported incidences of heteroresistance against bacteriostatic antibiotics. One of them involved *S. aureus* strains heteroresistant to fusidic acid (45), but PAP was performed using a narrow range of antibiotic concentrations in small increments. The other study reported *Bordetella pertussis* strains being heteroresistant to erythromycin (88), which appear as discrete colonies in the clear zones of inhibition after 7 days of incubation in Etest and disc diffusion assays.

Heteroresistance in Gram-positive bacteria was reported for *S. aureus*, as well as for other Staphylococci, Enterococci and *Clostridium difficile*. The earliest reports of heteroresistance in *S. aureus* were on the response to methicillin (3, 4), but this extended to other β-lactams, which accounted for the majority of research on heteroresistance until late 1990s (Table 1).

Heteroresistance to vancomycin and other glycopeptides was first detected in Japanese vancomycin-resistant *S. aureus* (13). This also initiated a trend of PAP testing with a narrow range of antibiotic concentrations in very small increments, which were used to determine the clinical relevance and spread of vancomycin resistance in MRSA infections. However, controversial findings, originating from similar time range and geographical distribution, indicated that "heterogeneity" in response to vancomycin is common among *S. aureus* strains (47, 50, 61, 63, 70, 79, 95). Others reported that heteroresistance to vancomycin was not prevalent (51, 64, 72, 73, 80, 81, 102, 106). These studies promoted the assessment of heteroresistance in clinical laboratories as a standard procedure, but the results were conflicting

since different criteria to define heteroresistance were adopted and improper methods to detect heterogeneity were mostly used (discussed above under 'Measuring Heteroresistance').

Fewer reports described heteroresistance in Gram-negative bacteria. Table 2 summarizes the incidences of heteroresistance in *Pseudomonas aeruginosa*, *Klebsiella*, *Acinetobacter*, and *B. cenocepacia*.

Antibiotic resistance generally can be intrinsic or acquired (107), and the same applies to heteroresistance. Intrinsic heteroresistance occurs without pre-exposure to the antibiotic, but may also be acquired or induced after initial exposure to antibiotics. For example, repeated exposure of homogenously sensitive *Staphylococci* to methicillin resulted in mixed populations resembling the intrinsically heteroresistant strains (3). Similarly, *B. cenocepacia* displayed intrinsic heteroresistance to several bactericidal antibiotics including polymyxin B (6). However, acquired resistance after exposure to multiple rounds of selection in polymyxin B was shown for a *B. cenocepacia hldA* mutant possessing truncated lipopolysaccharide, which developed highly resistant subpopulations at polymyxin B levels not even tolerated by the most resistant members of the original population (108). A similar selection for MRSA involving step-wise exposure to vancomycin, led to acquired heteroresistance (109). Acquired heteroresistance may also originate from genetic events such as transposition (110, 111) or conjugation (112). The generated progenies include cells having different MIC due to differences in the number of copies of the inserted resistance genes or random disruption of genes involved in the bacterial response to antibiotics.

Molecules besides antibiotics can also induce heteroresistance. For example, exogenous glycine led to heterogeneous response to methicillin in the highly homogeneous MRSA COL strain (31). The heterogeneous resistance phenotype in this case was decreased methicillin

253 resistance in subsets of the population, as increasing glycine concentration in the medium
254 resulted in replacement of the D-alanyl-D-alanine peptidoglycan muropeptides with D-alanyl-
255 glycine muropeptides.

256 Bacteria growing as biofilms are physiologically distinct from their planktonic counterparts
257 and generally more resistant to antibiotics (113). Biofilms are populations of microorganisms
258 that are concentrated at an interface (usually solid-liquid) on biotic or abiotic surfaces and
259 typically surrounded by an extracellular polymeric matrix (113). Bacterial cells within a biofilm
260 display a wide range of physiological states; these states arise from genotypic and phenotypic
261 variations leading to distinct metabolic pathways, stress responses and other differences (114).
262 Variation in levels of resistance across a bacterial population together with enhanced ability to
263 form biofilm acted synergistically in *P. aeruginosa* infection (115). While biofilms occur in
264 many infectious diseases, standard antimicrobial susceptibility testing procedures rely on
265 planktonic cells. Thus, whether biofilms and the inherent variability among their populations
266 contribute to the detection of heteroresistance remains to be explored.

267

268 **MECHANISMS OF HETERORESISTANCE**

269 Non-genetic individuality in bacterial populations has been observed in differentiation and cell
270 division (116), chemotaxis (117), enzymatic activity (118), sporulation (119), stress responses,
271 and antibiotic resistance (120-122). These variations can be attributed to genetic, epigenetic, and
272 non-genetic mechanisms. Genetic mechanisms explain many cases of variation across a bacterial
273 population since increased resistance may be due to mutations or gene duplications of key
274 resistance genes or regulatory systems. Long-term infection could result in instability of bacterial
275 genomic DNA potentially leading to heteroresistance. For example, mutations in gene products

having metronidazole nitroreductase activities, mainly oxygen-insensitive NADPH nitroreductase (RdxA) and NADPH flavin oxidoreductase (FrxA), occurred in *H. pylori* heteroresistant to metronidazole (21). Epigenetic variation across the bacterial population can also occur. In this case, one or more genes whose products are involved in resistance to antibiotics are differentially expressed among cells within a bacterial population. Other non-genetic mechanisms involved in heteroresistance include chemicals in the bacterial milieu that may modulate the response to antibiotics across the bacterial population. For example, putrescine mediates heteroresistance of *B. cenocepacia* to multiple antibiotics (6), and glycine leads to heterogeneous response to methicillin in *S. aureus* (31). These mechanisms will be discussed below with more details specific to each antibiotic class.

Heteroresistance to β-lactams

Chambers *et al.* showed that increased production of PBP2a, encoded by *mecA*, was responsible for increased methicillin resistance of a subset of the population (27). However, further studies by the same group revealed that high levels of resistance require other factors acting within the autolysis pathway (123). Differences in regulation of autolysins in homogeneous vs. heterogeneous resistant strains were suggested (124). However, subsequent reports argued against the involvement of *mecA* (8, 34) and penicillinase (34) in methicillin heteroresistance. Regulatory systems contribute to heteroresistance. Inactivation of transcription regulators, such as Sar (125) and the Sigma-B operon (126) were other factors suggested to underlie heteroresistance in MRSA (127). Nevertheless, Sigma-B contributed to methicillin resistance but not heteroresistance in *S. epidermidis*; inactivation of the anti-sigma factor RsbW switched heteroresistance to homogeneous high-level resistance (128). Heteroresistance to homogeneous

high resistance selection (HeR-HoR selection) by oxacillin was associated with increased mutation rate and expression of *mecA* and SOS response *lexA*/*recA* gene regulators (129). Increased expression of the *agr* (accessory gene regulator) system during HeR-HoR selection was required to tightly modulate SOS-mediated mutation rates, which then leads to full expression of oxacillin homogeneous resistance in very heterogeneous clinical MRSA strains (130). The PBP1 protein played a role in SOS-mediated RecA activation and HeR-HoR selection (131). Conversely, a mutation in the less resistant cells of a heterogeneous population seemed to be responsible of their increased susceptibility. Single nucleotide polymorphism in the *dacA* (diadenylate cyclase) gene, which synthesizes the second messenger cyclic diadenosine monophosphate (c-di-AMP), was detected in the more sensitive cells. Thus, decreasing c-di-AMP levels resulted in reduced autolysis, increased salt tolerance and reduced basal expression of the cell wall stress stimulon (132). Interestingly, the Eagle-type heteroresistance was explained based on reduced repression of *mecA* transcription and penicillin-binding protein 2' production at high concentration (128 µg/ml) of methicillin, which did not occur at lower concentrations (1 and 8 µg/ml). Deletion of *mecI*, the repressor of *mecA*, converted the Eagle-type resistance to homogeneous high methicillin resistance (16). In *Streptococcus pneumoniae*, the penicillin binding protein PBP2x, but not PBP2b or PBP1a, from a heteroresistant strain conferred heteroresistance in a homogenous strain (133). Counterintuitively, PBP2x expression was not altered in the more resistant cells, but the expression of certain phosphate ABC transporter subunits (PstS, PstB, PstC and PhoU) was upregulated, which may represent a form of adaption to antibiotic stress (133).

Heteroresistance to β-lactams occurs in several Gram-negative bacterial species. Increased cephalothinase activity of the more resistant subpopulation was reported for *Enterobacter*

322 *cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, and *Morganella morganii* (28). The New-Delhi
323 β-lactamase (NDM-1) conferred heteroresistance in *Providencia rettgeri* (134). Similarly,
324 elevated expression of the β-lactamase gene in resistant subpopulation compared to the native
325 populations was detected in *Klebsiella pneumoniae* heteroresistant to meropenem (40), and
326 imipenem-heteroresistant *A. baumannii* (91). However, certain carbapenem-heteroresistant *A.*
327 *baumannii* isolates were carbapenemase negative, suggesting that other factors are involved in
328 the phenomenon (90). Differences in transcriptional levels may also underlie heteroresistance in
329 *P. aeruginosa* to carbapenems; the resistant subpopulations, compared to native ones, had
330 significantly increased transcription levels of the *mexB* and *mexY* genes whose protein products
331 are involved in multidrug efflux, and decreased expression of the *oprD* gene encoding an outer
332 membrane porin (57). Slower growth in β-lactam resistant subpopulations of *A. baumannii* may
333 protect against antibiotic challenge (18). In *Enterobacter* species, mutation of *ampD* which is
334 involved in the regulation of production of a class C β-lactamase, at rates as high as 10^{-4} to 10^{-6},
335 resulted in a heterogeneous population of bacterial cells with differing levels of β-lactam
336 resistance (135). Heteroresistance of invasive non-typeable *H. influenzae* to imipenem depended
337 in part on the penicillin binding protein PBP3 encoded by *ftsI*, PBP4 encoded by *dacB*, or AcrAB
338 efflux system; with a potential role of regulatory networks in the control of the heterogeneous
339 expression of the resistance phenotype (35). In *B. cenocepacia*, an ornithine decarboxylase
340 homologue and YceI, a small conserved protein, played a role in heteroresistance to ceftazidime
341 (6).

342 A model for heteroresistance was constructed by introducing into a sensitive *Escherichia coli*
343 strain the $bla_{\text{CTX-M-14}}$ gene encoding a cephalosporin hydrolase on a plasmid carrying the green
344 fluorescent protein. This permitted to follow heteroresistant bacteria since a subset of the cells

expressed more hydrolase and hence exhibited higher level of resistance to ceftriaxone (136). Heteroresistance was followed on a single-cell level owing to the fusion with the green fluorescent protein. This study showed that cells with hydrolase overexpression formed the majority of the population upon increasing antibiotic concentrations due to decreased growth rates rather than selection for resistant cells (136).

Heteroresistance to glycopeptides

Heteroresistance to glycopeptides has not been directly linked to a particular mechanism. Some studies reported increased incidence of mutations of regulatory genes in the heteroresistant populations. For example, *agr* was dysfunctional in 58% of hVISA strains while in only 21% of MRSA strains (84); hence *agr* dysfunction seems advantageous to *S. aureus* clinical isolates toward the development of vancomycin heteroresistance (49, 137). Similarly, compared to vancomycin susceptible MRSA, 13 of 38 (34%) hVISA possessed at least 1 non-synonymous mutation: 6 in *vraSR*, 7 in *walRK*, and 2 in *rpoB* genes (138).

Several mutations increase resistance to glycopeptides, but whether these are involved in population-wide variation in resistance is yet to be determined. Mutation of the *vraS* gene led to upregulation of the VraSR two-component system and conversion to the hVISA phenotype (38). Various mutations within the essential *walKR* two-component regulatory locus involved in control of cell wall metabolism conferred increased resistance to vancomycin and daptomycin among several VISA strains (139). Also, a mutation in the response regulator of the GraSR two-component regulatory system could increase resistance of hVISA to VISA, suggesting this is a mechanism of increased resistance in general rather than of heteroresistance (140). The *rpoB* mutation but not *graR* mutation was involved in hVISA (62), while in *S. aureus rpoB*-mediated

resistance to vancomycin was accompanied by a thickened cell wall and reduced cell surface negative charge (141). Furthermore, cell wall thickening was proportional to increased resistance to glycopeptides in coagulase-negative Staphylococci (53, 142) and in *S. aureus* (143), and rapid cell wall turnover with increasing positive charges through *dltA* over-expression led to repulsion of vancomycin and daptomycin (137). The expression of *atlE* (encoding an autolysin with an adhesive function) also increased proportionally with the vancomycin concentration in the culture of *S. epidermidis* (142).

Independent novel mutations in the *vanR*, *vanS*, *vanH*, *vanA*, *vanX* and *vanY* genes occurring upon continuous exposure to antibiotics can give rise to heteroresistance among vancomycin-resistant Enterococci strains (15, 97). Subpopulations of *Enterococcus faecalis* with different surface charges, expressed as bimodal zeta potential distributions were reported (144), a phenotype that may lead to heteroresistance similar to Staphylococci.

Heteroresistance to antimicrobial peptides

The mechanism of colistin heteroresistance in *A. baumannii* was attributed to loss of lipopolysaccharide production in subpopulations displaying high-level of colistin resistance, which were selected by serial passages on colistin plates at increasing concentrations (145). Loss of lipopolysaccharide was caused by an insertion sequence inactivating lipid A biosynthesis genes *lpxA* and *lpxC* (146). In contrast, heteroresistance to polymyxin B in *B. cenocepacia* depends on differences in the level of secretion of putrescine and YceI being differentially expressed across the different subpopulations (6). Moreover, a periplasmic component of an ABC transporter involved in biosynthesis of hopanoids was overexpressed in the more resistant subpopulation exposed to polymyxin B (6). While the role of this transporter in heteroresistance

was not directly evaluated, hopanoids contribute to polymyxin B resistance in *B. cenocepacia* (147).

Heteroresistance to fluoroquinolones

Heterogeneity of *Bartonella* sp. to ciprofloxacin was linked to a natural mutation Ser-83 to Ala (*E. coli* numbering) in the quinolone-resistance-determining region of *gyrA* (96). Similarly, *gyrA* and *gyrB* mutations were associated with levofloxacin heteroresistance in *H. pylori*; three amino acid mutation sites (87, 91, and 143) were found in GyrA of levofloxacin-resistant strains and an A406G amino acid substitution in GyrB was only found once (21). Putrescine, and to a less extent YceI, contributed to heteroresistance of *B. cenocepacia* to norfloxacin where mutants unable to produce either of them showed more homogeneous response to norfloxacin (6).

Heteroresistance to fosfomycin

Heteroresistance to fosfomycin is predominant among *S. pneumoniae* isolates (44). The UDP-N-acetylglucosamine enolpyruvyltransferase MurA1, which catalyzes the first step of peptidoglycan synthesis, contributes to heteroresistance against fosfomycin; however, this is not the only factor involved and potentially such heteroresistance is multifactorial (44).

Heteroresistance to rifampicin

The small protein YceI and, to a less extent putrescine produced by the antibiotic-responsive ornithine decarboxylase are involved in heteroresistance of *B. cenocepacia* to rifampicin (6). The deletion of genes encoding them individually showed less heterogeneous phenotype compared to the wild type strain.

CLINICAL SIGNIFICANCE OF HETERORESISTANCE

While some reports question the clinical significance of heteroresistance (51, 63, 76, 148), others argue for deterioration in clinical outcomes due to heteroresistant bacteria (46, 50, 64, 71, 77, 78, 149-152). Lack of a standard definition of heteroresistance may lead to misidentification of homogenous strains as heteroresistant hindering proper assessment of its clinical relevance. Heteroresistance may also be misinterpreted when only a single colony, picked from primary bacterial populations isolated from patients, is analyzed for its susceptibility to antibiotics (86). Heteroresistance was relevant in recurrent infections (46, 71), chronic infections (78), and infections with increased mortality rates (64, 77, 150, 151). Underlying mechanisms for these therapeutic failures could be antibiotic selection for the more resistant cells within the bacterial population and chemical communication of resistance, as described in more detail below.

Selection for the more resistant cells in the population

Therapeutic dosing of antibiotics without considering the highly resistant subpopulations of a heteroresistant isolate would select for the more resistant subpopulations. This is particularly the case when the majority of the population is sensitive to antibiotics while only a small subset, undetectable through criteria set for traditional *in vitro* antibiotic susceptibility testing, displays resistance above the clinical breakpoint (Fig. 1). In these situations, antibiotic therapy would lead to eradication of the more sensitive members of the bacterial population and their replacement by the more resistant cells. For example, colistin treatment of a patient with meningitis due to a colistin-heteroresistant *A. baumannii* resulted in selection of colistin-resistant derivatives (149). Moreover, *A. baumannii* isolates transitioned *in vivo* from susceptibility to full-resistance to

carbapenems, with heteroresistance as an intermediate stage due to administration of meropenem (90). Meropenem pressure can produce meropenem-heteroresistant subpopulations of *A. baumannii* that could be selected for by suboptimal therapeutic drug dosages, giving rise to highly resistant strains (39). Evidence of *in vivo* development of heteroresistance from antibiotic therapy was also seen in a patient with MRSA (98). Initial treatment with glycopeptides led to the development of heterogeneous glycopeptide resistance, which transformed to full resistance following daptomycin treatment. A similar switch from susceptibility to heteroresistance occurred in *A. baumannii* infections after prolonged exposure to imipenem (91).

Chemical communication of antibiotic resistance

Highly resistant subpopulations of heteroresistant bacteria could further complicate the clinical picture of polymicrobial infections by providing protection to more sensitive bacteria through chemical signals. For example, *P. aeruginosa* could be protected from the antimicrobial peptide polymyxin B by a highly resistant subpopulation of the heteroresistant cystic fibrosis pathogen *B. cenocepacia* (6). Simultaneous infection by both organisms is not uncommon since cystic fibrosis patients often have polymicrobial infection (153). The polyamine putrescine and the YceI protein, a small conserved protein with a lipocalin fold, mediated protection. These chemicals were released from *B. cenocepacia* in the presence of the antibiotic and resulted in survival of *P. aeruginosa* at a polymyxin B concentration equivalent to recommended therapeutic breakpoints at which *P. aeruginosa* should be killed in pure culture (6). Exposure to host derived putrescine and other polyamines led to a transient increase in resistance to antimicrobial peptides in the urogenital pathogen *Neisseria gonorrhoeae* (154), suggesting that communication of resistance mediated by polyamines is likely a general phenomenon. Putrescine

460 protected the surface of bacteria from the initial binding of polymyxin B (6) and reduced
461 antibiotic-induced oxidative stress (155), while YceI could bind and sequester polymyxin B thus
462 potentially reducing its levels in the bacterial milieu (6).

463 Indole is another chemical signal implicated in the communication of antibiotic resistance.
464 More resistant *E. coli* mutants arising from continuous antibiotic treatment protected less
465 resistant cells of the same population from norfloxacin and gentamicin (156). Such mutants
466 could maintain same levels of indole production in the presence of antibiotic treatment, which
467 could protect less resistant cells that produced lower concentration of indole under antibiotic
468 stress. These mutants cannot be considered highly resistant as their MIC is around the MIC
469 breakpoint for antibiotic sensitivity especially for norfloxacin, hence questioning their survival *in*
470 *vivo* at therapeutic doses of antibiotics. Moreover, this *E. coli* bacterial population may not be
471 truly heteroresistant owing to the lack of significant variation in concentrations tolerated by its
472 members. Although indole production is not common among bacteria (157), indole produced by
473 *E. coli* conferred antibiotic resistance to indole-negative *Salmonella enterica* serovar
474 Typhimurium (158), demonstrating another example of chemical communication. Protection
475 from antibiotics also occurred through antibiotic degrading enzymes. Protection of sensitive
476 bacteria was mediated by beta-lactamases produced from resistant *E. coli* cells against beta-
477 lactamase sensitive agents as cefamandole, but not cefotaxime, cefoxitin or imipenem which are
478 more resistant to beta-lactamases (43).

479

480 **CONCLUSIONS AND RECOMMENDATIONS**

481 Despite being recognized since 1947, heteroresistance is often used indiscriminately to describe
482 observations unrelated to population-wide responses to antibiotics. Lack of standard test formats

and global guidelines for determining heteroresistance contribute to disagreements between outcomes of different methods and diverse results from different laboratories (69, 74, 75). Since heteroresistance could have serious implications in antimicrobial therapy, a standard operational definition and methods to assess its clinical importance are essential.

We recommend defining heteroresistance as the population-wide variation of antibiotic resistance, where different subpopulations within an isolate exhibit varying susceptibilities to a particular antimicrobial agent. Concerning methods, PAP remains the gold standard for detecting heteroresistance by CFU counts. Turbidimetric PAP is also an acceptable alternative if antibiotic concentration increments are set at 2-fold; however, monitoring bacterial growth at time points earlier than 24 h (and after reaching the late log-phase/early stationary phase) may be advisable to watch for outgrowth of the more resistant subpopulation. Therefore, an isolate can be considered heteroresistant when the lowest antibiotic concentration giving maximum growth inhibition is greater than 8-fold higher than the highest non-inhibitory concentration. A 8-fold difference could be regarded as intermediate heteroresistance, while a smaller difference would denote homogenous response to the antibiotic. In homogeneous cultures, the entire population is usually inhibited over a narrow increment of antibiotic in standard MIC broth or agar dilution assay, with cases of intermediate growth before reaching maximal inhibition at only one antibiotic concentration increment above the highest non-inhibitory concentration. This general observation of homogeneous response to antibiotics has been documented by PAP assays in our recent study (6). Since 2-fold fluctuations in antibiotic sensitivity could normally occur, further increase in the transition from no inhibition to full inhibition by 2-fold relative to the homogeneous response was considered intermediate heteroresistance; greater differences (>8-fold) indicated heteroresistance as previously shown (6). This is similar to results observed in

previous reports but with more standardization; for example, the concentration inhibiting the entire population in PAP assays was 8-fold higher than MIC values (which cannot detect the more resistant minority) as opposed to homogeneous bacteria where it is the same concentration or just 2-fold higher (41). Disc diffusion or Etest assays, where growth of discrete colonies within the clear zone of inhibition indicates heteroresistance, could be an alternative to PAP. These discrete colonies represent subpopulations growing at concentrations that are inhibitory to the rest of the bacterial population suggesting population-wide variation in resistance. Antibiotic diffusion methods may therefore speed up screening of clinical isolates, but cannot replace PAP assays. In the absence of specific recommendations to address heteroresistance from agencies concerned with antibiotic resistance such as CLSI, BSAC and others, we propose a workflow scheme and interpretation criteria based on standard antibiotic sensitivity testing recommended by the same agencies (Fig. 2). This scheme includes modifications in the read-out and existing standard assays for detection of population-wide variation in antibiotic resistance (Fig. 2). Having worldwide standard criteria to define and assess heteroresistance will facilitate assessing its prevalence, clinical relevance, and impact on healthcare. Consequently, effective therapeutic strategies should be explored to counteract heteroresistance, which may include testing synergistic combinations of antibiotics (159) and using antibiotic adjuvants inhibiting key pathways involved in antibiotic resistance in conjunction with frontline antibiotics (6). A standard definition of heteroresistance would also help elucidate its nature by determining whether common mechanisms exist among different bacteria and against different antibiotic classes, and finding new targets for its disruption.

We urge global organizations concerned with antimicrobial resistance to advocate for harmonized recommendations and coordinate general consensus concerning heteroresistance.

We believe this is of utmost importance especially in clinical practice where currently thousands of clinical isolates are screened for heteroresistance, but with non-standardized methods that differ among laboratories, precluding obtaining a global picture of this problem. We anticipate that accurate and standardized detection of heteroresistance will translate to superior therapeutic outcomes based on improved identification of heteroresistant bacteria and optimized strategies to eradicate them.

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Authors' Biographies

Omar M. El-Halfawy has recently completed a Ph.D. in Microbiology and Immunology at the University of Western Ontario. He received his B.Sc. in Pharmaceutical Sciences from Alexandria University, Egypt in 2005. After working as a Community Pharmacist for few months, he accepted a Teaching Assistant position at the Faculty of Pharmacy, Alexandria University, Egypt in 2006. He received his M.Sc. in Pharmaceutical Microbiology from Alexandria University, Egypt, in 2009, and hence became an Assistant Lecturer at the same university since 2009. His current interests involve mechanisms of intrinsic antibiotic resistance, in particular heteroresistance and antibiotic resistance mediated by metabolites and other bacterial components.

Miguel A. Valvano received his MD degree in 1976 from the University of Buenos Aires, Argentina. He specialized in Pediatrics, also in Buenos Aires, and trained in molecular microbiology as a fellow with Jorge H. Crosa at the Oregon Health Sciences University (1983-1988). In 1988, Dr. Valvano accepted a faculty position at the University of Western Ontario where he progressed through the ranks to Full Professor and also held a Tier I Canada Research Chair from 1992 to 2012. In 2012, he accepted a position as Professor at Queen's University Belfast. Dr. Valvano and his colleagues investigate the assembly of lipopolysaccharide in particular the O antigen, in several Gram-negative bacteria, and also the molecular pathogenesis of opportunistic, non-fermentative Gram-negative bacteria such as *Burkholderia cenocepacia*. This research also involves studying mechanisms of bacterial intracellular survival in macrophages and intrinsic antibiotic resistance. He is the recipient of a CSM/Roche Award from the Canadian Society of Microbiologists, the Zeller's Award from Cystic Fibrosis Canada, and a Chair in Microbiology and Infectious Diseases from Queen's University Belfast.

1098 **Table 1: Cases of Heteroresistance in Gram-positive bacteria**

Organism	Antibiotic	Method	Comments	Reference
S. aureus (MRSA isolates)	Methicillin	PAP by CFU using 2-fold increments and presence of colonies in the inhibition zone of disc diffusion tests	Cultures consisted of mixed populations; the majority of cells were sensitive with a minority showing resistance	(3)
S. aureus	Cephalexin; Oxacillin	PAP by CFU using 2-fold increments	The population comprised cells with differing levels of resistance	(4)
S. aureus	Cephalothin; Methicillin; Cephalexin	PAP by CFU using 2-fold increments and presence of colonies in the inhibition zone of disc diffusion tests	Decreasing proportion of resistant organisms with increasing antibiotic concentration. Improper criterion for heteroresistance in diffusion assay based on diameter	(14)
S. epidermidis and *S. haemolyticus*	Methicillin	PAP by CFU using 2-fold increments	Only a minority of cells in a culture had significant resistance	(26)
S. aureus	Nafcillin	PAP by CFU using 2-fold increments and MICs at 48 h giving greater values than at 24 h	Susceptible cells represent the vast majority with a very small number (1 in 10^6 cells) of highly resistant cells	(27)
S. aureus	Methicillin "Thermosensitive"	PAP by CFU using 2-fold increments	Ability to grow in high concentrations of methicillin, at 30°C but not at 37°C.	(19)
S. aureus	Methicillin	PAP by CFU		(8, 30-34, 132)
S. aureus	Methicillin "Eagle-type"	PAP by CFU using 2-fold increments	Resistance to high concentrations of methicillin (64-512 μg/ml) and susceptibility to low concentrations (2-16 μg/ml)	(16)
S. epidermidis	Methicillin; Oxacillin	PAP		(128)
S. pneumoniae	Penicillin	Etest (complicated by zone of hemolysis), and PAP by CFU using very small increments of 0.1 μg/ml)	Potential misidentification of heteroresistance	(66)
S. aureus	Oxacillin			(7)
S. aureus	Cefazolin; Methicillin	PAP by CFU using 2-fold increments	Detected heteroresistant MRSA with low cefazolin MIC; genetically distinct from 1980s hetero-MRSA	(37)
S. aureus	Methicillin	Flow cytometry using Bocillin FL comparing with known heteroresistant MRSA as reference	New method but not compared to other methods	(104)
S. aureus	Methicillin; Oxacillin	PAP and selection of high resistance by growing at subinhibitory concentration of Oxacillin	Selection led to conversion from heteroresistant to homogeneous highly resistant	(129)
S. pneumoniae	Penicillin	PAP by CFU		(133)
S. aureus	Ceftaroline	PAP by CFU	The frequency of resistant subpopulations: 1 in 10^4-10^5	(160)
S. epidermidis	Methicillin; Vancomycin; Teicoplanin	PAP by CFU using 2-fold increments	Isolates tested from recurrent infection in dialysis patients	(46)
S. aureus	Methicillin; Vancomycin	PAP by CFU (compared spread-plate to spotting of 10 μl techniques)	Spotting reproduces the standard spread-plate while saving plates and time.	(42)
S. aureus	Methicillin; Vancomycin	PAP	Argued against a major role of resistant subpopulations in persistence or relapse in bacteremia	(51)
S. aureus	Vancomycin	PAP using 1 μg/ml increments	The first report of narrow increments in PAP was in 1997 using vancomycin	(13, 47, 48, 140)
S. aureus	Vancomycin	PAP Disc diffusion examining for satellitism	Vancomycin heteroresistance is induced by β-lactams; sequential use of the 2 antibiotics may facilitate the emergence of glycopeptide resistance	(87)
S. aureus	Vancomycin	CFU on plates with 4 μg/ml Vancomycin	Method is not reliable and may select for rather than detect heteroresistance	(82, 85)
Enterococcus faecium	Vancomycin	E-tests (growth in zone of inhibition)		(93)
S. aureus	Vancomycin	PAP by CFU (narrow increments)		(12, 49, 52, 62, 148)
Coagulase negative	Vancomycin, Teicoplanin	PAP by CFU (narrow increments)		(53)

staphylococci				
S. aureus	Vancomycin	Etest		(95, 100)
Staphylococcus spp.	Vancomycin; Teicoplanin	BHI Agar screening method with 4 or 6 µg/ml; PAP (narrow increments)		(56)
S. aureus	Vancomycin and Teicoplanin	BHI agar + 6 µg/ml Vancomycin, Mueller Hinton agar (MH) + 5 µg/ml Vancomycin and MH + 5 µg/ml Teicoplanin); Etest macromethod (using a 2 McFarland)	Multi-Centre study of the methods: Intra- and inter-laboratory reproducibility varied between methods with poorest performance seen with screening plates compared to Etest	(83)
Enterococcus faecium	Vancomycin	MIC by broth dilution; Etest (colonies in inhibition zone)	High level of resistance (MIC>256 µg/mL) by broth dilution but sensitivity by Etest (MIC=1.8 µg/ml)	(15)
S. aureus	Vancomycin	Modified PAP by CFU on BHI agar +0.25, 0.5, 1, 1.5, 2, 4, 6 and 8 µg/ml Vancomycin. The area under the curve (AUC) was calculated	Attempt to develop a new method that relies on comparison to a previously identified hVISA	(67)
S. capitis	Vancomycin	PAP (1 µg/ml increments) and calculating (AUC test/AUC Mu3) ratios; Etest (colonies in inhibition zone); BHI agar +4 µg/ml Vancomycin	Etest is more reliable and sensitive for detection of heteroresistance. Results suggest that PAP method should be revised and standardized	(68)
Enterococcus faecium	Teicoplanin	Etest		(97)
S. aureus	Glycopeptides	Etest GRD strips, with one incorporated with nutrients to enhance growth of hGISA; BHI agar + 6 µg/ml Vancomycin; MH agar + 5 µg/ml Teicoplanin; PAP-AUC	Etest GRD strip utilizing standard media and inocula, proved to be a simple and acceptable tool for detection of hGISA/GISA for clinical and epidemiologic purposes. Glycopeptide screening plates performed poorly.	(69)
S. aureus	Vancomycin	PAP by CFU using 2-fold increments		(38)
S. aureus	Vancomycin	PAP-AUC; Screening cascade: BHI agar +5 µg/ml teicoplanin then MET for positive isolates	Suggests a screening cascade to substitute PAP-AUC since not suitable in clinical practice.	(70)
S. aureus	Vancomycin	MET; PAP (narrow increments)	Etest criteria were based on a cutoff concentration: MET readings ≥8 µg/ml for vancomycin and teicoplanin or ≥12 µg/ml for teicoplanin only indicate hVISA	(58)
S. aureus	Vancomycin	Etest; PAP-AUC compared to Mu3		(64, 71, 73, 76, 161)
S. aureus	Vancomycin	PAP-AUC		(72, 78, 79)
S. aureus	Vancomycin, but not Telavancin (bactericidal lipoglycopeptide)	PAP (narrow increments)	Telavancin was efficacious in infections caused by hVISA in a murine bacteraemia model	(59)
S. aureus	Vancomycin	PAP by CFU compared to Mu3 (hVISA) and Mu50 (VISA)	Included a reported homogeneous strain and not only a heterogeneous one as controls	(60)
S. aureus	Vancomycin/ glycopeptides	MET; PAP		(61, 77)
S. aureus	Vancomycin	PAP-AUC; MET; GRD Etest; broth microdilution, BMD (MIC cutoff ≥ 2 µg/ml); standard Vancomycin Etest (MIC cutoff ≥ 2 µg/ml): Methods comparison with PAP-AUC as standard	The most cost-effective strategy was BMD as a standalone assay or in combination with PAP-AUC. GRD Etest remained an alternative, but a single cut-off value was used in all cases	(74)
S. aureus	Vancomycin	PAP/AUC; MET; GRD Etest; BHI agars + 3 or 4 µg/ml Vancomycin: Methods comparison with PAP-AUC as standard	Both Etest screening methods have excellent negative predictive values, but positive results require confirmation. BHI+ 3 and 4 µg/ml screening agars provided precise identification of hVISA and VISA, respectively	(75)
S. aureus	Vancomycin	Broth microdilution; GRD Etest on 4,210 clinical isolates from 43 U.S. centers; PAP-AUC for GRD-positive	Low reproducibility between test methods. The overall prevalence of hVISA was low (0.3%)	(102)
S. aureus	Vancomycin	Broth microdilution; MET; Standard Etest on 220 clinical isolates (121	MET identified 5.5% as hVISA isolates; with higher percentage among	(84)

		MSSA, 99 MRSA) from bloodstream infections. PAP-AUC; BHI agar +4 μg/ml Vancomycin	MRSA (9.1%) versus MSSA (2.5%)	
S. aureus	Vancomycin	PAP on 750 MRSA clinical strains isolated from Japan in 1990, before the introduction of injectable Vancomycin into clinical use in Japan in 1991	Identified 5.1% as hVISA strains from 19 hospitals. hVISA was present in Japanese hospitals before clinical introduction of vancomycin	(138)
S. aureus	Vancomycin	Etest; PAP-AUC on 288 MRSA isolates from a Connecticut Veterans Hospital	Low prevalence of hVISA arguing against routine screening	(80)
S. aureus	Vancomycin	PAP on 268 MRSA isolates from Seoul, Republic of Korea	37.7% were identified as hVISA. However, overall mortality was similar in hVISA and VSSA-infected patients	(63)
S. aureus	Vancomycin	GRD Etest; PAP-AUC on 43 MRSA isolates from Malaysia	Two isolates were hVISA.	(81)
S. aureus MRSA	Glycopeptides; Daptomycin	Etest.	*In vivo* development to heteroresistance	(98)
S. aureus	Daptomycin	PAP by CFU (narrow increments)	Despite using narrow increments, would be still heterogeneous if used with 2-fold increments	(54)
S. aureus	Daptomycin	PAP	PAP demonstrated daptomycin heteroresistance among tested hVISA and VISA strains	(162)
Toxigenic *Clostridium difficile*	Metronidazole	Etest and disc diffusion (appearance of colonies in clear zone)	Prolonged exposure to metronidazole can select for resistance *in vitro*. Routine disk diffusion assay (5 μg metronidazole disk) with primary fresh *C. difficile* isolates was recommended	(89)
C. difficile	Metronidazole		Heteroresistance to metronidazole was detected (~24% of 110 isolates)	(163)
Staphylococcus	Ciprofloxacin but not nalidixic acid	PAP & MIC	MIC for Ciprofloxacin of cells selected from plates with the highest concentration allowing growth was higher than that of the parental strains	(29)
S. pneumoniae	Fosfomycin	PAP (wide scale of increments higher than 2-fold)	10 out of 11 strains tested displayed heteroresistance	(44)
S. aureus	Fusidic acid	PAP by CFU (narrow increments)	Cell populations have cells with different levels of resistance. More resistant subpopulations exhibited homogeneous resistance compared to their respective parental strains	(45)

BHI, Brain heart infusion; GRD Etest, glycopeptide resistance detection Etest; MET, Macro-Etest (referring to an Etest in which higher inoculum sizes increase the probability of detection of more resistant members of the bacterial population); hGISA, heterogeneous glycopeptides intermediate *S. aureus*; hVISA, heterogeneous vancomycin intermediate *S. aureus*; MSSA, Methicillin-sensitive *S. aureus*; PAP-AUC, population analysis profiling-area under the curve method.

1104 **Table 2: Cases of Heteroresistance in Gram-negative bacteria**

Organism	Antibiotic	Method	Comments	Reference
Type b *H. influenzae*	Streptomycin	PAP by CFU count (concentrations <10-1000 U/ml)	Most of the culture was inhibited at 10 units/ml. Few resistant cells survived 10-100 U/ml and fewer at 1000 U/ml	(2)
Enterobacter aerogenes; E. coli; other Enterobacteria	Cefamandole; Cefoxitin,; Carbenicillin; Nalidixic acid.	PAP by CFU (2-fold increments)	This assay format was used to determine antibiotic resistance frequency	(25)
E. coli	Cefamandole; Cefotaxime; Cefoxitin; Imipenem	Turbidimetric PAP (2 fold increments or more)	Co-culture assays showed protection of sensitive cells by β-lactamases produced from resistant cells from β-lactamase sensitive agents (cefamandole, but not cefotaxime, cefoxitin or imipenem)	(43)
8 species of Enterobacteriaceae	Cefotaxime	PAP: *E. coli* and *Proteus mirabilis*: homogeneous; *Klebsiella oxytoca* and *Citrobacter koseri*: less homogeneous; *Enterobacter cloacae*, *Citrobacter freundii, Proteus vulgaris*, and *Morganella morganii*: heterogeneous	More resistant subpopulations from the 4 heteroresistant species had a very high increase in cephalothinase activity compared to parental strains	(28)
P. aeruginosa, and 7 strains from 5 genera of Enterobacteriaceae	Ciprofloxacin	PAP and MIC	MIC for Ciprofloxacin of cells selected from the plates with the highest concentration allowing growth was higher than that of the parental strains	(29)
Helicobacter pylori	Metronidazole	Etest and disc diffusion (small or large colonies were growing within the zone of inhibition)	Risk of misinterpretations when antibiotic susceptibility testing is based on a single colony picked from the populations isolated from patients	(86)
A. baumannii	Imipenem; Meropenem	Etest (Colonies in the clear zone of inhibition)	Warned that using carbapenems may lead to selection of resistant subpopulations subsequently causing dissemination of resistant strains and to therapeutic failure	(94)
A. baumannii	Colistin	PAP by CFU (narrow increments, but would still be heteroresistant if tested using 2-fold increments); Time kill curves (regrowth at late time point ~24 h, after rapid early killing indicates heteroresistance)	Subpopulations (<0.1% from 10^8 -10^9 CFU/ml) grew in the presence of colistin 3 to 10 μg/ml while the MIC of entire populations ranged from 0.25-2 μg/ml. Warned that recommended dosing is suboptimal for heteroresistant strains	(9)
P. aeruginosa	Imipenem; Meropenem	Disc diffusion (colonies in inhibition zone); PAP by CFU: (narrow increments and low initial inoculum)	Subpopulations growing at high concentration at frequencies 6.9 x 10^{-5} - 1.1 x 10^{-7}, suggest that these cells might not be detected by standard agar dilution MIC assay	(55)
Invasive nontypeable *H. influenzae*	Imipenem	PAP by CFU using 2-fold increments and Etest to determine MIC		(35)
Enterobacter cloacae and *A. baumannii*	Colistin	Disk diffusion; Etest; agar dilution; broth microdilution	Isosensitest agar was better than Mueller Hinton agar in detection of heteroresistance	(11)
A. baumannii-calcoaceticus complex	Colistin	PAP by CFU using 2-fold increments	Heteroresistance was defined by growth of colonies on plates containing 8 μg/ml of colistin, while the MIC=8 μg/ml by broth microdilution	(36)
P. aeruginosa	Carbapenems	Agar dilution according to CLSI. Increments of 2 μg/ml for concentrations ranging from 2 to 32 μg/ml and of 8 μg/ml from 32 to 64 μg/ml	Mutant subpopulations had at least 4-fold higher MIC than those of native cells for imipenem and meropenem	(57)
Bartonella sp.	Ciprofloxacin	Etest		(96)
A. baumannii	Ampicillin/ Sulbactam	Etest (incubation for ≥48 h)	Resistance could be induced after ≥48 h of antimicrobial exposure; hence 24 h incubation of test plates may not be enough to screen for heteroresistance	(99)
A. baumannii	Carbapenem	Disk-diffusion; Etest: colonies in clear zone of inhibition	*in vivo* evolution of an antimicrobial profile from susceptibility to full-resistance to carbapenems, with heteroresistance as an intermediate stage	(90)
E. aerogenes	Carbapenem	Etest	Automated MicroScanWalkAway system	(164)

			failed to detect heteroresistance detected by Etest	
A. baumannii	Meropenem	PAP by CFU using 2-fold increments	Suggests that *A. baumannii* isolates that are apparently meropenem susceptible by standard susceptibility testing may contain resistant subpopulations that could be selected for by suboptimal therapeutic drug dosages	(39)
K. pneumoniae	Meropenem	MIC & PAP (2 fold increments); Time kill assays	Re-growth of heteroresistant strains after initial killing phase	(40)
K. pneumonia	Carbapenem	Etest (colonies in inhibition zone); PAP.	Low reproducibility of MIC led to investigation of heteroresistance	(101)
A. baumannii	Imipenem	Etest; disk diffusion (colonies in the inhibition zone)	Switch from imipenem susceptibility to heteroresistance was more likely to occur in strains successively isolated from patients who had been exposed to imipenem (10.9 ± 6.5 days exposure vs. 5.3 ± 4.8 days for controls)	(91)
Carbapenemase-producing *K. pneumoniae*	Colistin	PAP by CFU using 2-fold increments and MIC		(41)
A. baumannii	Cefepime	PAP by CFU using 2-fold increments, Etest, and disc diffusion	PAP of an isolate had 2 peaks of growth at different cefepime concentrations	(18)
A. baumannii	Carbapenems	Disc diffusion (colonies in zone of inhibition)	Heteroresistance was referred to as phenotypic heterogeneous resistance	(92)
P. aeruginosa	Polymyxin B	PAP by CFU (PmB concentrations from 0 to 8 μg/ml)	Isolates presenting subpopulations that exhibited growth at Polymyxin B concentrations ≥2 μg/ml were considered heteroresistant. Isolates containing subpopulations that grew at Polymyxin B concentrations at least twice higher than the original MIC but <2 μg/ml were considered heterogeneous	(65)
B. cenocepacia	Polymyxin B; Norfloxacin; Rifampicin; Ceftazidime; Gentamicin;	Etest; PAP by CFU and turbidimetric (2 fold increments)	Detailed comparison of population-wide response to bacteriostatic vs. bactericidal antibiotics showing heteroresistance only against bactericidal agents. Criteria adopted for interpretation of heteroresistance are similar to those recommended here	(6)
E. cloacae; E. aerogenes	Polymyxin B	PAP	Multiple skip wells were observed in polymyxin susceptibility testing of *Enterobacter* species leading to uninterpretable results	(105)
H. pylori	Levofloxacin; Clarithromycin; Metronidazole	MIC by Etest and agar dilution for 19 pairs of clinical isolates. Each pair was isolated from the same patient	Heteroresistance was reported when pairs showed difference in resistance in 5, 1 and 19 cases for levofloxacin, clarithromycin and metronidazole respectively.	(21)
Providencia rettgeri	Carbapenems	PAP by CFU		(134)
Bordetella pertussis	Erythromycin	Disc diffusion and Etest	Heteroresistance was not detected except after 7 days of incubation when colonies appeared in clear zone. Degradation of erythromycin from the disc on long incubation was ruled out	(88)

Figure legends

FIG. 1. Heteroresistant vs. homogenous response to antibiotics. Dotted lines represent breakpoints for resistance. Homogenous bacterial cultures (A-C) can either be A, susceptible, B, of intermediate susceptibility, or C, resistant to an antibiotic according to traditional *in vitro* susceptibility testing. Heteroresistant bacteria (D-F) may be: D, completely susceptible to an antibiotic, whereby the different subpopulations respond to antibiotic concentrations extending below the breakpoints. This form is less likely to be detected and is probably the least clinically important (unless the least responsive subpopulations develop resistance to the antibiotic). E, the more classical form of heteroresistance in which the majority of the bacterial population is susceptible to an antibiotic with a highly resistant minority. Antibiotic treatment guided by the traditional susceptibility testing breakpoints would select for the resistant subpopulation, leading to therapeutic failure. F, the entire bacterial population, including the least resistant subpopulations, is resistant to the antibiotic. Chemical communication of antibiotic resistance from the more resistant members of the population protecting less resistant bacteria is the major concern of such bacterial populations.

FIG. 2. Recommended scheme for determination of heteroresistance and interpretation criteria. Disc diffusion assays should be performed according to standardized procedures for antimicrobial susceptibility testing as recommended by agencies such as CLSI or BSAC. These procedures may be applied to Etest assays while taking into consideration the manufacturer guidelines. PAP by CFU counts should be performed by plating aliquots of 10-fold serially diluted bacterial cultures on antibiotic-containing agar plates. Agar plate preparation should follow standardized guidelines used for MIC by agar dilution assays. Turbidimetric PAP should follow the standard MIC by broth dilution technique, with the exception of turbidimetric quantification of bacterial growth at each antibiotic concentration.

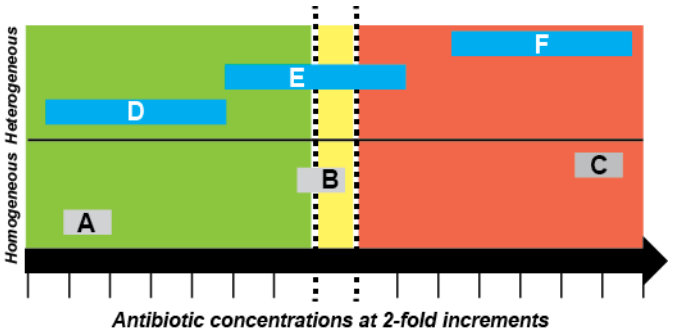
Homogeneous Heterogeneous
A
B
C
D
E
F
Antibiotic concentrations at 2-fold increments

Figure 1

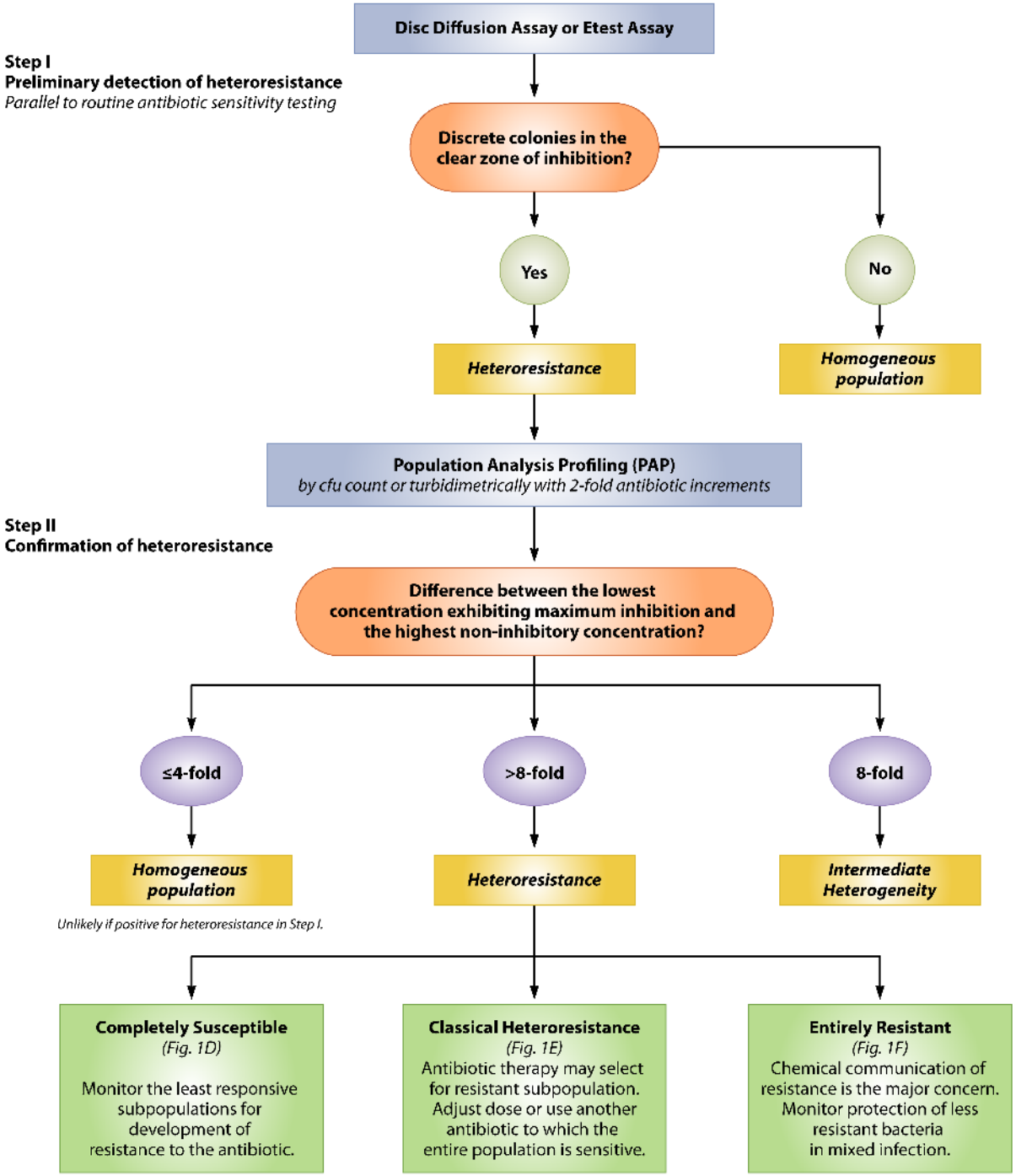
Step I
Preliminary detection of heteroresistance
Parallel to routine antibiotic sensitivity testing
Disc Diffusion Assay or Etest Assay
Discrete colonies in the clear zone of inhibition?
Yes
No
Heteroresistance
Homogeneous population
Population Analysis Profiling (PAP)
by cfu count or turbidimetrically with 2-fold antibiotic increments
Step II
Confirmation of heteroresistance
Difference between the lowest concentration exhibiting maximum inhibition and the highest non-inhibitory concentration?
≤4-fold
>8-fold
8-fold
Homogeneous population
Unlikely if positive for heteroresistance in Step I.
Heteroresistance
Intermediate Heterogeneity
Completely Susceptible
(Fig. 1D)
Monitor the least responsive subpopulations for development of resistance to the antibiotic.
Classical Heteroresistance
(Fig. 1E)
Antibiotic therapy may select for resistant subpopulation. Adjust dose or use another antibiotic to which the entire population is sensitive.
Entirely Resistant
(Fig. 1F)
Chemical communication of resistance is the major concern. Monitor protection of less resistant bacteria in mixed infection.

Figure 2