

# Heteroresistance of opportunistic bacteria to antimicrobial peptides: a new challenge to antimicrobial therapy of cystic fibrosis infections

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**KEYWORDS:** *Acinetobacter* ■ antibiotic resistance ■ antimicrobial peptides ■ *Burkholderia cepacia* ■ cystic fibrosis ■ heteroresistance ■ nosocomial infections ■ opportunistic bacteria ■ *Pseudomonas aeruginosa*

Since the discovery of penicillin and the synthesis of prontosil, the prototype of sulfonamide antimicrobials, the development of new antimicrobial agents has expanded considerably. However, microbial infections are still among the top causes of fatalities worldwide [101]. The successful therapeutic outcome of such infections is hampered by the continuous emergence of antibiotic resistance mechanisms in bacteria. This represents a major challenge that aggravates the problems posed by microbial infections, which in many cases further complicate existing conditions resulting in the deterioration of health. This is of particular importance in case of immunocompromised patients such as patients with cystic fibrosis (CF), which is of special interest in this editorial. A new challenge faced by antimicrobial therapy is the phenomenon of heteroresistance of bacteria to antibiotics.

Heteroresistance could be defined as resistance to certain antibiotics, expressed by a subset of the microbial population that is generally considered to be susceptible to these antibiotics, according to traditional *in vitro* susceptibility testing [1]. However, there is no distinct and precise definition that encompasses the phenomenon of heteroresistance, which has been described in previous reports [2–6]. FIGURE 1A illustrates the notion of heteroresistance in contrast to a bacterial population homogeneously responding to an antibiotic (FIGURE 1B). This phenomenon is distinct from bacterial persistence. Persisters neither die nor grow in the presence of an antibiotic (FIGURE 1C), suggesting they are dormant [7], and they only grow after the removal of the antibiotic. Furthermore, the progeny of persisters do not exhibit increased resistance to the antibiotic; rather they show the same pattern of sensitivity to the antibiotic as that of the original bacterial population [8].

The phenomenon of heteroresistance challenges the traditional concept that an isogenic population of bacteria should display identical phenotypes. Indeed, isogenic bacteria exhibit a range of phenotypes, even in homogeneous environmental conditions. Nongenetic individuality has been observed in a wide range of biological processes, including differentiation, stress response and antibiotic resistance [8–12]. This phenomenon could be attributed to various reasons, including heterogeneity of plasmid copy number within a monoclonal population of bacteria [9] and variant gene expression patterns within an isogenic population [10].

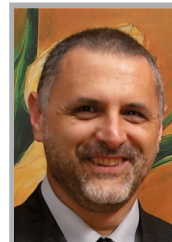
In this editorial, we will focus on discussing incidences of heteroresistance in nonfermenting Gram-negative bacteria. These microorganisms pose a significant emerging medical problem, being a common cause of nosocomial infections associated with multiple antibiotic resistances [13]. The major opportunistic pathogens from this group are *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Burkholderia* and *Ralstonia* spp., but there are no reasons precluding that other pathogens may arise, especially taking into account the evolving improvements in medical treatments of chronic conditions that result in an increasing number of individuals with various degrees of immunosuppression. These bacteria are not commonly found in the human microflora, but rather originate from aquatic habitats and terrestrial ecosystems, such as the rhizosphere. They have the capacity to form biofilms, and in hospitals, these bacteria can grow in humidifiers, ventilators, mattresses and even disinfectant solutions. They are especially dangerous to patient populations immunosuppressed by disease or medical treatments.

Cystic fibrosis patients are among those populations immunosuppressed by disease, as



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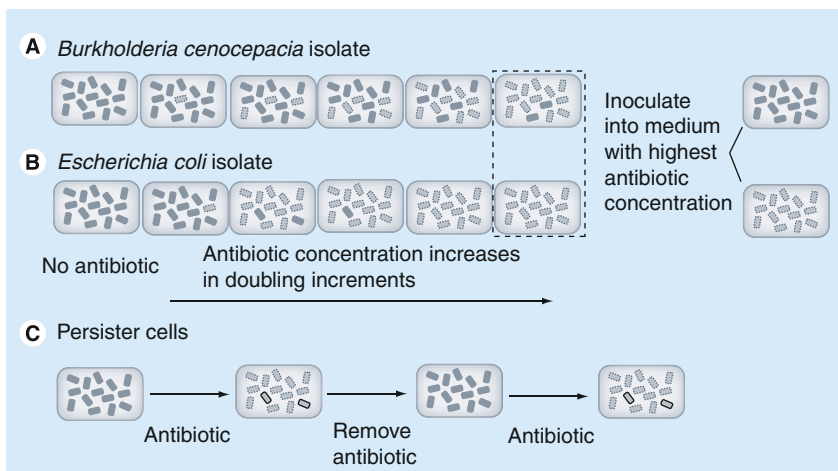
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**Figure 1. Illustration of the phenomenon of heteroresistance to antibiotics displayed by a *Burkholderia cenocepacia* isolate (A) in contrast to the homogeneous response to an antibiotic exhibited by an *Escherichia coli* isolate (B) and compared to the phenomenon of persistence (C).**

they suffer from an autosomal recessive disorder caused by mutations in a single gene on the long arm of chromosome 7 that encodes the CF transmembrane conductance regulator (CFTR) protein [14]. Despite impressive advances in understanding the molecular basis and pathophysiology of this disorder, it remains one of the most common life-shortening genetic disorder [14].

### Heteroresistance in *Burkholderia*

*Burkholderia cenocepacia* and other members of the *Burkholderia cepacia* complex are Gram-negative opportunistic pathogens ubiquitously found in the environment [15,16]. Although generally harmless to healthy individuals, *B. cenocepacia* affects immunocompromised patients [16], such as those with CF and chronic granulomatous disease. CF patients infected with *B. cenocepacia* commonly develop chronic lung infections that are very difficult to treat because these bacteria are extremely resistant to virtually all clinically useful antibiotics as well as antimicrobial peptides (APs) [15,17]. Infection of CF patients with *B. cenocepacia* leads to greater overall number of deaths and significantly shortened survival for those patients in comparison with infections caused by other pathogens [18]. *B. cenocepacia* is intrinsically resistant to human and nonhuman APs such as those produced by airway epithelial cells [19,20], human  $\beta$ -defensin 3 [21], human neutrophil peptides [20] and polymyxin B (PmB) [22,23]. *B. cenocepacia* resistance to APs originates, for the most part, from ineffective binding to the outer membrane as a consequence of the low number of phosphate and

carboxylate groups in the lipopolysaccharide (LPS) [24]. LPS structure plays an important role in resistance to polymyxins and other peptides in *B. cenocepacia* [22]. Other mechanisms of bacterial resistance to antimicrobial peptides have been described, including alteration of surface charges, changes in membrane proteins, efflux pumps/transporters and proteolytic enzymes [25,26].

To date, the heterogeneity of *B. cenocepacia* populations has not been investigated. In our preliminary studies, we were able to detect that the resistance of *B. cenocepacia* to APs is heterogeneous across an isogenic population (FIGURE 1A); the more resistant subpopulations displayed a stable and more homogenous higher-level resistance to APs. This is opposite to homogenous bacterial populations whose individual cells respond more or less similarly to a range of increasing antibiotic concentrations as exemplified in an *Escherichia coli* strain (FIGURE 1B). Nonetheless, *B. cenocepacia* and all mutants tested so far are generally extremely resistant to PmB (as a representative of APs) where its minimum inhibitory concentration (MIC) is more than 1024  $\mu\text{g/ml}$  [27], whereas the resistance breakpoint applied to the MIC of PmB, based on the level that is easily achievable in serum following intravenous administration, is set to, at least 4  $\mu\text{g/ml}$  [28].

### Heteroresistance in *Acinetobacter*

*Acinetobacter baumannii* are multidrug-resistant, opportunistic pathogens that may cause pneumonia, bacteremia, infection in burn wounds, meningitis and urinary tract infections. Heterogeneous colistin-resistant *A. baumannii* was demonstrated to exist in 'colistin susceptible' clinical isolates. The MICs of colistin against various isolates were within 0.25–2  $\mu\text{g/ml}$ . However, subpopulations grew in the presence of colistin 3–10  $\mu\text{g/ml}$  [6]. The mechanism of such heteroresistance in *A. baumannii* was due to complete loss of LPS production in subpopulations displaying a high level of colistin resistance [29]. This was caused by an insertion sequence that inactivates lipid-A biosynthesis genes; *lpxA* and *lpxC* [30].

Heteroresistance to the carbapenems, imipenem and meropenem, was also reported in *A. baumannii* clinical isolates. Subpopulations showing a higher level of resistance to these antibiotics could grow at concentrations four-fold or higher than the MICs of the entire population [5]. Prior to that report, resistance to carbapenems among *Acinetobacter* strains

was known to be homogeneous within a culture. However, the molecular basis of this phenomenon remains to be elucidated.

### Heteroresistance in *Pseudomonas*

*P. aeruginosa* is by far the most prevalent pathogen in CF. Up to 80% of patients with CF are eventually infected with this organism, and acquisition of the organism is associated with clinical deterioration [14]. There is a wide distribution of *P. aeruginosa* genotypes that have been demonstrated in young children, suggesting acquisition from environmental reservoirs [14].

In a leukopenic mouse model 'susceptible' *P. aeruginosa* could not be eradicated with gentamicin alone [4]. Indeed, the bacterial subpopulations of gentamicin-resistant, slower-growing small-colony variants, lead to the 'aminoglycoside escape phenomenon of *Pseudomonas*'. This phenomenon is characterized by regrowth of the more resistant variants after the initial killing of the test organisms. This problem was solved after combining gentamicin with ticarcillin. The two drugs could eradicate different subpopulations of the infecting pathogen.

### The problem of heteroresistance in therapy

The use of the current antibiotic dosage regimen, which is guided by the traditional *in vitro* susceptibility testing for heteroresistant bacteria, may lead to disastrous outcomes. There may be substantial potential for the rapid development of resistance and subsequent therapeutic failure. In such cases, the antibiotic therapy will select for the more resistant subpopulations, which would aggravate the overall clinical situation. Novel therapeutic strategies and extra precautions are hence required to ensure the judicious use of antibiotics in appropriate dosage regimens.

### Can it be worse? Transfer of resistance across the subpopulations

The phenomenon of heteroresistance could pose a new challenge to antimicrobial therapy, especially if the high level of resistance was transferred from the more resistant subpopulations to the rest of the bacterial culture. Interbacterial communication is common, where microorganisms often use small chemicals or secondary metabolites as informational cues [31]. Increasing the overall resistance of a bacterial culture can be induced by either

of two possibilities. Neutralizing elements may be released from the more resistant cells, which would eliminate the antibiotic from the medium thus protecting the rest of the culture. Another possibility is that the more resistant cells secrete signaling molecules that would stimulate the gene expression in the other cells, allowing them to display increased resistance to the antibiotic. It has been shown previously that small signaling molecules secreted by subsets of the entire population could increase virulence or resistance to antibiotics [32–35].

*B. cenocepacia* possesses a signal transduction machinery that enables cells to communicate with one another and coordinate multicellular behavior [36]. This generally takes place through quorum sensing, which allows for regulation of gene expression based on the density of the bacterial population. *B. cenocepacia* possesses genes for the homoserine lactone-producing quorum sensing systems *cepRI*, *cciRI* and an orphan regulator designated as *cepR2*, which is not encoded with a homoserine lactone synthase gene. *B. cenocepacia* also utilizes a nonhomoserine lactone compound, the *Burkholderia* diffusible signal factor (BDSF), which produces *cis*-2-dodecenoic acid in a cell-density dependent manner. These systems show overlapping functions in altering many of the same genes required for virulence and form a complex network of gene regulation in response to bacterial cell density. Some of these systems are involved in cross-species communication, for example with *P. aeruginosa* [36]. However, the mechanisms by which these systems communicate the high levels of resistance across heteroresistant populations is unknown.

### Future perspective

The molecular mechanism of heterogeneity of resistance to antibiotics should be identified. Moreover, the transferability of such resistance across the same bacterial culture or in an interspecies manner needs to be explored. Of special interest, the possibility of transferring the high level of resistance from *B. cenocepacia* to *P. aeruginosa* should be investigated. This is clinically important since *P. aeruginosa* is the most common causative agent of chronic respiratory tract infections in CF patients [14], while *B. cenocepacia* is the most resistant to antibiotics and is responsible for the highest rates of mortality and morbidity among CF patients [18]. The signals involved in any transfer of resistance should be identified and their effect on the less-resistant cells should be

examined at the molecular level. Finally, therapeutic solutions should be presented to counteract this emerging challenge that complicates the therapeutic outcome of antibiotics.

### Questions for future research

- What is the molecular mechanism(s) of heteroresistance?
- Can heteroresistance be transferred across different bacteria?
- Can combination therapies contribute to eradicate the heteroresistant bacterial populations?

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