The last bastion of AMR: heteroresistance in multidrug-resistant Gram-negative pathogens

Heteroresistance: selection of mutants versus persistence

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HETERORESISTANCE
A poorly defined concept

A variable response to antibiotics from individuals within the same bacterial population
HETERORESISTANCE IN THE CLINICAL LABORATORY
Detection is not standardized

E. coli - Ciprofloxacin

K. pneumoniae - Imipenem

S. haemolyticus PAP using plates with vancomycin (black) and vancomycin plus oxacillin at 6 mg/L (white)

Martínez-Martínez L. Unpublished.
Guzmán Puche et al. J. ECCMID 2019, P1473
Climo MW et al. AAC 1999, 1747
Modified PAP method

Applied to *S. aureus* and vancomycin

Comparison of the area under the curve (PAP-AUC) for:
- Strain to be characterized
- Reference heterogeneous strain

- <0.9: Susceptible strain
- 0.9-1.3: Heterogeneous strain
- >1.3: Intermediate *S. aureus* (VISA)

El-Halfawy OM, Valvano MA. Clin Microbiol Rev 2015, 191
PAP
Definition of heteroresistance

When the antibiotic concentration exhibiting the highest inhibitory effect is >=8-fold higher than the highest noninhibitory concentration

No heteroresistance
Heteroresistance

El-Halfawy OM, Valvano MA. Plos One 2013, 8:e68874
Resistant mutants vs. Persisters

Abraham EP, Chain E. (1940)
An Enzyme from Bacteria able to Destroy Penicillin. Nature 146, 837

Hobby GL et al. (1942)
Observations on the Mechanism of Action of Penicillin.

Bigger JW. (1944)
Treatment of Staphyloccal Infections with Penicillin by Intermittent Sterilisation. Lancet ii, 497–500
Resistance

Resistant bacteria replicate in a given concentration of antibiotic because of well-known INHERITED mutations responsible of mechanisms that prevent the antibiotic interacts with its target.

The MIC is higher (or the inhibition zone is smaller) for Resistant than for Susceptible organisms.

Increased concentration of antibiotic will again kill the resistant mutants.

Resistance is defined with independence of the degree of the MIC increase.
When the MIC is higher than a predefined breakpoint this is considered clinical resistance.
“Biologically” resistant bacteria can still be considered clinically susceptible (i.e. FQ-R, ESBL/Carbapenemase producers).
Persistence

Temporary survival of a non-majority fraction of the whole population to a concentration of antibiotic that eliminates all other members.

Persisters switch back to usual growth pattern when antibiotic is not present.

The transient survival of persisters is NOT INHERITED by the whole regenerated population…

[…but genes related to persistence and mutations leading to increased level of persisters have been described].
Tolerance

Slow-growing and dormant/nongrowing bacterial cultures can escape the killing activity of bactericidal antibiotic.

[Cell-wall acting agents: Killing is not related to antibiotic concentration, but to duration of treatment].

In Clinical Microbiology, a strain is defined as tolerant to an agent when the MBC is >=32 times higher than the MIC
- No change in MIC
- Killing of 99.9% of the initial inoculum (10^5 cfu/mL)
- 24 h incubation!

Tolerance is also defined after the Minimum Duration for Killing (MDK):
- “Duration of antibiotic treatment that is required to kill a given proportion of the bacterial population” (Brauner A et al. NRM 2016,320).
- MDK is deduced from a time-kill curve.
- Tolerance: An increase in MDK.
Tolerance by lag [time to resume exponential growth]
Growth arrest longer than antibiotic exposure
Broad effect on antibiotic tolerance

Tolerance by slow growth
Inherited (i.e. auxotrophs)
Non-inherited (external factors)
Persistence is related to Tolerance

When “tolerance” occurs in just a portion of a whole clonal population, the implicated subpopulation corresponds to persister cells.

Brauner A et al. Nat Rev Microbiol 2016, 320
A PROPOSAL TO DIFFERENTIATE TWO TYPES OF PERSISTERS

Time-dependent persisters
Subpopulation presenting:
   Lag-type tolerance (Type I)
   Slow growth-type tolerance (Type II)
   Longer exposure to antibiotic will decrease survival

Dose-dependent persisters
Transient expression of resistance mechanism
(increased efflux, porin loss,…) in a bacterial subpopulation
Higher doses of antibiotic will decrease survival

Brauner A et al. Nat Rev Microbiol 2016, 320
Persisters and Viable but Non-Culturable Cells (VBNC): The dormancy continuum

- Active Cells
- Persister Cells
- VBNC Cells
- Resuscitated Cells

Growth Inhibition

Resuscitation Over Time
Repair of oxidative damage, regained metabolic competence, normalization of T-A ratios

Ayrapetyan M et al. JB 2018, e00249-18
Ayrapetyan M et al. Trends Microbiol 2015, 7
<table>
<thead>
<tr>
<th></th>
<th>Resistant Mutants</th>
<th>Persisters</th>
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</thead>
<tbody>
<tr>
<td>Proliferation at MIC of the original population</td>
<td>Yes</td>
<td>Surviving [not entirely] dormant cells</td>
</tr>
<tr>
<td>MIC after re-inoculation</td>
<td>Increased</td>
<td>Unvariable</td>
</tr>
<tr>
<td>Type of change</td>
<td>Genetic</td>
<td>Phenotypic</td>
</tr>
<tr>
<td>Inherited trait</td>
<td>Yes</td>
<td>[No]</td>
</tr>
<tr>
<td>Proportion in a new population</td>
<td>Homogeneous</td>
<td>0.0001-0.1-1%</td>
</tr>
<tr>
<td>Antibiotic-Target</td>
<td>Not binding</td>
<td>Target Inactive</td>
</tr>
<tr>
<td>Standardized detection</td>
<td>Yes</td>
<td>[No]</td>
</tr>
<tr>
<td>Clinical relevance</td>
<td>Demonstrated</td>
<td>Quite likely (...but not completely proved in all cases)</td>
</tr>
</tbody>
</table>
How do resistant mutants and persisters are generated within bacterial populations?

Common pathways?
Resistant mutants causing Heteroresistance

Selection of
Mutations in structural genes, regulators
[Horizontal gene transfer]
Resistant mutants causing Heteroresistance

Heteroresistance can occur because of the selection of susceptible mutants in the background of a resistant population.

*K. pneumoniae*: Heteroresistance to colistin
Mutations in PhoP

Jayol A et al. AAC 2015, 2780

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Resistant mutants causing Heteroresistance

Methicillin resistance in *S. aureus*
Mutation in *dacA*
Decreased cyclic-di-AMP
Lower resistance […] but increased fitness!

Dengler V et al. PLoS ONE 2013, 8(8): e73512
Persisters causing Heteroresistance
Generation of persisters

a) Bet-hedging strategy (Random switching)
Stochastic variation in the expression of some genes

Persisters appear BEFORE bacteria have been exposed to antibiotics [Balaban NQ et al. Science 2004,1622]

b) Responsive diversification
Related to environmental factors (sublethal stressing factors)
A Positive feedback contribute to fixing biological noise
HipB-HipA: Type II Toxin-Antitoxin module

HipA:
Phosphorylates and inactivates GltX (glutamyl tRNA synthetase):
Accumulation of tRNAGlu
Increased levels of ppGpp (mediator of stringent response)

HipB: Inhibits HipA activity (and autorepressor of hipBA transcription)

[Persisters related to a certain threshold of HipA, as well as to affinity and concentration of HipB]

hipA7 (G22S, D291A): increased persistence x1000
Reduced interaction of HipB with HipA7 (in comparison with HipA)
A role for [type II] Toxin-Antitoxin modules and persistence in *E. coli*?

Deletion of a single locus minimally affects persistence

**Deletion of all 10 type II T-AT loci reduces persistence**

Some strains (including Δ10) were infected with prophages phi80 (likely involved in the observed phenotype)

New studies indicate that induction of T-AT systems is not directly linked to persistence

Maisonneuve E et al. PNAS 2011, 13206 (Retracted)
Harms et al. mBio 2017, e01964-17
Goormaghtigh F et al. mBio 2018, e00640-18
In *E. coli*, Persisters (to ciprofloxacin) are related to SOS and *tisB-istR-1* (type I T-AT module)

Kint CI et al. Trends Microbiol 2012, 577
Environment and Persistence

Increased levels of persisters:

- SubMICs of antibiotics
- Oxidative stress
- Heat shock
- DNA-damaging agents
- Nutrient limitation
- Diauxic carbon-source transition
- Phagocyte vacuoles
- Stringent response
- SOS response
A link between Persistence and Resistance...
Heteroresistance to carbapenems in clinical isolates of *A. baumannii*

Related to persistence not to selection of resistant mutants

<table>
<thead>
<tr>
<th></th>
<th>% of strains with</th>
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<tr>
<td></td>
<td>$bla_{\text{oxa-58-like}}$</td>
</tr>
<tr>
<td>HeteroR</td>
<td>57</td>
</tr>
<tr>
<td>NO HeteroR</td>
<td>0</td>
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</table>
Resistance mechanisms also related to the emergence of persisters

Paraquat induces SoxRS leading to AcrAB–TolC expression

AcrAB-TolC decreases intracellular concentrations of fluoroquinolones

More bacteria (x1000) become persisters in the presence of fluoroquinolone (but not ampicillin or kanamycin) after paraquat preincubation
Tolerance preceding Resistance

Tolerance can give the bacteria an opportunity to survive, allowing the selection of uncommon mutations coding for resistance.

Intermittent exposures of *E. coli* cultures to ampicillin (with intercalated growth in fresh medium) results in tolerance, because of an extended lag phase related to mutations in tolerome genes.

These tolerant bacteria allow selection of conventional resistant mutants in β-lactamase encoding gene *ampC*.
Heteroresistance and gene amplification
In clinical isolates

41 clinical isolates: *E. coli*, *S. enterica*, *K. pneumoniae*, *A. baumannii*

28 antibiotics.

766 bacteria–antibiotic combinations

27.4% of the combinations corresponded to heteroresistance.

Heteroresistance: Tetracyline, cephalosporins, carbapenems, trimethroprin
No Heteroresistance: (Fluoro)quinolones, erythromycin, rifampin, polymyxins

In ca. 90% of cases heteroresistance was unstable (...and costly)

Subpopulations with increased resistance:
- Spontaneous tandem amplifications of known resistance genes (chromosome and plasmids)
- Point mutations, IS, small deletions (chromosomal genes)

Different heteroresistance phenotypes can coexist in the same culture (implications for detection!)

Nicoloff H et al. Nat Microbiol 2019,
Heteroresistance is caused by… resistant mutants [and]/or persisters?

Conceptual basic issue
Microbiological aspects
Clinical consequences
Heteroresistance to carbapenems in clinical isolates of wild-type *K. pneumoniae* (n=6)
Bacteria from broth (any time) or solid medium (4-24h): HR to imipenem and ceftazidime

Bacteria grown in solid medium for 48h-3d-5d:
HR: carbapenems, mecillinam, ceftazidime (+/- avibactam), cefepime, amoxicillin/clavulanate
NO HR: aztreonam, cefotaxime, ceftriaxone, amikacin, gentamicin, ciprofloxacin
In all WT-Kpn we studied, heteroresistant colonies corresponded to PERSISTERS
Heteroresistance to carbapenems in clinical isolates of *K. pneumoniae* producing OXA-48

Colonies in disk and gradient diffusion assays: Persisters

Colonies grown on PAP plates with ≥4 μg/mL of meropenem: Stable resistant mutants (*ompK36*)

Methodological differences between PAP and diffusion assays (inoculum, antibiotic exposure, solid/liquid medium,...) can explain these observations.

López-Camacho E et al. Diagn Microbiol Infect Dis 2019, 162
CONCLUSIONS

• Needs in the field for better understanding of biological aspects and clinical relevance:
  Reference definition of heteroresistance.
  Standardization of the methodology to study heteroresistance

• Heteroresistance can be the consequence of multiple, not totally related processes, including selection of (stable and unstable) resistant mutants and emergence of persisters.

• While there is considerable information about mechanisms of resistance, this is not the cases when considering persistence, where conflicting observations have been made by different research groups.

• Recent data suggest that some “classical” mechanisms of resistance are also important for understanding the transitory survival of bacteria to antimicrobial agents.