CMR
Journals.ASM.org

# Antimicrobial Heteroresistance: an Emerging Field in Need of Clarity

**Omar M. El-Halfawy,[a,b] Miguel A. Valvano[a,c]**

Centre for Human Immunology and Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada[a]; Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt[b]; Centre for Infection and Immunity, Queen's University Belfast, Belfast, United Kingdom[c]



## SUMMARY

"Heteroresistance" describes a phenomenon where subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic. Unfortunately, a 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 may be considerable, since more resistant subpopulations may be selected during antimicrobial therapy. However, the use of nonstandard 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 in 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 societies and economies and account for increasing global morbidity and mortality (1). The occurrence of various 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 determination 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 applied indiscriminately to describe not only population-wide variation in antibiotic resistance but also other observations, and methods to determine heteroresistance vary significantly among laborato-

**Citation** El-Halfawy OM, Valvano MA. 2015. Antimicrobial heteroresistance: an emerging field in need of clarity. Clin Microbiol Rev 28:191–207. doi:10.1128/CMR.00058-14.

Address correspondence to Miguel A. Valvano, m.valvano@qub.ac.uk.



doi:10.1128/CMR.00058-14

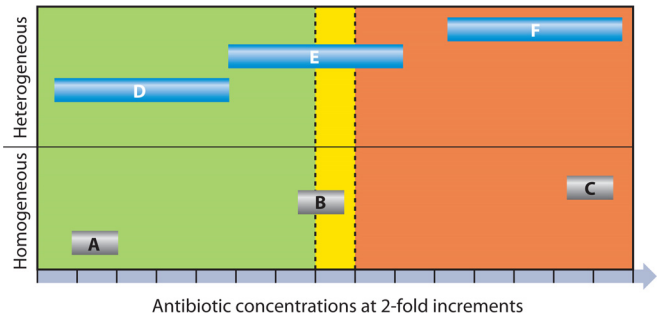


**FIG 1** Heteroresistant versus homogeneous responses to antibiotics. Dotted lines represent breakpoints for resistance. Homogeneous bacterial cultures can be either susceptible (A), of intermediate susceptibility (B), or resistant (C) to an antibiotic according to traditional *in vitro* susceptibility testing. Heteroresistant bacteria may be any of the following. (D) Bacteria are 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) Bacteria exhibit 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.

ries. 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 for assessment of heteroresistant bacteria.

## MULTIPLE DEFINITIONS OF HETERORESISTANCE

Heteroresistance means that there are population-wide variable responses 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 and 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 various 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 misconceptions about the nature of the phenomenon. Some of them were based on single cutoff 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 a MIC of 8 μg/ml by a 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 a MIC of >4 μg/ml for vancomycin or ≥16 μg/ml for teicoplanin (12), or simply with a MIC above those specified in the CLSI guidelines for breakpoints of vancomycin or teicoplanin (13). A similar definition was adopted by setting a cutoff 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 a high MIC of *Enterococcus faecium* against vancomycin (>256 μg/ml) by broth dilution but a low MIC (1.8 μg/ml) by Etest (15).

Other forms of heterogeneous bacterial behavior against antibiotics were reported as heteroresistance. Certain *Staphylococcus 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 for *A. baumannii* with cefepime, where growth inhibition after an initial peak of growth at a low antibiotic concentration was followed by another peak of growth at a higher concentration (18). Certain *S. aureus* strains displayed "thermosensitive" heteroresistance, where cultures growing at high methicillin concentrations at 30°C lost this ability within 30 min after shifting of the growth temperature to 37°C (19). A temperature shift in the reverse direction caused an equally rapid expression of methicillin resistance (19).

Adding to the confusion, the term "heteroresistance" has been 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 biopsy specimens from one patient, displaying what was described as "interniche" heteroresistance (20). More recently, pairs of *H. pylori* isolates obtained from the same patients had different levels of resistance to levofloxacin, metronidazole, and (in only one case) clarithromycin; the antibiotic-resistant strains were mostly derived from a preexisting 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 antituberculosis 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

The population analysis profiling (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. PAP is typically performed using the format of standard MIC determina-

tion, with 2-fold antibiotic increments, and by use of spread plate techniques for CFU counting (3, 4, 6, 8, 14, 16, 18, 19, 25–41). Counting of CFU by dropping smaller aliquots is as efficient as spread plate techniques (6, 42). Turbidimetric PAP assays are also performed using 2-fold antibiotic increments (6, 43) and antibiotic increments of >2-fold (2, 44).

Recently, heteroresistance was considered if the antibiotic concentration exhibiting the highest inhibitory effects was at least 8-fold higher than the highest noninhibitory concentration (6), which allowed comparisons of the isolate's behavior against different antibiotics. However, most studies lacked criteria to define homogeneous versus 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 by 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 inaccurately be considered heteroresistant, and sometimes the same strain appeared to be homogeneous by one curve and heterogeneous by 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). PAP-AUC ratios between the test and control strains 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 uses 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 assays (3, 14, 18, 55, 86–92) and Etest strips have been 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 glycopeptide resistance detection (GRD Etest) (69, 74, 75, 81, 102). These are double-sided strips that contain vancomycin on one side and teicoplanin on the other. As with PAP, the lack of standard guidelines hampers detection of heteroresistance by use of 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 assay. However, many reports set cutoff concentrations or inhibition zone diameters to decide on the heterogeneity of the response of the bacterial population to antibiotics, as discussed before, but such cutoff values cannot sufficiently describe the population-wide behavior.

### Additional Methods To Characterize Heteroresistance

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

## HETERORESISTANCE IN DIFFERENT BACTERIAL SPECIES

Heteroresistance denotes the presence of subpopulations of bacterial cells with higher levels of antibiotic resistance than those of the rest of the population in the same culture. Individual subpopulations of more resistant bacteria are often isolated, but their stability differs. Typically, after 5 to 10 serial passages in antibiotic-free medium, some highly resistant subpopulations revert to the heterogeneous resistance phenotype displayed by the original population (3, 30, 40), whereas others retain their high-level resistance (6, 28). Most of the reported incidences of heteroresistance involve bactericidal antibiotics, including β-lactams, glycopeptides, antimicrobial peptides, fluoroquinolones, aminoglycosides, and the nitroimidazole antibiotic metronidazole, which acts on anaerobic bacteria (Tables 1 and 2). No systematic comparisons of the responses of heteroresistant bacteria to bacteriostatic versus bactericidal antibiotics have been reported, except for one study of *Burkholderia cenocepacia* (6) showing heteroresistance to different classes of bactericidal antibiotics and homogeneous 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), appearing 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 has been reported for *S. aureus* as well as for other staphylococci, enterococci, and *Clostridium difficile*. The earliest reports of heteroresistance in *S. aureus* were based on the response to methicillin (3, 4), but this extended to other β-lactams, which accounted for the majority of research on heteroresistance until the late 1990s (Table 1).

Heteroresistance to vancomycin and other glycopeptides was first detected in Japanese vancomycin-resistant *S. aureus* strains (13). This also initiated a trend of PAP testing with a narrow range of antibiotic concentrations in very small increments, which was used to determine the clinical relevance and spread of vancomycin resistance in MRSA infections. However, controversial findings, originating from similar time ranges and geographical distributions, indicated that "heterogeneity" in response to vancomycin is

**TABLE 1** Cases of heteroresistance in Gram-positive bacteria[a]

| Organism(s) | Antibiotic(s) | Method(s) | Comments | Reference(s) |
|---|---|---|---|---|
| *S. aureus* (MRSA isolates) | Methicillin | PAP by CFU counts, 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 counts, using 2-fold increments | The population comprised cells with differing levels of resistance. | 4 |
| | Cephalothin, methicillin, cephalexin | PAP by CFU counts, 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 counts, using 2-fold increments | Only a minority of cells in a culture had significant resistance. | 26 |
| *S. aureus* | Nafcillin | PAP by CFU counts, using 2-fold increments, and MICs at 48 h being greater than those at 24 h | Susceptible cells represent the vast majority, with a very small number (1 in $10^6$ cells) of highly resistant cells. | 27 |
| | Methicillin ("thermosensitive") | PAP by CFU counts, using 2-fold increments | Ability to grow in high concentrations of methicillin at 30°C but not at 37°C. | 19 |
| | Methicillin | PAP by CFU counts | | 8, 30–34, 132 |
| | Methicillin ("Eagle-type" resistance) | PAP by CFU counts, 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 counts, using very small increments (0.1 μg/ml) | Potential misidentification of heteroresistance. | 66 |
| *S. aureus* | Oxacillin | | | 7 |
| | Cefazolin, methicillin | PAP by CFU counts, using 2-fold increments | Detection of heteroresistant MRSA with a low cefazolin MIC; genetically distinct from 1980s hetero-MRSA. | 37 |
| | Methicillin | Flow cytometry using Bocillin FL, with comparison to known heteroresistant MRSA strains as a reference | New method, has not been compared to other methods. | 104 |
| | Methicillin, oxacillin | PAP and selection of high resistance by growing at subinhibitory concentration of oxacillin | Selection led to conversion from heteroresistant to homogeneously highly resistant. | 129 |
| *S. pneumoniae* | Penicillin | PAP by CFU counts | | 133 |
| *S. aureus* | Ceftaroline | PAP by CFU counts | The frequency of resistant subpopulations was 1 in $10^4$ to 1 in $10^5$. | 160 |
| *S. epidermidis* | Methicillin, vancomycin, teicoplanin | PAP by CFU counts, using 2-fold increments | Isolates tested were obtained from recurrent infections of dialysis patients. | 46 |
| *S. aureus* | Methicillin, vancomycin | PAP by CFU counts (comparison of spread plate technique to spotting of 10-μl samples) | Spotting reproduces the standard spread plate technique while saving plates and time. | 42 |
| | Methicillin, vancomycin | PAP | Argued against a major role of resistant subpopulations in persistence or relapse in bacteremia. | 51 |
| | Vancomycin | PAP using 1-μg/ml increments | The first report of using narrow increments for PAP was in 1997, using vancomycin. | 13, 47, 48, 140 |
| | Vancomycin | PAP and disc diffusion assay to examine satellitism | Vancomycin heteroresistance is induced by β-lactams; sequential use of 2 antibiotics may facilitate the emergence of glycopeptide resistance. | 87 |
| | Vancomycin | CFU counts on plates with 4 μg/ml vancomycin | The method is not reliable and may select for rather than detect heteroresistance. | 82, 85 |
| *Enterococcus faecium* | Vancomycin | Etests (growth in zone of inhibition) | | 93 |
| *S. aureus* | Vancomycin | PAP by CFU counts (narrow increments) | | 12, 49, 52, 62, 148 |
| Coagulase-negative staphylococci | Vancomycin, teicoplanin | PAP by CFU counts (narrow increments) | | 53 |
| *S. aureus* | Vancomycin | Etest | | 95, 100 |
| *Staphylococcus* spp. | Vancomycin, teicoplanin | BHI agar screening method with 4 or 6 μg/ml antibiotic; PAP (narrow increments) | | 56 |
| *S. aureus* | Vancomycin and teicoplanin | BHI agar plus 6 μg/ml vancomycin, MH agar plus 5 μg/ml vancomycin, and MH agar plus 5 μg/ml teicoplanin; Etest macromethod (using a 2 McFarland standard) | In this multicenter study of methods, intra- and interlaboratory reproducibilities varied between methods, with the poorest performance seen with screening plates compared to Etest. | 83 |
| *Enterococcus faecium* | Vancomycin | MIC by broth dilution and Etest (colonies in inhibition zone) | High level of resistance (MIC of >256 μg/ml) by broth dilution but sensitivity by Etest (MIC = 1.8 μg/ml). | 15 |
| *S. aureus* | Vancomycin | Modified PAP by CFU counts on BHI agar plus 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 μg/ml vancomycin, with calculation of the AUC | Attempt to develop a new method that relies on comparison to a previously identified hVISA strain. | 67 |
| *S. capitis* | Vancomycin | PAP (1-μg/ml increments) and calculation of AUC test/AUC Mu3 ratios; Etest (colonies in inhibition zone); BHI agar plus 4 μg/ml vancomycin | Etest is more reliable and sensitive for detection of heteroresistance. Results suggest that the PAP method should be revised and standardized. | 68 |

| | | | | |
|---|---|---|---|---|
| *Enterococcus faecium* | Teicoplanin | Etest | | 97 |
| *S. aureus* | Glycopeptides | GRD Etest strips, with one incorporated with nutrients to enhance growth of hGISA; BHI agar plus 6 μg/ml vancomycin; MH agar plus 5 μg/ml teicoplanin; PAP-AUC | A GRD Etest 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 |
| | Vancomycin | PAP by CFU counts, using 2-fold increments | | 38 |
| | Vancomycin | PAP-AUC; screening cascade, i.e., BHI agar plus 5 μg/ml teicoplanin and then MET for positive isolates | The study suggests that a screening cascade should replace PAP-AUC, since that method is not suitable for clinical practice. | 70 |
| | Vancomycin | MET; PAP (narrow increments) | Etest criteria were based on cutoff concentrations: MET readings of ≥8 μg/ml for vancomycin and teicoplanin or ≥12 μg/ml for teicoplanin only indicate hVISA. | 58 |
| | Vancomycin | Etest; PAP-AUC compared to Mu3 | | 64, 71, 73, 76, 161 |
| | Vancomycin | PAP-AUC | | 72, 78, 79 |
| | Vancomycin but not telavancin (bactericidal lipoglycopeptide) | PAP (narrow increments) | Telavancin was efficacious against infections caused by hVISA in a murine bacteremia model. | 59 |
| | Vancomycin | PAP by CFU counts compared to Mu3 (hVISA) and Mu50 (VISA) | Included a reported homogeneous strain, not just a heterogeneous one, as a control. | 60 |
| | Vancomycin, glycopeptides | MET; PAP | | 61, 77 |
| | Vancomycin | PAP-AUC; MET; GRD Etest; broth microdilution (MIC cutoff, ≥2 μg/ml); standard vancomycin Etest (MIC cutoff, ≥2 μg/ml); method comparison with PAP-AUC as the standard | The most cost-effective strategy was broth microdilution as a stand-alone assay or in combination with PAP-AUC. GRD Etest remained an alternative, but a single cutoff value was used in all cases. | 74 |
| | Vancomycin | PAP-AUC; MET; GRD Etest; BHI agar plus 3 or 4 μg/ml vancomycin; method comparison with PAP-AUC as the standard | Both Etest screening methods have excellent negative predictive values, but positive results require confirmation. BHI screening agars with 3 and 4 μg/ml vancomycin provided precise identification of hVISA and VISA, respectively. | 75 |
| | Vancomycin | Broth microdilution; GRD Etest on 4,210 clinical isolates from 43 U.S. centers; PAP-AUC for GRD-positive isolates | Low reproducibility between test methods. The overall prevalence of hVISA was low (0.3%). | 102 |
| | Vancomycin | Broth microdilution; MET; standard Etest on 220 clinical isolates (121 MSSA, 99 MRSA) from bloodstream infections; PAP-AUC; BHI agar plus 4 μg/ml vancomycin | MET identified 5.5% of isolates as hVISA isolates, with a higher percentage among MRSA isolates (9.1%) than among MSSA isolates (2.5%). | 84 |
| | 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% of strains as hVISA strains from 19 hospitals. hVISA was present in Japanese hospitals before clinical introduction of vancomycin. | 138 |
| | Vancomycin | Etest; PAP-AUC on 288 MRSA isolates from a Connecticut veterans hospital | A low prevalence of hVISA argues against routine screening. | 80 |
| | Vancomycin | PAP on 268 MRSA isolates from Seoul, Republic of Korea | A total of 37.7% of isolates were identified as hVISA. However, overall mortalities were similar in hVISA- and VSSA-infected patients. | 63 |
| | Vancomycin | GRD Etest; PAP-AUC on 43 MRSA isolates from Malaysia | Two isolates were hVISA. | 81 |
| MRSA | Glycopeptides, daptomycin | Etest | *In vivo* development to heteroresistance. | 98 |
| *S. aureus* | Daptomycin | PAP by CFU counts (narrow increments) | Would still be heterogeneous if 2-fold increments were used. | 54 |
| | Daptomycin | PAP | PAP demonstrated daptomycin heteroresistance among tested hVISA and VISA strains. | 162 |
| Toxigenic *Clostridium difficile* | Metronidazole | Etest and disc diffusion assay (appearance of colonies in clear zone) | Prolonged exposure to metronidazole can select for resistance *in vitro*. Routine disc diffusion assays (5-μg metronidazole disc) with primary fresh *C. difficile* isolates were recommended. | 89 |
| *C. difficile* | Metronidazole | | Heteroresistance to metronidazole was detected in ~24% of 110 isolates. | 163 |
| *Staphylococcus* | Ciprofloxacin but not nalidixic acid | PAP and MIC determination | The MIC of ciprofloxacin for cells selected from plates with the highest concentration allowing growth was higher than that for the parental strains. | 29 |
| *S. pneumoniae* | Fosfomycin | PAP (wide scale of increments [>2-fold]) | Ten of 11 strains tested displayed heteroresistance. | 44 |
| *S. aureus* | Fusidic acid | PAP by CFU counts (narrow increments) | Cell populations had cells with different levels of resistance. More resistant subpopulations exhibited homogeneous resistance compared to their respective parental strains. | 45 |

[a] BHI, brain heart infusion; MH, Mueller-Hinton; GRD Etest, glycopeptide resistance detection Etest; MET, macro-Etest (referring to an Etest in which larger inoculum sizes increase the probability of detection of more resistant members of the bacterial population); hGISA, heterogeneous glycopeptide-intermediate *S. aureus*; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; VSSA, vancomycin-sensitive *S. aureus*; PAP-AUC, population analysis profiling-area under the curve method.

TABLE 2 Cases of heteroresistance in Gram-negative bacteria

| Organism(s) | Antibiotic(s) | Method(s) | Comments | Reference |
|---|---|---|---|---|
| Type b *H. influenzae* | Streptomycin | PAP by CFU counts (concentrations of <10–1,000 U/ml) | Most of the culture was inhibited at 10 U/ml. Few resistant cells survived at 10–100 U/ml, and fewer still at 1,000 U/ml. | 2 |
| *Enterobacter aerogenes*, *E. coli*, other enterobacteria | Cefamandole, cefoxitin, carbenicillin, nalidixic acid | PAP by CFU counts (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) | Coculture assays showed protection of sensitive cells by β-lactamases produced from resistant cells against β-lactamase-sensitive agents (cefamandole, but not cefotaxime, cefoxitin, or imipenem) | 43 |
| 8 species of *Enterobacteriaceae* | Cefotaxime | PAP (*E. coli* and *Proteus mirabilis* were homogeneous, *Klebsiella oxytoca* and *Citrobacter koseri* were less homogeneous, and *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, and *Morganella morganii* were heterogeneous) | More resistant subpopulations from the 4 heteroresistant species had very high increases in cephalothinase activity compared to parental strains. | 28 |
| *P. aeruginosa* and 7 strains from 5 genera of *Enterobacteriaceae* | Ciprofloxacin | PAP and MIC | The MIC of ciprofloxacin for 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 assay (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) | The study warns that using carbapenems may lead to selection of resistant subpopulations, subsequently causing dissemination of resistant strains, and to therapeutic failure. | 94 |
| | Colistin | PAP by CFU counts (narrow increments, but would still be heteroresistant if tested using 2-fold increments); time-kill curves (regrowth at a late time point, i.e., ~24 h, after rapid early killing indicates heteroresistance) | Subpopulations (<0.1% of $10^8$ to $10^9$ CFU/ml) grew in the presence of colistin at 3 to 10 μg/ml, while the MIC of entire populations ranged from 0.25 to 2 μg/ml. The study warns that the recommended dosing is suboptimal for heteroresistant strains. | 9 |
| *P. aeruginosa* | Imipenem, meropenem | Disc diffusion assay (colonies in inhibition zone); PAP by CFU counts (narrow increments and low initial inoculum) | The presence of subpopulations growing at high antibiotic concentrations, at frequencies of $6.9 \times 10^{-5}$ to $1.1 \times 10^{-7}$, suggests that these cells might not be detected by standard agar dilution MIC assay. | 55 |
| Invasive nontypeable *H. influenzae* | Imipenem | PAP by CFU counts, using 2-fold increments, and Etest to determine MIC | | 35 |
| *Enterobacter cloacae* and *A. baumannii* | Colistin | Disk diffusion; Etest; agar dilution; broth microdilution | Iso-Sensitest agar was better than Mueller-Hinton agar for detection of heteroresistance. | 11 |
| *A. baumannii-calcoaceticus* complex | Colistin | PAP by CFU counts, using 2-fold increments | Heteroresistance was defined by growth of colonies on plates containing 8 μg/ml of colistin, while the MIC was 8 μg/ml by broth microdilution. | 36 |
| *P. aeruginosa* | Carbapenems | Agar dilution according to CLSI method, using increments of 2 μg/ml for concentrations ranging from 2 to 32 μg/ml and of 8 μg/ml for concentrations of 32 to 64 μg/ml | Mutant subpopulations had at least 4-fold higher MICs 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 of incubation of test plates may not be enough to screen for heteroresistance. | 99 |
| | Carbapenems | Disc diffusion assay; Etest (colonies in clear zone of inhibition) | *In vivo* evolution of an antimicrobial profile from susceptibility to full resistance to carbapenems was observed, with heteroresistance as an intermediate stage. | 90 |
| *E. aerogenes* | Carbapenems | Etest | Automated MicroScan WalkAway system failed to detect heteroresistance detected by Etest. | 164 |
| *A. baumannii* | Meropenem | PAP by CFU counts, using 2-fold increments | The study suggests that *A. baumannii* isolates that are apparently meropenem susceptible by standard susceptibility testing may contain resistant subpopulations that can be selected for by suboptimal therapeutic drug dosages. | 39 |
| *K. pneumoniae* | Meropenem | MIC determination and PAP (2-fold increments); time-kill assays | Regrowth of heteroresistant strains after initial killing phase. | 40 |
| | Carbapenems | Etest (colonies in inhibition zone); PAP | Low reproducibility of MIC led to investigation of heteroresistance. | 101 |
| *A. baumannii* | Imipenem | Etest; disc diffusion assay (colonies in 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 for exposure versus 5.3 ± 4.8 days for controls). | 91 |

| | | | | |
|---|---|---|---|---|
| Carbapenemase-producing *K. pneumoniae* | Colistin | PAP by CFU counts, using 2-fold increments, and MIC determination | | 41 |
| *A. baumannii* | Cefepime | PAP by CFU counts, using 2-fold increments; Etest; disc diffusion assay | PAP of an isolate showed 2 peaks of growth at different cefepime concentrations. | 18 |
| | Carbapenems | Disc diffusion assay (colonies in zone of inhibition) | Heteroresistance was referred to as phenotypic heterogeneous resistance. | 92 |
| *P. aeruginosa* | Polymyxin B | PAP by CFU counts (polymyxin B concentrations of 0 to 8 μg/ml) | Isolates presenting subpopulations that exhibited growth at polymyxin B concentrations of ≥2 μg/ml were considered heteroresistant. Isolates containing subpopulations that grew at polymyxin B concentrations of at least twice the original MIC but <2 μg/ml were considered heterogeneous. | 65 |
| *B. cenocepacia* | Polymyxin B, norfloxacin, rifampin, ceftazidime, gentamicin | Etest; PAP by CFU counts and turbidimetric PAP (2-fold increments) | Detailed comparison of population-wide responses to bacteriostatic versus 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 determination 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 differences in resistance in 5, 1, and 19 cases for levofloxacin, clarithromycin, and metronidazole, respectively. | 21 |
| *Providencia rettgeri* | Carbapenems | PAP by CFU counts | | 134 |
| *Bordetella pertussis* | Erythromycin | Disc diffusion assay and Etest | Heteroresistance was not detected except for after 7 days of incubation, when colonies appeared in the clear zone. Degradation of erythromycin from the disc upon long incubation was ruled out. | 88 |

common among *S. aureus* strains (47, 50, 61, 63, 70, 79, 95). Others reported that heteroresistance to vancomycin is 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 because different criteria to define heteroresistance were adopted and improper methods to detect heterogeneity were mostly used (see Measuring Heteroresistance, above).

Fewer reports have 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 preexposure to the antibiotic but may also be acquired or induced after initial exposure to antibiotics. For example, repeated exposure of homogeneously sensitive staphylococci to methicillin resulted in mixed populations resembling 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 a 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 stepwise 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 MICs 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 responses to methicillin in the highly homogeneous MRSA COL strain (31). The heterogeneous resistance phenotype in this case was decreased methicillin resistance in subsets of the population, as increasing glycine concentrations in the medium resulted in replacement of the D-alanyl-D-alanine peptidoglycan muropeptides with D-alanyl-glycine muropeptides.

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

## MECHANISMS OF HETERORESISTANCE

Nongenetic individuality in bacterial populations has been observed in differentiation and cell division (116), chemotaxis (117), enzymatic activity (118), sporulation (119), stress responses, and antibiotic resistance (120–122). These variations can be attributed to genetic, epigenetic, and nongenetic mechanisms. Genetic mechanisms explain many cases of variation across a bacterial population, since increased resistance may be due to mutations or gene duplications of key resistance genes or regulatory systems. Long-term infection may result in instability of bacterial 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* strains that were 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 nongenetic 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 responses to methicillin in *S. aureus* (31). These mechanisms are 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 versus 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), was another factor suggested to underlie heteroresistance in MRSA (127). Nevertheless, Sigma B contributed to methicillin resistance but not heteroresistance in *Staphylococcus 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 an increased mutation rate and expression of *mecA* and the 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 led to full expression of homogeneous oxacillin 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 for their increased susceptibility. A single nucleotide polymorphism in the *dacA* (diadenylate cyclase) gene, which synthesizes the second messenger cyclic di-AMP (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, Eagle-type heteroresistance was explained based on reduced repression of *mecA* transcription and penicillin-binding protein 2′ production at a 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-level methicillin resistance (16). In *Streptococcus pneumoniae*, the penicillin-binding protein PBP2x, but not PBP2b or PBP1a, from a heteroresistant strain conferred heteroresistance in a homogeneous 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 cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, and *Morganella morganii* (28). The New Delhi β-lactamase (NDM-1) conferred heteroresistance in *Providencia rettgeri* (134). Similarly, elevated expression of the β-lactamase gene in resistant subpopulations compared to native populations was detected in *Klebsiella pneumoniae* isolates heteroresistant to meropenem (40) and in imipenem-heteroresistant *A. baumannii* (91). However, certain carbapenem-heteroresistant *A. baumannii* isolates were carbapenemase negative, suggesting that other factors are involved in the phenomenon (90). Differences in transcriptional levels may also underlie heteroresistance to carbapenems in *P. aeruginosa*; compared to the native populations, the resistant subpopulations had significantly increased transcription levels of the *mexB* and *mexY* genes, whose protein products are involved in multidrug efflux, and decreased expression of the *oprD* gene, encoding an outer membrane porin (57). Slower growth of β-lactam-resistant subpopulations of *A. baumannii* may protect against antibiotic challenge (18). In *Enterobacter* species, mutation of *ampD*, which is involved in the regulation of production of a class C β-lactamase, at rates as high as $10^{-4}$ to $10^{-6}$, resulted in a heterogeneous population of bacterial cells with differing levels of β-lactam resistance (135). Heteroresistance of invasive nontypeable *H. influenzae* to imipenem depended in part on the penicillin-binding protein PBP3, encoded by *ftsI*, on PBP4, encoded by *dacB*, or on the AcrAB efflux system, with a potential role of regulatory networks in the control of the heterogeneous expression of the resistance phenotype (35). In *B. cenocepacia*, an ornithine decarboxylase homologue and YceI, a small conserved protein, played a role in heteroresistance to ceftazidime (6).

A model of heteroresistance was constructed by introducing into a sensitive *Escherichia coli* strain the $bla_{\text{CTX-M-14}}$ gene, encoding a cephalosporin hydrolase, on a plasmid carrying the green fluorescent protein gene. This permitted monitoring of heteroresistant bacteria, since a subset of the cells expressed more hydrolase and hence exhibited a higher level of resistance to ceftriaxone (136). Heteroresistance was followed on a single-cell level owing to the fusion with 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 linked directly to a particular mechanism. Some studies reported an increased incidence of mutations of regulatory genes in the heteroresistant populations. For example, *agr* was dysfunctional in 58% of hVISA strains but only 21% of MRSA strains (84); hence, *agr* dysfunction seems to be advantageous to *S. aureus* clinical isolates for the development of vancomycin heteroresistance (49, 137). Similarly, compared to vancomycin-susceptible MRSA, 13 of 38 (34%) hVISA isolates possessed at least 1 nonsynonymous mutation: 6 in *vraSR*, 7 in *walRK*, and 2 in *rpoB* (138).

Several mutations increase resistance to glycopeptides, but whether these are involved in population-wide variations in resistance has 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 the resistance of hVISA to that of VISA, suggesting that 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 a 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* overexpression 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 a 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 enterococcal strains (15, 97). Subpopulations of *Enterococcus faecalis* with different surface charges, expressed as bimodal zeta potential distributions, were reported (144), and this phenotype may lead to heteroresistance similar to that of staphylococci.

### Heteroresistance to Antimicrobial Peptides

The mechanism of colistin heteroresistance in *A. baumannii* was attributed to a loss of lipopolysaccharide production in subpopulations displaying high-level 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 the lipid A biosynthesis genes *lpxA* and *lpxC* (146). In contrast, heteroresistance to polymyxin B in *B. cenocepacia* depends on differences in the levels of secretion of putrescine and YceI, which are 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

The heterogeneity of *Bartonella* sp. susceptibility 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 (positions 87, 91, and 143) were found in GyrA of levofloxacin-resistant strains, and an A406G amino acid substitution in GyrB was found only once (21). Putrescine and, to a lesser extent, YceI contributed to heteroresistance of *B. cenocepacia* to norfloxacin, as mutants unable to produce either of them showed a 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 such heteroresistance is potentially multifactorial (44).

### Heteroresistance to Rifampin

The small protein YceI and, to a lesser extent, putrescine, produced by the antibiotic-responsive ornithine decarboxylase, are involved in heteroresistance of *B. cenocepacia* to rifampin (6). Deletion of the genes encoding these proteins individually showed less heterogeneous phenotypes than that of the wild-type strain.

## CLINICAL SIGNIFICANCE OF HETERORESISTANCE

While some reports question the clinical significance of heteroresistance (51, 63, 76, 148), others argue that there is a 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 homogeneous 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 susceptibility to antibiotics (86). Heteroresistance has been 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 may have been 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 will select for the more resistant subpopulations. This is particularly the case when the majority of the population is sensitive to antibiotics and 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 will 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* strain resulted in selection of colistin-resistant derivatives (149). More-

over, *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 may 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 may 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 infections (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 protected the surfaces of bacteria from the initial binding of polymyxin B (6) and reduced antibiotic-induced oxidative stress (155), while YceI could bind and sequester polymyxin B, thus potentially reducing its levels in the bacterial milieu (6).

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

## CONCLUSIONS AND RECOMMENDATIONS

Despite being recognized since 1947, heteroresistance is often used indiscriminately to describe observations unrelated to population-wide responses to antibiotics. The lack of standard test formats and global guidelines for determining heteroresistance contributes to disagreements between outcomes of different methods and diverse results from different laboratories (69, 74, 75). Since heteroresistance may have serious implications in antimicrobial therapy, a standard operational definition and methods to assess its clinical importance are essential.

We recommend defining heteroresistance as a population-wide variation of antibiotic resistance, where different subpopulations within an isolate exhibit various 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 >8-fold higher than the highest noninhibitory concentration. An 8-fold difference may be regarded as intermediate heteroresistance, while a smaller difference denotes a homogeneous response to the antibiotic. In homogeneous cultures, the entire population is usually inhibited over a narrow increment of antibiotic in a 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 noninhibitory concentration. This general observation of a homogeneous response to antibiotics was documented for PAP assays in our recent study (6). Since 2-fold fluctuations in antibiotic sensitivity may normally occur, a further 2-fold increase in the transition from no inhibition to full inhibition relative to the homogeneous response was considered to indicate 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 the MIC value (which cannot detect the more resistant minority), as opposed to the case for homogeneous bacteria, where it was 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, may be an alternative to PAP. The discrete colonies represent subpopulations growing at concentrations that are inhibitory to the rest of the bacterial population, suggesting a population-wide variation in resistance. Antibiotic diffusion methods may therefore speed up screening of clinical isolates, but they 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 readout and existing standard assays for detection of population-wide vari-

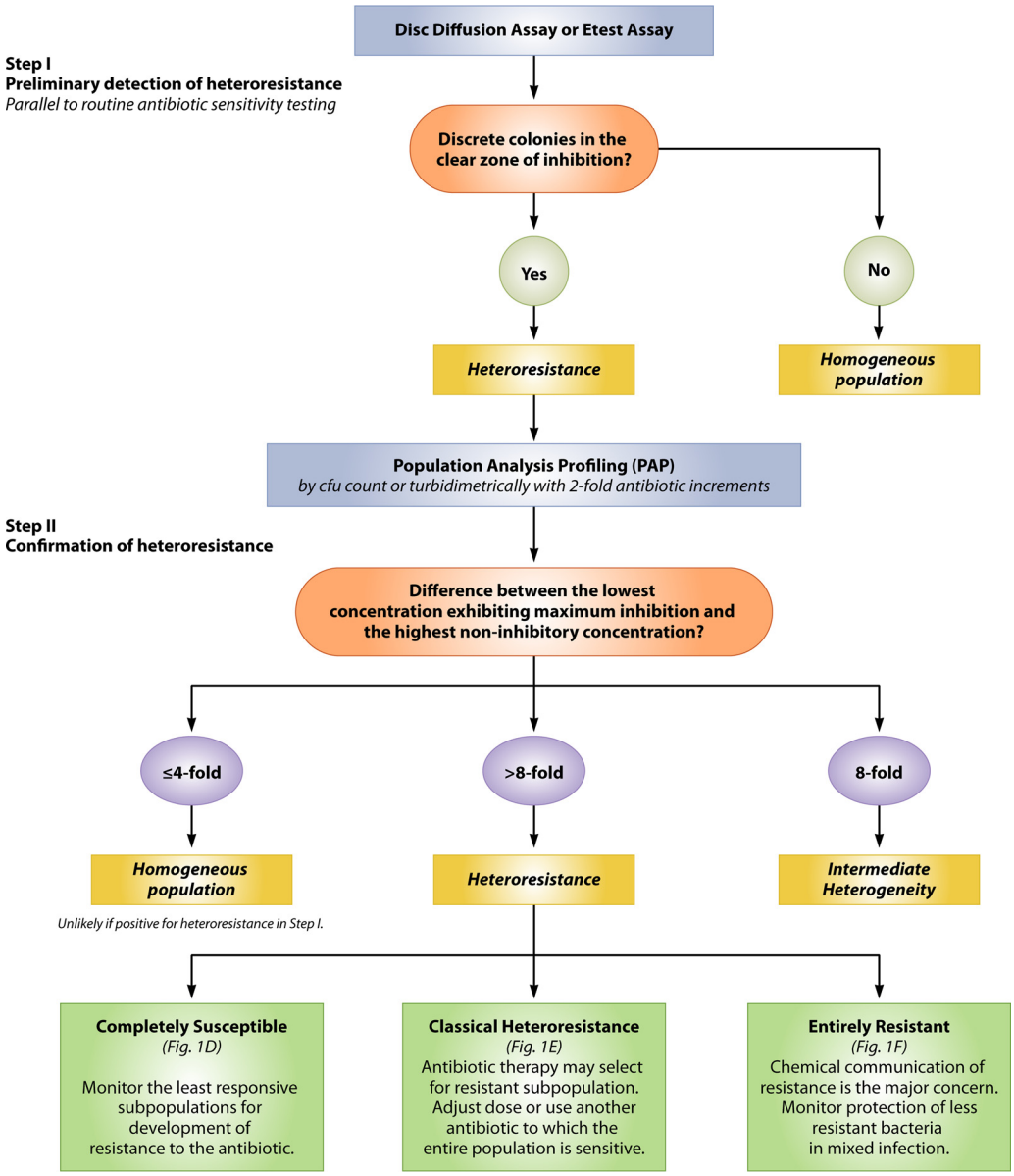


**FIG 2** Recommended scheme for determination of heteroresistance and interpretation criteria. Disc diffusion assays should be performed according to standardized procedures for antimicrobial susceptibility testing as recommended by agencies such as CLSI and BSAC. These procedures may be applied to Etest assays while taking into consideration the manufacturer's 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 for MIC determination by agar dilution assays. Turbidimetric PAP should follow standard guidelines for MIC determination by the broth dilution technique, with the exception of turbidimetric quantification of bacterial growth at each antibiotic concentration.

ation in antibiotic resistance (Fig. 2). Having worldwide standard criteria to define and assess heteroresistance will facilitate assessing its prevalence, clinical relevance, and impact on health care. 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 to elucidate its nature by determining whether common mechanisms exist among different bacteria and against different antibiotic classes and by finding new targets for its disruption.

We urge global organizations concerned with antimicrobial resistance to advocate for harmonized recommendations and to coordinate a general consensus concerning heteroresistance. We believe that this is of utmost importance, especially in clinical practice, where thousands of clinical isolates are currently screened for heteroresistance, but with nonstandardized methods that differ among laboratories, precluding a global picture of this problem. We anticipate that accurate and standardized detection of heteroresistance will translate into superior therapeutic outcomes based on improved identification of heteroresistant bacteria and optimized strategies to eradicate them.

## ACKNOWLEDGMENTS

Our research is funded by grants from Cystic Fibrosis Canada and The Cystic Fibrosis Trust and by a Marie Curie Career Integration Grant (project 618095). O.M.E. was supported by an Ontario Graduate Scholarship. M.A.V. acknowledges participation in COST action BM1003 ("Microbial Cell Surface Determinants of Virulence as Targets for New Therapeutics in Cystic Fibrosis").

We thank Patrick Lane for graphical enhancement of Fig. 1 and 2.

## REFERENCES

1. **CDC.** 2013. Antibiotic resistance threats in the United States, 2013. US Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, GA.
2. **Alexander HE, Leidy G.** 1947. Mode of action of streptomycin on type b *Haemophilus influenzae*. II. Nature of resistant variants. J Exp Med **85:**607–621.
3. **Sutherland R, Rolinson GN.** 1964. Characteristics of methicillin-resistant staphylococci. J Bacteriol **87:**887–899.
4. **Kayser FH, Benner EJ, Hoeprich PD.** 1970. Acquired and native resistance of *Staphylococcus aureus* to cephalexin and other beta-lactam antibiotics. Appl Microbiol **20:**1–5.
5. **Wright GD, Sutherland AD.** 2007. New strategies for combating multidrug-resistant bacteria. Trends Mol Med **13:**260–267. http://dx.doi.org/10.1016/j.molmed.2007.04.004.
6. **El-Halfawy OM, Valvano MA.** 2013. Chemical communication of antibiotic resistance by a highly resistant subpopulation of bacterial cells. PLoS One **8:**e68874. http://dx.doi.org/10.1371/journal.pone.0068874.
7. **Markova N, Haydoushka I, Michailova L, Ivanova R, Valcheva V, Jourdanova M, Popova T, Radoucheva T.** 2008. Cell wall deficiency and its effect on methicillin heteroresistance in *Staphylococcus aureus*. Int J Antimicrob Agents **31:**255–260. http://dx.doi.org/10.1016/j.ijantimicag.2007.09.015.
8. **Ryffel C, Strassle A, Kayser FH, Berger-Bachi B.** 1994. Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **38:**724–728. http://dx.doi.org/10.1128/AAC.38.4.724.
9. **Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L.** 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother **50:**2946–2950. http://dx.doi.org/10.1128/AAC.00103-06.
10. **Falagas ME, Makris GC, Dimopoulos G, Matthaiou DK.** 2008. Heteroresistance: a concern of increasing clinical significance? Clin Microbiol Infect **14:**101–104. http://dx.doi.org/10.1111/j.1469-0691.2007.01912.x.
11. **Lo-Ten-Foe JR, de Smet AM, Diederen BM, Kluytmans JA, van Keulen PH.** 2007. Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. Antimicrob Agents Chemother **51:**3726–3730. http://dx.doi.org/10.1128/AAC.01406-06.
12. **Nakipoglu Y, Derbentli S, Cagatay AA, Katranci H.** 2005. Investigation of Staphylococcus strains with heterogeneous resistance to glycopeptides in a Turkish university hospital. BMC Infect Dis **5:**31. http://dx.doi.org/10.1186/1471-2334-5-31.
13. **Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–1673. http://dx.doi.org/10.1016/S0140-6736(97)07324-8.
14. **Hallander HO, Laurell G.** 1972. Identification of cephalosporin-resistant *Staphylococcus aureus* with the disc diffusion method. Antimicrob Agents Chemother **1:**422–426. http://dx.doi.org/10.1128/AAC.1.5.422.
15. **Khan SA, Sung K, Layton S, Nawaz MS.** 2008. Heteroresistance to vancomycin and novel point mutations in Tn1546 of *Enterococcus faecium* ATCC 51559. Int J Antimicrob Agents **31:**27–36. http://dx.doi.org/10.1016/j.ijantimicag.2007.08.007.
16. **Kondo N, Kuwahara-Arai K, Kuroda-Murakami H, Tateda-Suzuki E, Hiramatsu K.** 2001. Eagle-type methicillin resistance: new phenotype of high methicillin resistance under *mec* regulator gene control. Antimicrob Agents Chemother **45:**815–824. http://dx.doi.org/10.1128/AAC.45.3.815-824.2001.
17. **Eagle H, Musselman AD.** 1948. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J Exp Med **88:**99–131. http://dx.doi.org/10.1084/jem.88.1.99.
18. **Hung KH, Wang MC, Huang AH, Yan JJ, Wu JJ.** 2012. Heteroresistance to cephalosporins and penicillins in *Acinetobacter baumannii*. J Clin Microbiol **50:**721–726. http://dx.doi.org/10.1128/JCM.05085-11.
19. **Hartman BJ, Tomasz A.** 1986. Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. Antimicrob Agents Chemother **29:**85–92. http://dx.doi.org/10.1128/AAC.29.1.85.
20. **Matteo MJ, Granados G, Olmos M, Wonaga A, Catalano M.** 2008. *Helicobacter pylori* amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. J Antimicrob Chemother **61:**474–477. http://dx.doi.org/10.1093/jac/dkm504.
21. **Kao CY, Lee AY, Huang AH, Song PY, Yang YJ, Sheu SM, Chang WL, Sheu BS, Wu JJ.** 2014. Heteroresistance of *Helicobacter pylori* from the same patient prior to antibiotic treatment. Infect Genet Evol **23:**196–202. http://dx.doi.org/10.1016/j.meegid.2014.02.009.
22. **Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova GT, Murmusaeva G, van Soolingen D, Hoffmann H.** 2009. Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan. Eur Respir J **33:**368–374. http://dx.doi.org/10.1183/09031936.00089808.
23. **Folkvardsen DB, Thomsen VO, Rigouts L, Rasmussen EM, Bang D, Bernaerts G, Werngren J, Toro JC, Hoffner S, Hillemann D, Svensson E.** 2013. Rifampin heteroresistance in *Mycobacterium tuberculosis* cultures as detected by phenotypic and genotypic drug susceptibility test methods. J Clin Microbiol **51:**4220–4222. http://dx.doi.org/10.1128/JCM.01602-13.
24. **Eilertson B, Maruri F, Blackman A, Herrera M, Samuels DC, Sterling TR.** 2014. High proportion of heteroresistance in *gyrA* and *gyrB* in fluoroquinolone resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother. **58:**3270–3275. http://dx.doi.org/10.1128/AAC.02066-13.
25. **Ott JL, Turner JR, Mahoney DF.** 1979. Lack of correlation between beta-lactamase production and susceptibility to cefamandole or cefoxitin among spontaneous mutants of Enterobacteriaceae. Antimicrob Agents Chemother **15:**14–19. http://dx.doi.org/10.1128/AAC.15.1.14.
26. **Hamilton-Miller JM, Iliffe A.** 1985. Antimicrobial resistance in coagulase-negative staphylococci. J Med Microbiol **19:**217–226. http://dx.doi.org/10.1099/00222615-19-2-217.
27. **Chambers HF, Hartman BJ, Tomasz A.** 1985. Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. J Clin Invest **76:**325–331. http://dx.doi.org/10.1172/JCI111965.
28. **Søgaard P.** 1985. Population analysis of susceptibility to cefotaxime in Enterobacteriaceae. Acta Pathol Microbiol Immunol Scand B **93:**365–369.
29. **Søgaard P, Gahrn-Hansen B.** 1986. Population analysis of susceptibility to ciprofloxacin and nalidixic acid in Staphylococcus, Pseudomonas aeruginosa, and Enterobacteriaceae. Acta Pathol Microbiol Immunol Scand B **94:**351–356.
30. **de Lencastre H, Figueiredo AM, Tomasz A.** 1993. Genetic control of population structure in heterogeneous strains of methicillin resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis **12**(Suppl 1)**:**S13–S18. http://dx.doi.org/10.1007/BF02389872.
31. **de Jonge BL, Chang YS, Xu N, Gage D.** 1996. Effect of exogenous glycine on peptidoglycan composition and resistance in a methicillin-resistant *Staphylococcus aureus* strain. Antimicrob Agents Chemother **40:**1498–1503.
32. **Stranden AM, Roos M, Berger-Bachi B.** 1996. Glutamine synthetase and heteroresistance in methicillin-resistant *Staphylococcus aureus*. Microb Drug Resist **2:**201–207. http://dx.doi.org/10.1089/mdr.1996.2.201.
33. **Tassios PT, Vatopoulos AC, Xanthaki A, Mainas E, Goering RV, Legakis NJ.** 1997. Distinct genotypic clusters of heterogeneously and homogeneously methicillin-resistant *Staphylococcus aureus* from a Greek hospital. Eur J Clin Microbiol Infect Dis **16:**170–173. http://dx.doi.org/10.1007/BF01709481.
34. **Yoshida R, Kuwahara-Arai K, Baba T, Cui L, Richardson JF, Hiramatsu K.** 2003. Physiological and molecular analysis of a *mecA*-negative *Staphylococcus aureus* clinical strain that expresses heterogeneous methicillin resistance. J Antimicrob Chemother **51:**247–255. http://dx.doi.org/10.1093/jac/dkg036.

35. **Cerquetti M, Giufre M, Cardines R, Mastrantonio P.** 2007. First characterization of heterogeneous resistance to imipenem in invasive nontypeable *Haemophilus influenzae* isolates. Antimicrob Agents Chemother **51:**3155–3161. http://dx.doi.org/10.1128/AAC.00335-07.
36. **Hawley JS, Murray CK, Jorgensen JH.** 2008. Colistin heteroresistance in Acinetobacter and its association with previous colistin therapy. Antimicrob Agents Chemother **52:**351–352. http://dx.doi.org/10.1128/AAC.00766-07.
37. **Kishii K, Ito T, Watanabe S, Okuzumi K, Hiramatsu K.** 2008. Recurrence of heterogeneous methicillin-resistant *Staphylococcus aureus* (MRSA) among the MRSA clinical isolates in a Japanese university hospital. J Antimicrob Chemother **62:**324–328. http://dx.doi.org/10.1093/jac/dkn186.
38. **Katayama Y, Murakami-Kuroda H, Cui L, Hiramatsu K.** 2009. Selection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* by imipenem. Antimicrob Agents Chemother **53:**3190–3196. http://dx.doi.org/10.1128/AAC.00834-08.
39. **Ikonomidis A, Neou E, Gogou V, Vrioni G, Tsakris A, Pournaras S.** 2009. Heteroresistance to meropenem in carbapenem-susceptible *Acinetobacter baumannii*. J Clin Microbiol **47:**4055–4059. http://dx.doi.org/10.1128/JCM.00959-09.
40. **Pournaras S, Kristo I, Vrioni G, Ikonomidis A, Poulou A, Petropoulou D, Tsakris A.** 2010. Characteristics of meropenem heteroresistance in *Klebsiella pneumoniae* carbapenemase (KPC)-producing clinical isolates of *K. pneumoniae*. J Clin Microbiol **48:**2601–2604. http://dx.doi.org/10.1128/JCM.02134-09.
41. **Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D.** 2011. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. J Antimicrob Chemother **66:**946–947. http://dx.doi.org/10.1093/jac/dkr007.
42. **Pfeltz RF, Schmidt JL, Wilkinson BJ.** 2001. A microdilution plating method for population analysis of antibiotic-resistant staphylococci. Microb Drug Resist **7:**289–295. http://dx.doi.org/10.1089/10766290152652846.
43. **Baquero F, Vicente MF, Perez-Diaz JC.** 1985. Beta-lactam coselection of sensitive and TEM-1 beta-lactamase-producing subpopulations in heterogeneous *Escherichia coli* colonies. J Antimicrob Chemother **15:**151–157. http://dx.doi.org/10.1093/jac/15.2.151.
44. **Engel H, Gutierrez-Fernandez J, Fluckiger C, Martinez-Ripoll M, Muhlemann K, Hermoso JA, Hilty M, Hathaway LJ.** 2013. Heteroresistance to fosfomycin is predominant in *Streptococcus pneumoniae* and depends on the *murA1* gene. Antimicrob Agents Chemother **57:**2801–2808. http://dx.doi.org/10.1128/AAC.00223-13.
45. **O'Brien FG, Botterill CI, Endersby TG, Lim RL, Grubb WB, Gustafson JE.** 1998. Heterogeneous expression of fusidic acid resistance in *Staphylococcus aureus* with plasmid or chromosomally encoded fusidic acid resistance genes. Pathology **30:**299–303. http://dx.doi.org/10.1080/00313029800169486.
46. **Sieradzki K, Roberts RB, Serur D, Hargrave J, Tomasz A.** 1999. Heterogeneously vancomycin-resistant *Staphylococcus epidermidis* strain causing recurrent peritonitis in a dialysis patient during vancomycin therapy. J Clin Microbiol **37:**39–44.
47. **Marchese A, Balistreri G, Tonoli E, Debbia EA, Schito GC.** 2000. Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. J Clin Microbiol **38:**866–869.
48. **Bobin-Dubreux S, Reverdy ME, Nervi C, Rougier M, Bolmstrom A, Vandenesch F, Etienne J.** 2001. Clinical isolate of vancomycin-heterointermediate *Staphylococcus aureus* susceptible to methicillin and in vitro selection of a vancomycin-resistant derivative. Antimicrob Agents Chemother **45:**349–352. http://dx.doi.org/10.1128/AAC.45.1.349-352.2001.
49. **Sakoulas G, Eliopoulos GM, Moellering RC, Jr, Wennersten C, Venkataraman L, Novick RP, Gold HS.** 2002. Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob Agents Chemother **46:**1492–1502. http://dx.doi.org/10.1128/AAC.46.5.1492-1502.2002.
50. **Bert F, Clarissou J, Durand F, Delefosse D, Chauvet C, Lefebvre P, Lambert N, Branger C.** 2003. Prevalence, molecular epidemiology, and clinical significance of heterogeneous glycopeptide-intermediate *Staphylococcus aureus* in liver transplant recipients. J Clin Microbiol **41:**5147–5152. http://dx.doi.org/10.1128/JCM.41.11.5147-5152.2003.
51. **Khosrovaneh A, Riederer K, Saeed S, Tabriz MS, Shah AR, Hanna MM, Sharma M, Johnson LB, Fakih MG, Khatib R.** 2004. Frequency of reduced vancomycin susceptibility and heterogeneous subpopulation in persistent or recurrent methicillin-resistant *Staphylococcus aureus* bacteremia. Clin Infect Dis **38:**1328–1330. http://dx.doi.org/10.1086/383036.
52. **Rybak MJ, Cha R, Cheung CM, Meka VG, Kaatz GW.** 2005. Clinical isolates of *Staphylococcus aureus* from 1987 and 1989 demonstrating heterogeneous resistance to vancomycin and teicoplanin. Diagn Microbiol Infect Dis **51:**119–125. http://dx.doi.org/10.1016/j.diagmicrobio.2004.09.005.
53. **Nunes AP, Teixeira LM, Iorio NL, Bastos CC, de Sousa Fonseca L, Souto-Padron T, dos Santos KR.** 2006. Heterogeneous resistance to vancomycin in *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri* clinical strains: characterisation of glycopeptide susceptibility profiles and cell wall thickening. Int J Antimicrob Agents **27:**307–315. http://dx.doi.org/10.1016/j.ijantimicag.2005.11.013.
54. **Sakoulas G, Alder J, Thauvin-Eliopoulos C, Moellering RC, Jr, Eliopoulos GM.** 2006. Induction of daptomycin heterogeneous susceptibility in *Staphylococcus aureus* by exposure to vancomycin. Antimicrob Agents Chemother **50:**1581–1585. http://dx.doi.org/10.1128/AAC.50.4.1581-1585.2006.
55. **Pournaras S, Ikonomidis A, Markogiannakis A, Spanakis N, Maniatis AN, Tsakris A.** 2007. Characterization of clinical isolates of *Pseudomonas aeruginosa* heterogeneously resistant to carbapenems. J Med Microbiol **56:**66–70. http://dx.doi.org/10.1099/jmm.0.46816-0.
56. **Nunes AP, Schuenck RP, Bastos CC, Magnanini MM, Long JB, Iorio NL, Santos KR.** 2007. Heterogeneous resistance to vancomycin and teicoplanin among Staphylococcus spp. isolated from bacteremia. Braz J Infect Dis **11:**345–350. http://dx.doi.org/10.1590/S1413-86702007000300009.
57. **Ikonomidis A, Tsakris A, Kantzanou M, Spanakis N, Maniatis AN, Pournaras S.** 2008. Efflux system overexpression and decreased OprD contribute to the carbapenem heterogeneity in *Pseudomonas aeruginosa*. FEMS Microbiol Lett **279:**36–39. http://dx.doi.org/10.1111/j.1574-6968.2007.00997.x.
58. **Sader HS, Jones RN, Rossi KL, Rybak MJ.** 2009. Occurrence of vancomycin-tolerant and heterogeneous vancomycin-intermediate strains (hVISA) among *Staphylococcus aureus* causing bloodstream infections in nine USA hospitals. J Antimicrob Chemother **64:**1024–1028. http://dx.doi.org/10.1093/jac/dkp319.
59. **Hegde SS, Skinner R, Lewis SR, Krause KM, Blais J, Benton BM.** 2010. Activity of telavancin against heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) *in vitro* and in an *in vivo* mouse model of bacteraemia. J Antimicrob Chemother **65:**725–728. http://dx.doi.org/10.1093/jac/dkq028.
60. **Cafiso V, Bertuccio T, Spina D, Campanile F, Bongiorno D, Santagati M, Sciacca A, Sciuto C, Stefani S.** 2010. Methicillin resistance and vancomycin heteroresistance in *Staphylococcus aureus* in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis **29:**1277–1285. http://dx.doi.org/10.1007/s10096-010-1000-5.
61. **Campanile F, Borbone S, Perez M, Bongiorno D, Cafiso V, Bertuccio T, Purrello S, Nicolosi D, Scuderi C, Stefani S.** 2010. Heteroresistance to glycopeptides in Italian meticillin-resistant *Staphylococcus aureus* (MRSA) isolates. Int J Antimicrob Agents **36:**415–419. http://dx.doi.org/10.1016/j.ijantimicag.2010.06.044.
62. **Matsuo M, Hishinuma T, Katayama Y, Cui L, Kapi M, Hiramatsu K.** 2011. Mutation of RNA polymerase beta subunit (*rpoB*) promotes hVISA-to-VISA phenotypic conversion of strain Mu3. Antimicrob Agents Chemother **55:**4188–4195. http://dx.doi.org/10.1128/AAC.00398-11.
63. **Park KH, Kim ES, Kim HS, Park SJ, Bang KM, Park HJ, Park SY, Moon SM, Chong YP, Kim SH, Lee SO, Choi SH, Jeong JY, Kim MN, Woo JH, Kim YS.** 2012. Comparison of the clinical features, bacterial genotypes and outcomes of patients with bacteraemia due to heteroresistant vancomycin-intermediate *Staphylococcus aureus* and vancomycin-susceptible *S. aureus*. J Antimicrob Chemother **67:**1843–1849. http://dx.doi.org/10.1093/jac/dks131.
64. **Lin SY, Chen TC, Chen FJ, Chen YH, Lin YI, Siu LK, Lu PL.** 2012. Molecular epidemiology and clinical characteristics of hetero-resistant vancomycin intermediate *Staphylococcus aureus* bacteremia in a Taiwan medical center. J Microbiol Immunol Infect **45:**435–441. http://dx.doi.org/10.1016/j.jmii.2012.05.004.
65. **Hermes DM, Pormann Pitt C, Lutz L, Teixeira AB, Ribeiro VB, Netto B, Martins AF, Zavascki AP, Barth AL.** 2013. Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -resistant *Pseudomonas aeruginosa*. J Med Microbiol **62:**1184–1189. http://dx.doi.org/10.1099/jmm.0.059220-0.
66. **Morand B, Muhlemann K.** 2007. Heteroresistance to penicillin in *Strep-*

*tococcus pneumoniae*. Proc Natl Acad Sci U S A **104:**14098–14103. http://dx.doi.org/10.1073/pnas.0702377104.
67. **Iyer RN, Hittinahalli V.** 2008. Modified PAP method to detect heteroresistance to vancomycin among methicillin resistant *Staphylococcus aureus* isolates at a tertiary care hospital. Indian J Med Microbiol **26:**176–179. http://dx.doi.org/10.4103/0255-0857.40537.
68. **D'Mello D, Daley AJ, Rahman MS, Qu Y, Garland S, Pearce C, Deighton MA.** 2008. Vancomycin heteroresistance in bloodstream isolates of *Staphylococcus capitis*. J Clin Microbiol **46:**3124–3126. http://dx.doi.org/10.1128/JCM.00592-08.
69. **Yusof A, Engelhardt A, Karlsson A, Bylund L, Vidh P, Mills K, Wootton M, Walsh TR.** 2008. Evaluation of a new Etest vancomycin-teicoplanin strip for detection of glycopeptide-intermediate *Staphylococcus aureus* (GISA), in particular, heterogeneous GISA. J Clin Microbiol **46:**3042–3047. http://dx.doi.org/10.1128/JCM.00265-08.
70. **Sun W, Chen H, Liu Y, Zhao C, Nichols WW, Chen M, Zhang J, Ma Y, Wang H.** 2009. Prevalence and characterization of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates from 14 cities in China. Antimicrob Agents Chemother **53:**3642–3649. http://dx.doi.org/10.1128/AAC.00206-09.
71. **Fusco DN, Alexander EL, Weisenberg SA, Mediavilla JR, Kreiswirth BN, Schuetz AN, Jenkins SG, Rhee KY.** 2009. Clinical failure of vancomycin in a dialysis patient with methicillin-susceptible vancomycin-heteroresistant *S. aureus*. Diagn Microbiol Infect Dis **65:**180–183. http://dx.doi.org/10.1016/j.diagmicrobio.2009.05.017.
72. **Adam HJ, Louie L, Watt C, Gravel D, Bryce E, Loeb M, Matlow A, McGeer A, Mulvey MR, Simor AE.** 2010. Detection and characterization of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates in Canada: results from the Canadian Nosocomial Infection Surveillance Program, 1995–2006. Antimicrob Agents Chemother **54:**945–949. http://dx.doi.org/10.1128/AAC.01316-09.
73. **Pitz AM, Yu F, Hermsen ED, Rupp ME, Fey PD, Olsen KM.** 2011. Vancomycin susceptibility trends and prevalence of heterogeneous vancomycin-intermediate *Staphylococcus aureus* in clinical methicillin-resistant *S. aureus* isolates. J Clin Microbiol **49:**269–274. http://dx.doi.org/10.1128/JCM.00914-10.
74. **van Hal SJ, Wehrhahn MC, Barbagiannakos T, Mercer J, Chen D, Paterson DL, Gosbell IB.** 2011. Performance of various testing methodologies for detection of heteroresistant vancomycin-intermediate *Staphylococcus aureus* in bloodstream isolates. J Clin Microbiol **49:**1489–1494. http://dx.doi.org/10.1128/JCM.02302-10.
75. **Riederer K, Shemes S, Chase P, Musta A, Mar A, Khatib R.** 2011. Detection of intermediately vancomycin-susceptible and heterogeneous *Staphylococcus aureus* isolates: comparison of Etest and agar screening methods. J Clin Microbiol **49:**2147–2150. http://dx.doi.org/10.1128/JCM.01435-10.
76. **Khatib R, Jose J, Musta A, Sharma M, Fakih MG, Johnson LB, Riederer K, Shemes S.** 2011. Relevance of vancomycin-intermediate susceptibility and heteroresistance in methicillin-resistant *Staphylococcus aureus* bacteraemia. J Antimicrob Chemother **66:**1594–1599. http://dx.doi.org/10.1093/jac/dkr169.
77. **Cheong JW, Harris P, Oman K, Norton R.** 2011. Challenges in the microbiological diagnosis and management of hVISA infections. Pathology **43:**357–361. http://dx.doi.org/10.1097/PAT.0b013e3283464ca3.
78. **van Hal SJ, Jones M, Gosbell IB, Paterson DL.** 2011. Vancomycin heteroresistance is associated with reduced mortality in ST239 methicillin-resistant *Staphylococcus aureus* blood stream infections. PLoS One **6:**e21217. http://dx.doi.org/10.1371/journal.pone.0021217.
79. **Chen H, Liu Y, Sun W, Chen M, Wang H.** 2011. The incidence of heterogeneous vancomycin-intermediate *Staphylococcus aureus* correlated with increase of vancomycin MIC. Diagn Microbiol Infect Dis **71:**301–303. http://dx.doi.org/10.1016/j.diagmicrobio.2011.06.010.
80. **Fink SL, Martinello RA, Campbell SM, Murray TS.** 2012. Low prevalence of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates among Connecticut veterans. Antimicrob Agents Chemother **56:**582–583. http://dx.doi.org/10.1128/AAC.05024-11.
81. **Norazah A, Law NL, Kamel AG, Salbiah N.** 2012. The presence of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hetero-VISA) in a major Malaysian hospital. Med J Malaysia **67:**269–273.
82. **Chesneau O, Morvan A, Solh NE.** 2000. Retrospective screening for heterogeneous vancomycin resistance in diverse *Staphylococcus aureus* clones disseminated in French hospitals. J Antimicrob Chemother **45:**887–890. http://dx.doi.org/10.1093/jac/45.6.887.
83. **Voss A, Mouton JW, van Elzakker EP, Hendrix RG, Goessens W, Kluytmans JA, Krabbe PF, de Neeling HJ, Sloos JH, Oztoprak N, Howe RA, Walsh TR.** 2007. A multi-center blinded study on the efficiency of phenotypic screening methods to detect glycopeptide intermediately susceptible *Staphylococcus aureus* (GISA) and heterogeneous GISA (h-GISA). Ann Clin Microbiol Antimicrob **6:**9. http://dx.doi.org/10.1186/1476-0711-6-9.
84. **Harigaya Y, Ngo D, Lesse AJ, Huang V, Tsuji BT.** 2011. Characterization of heterogeneous vancomycin-intermediate resistance, MIC and accessory gene regulator (*agr*) dysfunction among clinical bloodstream isolates of *Staphylococcus aureus*. BMC Infect Dis **11:**287. http://dx.doi.org/10.1186/1471-2334-11-287.
85. **Howe RA, Wootton M, Walsh TR, Bennett PM, MacGowan AP.** 1999. Expression and detection of hetero-vancomycin resistance in *Staphylococcus aureus*. J Antimicrob Chemother **44:**675–678. http://dx.doi.org/10.1093/jac/44.5.675.
86. **Weel JF, van der Hulst RW, Gerrits Y, Tytgat GN, van der Ende A, Dankert J.** 1996. Heterogeneity in susceptibility to metronidazole among *Helicobacter pylori* isolates from patients with gastritis or peptic ulcer disease. J Clin Microbiol **34:**2158–2162.
87. **Wong SS, Ho PL, Woo PC, Yuen KY.** 1999. Bacteremia caused by staphylococci with inducible vancomycin heteroresistance. Clin Infect Dis **29:**760–767. http://dx.doi.org/10.1086/520429.
88. **Wilson KE, Cassiday PK, Popovic T, Sanden GN.** 2002. *Bordetella pertussis* isolates with a heterogeneous phenotype for erythromycin resistance. J Clin Microbiol **40:**2942–2944. http://dx.doi.org/10.1128/JCM.40.8.2942-2944.2002.
89. **Pelaez T, Cercenado E, Alcala L, Marin M, Martin-Lopez A, Martinez-Alarcon J, Catalan P, Sanchez-Somolinos M, Bouza E.** 2008. Metronidazole resistance in *Clostridium difficile* is heterogeneous. J Clin Microbiol **46:**3028–3032. http://dx.doi.org/10.1128/JCM.00524-08.
90. **Superti SV, Martins DS, Caierao J, Soares FS, Prochnow T, Zavascki AP.** 2009. Indications of carbapenem resistance evolution through heteroresistance as an intermediate stage in *Acinetobacter baumannii* after carbapenem administration. Rev Inst Med Trop Sao Paulo **51:**111–113. http://dx.doi.org/10.1590/S0036-46652009000200010.
91. **Lee HY, Chen CL, Wang SB, Su LH, Chen SH, Liu SY, Wu TL, Lin TY, Chiu CH.** 2011. Imipenem heteroresistance induced by imipenem in multidrug-resistant *Acinetobacter baumannii*: mechanism and clinical implications. Int J Antimicrob Agents **37:**302–308. http://dx.doi.org/10.1016/j.ijantimicag.2010.12.015.
92. **Fernandez-Cuenca F, Gomez-Sanchez M, Rodriguez-Bano J, Martinez-Martinez L, Vila J, Bou G, Pascual A.** 2012. Epidemiological and clinical features associated with colonisation/infection by *Acinetobacter baumannii* with phenotypic heterogeneous resistance to carbapenems. Int J Antimicrob Agents **40:**235–238. http://dx.doi.org/10.1016/j.ijantimicag.2012.05.005.
93. **Alam MR, Donabedian S, Brown W, Gordon J, Chow JW, Zervos MJ, Hershberger E.** 2001. Heteroresistance to vancomycin in *Enterococcus faecium*. J Clin Microbiol **39:**3379–3381. http://dx.doi.org/10.1128/JCM.39.9.3379-3381.2001.
94. **Pournaras S, Ikonomidis A, Markogiannakis A, Maniatis AN, Tsakris A.** 2005. Heteroresistance to carbapenems in *Acinetobacter baumannii*. J Antimicrob Chemother **55:**1055–1056. http://dx.doi.org/10.1093/jac/dki115.
95. **Maor Y, Rahav G, Belausov N, Ben-David D, Smollan G, Keller N.** 2007. Prevalence and characteristics of heteroresistant vancomycin-intermediate *Staphylococcus aureus* bacteremia in a tertiary care center. J Clin Microbiol **45:**1511–1514. http://dx.doi.org/10.1128/JCM.01262-06.
96. **Angelakis E, Biswas S, Taylor C, Raoult D, Rolain JM.** 2008. Heterogeneity of susceptibility to fluoroquinolones in Bartonella isolates from Australia reveals a natural mutation in *gyrA*. J Antimicrob Chemother **61:**1252–1255. http://dx.doi.org/10.1093/jac/dkn094.
97. **Park IJ, Lee WG, Shin JH, Lee KW, Woo GJ.** 2008. VanB phenotype-*vanA* genotype *Enterococcus faecium* with heterogeneous expression of teicoplanin resistance. J Clin Microbiol **46:**3091–3093. http://dx.doi.org/10.1128/JCM.00712-08.
98. **Kirby A, Mohandas K, Broughton C, Neal TJ, Smith GW, Pai P, Nistal de Paz C.** 2009. *In vivo* development of heterogeneous glycopeptide-intermediate *Staphylococcus aureus* (hGISA), GISA and daptomycin resistance in a patient with meticillin-resistant *S aureus* endocarditis. J Med Microbiol **58:**376–380. http://dx.doi.org/10.1099/jmm.0.006486-0.
99. **Savini V, Catavitello C, Talia M, Febbo F, Balbinot A, Pompilio A, Di**

**Bonaventura G, Piccolomini R, D'Antonio D.** 2009. Misidentification of ampicillin-sulbactam heteroresistance in *Acinetobacter baumannii* strains from ICU patients. J Infect **58:**316–317. http://dx.doi.org/10.1016/j.jinf.2009.02.001.

100. **Musta AC, Riederer K, Shemes S, Chase P, Jose J, Johnson LB, Khatib R.** 2009. Vancomycin MIC plus heteroresistance and outcome of methicillin-resistant *Staphylococcus aureus* bacteremia: trends over 11 years. J Clin Microbiol **47:**1640–1644. http://dx.doi.org/10.1128/JCM.02135-08.
101. **Tato M, Morosini M, Garcia L, Alberti S, Coque MT, Canton R.** 2010. Carbapenem heteroresistance in VIM-1-producing *Klebsiella pneumoniae* isolates belonging to the same clone: consequences for routine susceptibility testing. J Clin Microbiol **48:**4089–4093. http://dx.doi.org/10.1128/JCM.01130-10.
102. **Richter SS, Satola SW, Crispell EK, Heilmann KP, Dohrn CL, Riahi F, Costello AJ, Diekema DJ, Doern GV.** 2011. Detection of *Staphylococcus aureus* isolates with heterogeneous intermediate-level resistance to vancomycin in the United States. J Clin Microbiol **49:**4203–4207. http://dx.doi.org/10.1128/JCM.01152-11.
103. **Liu Y, Li J, Du J, Hu M, Bai H, Qi J, Gao C, Wei T, Su H, Jin J, Gao P.** 2011. Accurate assessment of antibiotic susceptibility and screening resistant strains of a bacterial population by linear gradient plate. Sci China Life Sci **54:**953–960. http://dx.doi.org/10.1007/s11427-011-4230-6.
104. **Jarzembowski T, Wisniewska K, Jozwik A, Witkowski J.** 2009. Heterogeneity of methicillin-resistant *Staphylococcus aureus* strains (MRSA) characterized by flow cytometry. Curr Microbiol **59:**78–80. http://dx.doi.org/10.1007/s00284-009-9395-x.
105. **Landman D, Salamera J, Quale J.** 2013. Irreproducible and uninterpretable polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*. J Clin Microbiol **51:**4106–4111. http://dx.doi.org/10.1128/JCM.02129-13.
106. **Goldman JL, Harrison CJ, Myers AL, Jackson MA, Selvarangan R.** 2014. No evidence of vancomycin minimal inhibitory concentration creep or heteroresistance identified in pediatric *Staphylococcus aureus* blood isolates. Pediatr Infect Dis J **33:**216–218. http://dx.doi.org/10.1097/01.inf.0000436281.18687.0c.
107. **Alekshun MN, Levy SB.** 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell **128:**1037–1050. http://dx.doi.org/10.1016/j.cell.2007.03.004.
108. **Loutet SA, Di Lorenzo F, Clarke C, Molinaro A, Valvano MA.** 2011. Transcriptional responses of *Burkholderia cenocepacia* to polymyxin B in isogenic strains with diverse polymyxin B resistance phenotypes. BMC Genomics **12:**472. http://dx.doi.org/10.1186/1471-2164-12-472.
109. **Plipat N, Livni G, Bertram H, Thomson RB, Jr.** 2005. Unstable vancomycin heteroresistance is common among clinical isolates of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **43:**2494–2496. http://dx.doi.org/10.1128/JCM.43.5.2494-2496.2005.
110. **Jahn G, Laufs R, Kaulfers PM, Kolenda H.** 1979. Molecular nature of two *Haemophilus influenzae* R factors containing resistances and the multiple integration of drug resistance transposons. J Bacteriol **138:**584–597.
111. **Wu S, de Lencastre H, Sali A, Tomasz A.** 1996. A phosphoglucomutase-like gene essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*: molecular cloning and DNA sequencing. Microb Drug Resist **2:**277–286. http://dx.doi.org/10.1089/mdr.1996.2.277.
112. **Hayden MK, Picken RN, Sahm DF.** 1997. Heterogeneous expression of glycopeptide resistance in enterococci associated with transfer of *vanB*. Antimicrob Agents Chemother **41:**872–874.
113. **Hall-Stoodley L, Costerton JW, Stoodley P.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol **2:**95–108. http://dx.doi.org/10.1038/nrmicro821.
114. **Stewart PS, Franklin MJ.** 2008. Physiological heterogeneity in biofilms. Nat Rev Microbiol **6:**199–210. http://dx.doi.org/10.1038/nrmicro1838.
115. **Drenkard E, Ausubel FM.** 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature **416:**740–743. http://dx.doi.org/10.1038/416740a.
116. **Powell EO.** 1958. An outline of the pattern of bacterial generation times. J Gen Microbiol **18:**382–417. http://dx.doi.org/10.1099/00221287-18-2-382.
117. **Spudich JL, Koshland DE, Jr.** 1976. Non-genetic individuality: chance in the single cell. Nature **262:**467–471. http://dx.doi.org/10.1038/262467a0.
118. **Novick A, Weiner M.** 1957. Enzyme induction as an all-or-none phenomenon. Proc Natl Acad Sci U S A **43:**553–566. http://dx.doi.org/10.1073/pnas.43.7.553.
119. **Gonzalez-Pastor JE, Hobbs EC, Losick R.** 2003. Cannibalism by sporulating bacteria. Science **301:**510–513. http://dx.doi.org/10.1126/science.1086462.
120. **Adam M, Murali B, Glenn NO, Potter SS.** 2008. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. BMC Evol Biol **8:**52. http://dx.doi.org/10.1186/1471-2148-8-52.
121. **Gefen O, Balaban NQ.** 2009. The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol Rev **33:**704–717. http://dx.doi.org/10.1111/j.1574-6976.2008.00156.x.
122. **Pearl S, Gabay C, Kishony R, Oppenheim A, Balaban NQ.** 2008. Nongenetic individuality in the host-phage interaction. PLoS Biol **6:**e120. http://dx.doi.org/10.1371/journal.pbio.0060120.
123. **Chambers HF, Hackbarth CJ.** 1987. Effect of NaCl and nafcillin on penicillin-binding protein 2a and heterogeneous expression of methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother **31:**1982–1988. http://dx.doi.org/10.1128/AAC.31.12.1982.
124. **Gustafson JE, Wilkinson BJ.** 1989. Lower autolytic activity in a homogeneous methicillin-resistant *Staphylococcus aureus* strain compared to derived heterogeneous-resistant and susceptible strains. FEMS Microbiol Lett **50:**107–111.
125. **Piriz Duran S, Kayser FH, Berger-Bachi B.** 1996. Impact of *sar* and *agr* on methicillin resistance in *Staphylococcus aureus*. FEMS Microbiol Lett **141:**255–260. http://dx.doi.org/10.1111/j.1574-6968.1996.tb08394.x.
126. **Wu S, de Lencastre H, Tomasz A.** 1996. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J Bacteriol **178:**6036–6042.
127. **Frebourg NB, Nouet D, Lemee L, Martin E, Lemeland JF.** 1998. Comparison of ATB staph, rapid ATB staph, Vitek, and E-test methods for detection of oxacillin heteroresistance in staphylococci possessing *mecA*. J Clin Microbiol **36:**52–57.
128. **Knobloch JK, Jager S, Huck J, Horstkotte MA, Mack D.** 2005. *mecA* is not involved in the sigma B-dependent switch of the expression phenotype of methicillin resistance in *Staphylococcus epidermidis*. Antimicrob Agents Chemother **49:**1216–1219. http://dx.doi.org/10.1128/AAC.49.3.1216-1219.2005.
129. **Cuirolo A, Plata K, Rosato AE.** 2009. Development of homogeneous expression of resistance in methicillin-resistant *Staphylococcus aureus* clinical strains is functionally associated with a beta-lactam-mediated SOS response. J Antimicrob Chemother **64:**37–45. http://dx.doi.org/10.1093/jac/dkp164.
130. **Plata KB, Rosato RR, Rosato AE.** 2011. Fate of mutation rate depends on *agr* locus expression during oxacillin-mediated heterogeneous-homogeneous selection in methicillin-resistant *Staphylococcus aureus* clinical strains. Antimicrob Agents Chemother **55:**3176–3186. http://dx.doi.org/10.1128/AAC.01119-09.
131. **Plata KB, Riosa S, Singh CR, Rosato RR, Rosato AE.** 2013. Targeting of PBP1 by beta-lactams determines recA/SOS response activation in heterogeneous MRSA clinical strains. PLoS One **8:**e61083. http://dx.doi.org/10.1371/journal.pone.0061083.
132. **Dengler V, McCallum N, Kiefer P, Christen P, Patrignani A, Vorholt JA, Berger-Bachi B, Senn MM.** 2013. Mutation in the c-di-AMP cyclase DacA affects fitness and resistance of methicillin resistant *Staphylococcus aureus*. PLoS One **8:**e73512. http://dx.doi.org/10.1371/journal.pone.0073512.
133. **Engel H, Mika M, Denapaite D, Hakenbeck R, Muhlemann K, Heller M, Hathaway LJ, Hilty M.** 2014. A low-affinity penicillin-binding protein 2x variant is required for heteroresistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother **58:**3934–3941. http://dx.doi.org/10.1128/AAC.02547-14.
134. **Zavascki AP, Falci DR, da Silva RC, Dalarosa MG, Ribeiro VB, Rozales FP, Luz DI, Magagnin CM, Vieira FJ, Sampaio JM, Barth AL.** 2014. Heteroresistance to carbapenems in New Delhi metallo-beta-lactamase-1-producing isolates: a challenge for detection? Infect Control Hosp Epidemiol **35:**751–752. http://dx.doi.org/10.1086/676442.
135. **Medeiros AA.** 1997. Relapsing infection due to Enterobacter species: lessons of heterogeneity. Clin Infect Dis **25:**341–342. http://dx.doi.org/10.1086/516918.
136. **Wang X, Kang Y, Luo C, Zhao T, Liu L, Jiang X, Fu R, An S, Chen J, Jiang N, Ren L, Wang Q, Baillie JK, Gao Z, Yu J.** 2014. Heteroresistance at the single-cell level: adapting to antibiotic stress through a population-based strategy and growth-controlled interphenotypic coordination. mBio **5:**e00942–13. http://dx.doi.org/10.1128/mBio.00942-13.
137. **Cafiso V, Bertuccio T, Spina D, Purrello S, Campanile F, Di Pietro C, Purrello M, Stefani S.** 2012. Modulating activity of vancomycin and

daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. PLoS One **7:**e29573. http://dx.doi.org/10.1371/journal.pone.0029573.
138. **Yamakawa J, Aminaka M, Okuzumi K, Kobayashi H, Katayama Y, Kondo S, Nakamura A, Oguri T, Hori S, Cui L, Ito T, Jin J, Kurosawa H, Kaneko K, Hiramatsu K.** 2012. Heterogeneously vancomycin-intermediate *Staphylococcus aureus* (hVISA) emerged before the clinical introduction of vancomycin in Japan: a retrospective study. J Infect Chemother **18:**406–409. http://dx.doi.org/10.1007/s10156-011-0330-2.
139. **Howden BP, McEvoy CR, Allen DL, Chua K, Gao W, Harrison PF, Bell J, Coombs G, Bennett-Wood V, Porter JL, Robins-Browne R, Davies JK, Seemann T, Stinear TP.** 2011. Evolution of multidrug resistance during Staphylococcus aureus infection involves mutation of the essential two component regulator WalKR. PLoS Pathog **7:**e1002359. http://dx.doi.org/10.1371/journal.ppat.1002359.
140. **Neoh HM, Cui L, Yuzawa H, Takeuchi F, Matsuo M, Hiramatsu K.** 2008. Mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance. Antimicrob Agents Chemother **52:**45–53. http://dx.doi.org/10.1128/AAC.00534-07.
141. **Cui L, Isii T, Fukuda M, Ochiai T, Neoh HM, Camargo IL, Watanabe Y, Shoji M, Hishinuma T, Hiramatsu K.** 2010. An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **54:**5222–5233. http://dx.doi.org/10.1128/AAC.00437-10.
142. **Gazzola S, Cocconcelli PS.** 2008. Vancomycin heteroresistance and biofilm formation in *Staphylococcus epidermidis* from food. Microbiology **154:**3224–3231. http://dx.doi.org/10.1099/mic.0.2008/021154-0.
143. **Rose WE, Knier RM, Hutson PR.** 2010. Pharmacodynamic effect of clinical vancomycin exposures on cell wall thickness in heterogeneous vancomycin-intermediate *Staphylococcus aureus*. J Antimicrob Chemother **65:**2149–2154. http://dx.doi.org/10.1093/jac/dkq292.
144. **van Merode AE, van der Mei HC, Busscher HJ, Krom BP.** 2006. Influence of culture heterogeneity in cell surface charge on adhesion and biofilm formation by *Enterococcus faecalis*. J Bacteriol **188:**2421–2426. http://dx.doi.org/10.1128/JB.188.7.2421-2426.2006.
145. **Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD.** 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother **54:**4971–4977. http://dx.doi.org/10.1128/AAC.00834-10.
146. **Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD.** 2011. Insertion sequence ISAba11 is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. Antimicrob Agents Chemother **55:**3022–3024. http://dx.doi.org/10.1128/AAC.01732-10.
147. **Schmerk CL, Bernards MA, Valvano MA.** 2011. Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in *Burkholderia cenocepacia*. J Bacteriol **193:**6712–6723. http://dx.doi.org/10.1128/JB.05979-11.
148. **Bae IG, Federspiel JJ, Miro JM, Woods CW, Park L, Rybak MJ, Rude TH, Bradley S, Bukovski S, de la Maria CG, Kanj SS, Korman TM, Marco F, Murdoch DR, Plesiat P, Rodriguez-Creixems M, Reinbott P, Steed L, Tattevin P, Tripodi MF, Newton KL, Corey GR, Fowler VG, Jr.** 2009. Heterogeneous vancomycin-intermediate susceptibility phenotype in bloodstream methicillin-resistant *Staphylococcus aureus* isolates from an international cohort of patients with infective endocarditis: prevalence, genotype, and clinical significance. J Infect Dis **200:**1355–1366. http://dx.doi.org/10.1086/606027.
149. **Rodriguez CH, Bombicino K, Granados G, Nastro M, Vay C, Famiglietti A.** 2009. Selection of colistin-resistant *Acinetobacter baumannii* isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagn Microbiol Infect Dis **65:**188–191. http://dx.doi.org/10.1016/j.diagmicrobio.2009.05.019.
150. **Sola C, Lamberghini RO, Ciarlantini M, Egea AL, Gonzalez P, Diaz EG, Huerta V, Gonzalez J, Corso A, Vilaro M, Petiti JP, Torres A, Vindel A, Bocco JL.** 2011. Heterogeneous vancomycin-intermediate susceptibility in a community-associated methicillin-resistant *Staphylococcus aureus* epidemic clone, in a case of infective endocarditis in Argentina. Ann Clin Microbiol Antimicrob **10:**15. http://dx.doi.org/10.1186/1476-0711-10-15.
151. **Campanile F, Bongiorno D, Falcone M, Vailati F, Pasticci MB, Perez M, Raglio A, Rumpianesi F, Scuderi C, Suter F, Venditti M, Venturelli C, Ravasio V, Codeluppi M, Stefani S.** 2012. Changing Italian nosocomial-community trends and heteroresistance in *Staphylococcus aureus* from bacteremia and endocarditis. Eur J Clin Microbiol Infect Dis **31:**739–745. http://dx.doi.org/10.1007/s10096-011-1367-y.
152. **Charles PG, Ward PB, Johnson PD, Howden BP, Grayson ML.** 2004. Clinical features associated with bacteremia due to heterogeneous vancomycin-intermediate *Staphylococcus aureus*. Clin Infect Dis **38:**448–451. http://dx.doi.org/10.1086/381093.
153. **Gibson RL, Burns JL, Ramsey BW.** 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med **168:**918–951. http://dx.doi.org/10.1164/rccm.200304-505SO.
154. **Goytia M, Shafer WM.** 2010. Polyamines can increase resistance of *Neisseria gonorrhoeae* to mediators of the innate human host defense. Infect Immun **78:**3187–3195. http://dx.doi.org/10.1128/IAI.01301-09.
155. **El-Halfawy OM, Valvano MA.** 2014. Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*. Antimicrob Agents Chemother **58:**4162–4171. http://dx.doi.org/10.1128/AAC.02649-14.
156. **Lee HH, Molla MN, Cantor CR, Collins JJ.** 2010. Bacterial charity work leads to population-wide resistance. Nature **467:**82–85. http://dx.doi.org/10.1038/nature09354.
157. **Lee JH, Lee J.** 2010. Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev **34:**426–444. http://dx.doi.org/10.1111/j.1574-6976.2009.00204.x.
158. **Vega NM, Allison KR, Samuels AN, Klempner MS, Collins JJ.** 2013. *Salmonella typhimurium* intercepts *Escherichia coli* signaling to enhance antibiotic tolerance. Proc Natl Acad Sci U S A **110:**14420–14425. http://dx.doi.org/10.1073/pnas.1308085110.
159. **Tsuji BT, Rybak MJ.** 2006. Etest synergy testing of clinical isolates of *Staphylococcus aureus* demonstrating heterogeneous resistance to vancomycin. Diagn Microbiol Infect Dis **54:**73–77. http://dx.doi.org/10.1016/j.diagmicrobio.2005.08.014.
160. **Saravolatz SN, Martin H, Pawlak J, Johnson LB, Saravolatz LD.** 2014. Ceftaroline heteroresistant *Staphylococcus aureus*. Antimicrob Agents Chemother **58:**3133–3136. http://dx.doi.org/10.1128/AAC.02685-13.
161. **Sancak B, Yagci S, Gur D, Gulay Z, Ogunc D, Soyletir G, Yalcin AN, Dundar DO, Topcu AW, Aksit F, Usluer G, Ozakin C, Akalin H, Hayran M, Korten V.** 2013. Vancomycin and daptomycin minimum inhibitory concentration distribution and occurrence of heteroresistance among methicillin-resistant *Staphylococcus aureus* blood isolates in Turkey. BMC Infect Dis **13:**583. http://dx.doi.org/10.1186/1471-2334-13-583.
162. **Kelley PG, Gao W, Ward PB, Howden BP.** 2011. Daptomycin nonsusceptibility in vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous-VISA (hVISA): implications for therapy after vancomycin treatment failure. J Antimicrob Chemother **66:**1057–1060. http://dx.doi.org/10.1093/jac/dkr066.
163. **Huang H, Weintraub A, Fang H, Wu S, Zhang Y, Nord CE.** 2010. Antimicrobial susceptibility and heteroresistance in Chinese *Clostridium difficile* strains. Anaerobe **16:**633–635. http://dx.doi.org/10.1016/j.anaerobe.2010.09.002.
164. **Gordon NC, Wareham DW.** 2009. Failure of the MicroScan WalkAway system to detect heteroresistance to carbapenems in a patient with *Enterobacter aerogenes* bacteremia. J Clin Microbiol **47:**3024–3025. http://dx.doi.org/10.1128/JCM.01033-09.

**Omar M. El-Halfawy** 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 a 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 became an Assistant Lecturer at the same university, also in 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 M.D. 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 to 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, nonfermentative 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, Zeller's Award from Cystic Fibrosis Canada, and a Chair in Microbiology and Infectious Diseases from Queen's University Belfast.