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Research Article

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Levels of persisters influenced by aeration in *Acinetobacter calcoaceticus–baumannii*

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Aim: To evaluate the influence of aeration on persister levels from *Acinetobacter calcoaceticus–baumannii* isolates exposed to meropenem or tobramycin, as well as analyze morphological and structural changes in persisters. **Materials & methods:** Levels of persisters were determined after a 48-h exposure to tobramycin or meropenem under aerated or static conditions, and persisters were analyzed by scanning and transmission electron microscopy. **Results:** The fractions of persisters varied between isolates. Aeration reduced cell survival under each antibiotic treatment, and cell survival decreased as the tobramycin concentration was increased. Interestingly, division septa were observed in persisters by electron microscopy. **Conclusion:** Aeration may have stimulated bacterial growth, providing more targets for antibiotic action and leading to increased production of reactive oxygen species, which decreased levels of persisters.

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Keywords: *Acinetobacter* spp. • meropenem • oxygen availability • persistence • tobramycin

Acinetobacter spp. are opportunistic pathogens that have attracted great interest due to the high morbidity and mortality rates associated with *Acinetobacter* infections in hospitals and long-term care institutions and to the capacity of *Acinetobacter* to acquire and spread multiple antimicrobial resistance mechanisms [1,2]. The species *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* are genetically and phenotypically similar and are collectively referred to as the *A. calcoaceticus–baumannii* (ACB) complex [3]. Strains belonging to this complex are commonly associated with respiratory and surgical wound infections, sepsis and catheter contamination, especially affecting immunocompromised patients and those in intensive care units [4,5].

Carbapenems are the treatment of choice for *A. baumannii* infections. However, resistance to them has forced the use of other drugs, such as colistin, polymyxin B, tobramycin and tigecycline. The latter two can also be used as an adjunct treatment in aerosolized form in cases of ventilator-associated pneumonia, due to their superior ability to penetrate the lungs [6,7].

Even though antimicrobial resistance represents an important cause of treatment failure, another concern is persistence – a transitory phenotype comprising a genetically susceptible bacterial subpopulation that is able to survive an antibiotic treatment and, after its removal, is capable of regrowing [8,9]. These tolerant cells, also known as persisters, have already been observed in several species, such as *Staphylococcus aureus* [10], *Mycobacterium* spp. [11], *Escherichia coli* [12], *Pseudomonas aeruginosa* [13] and *A. baumannii* [14].

The exact mechanisms involved in the formation of persisters are unclear, but they are believed to be formed stochastically and/or induced by stressors, such as nutrient deprivation, oxidative stress and temperature and pH changes. They are also thought to be regulated by toxin–antitoxin systems, stringent response and quorum sensing [9,11–12,15]. Studies in Gram-negative bacteria also demonstrated that the production of reactive oxygen species (ROS), modulation of antioxidant enzymes and increased or limited oxygen availability can influence the levels of surviving cells [13,16–17]. These studies, however, did not evaluate the influence of aeration on ACB complex persister levels.

Because the ACB complex is a common cause of respiratory diseases in intensive care units, assessing the interference of environmental conditions found in the respiratory tract on the formation of these cells is necessary

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Table 1. Clinical origin of *Acinetobacter calcoaceticus–baumannii* complex isolates and their MICs to tobramycin and meropenem.

Isolates	Clinical origin	MIC to tobramycin (mg/l)	MIC to meropenem (mg/l)
Acb1 (*A. baumannii*)	Catheter tip	0.5	1
Acb2 (*A. baumannii*)	Skin wound	2	1
Acb3 (*A. baumannii*)	Surgical wound	1	1
Acb4 (*A. baumannii*)	Blood	1	0.5
Acb5 (*A. baumannii*)	Drain secretion	0.5	0.5
Acb6 (*A. baumannii*)	Skin wound	0.5	0.25
Acb7 (*A. baumannii*)	Tracheal aspirate	1	0.5
Acb8 (*A. baumannii*)	Nasopharynx secretion	1	0.5
Acb9 (*Acinetobacter nosocomialis*)	Urine	<0.25	0.5
Acb10 (*A. baumannii*)	Blood	0.5	0.25

MIC: Minimum inhibitory concentration.

in order to develop new therapeutic strategies appropriate for each circumstance. Therefore, the aim of this study was to investigate if aeration enhances killing in persisters treated with meropenem or tobramycin.

Materials & methods

ACB isolates

Ten ACB complex isolates obtained from the Department of Microbiology of the Clinical Pathology Laboratory (São Lucas Hospital, PUCRS, Porto Alegre, Brazil) were used in this study (Table 1). All isolates were previously identified by the VITEK-2 System (BioMérieux Inc, MO, USA), confirmed at the genus level by PCR targeting the 16S RNA [18] and characterized regarding the minimum inhibitory concentration (MIC) to meropenem by broth microdilution according to the Clinical and Laboratory Standards Institute's (CLSI) recommendations [19]. Susceptibility to tobramycin has already been evaluated by the disk diffusion method, and here we determined the MIC for this antimicrobial as described above. The identification of ACB complex species was performed for all isolates by multiplex PCR targeting the *gyr*B gene as previously described by Higgins *et al.* [20].

Evaluation of the influence of aeration on persister fractions

Isolates were cultured in Luria-Bertani (LB) broth (10 g/l tryptone [Oxoid, Basingstoke, UK], 5 g/l yeast extract [Himedia, Mumbai, India] and 5 g/l NaCl [Nuclear, Diadema, Brazil], pH 7.0) overnight at 37°C, and then the culture was diluted 30-fold in fresh LB broth and incubated for 3 h until the late exponential growth phase. Afterward, the initial cell concentration was measured by serial tenfold dilutions up to 10^5, followed by plating of three 10-μl drops of each dilution on nutrient agar (Oxoid) and a 24-h incubation. To determine the killing curve, tobramycin (20 mg/l or 10× the MIC) or meropenem (15 mg/l; both from Sigma-Aldrich, MO, USA) was added and incubated at room temperature under static or aerated (i.e., with shaking at 200 r.p.m.) conditions. Aliquots were collected 2, 4, 6, 24 and 48 h after tobramycin exposure and 6, 24 and 48 h after meropenem exposure, washed with 0.85% saline, and serially diluted 100,000-fold. 10 μl of each dilution was then spotted onto nutrient agar in triplicate. Plates were incubated at 37°C for 24 h and the surviving cells enumerated. All experiments were performed in biological triplicate. To confirm the persistence phenotype and discard the possible selection of resistant cells, the cells that survived after 48 h of treatment were grown on nutrient agar and then re-exposed to the antimicrobial at the previously determined MIC.

Scanning & transmission electron microscopy analyses

Planktonic cells from the Acb4 isolate were evaluated by scanning electron microscopy (SEM) before and after tobramycin (10 mg/l) or meropenem (15 mg/l) exposure, as well as in the absence of the antimicrobials, at the same time points. In addition, cells exposed to tobramycin (10 mg/l) and their controls (not treated) were analyzed by transmission electron microscopy (TEM). The isolate was grown in LB broth until the late exponential phase, treated with tobramycin or meropenem or untreated and incubated at room temperature under aerated or static conditions. Before antimicrobial exposure and after 24 h of treatment, 5-ml aliquots were removed for microscopy analysis. Samples were washed with 0.85% saline, pellets were immediately immersed in a specific

SEM (2.5% glutaraldehyde and 0.12 M phosphate buffer, pH 7.2–7.4) or TEM (2.5% glutaraldehyde, 2% paraformaldehyde and 0.12 M phosphate buffer, pH 7.2–7.4) fixative solution, and washed thrice in phosphate buffer. For SEM analyses, samples were dehydrated in a graded acetone series (30–100%), desiccated to remove acetone, coated in gold particles and visualized with a field emission scanning electron microscope (Inspect F50, FEI Company Inspect, Eindhoven, The Netherlands). Whereas for TEM analyses, samples were postfixed with 2% buffered osmium tetroxide, washed thrice with phosphate buffer, dehydrated in a graded acetone series (30–100%), pre-imbibed in a mix of resin and acetone, imbibed in resin and subjected to polymerization at 60°C for 3 days. Finally, 100-nm-thick ultrathin sections were cut using an ultramicrotome (EM UC7, Leica Microsystems, Wetzlar, Germany) and collected on 300-mesh copper grids. The samples were observed and photographed using a TEM (Tecnai G2 T20, FEI Company Inspect, OR, USA) at the Central Laboratory of Microscopy and Microanalysis (LabCEMM) from PUCRS.

Statistical analysis

Our statistical framework aimed to: evaluate the influence of aeration in persister formation with exposure to meropenem; evaluate the influence of tobramycin concentration, aeration and the interaction between these factors on persister fractions; and compare the fractions of surviving cells after exposure to both antibiotics under aerated or static conditions. In addition, we estimated the heterogeneity of persister fractions by estimating sample variation across different treatments and antibiotics.

We used analysis of variance with permutation (PERM-ANOVA) in all tests, controlling for repeated measures when testing for differences between exposure times. All tests were carried out in the statistical platform R [21], with built-in basic packages and package 'lmPerm' [22]. We considered p-values < 0.05 to indicate statistical significance.

Results

MIC determination

The tobramycin MIC values determined for all ten ACB isolates, ranging from <0.25 to 2 mg/l, are shown in Table 1, as well as the previously measured MIC values for meropenem [23].

Aeration can reduce persister levels after meropenem exposure

Late exponential-phase cultures exposed to a high dose of meropenem presented distinct cell survival fractions when static and aerated conditions were compared. Persister levels ranged from 0.0065 to 2.2399% and 0.0005 to 0.2068% under static and aerated conditions, respectively (Figure 1A). A reduction in the size of persister fractions was observed after meropenem exposure under aerated conditions in comparison with that obtained under static conditions. However, a statistically significant difference between the two culture conditions was verified after 24 h but not after 6 h ($p = 0.0590$) and 48 h ($p = 0.0586$) of meropenem treatment (Figure 1B & Supplementary Figure 1A).

Aeration & tobramycin concentration influence persister levels

A. baumannii persisters have been reported to be multidrug tolerant [14]. Therefore, we also evaluated the effect of tobramycin, an antibiotic with a different mechanism of action, on the levels of these cells. The isolates were exposed to two concentrations of this drug under static or aerated conditions, and rapid cell death followed by a maintenance plateau of surviving putative persisters was observed in all isolates, characterizing thusly the classic death curve of persistence (Supplementary Figure 1B & C). The heterogeneity of the persister levels among the isolates under different conditions of oxygen availability and antimicrobial concentration can be seen in Figure 2A and B, represented as the magnitude of surviving cells expressed in box plots at all times tested. Aeration resulted in a statistically significantly increased death rate at all-time points evaluated; therefore, smaller persister cell fractions were present in the cultures subjected to both tobramycin concentrations under aeration. Tobramycin treatment under aeration generated lower levels of surviving cells than meropenem treatment at all-time points evaluated. Exposure to 10× the MIC (<20 mg/l) of tobramycin led to surviving cell fractions ranging from 0.4231 to 0.0022% and 0.3 to 0.0003% under static and aerated conditions, respectively (Figure 2C), whereas treatment with 20 mg/l of tobramycin led to surviving cell fractions of 0.03% to not detectable and 0.0078% to not detectable under static and aerated conditions, respectively (Figure 2D).

In order to evaluate the influence of tobramycin concentration on persister fractions, the isolates were separated into three groups according to the tobramycin concentration to which they were exposed (10× the MIC) under

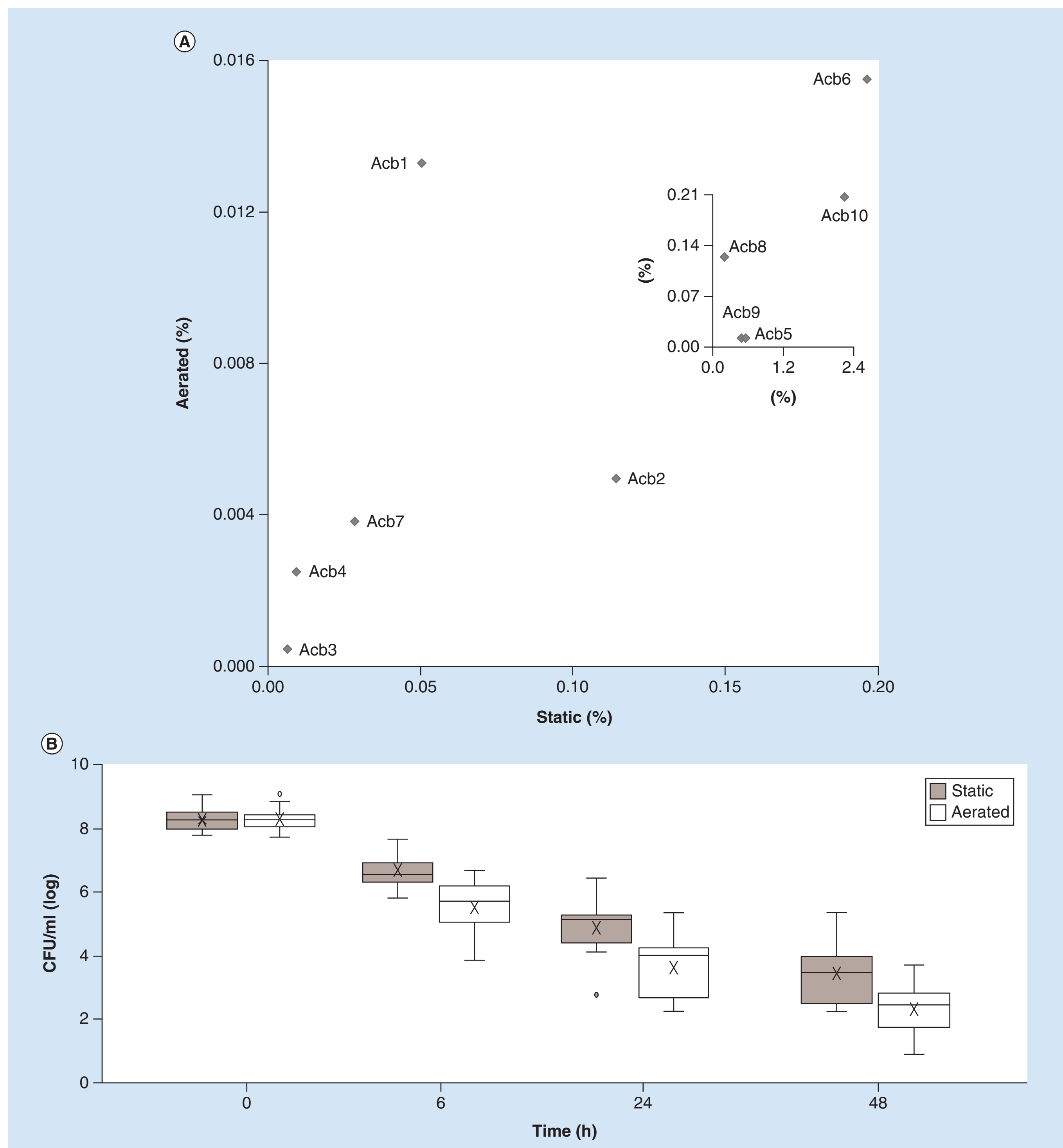

Figure 1. Persister fractions after a 48-h exposure to meropenem at 15 mg/l. Fractions of surviving cells under static and aerated conditions **(A)**. Box plots representing the average and variance of all ten isolates at each time point evaluated **(B)**. Bars represent the difference between averages of all ten isolates.
CFU: Colony-forming unit.

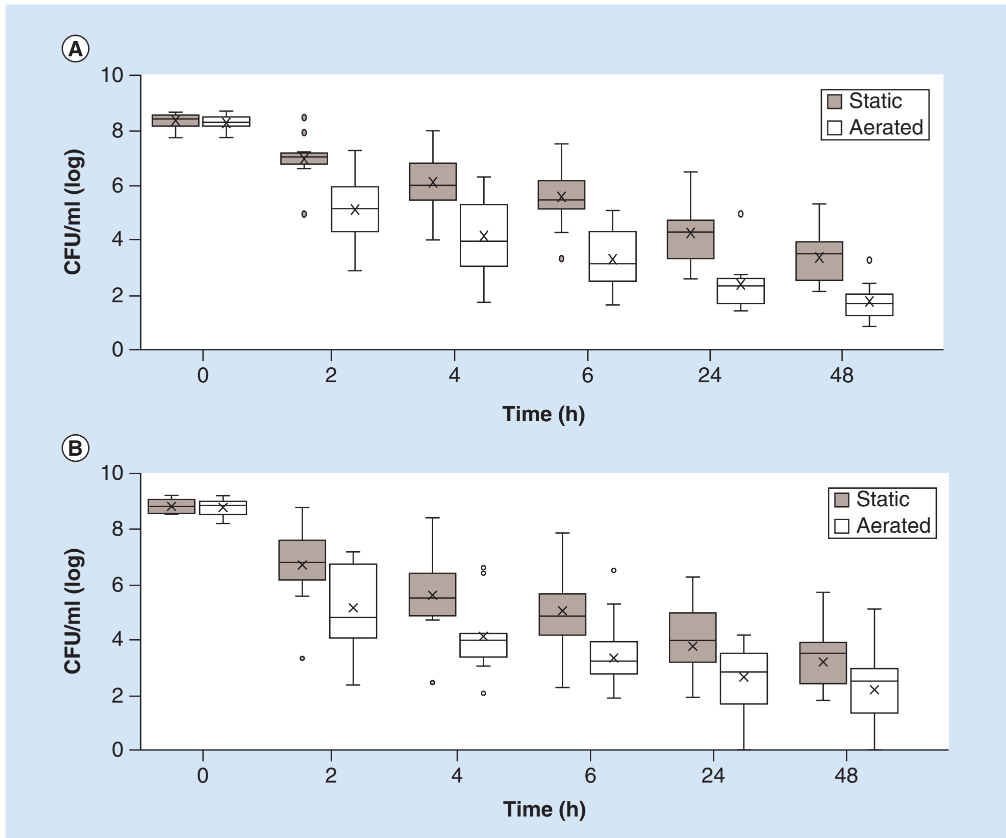

Figure 2. Persister fractions after exposure to 10× the minimum inhibitory concentration (MIC; <20 mg/l) or 20 mg/l of tobramycin. Box plots representing the average and variance of all ten isolates at each time evaluated after a 48-h exposure to 10× the MIC (<20 mg/l) **(A)** or 20 mg/l **(B)** of tobramycin under static and aerated conditions. Bars represent the difference between averages of all ten isolates. Surviving cell fractions after a 48-h exposure to 10× the MIC **(C)** or 20 mg/l **(D)** of tobramycin under static and aerated conditions are also shown.
CFU: Colony-forming unit.

both static and aerated conditions. In each group, we subtracted surviving cell fractions (colony-forming unit per milliliter) exposed to a fixed concentration of 20 mg/l from those exposed to each concentration (2.5, 5 and 10 mg/l; groups 1, 2 and 3, respectively). This analysis showed that as tobramycin concentration approached the maximum exposure value of 20 mg/l, the differences between fractions of persisters decreased accordingly, especially under aeration. Interestingly, the differences were lower under aerated compared with static conditions (Figure 3). This pattern suggests that persister levels are influenced by the tobramycin concentration used. However, there was no statistically significant interaction between tobramycin concentration and oxygen availability, suggesting that the two factors may be acting independently. Additionally, we verified that, under aeration, both tobramycin concentrations resulted in significantly lower persister fractions when compared with meropenem under the same conditions ($p = 0.0014$ for 10× the MIC and $p = 0.0065$ for 20 mg/l).

Heterogeneity variance depends on the treatment

As mentioned above, high heterogeneity in persister levels was found between isolates, especially after tobramycin exposure. Thus, in order to verify if aeration could influence this heterogeneity, a comparison between the variances found in the fractions detected under aerated and static conditions was performed for each antimicrobial exposure condition. After 48 h of meropenem treatment under aeration, a lower variance in the fractions was observed, that is, heterogeneity between persister fractions had decreased compared with those observed under static conditions. On the other hand, aeration did not seem to influence the heterogeneity of fractions detected after a 48-h tobramycin exposure (Figure 4).

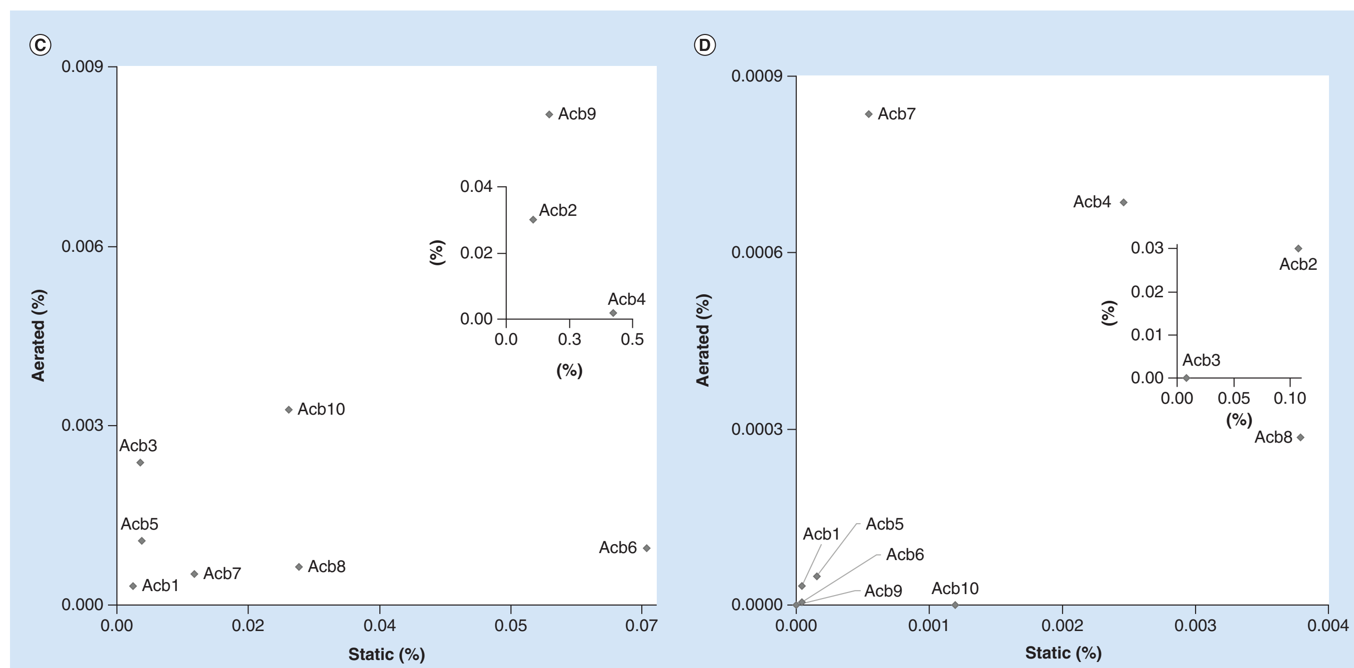

Figure 2. Persister fractions after exposure to 10× the minimum inhibitory concentration (MIC; <20 mg/l) or 20 mg/l of tobramycin (cont.). Box plots representing the average and variance of all ten isolates at each time evaluated after a 48-h exposure to 10× the MIC (<20 mg/l) **(A)** or 20 mg/l **(B)** of tobramycin under static and aerated conditions. Bars represent the difference between averages of all ten isolates. Surviving cell fractions after a 48-h exposure to 10× the MIC **(C)** or 20 mg/l **(D)** of tobramycin under static and aerated conditions are also shown.
CFU: Colony-forming unit.

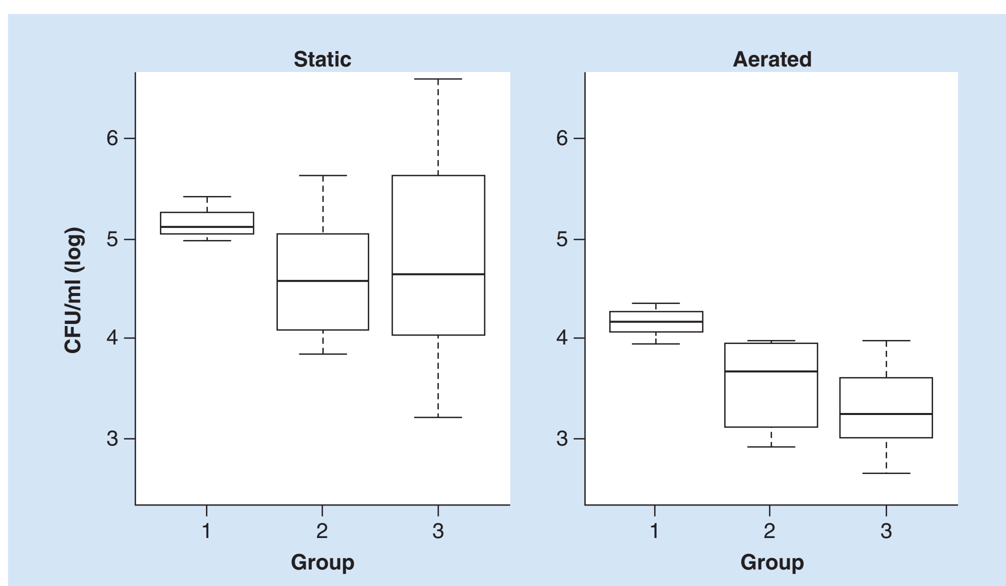

Figure 3. Box plots representing the influence of tobramycin concentration on persisters under static and aerated conditions. The difference in surviving fractions between isolates was obtained by subtraction of surviving fractions exposed to 20 mg/l from those exposed to each concentration (2.5, 5 and 10 mg/l; groups 1, 2 and 3, respectively).
CFU: Colony-forming unit.

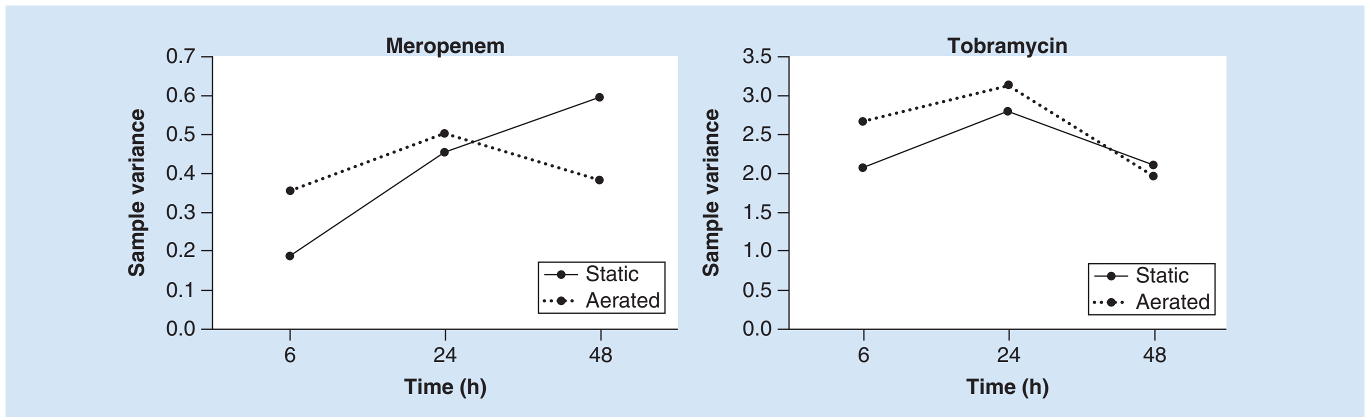

Figure 4. Heterogeneity in persister fractions after a 48-h exposure to meropenem (15 mg/l) or 10× the minimum inhibitory concentration (<20 mg/l) and 20 mg/l of tobramycin under static and aerated conditions evaluated by analysis of variance.

Persisters presenting division septa

Analyses of TEM and SEM images did not show morphological or size changes when persisters were compared with nontreated cells. Interestingly, persisters showing division septa at the midpoint of the cell were observed by TEM and SEM (Figure 5).

Surviving cells confirmed as persisters

Cells surviving 48 h of antimicrobial treatment were re-inoculated in LB broth containing antibiotic with its corresponding MIC, and no growth was observed, thereby confirming the persistence phenotype.

Discussion

Persisters are present in all bacteria studied to date; however, a definitive treatment to eradicate these cells has not yet been established, possibly due to the presence of multiple mechanisms of formation and regulation that can be affected by environmental conditions [24]. Among the factors involved, we can highlight aeration, which was associated, in this work, with tobramycin to verify whether it could affect the persister levels. The aminoglycosides act by hindering protein synthesis, leading to the production of aberrant proteins, hence disrupting the integrity of the bacterial cell membrane, as well as inducing the stress response system to trigger free-radical formation [25,26]. Concerning aeration, increased oxygen availability may generate higher levels of ROS [27,28], which has been reported to contribute to antibiotic-mediated killing [26,29]. Here, we report a significantly higher death rate under aeration, providing an average reduction of approximately 25-fold in the fraction of surviving cells, which may be due to the increased proton motive force promoting aminoglycoside uptake and ROS formation. These findings suggest that increased oxygen availability favors drug action, corroborating other studies using aminoglycosides, which may be of considerable relevance, as tobramycin can be administered by inhalation for ventilator-associated pneumonia [6,17,30–31]. Oxygen availability has also been reported to affect persister formation in *P. aeruginosa*, *Burkholderia pseudomallei* and *Mycobacterium tuberculosis*, when reduced levels of oxygen promotes increased survival of those cells and, most recently, to lead to depletion of or even prevent the appearance of persisters in *M. tuberculosis* by increasing oxygen consumption [11,17,31–33].

The possible influence of increasing tobramycin concentration on persister levels was also verified, showing the potentiation of tobramycin activity, as previously reported in the *E. coli*, *Listeria monocytogenes* and *Burkholderia cepacia* complex [16,34–35]. This result was already expected, both in regular and tolerant phenotypes, as aminoglycosides are bactericidal concentration-dependent antibiotics. However, to the best of our knowledge, the association between increased concentration and aeration had not yet been verified in the ACB complex [25,34].

Furthermore, we evaluated whether oxygen availability affects the action of another antibiotic in this study, meropenem, which is a carbapenem that acts by acylating penicillin-binding proteins – enzymes responsible for catalyzing the formation of peptidoglycans – weakening the peptidoglycan and leading the cell wall to burst from osmotic pressure [7]. Carbapenems, such as imipenem/cilastatin and meropenem, are broad spectrum antibiotics with aerobic and anaerobic activities, which are still widely used in spite of increasing resistance levels, and there

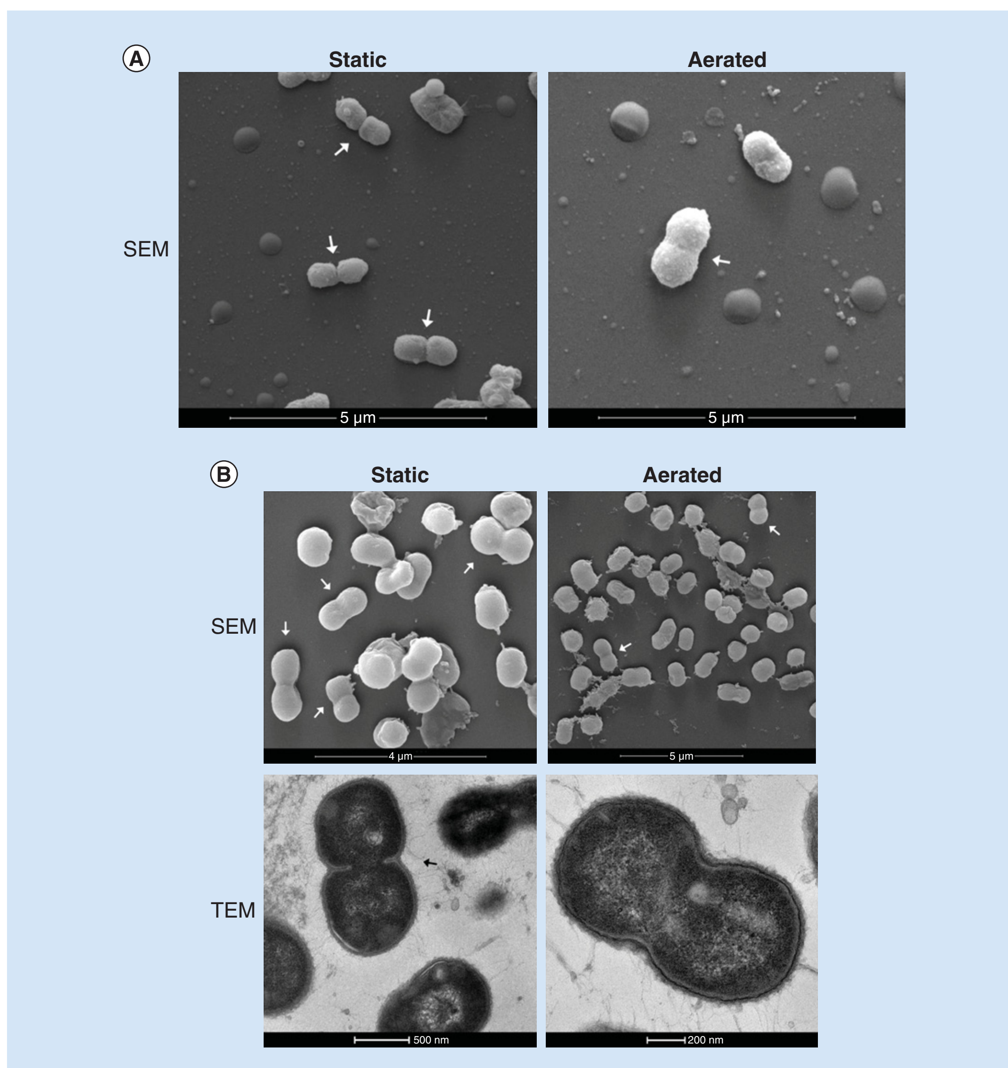

Figure 5. Division septa in persisters. Septum formation in persisters (indicated by arrows) surviving after a 24-h exposure to 15 mg/l of meropenem observed by SEM **(A)** or to 10 mg/l of tobramycin by SEM and TEM **(B)** under static or aerated conditions.
SEM: Scanning electron microscopy; TEM: Transmission electron microscopy.

are few studies of their action on persisters [7]. The data obtained in the meropenem assay showed a very similar pattern of cell death curves comparing static and aerated conditions, although oxygen availability seems to increase the antibiotic's effectiveness, especially after 24 h of exposure.

Taken together, these results suggest that aeration can influence ACB survival after exposure to the antimicrobials tested, especially tobramycin, which stimulates cells to multiply, thus allowing for a higher availability of the antibiotic targets. Additionally, the combination of tobramycin treatment with aeration could enhance the proton motive force and production of ROS, thus providing a greater effect of this drug on cell survival compared with that observed with meropenem treatment. In addition to the results obtained under each condition, we must also highlight the heterogeneity of the persister fractions detected in the ten isolates analyzed – a phenomenon already observed in *E. coli* and *A. baumannii* – which may hamper the eradication of these cells *in vivo* and represent a therapeutic challenge [14,23,36–37]. Interestingly, aeration decreased the amount of heterogeneity among the isolates treated with meropenem, whereas the same effect was not observed after exposure to tobramycin. This may have been due to the different level of heterogeneity in persister fractions verified after treatment with this antimicrobial, minimizing a possible influence of aeration.

It was not possible to observe different morphology or size in ACB cells surviving antimicrobial exposure under either aerated or static conditions. Nevertheless, a smaller size with a normal shape was found in persisters from *Staphylococcus epidermidis* [38]. However, division septa were seen in surviving cells exposed to both antibiotics and conditions, suggesting that persisters could be dividing cells, which agrees with a previous report that *Mycobaterium smegmatis* persists by dividing in the presence of isoniazid, characterizing persistence as stable numbers of cells, represented by a dynamic state of balanced division and death [39]. Likewise, dividing cells were found in antimicrobial-tolerant *S. epidermidis*, indicating the presence of metabolically active dividing bacteria [38].

Conclusion & future perspective

Considering that ACB isolates are a common cause of pneumonia and showed reduced levels of persisters after exposure to tobramycin under aeration, it would be important to evaluate the use of adjunctive treatments in aerosolized form combined with higher oxygen proportions in the inspiratory gas. The role of persisters in recalcitrant infections and their relation to failure of antibiotic therapies remain a cause for concern, and despite recent findings on the mechanisms involved in their formation, there are several unsolved issues yet to be explained.

Summary points

- All *Acinetobacter calcoaceticus–baumannii* (ACB) isolates presented persister fractions after exposure to tobramycin or meropenem.
- Treatments under aeration exhibited smaller persister fractions, especially with tobramycin.
- The action of tobramycin was shown to be concentration dependent, promoting reduced levels of persisters as the concentration increased.
- The ACB isolates presented high heterogeneity in the levels of persisters in all experiments.
- Electron microscopy revealed division septa in ACB persisters.
- Increased availability of oxygen can enhance tobramycin action.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fmb-2017-0153.

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Financial & competing interest disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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- **Reported *Mycobaterium smegmatis* persister cells divide in the presence of isoniazid.**