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# Discrepancies in susceptibility testing to colistin in *Acinetobacter baumannii*: The influence of slow growth and heteroresistance

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ABSTRACT

The increasing use of polymyxins as last-resort drugs for managing infections by *Acinetobacter baumannii* has led to the emergence of resistance. This study aimed to determine the resistance mechanisms in *Acinetobacter baumannii* isolates with colistin MIC ≥ 4 mg/L and to relate the mechanisms of resistance with the difficulties in detecting them. Absolute agreement among the different methodologies (Phoenix automatized system, broth and agar dilution, and a rapid colorimetric test) in the 140 colistin-susceptible isolates was observed; whereas in the 25 resistant isolates, the performance varied according to the colistin MIC value. Most of the discrepancies (irrespective of the methodology that was used) were observed in isolates with an MIC value close to the breakpoint. The number of errors in each method in the resistant isolates was as follows: rapid test, four of 25 (16%); agar dilution, eight of 25 (32%); Phoenix system, 13 of 25 (52%) and its manual reading at 24 h, eight of 25 (32%). Categorical errors were detected in 13 isolates: slow growth was the main reason in five isolates, whereas in the remaining eight isolates, slow growth was detected together with a low proportion of colistin-resistant subpopulations and the colistin MIC value was close to the breakpoint value. To understand the probable reason for the observed MIC values, sequencing of genes associated with colistin resistance was performed. Mutations at *lpx*A, *lpx*C, and *pmr*B genes were detected and it was observed that isolates carrying mutations in *lpx*C presented slow growth at killing curves.



## 1. Introduction

Colistin (COL) became available for clinical use in the 1960s but other antibiotics replaced it 10 years later due to its toxicity [1]. However, in the last few years, it has been re-introduced for managing infections by multidrug-resistant Gram-negative bacilli [2,3]. The rise in the use of polymyxins, both in hospital and veterinary settings, has led to an increase in the reports of acquired COL-resistant isolates [2]. This scenario has revealed the limitations of these drugs in in vitro susceptibility testing, as susceptibility testing fails to detect isolates with MIC values close to the breakpoint [1,4-6]. Thus, a joint CLSI (Clinical and Laboratory Standards Institute)/ EUCAST (European Committeee on Antimicrobial Susceptibility Testing) document recommends the use of broth dilution to perform susceptibility to COL [7]. Reference broth microdilution (BMD) is rarely performed by most clinical microbiology laboratories because it is laborious. Several methods have been designed in the last few years, including rapid tests for Enterobacteriaceae, which provide immediate results as the main advantage [8-10].

Due to the ability of *Acinetobacter baumannii* (*A. baumannii*) to acquire multiple antibiotic resistance traits, the Infectious Diseases Society of America has included it among the six pathogens resistant to antimicrobials (*Enterococcus faecium, Staphylococcus*

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*aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa and Enterobacter* spp) responsible for high morbidity and mortality in hospitalised patients. Regarding the known mechanisms of resistance to COL, opposed to what happens with Enterobacteriaceae, COL resistance in *A. baumannii* is only chromosomic [1].

Colistin resistance in *A. baumannii* is mediated by the addition of phosphoethanolamine to the lipid A of lipopolysaccharide as a result of mutations in the *pmr*AB two-component system or complete loss of lipopolysaccharide expression due to mutations in the *lpxA, lpxC* and *lpxD* genes [1,2]. Recently, efflux mechanisms have also been described [11]. Heteroresistance (HR) and COL dependence (CD) are also well-described phenomena in *A. baumannii* clinical isolates. Heteroresistance is defined by growth of subpopulations on plates containing 8 mg/L of colistin and CD as COL-resistant subpopulations that grow in presence of a COL concentration > 8 mg/L only near the COL disks [2,12-14]. Several authors have defined the influence of mutations in *pmrB* and/or *lpx* genes in growth rate, biofilm formation and fitness decrease [15,16]. Likewise, the regrowth observed in killing curves has been attributed to resistant subpopulations in HR isolates [14,17,18]. However, there are no studies that have evaluated the impact of the two principal COL-resistance mechanisms and the HR on the performance of the susceptibility methods.

The present study aimed to determine the resistance mechanisms in isolates with a COL MIC ≥ 4 mg/L and to correlate the mechanisms of resistance with the difficulties in detecting them with in vitro susceptibility testing. Furthermore, a rapid test to detect COL resistance in *A. baumannii* was evaluated.

## 2. Materials and methods

### 2.1. Bacterial isolates and species identification

A collection of 165 non-duplicated *A. baumannii* strains from different clinical specimens of hospitalised patients was studied during the period 2004–2017. A COL-dependent mutant (CAT-1D) obtained from *A. baumannii* strain (CAT-1), as previously described, was also included [10]. The isolates were recovered from 15 hospitals in six South American countries. These isolates were identified at the Hospital de Clínicas José de San Martín, Buenos Aires city, using standard biochemical tests. Genospecies were confirmed using matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS), and/or by *rpoB* sequencing [19].

### 2.2. Susceptibility testing

The COL MICs were determined using the Mueller-Hinton broth dilution method (BDM) (Oxoid, UK, lot 1900545), using glass tubes during all procedures, and also by the agar dilution method (ADM) (Oxoid, UK, lot 2253971). Dilution methods were performed according to CLSI procedures. The COL susceptibility levels were also determined by Phoenix 100 ID/AST system (Becton Dickinson Co., Sparks, Md.) using the Phoenix NMIC-406 panel, according to the manufacture's recommendations (Pho). In addition, a second reading, by visual examination of the COL-containing wells of the previously mentioned panel, was performed at 24 hours of incubation (Pho24). A change from bluish to pink indicated bacterial growth. Results were interpreted according to CLSI breakpoints [20].

### 2.3. Rapid colistin Acinetobacter spp

All of the isolates were subjected to a rapid colorimetric colistin sensitivity test. Briefly: 1 L of an alkaline solution was prepared containing 10 g peptone, 5 g sodium chloride, 3 g beef extract, and 0.05% rezarzurine, with a pH adjusted to 7.4 ± 0.2. Tubes containing 175 $\mu$l of this solution without (control tube) and with COL (test tube) in a final concentration of 3.8 mg/L were inoculated with 25 µl of a bacterial suspension with a turbidity compatible with 0.5 McFarland, and incubated for 3 hours at 35 °C. The test result was positive when the test tube turned from purple to pink, giving the same colour as the control tube, indicating growth in the presence of COL (COL resistance), whereas the test result was negative when the test tube remained purple (COL susceptible). A concentration of 3.8 mg/L was used as it is slightly lower than the breakpoint. For comparison of methodologies performance, BDM was taken as the reference method. Errors were ranked as follows: very major error, false susceptibility results by ADM, Pho, Pho24 or rapid colistin *Acinetobacter* spp. (RCA); or major errors, false resistant results by ADM, Pho, Pho24 or RCA.

### 2.4. Population analysis

Population analysis screening profiles were performed in duplicate in the 165 isolates according to a previous report, with several modifications [14]. Briefly, the Mueller-Hinton agar (Oxoid, UK, lot 2253971) plates with or without 6 mg/L of COL were inoculated with 50 µl of cell suspension (0.5 Mc Farland). Colonies were counted after 48 hours of incubation at 35 °C. The frequency of resistant subpopulations (HR) was calculated by dividing the growth (CFU) with 6 mg/L of COL by the growth (CFU) without COL.

### 2.5. Time-kill curves

In five selected isolates with different MIC values and gene mutations (2858 COL MIC 32 mg/L and *lpx* gene mutated; 8058 COL MIC 32 mg/L and *pmrB* gene mutated; 52SJ COL MIC 8 mg/L and *lpxC* gene mutated; 98726 COL MIC 4 mg/L and *lpxD* gene mutated; and 269 COL MIC 4 mg/L and *lpxD* gene mutated) the growth speed of resistant isolates with and without COL was evaluated by time-kill studies. The studies were performed in duplicate. Tubes containing cation-supplemented Mueller-Hinton broth (Oxoid, UK, lot 1900545) with and without antibiotic were inoculated with $5 \times 10^5$ CFU/mL of each isolate in log phase/mL. They were incubated at 37 °C and killing was assessed at 0, 4, 6, 16 and 24 hours by performing serial 10-fold dilutions and coating the aliquots onto nutrient agar. The COL concentration was 3 mg/L because it was included in the concentration range of the Phoenix NMIC-406 panel. The killing curves from the five isolates without COL were shown as an average curve (T). Colistin sulfate (Sigma Aldrich, St Louis, USA, lot 070M1499V) was used in all the methods performed in this study.

### 2.6. Detection of lpxACD and pmrB gene mutations

Detecting *lpxACD* and *pmrB* gene mutations in 17 *A. baumannii* isolates was performed by 16S ARN ribosomal amplification following capillary sequencing. Briefly: total DNA was extracted using ADN Puriprep B-kit (INBIO Highway, Argentina) and used as the template for PCR reactions, which were carried out using the 'Kit-T plus ADN polimerasa' enzyme according to manufacturer's instructions (INBIO Highway, Argentina), and the products were detected by agarose gel electrophoresis. The specific primers proposed by Moffatt et al. were used to analyse the presence of different mutations of *lpx*A, *lpx*C and *lpx*D genes [21]. The COL-susceptible *A. baumannii* ATCC19606 strain (GenBank accession number ACQB01000000) was used as the reference sequence. For detecting *pmr*B gene mutations, the specific primers reported by Beceiro et al. were used [22]. The *A. baumanni* ATCC 17978 strain (GenBank accession number CP000521.1) was included as the reference sequence. Sequencing was performed on both DNA

**Table 1**
The MIC distribution, performance of the different colistin-susceptibility tests, and detection methods compared with BMD in the 165 isolates. Categorical agreement analysis.

| BMD MIC (μg/mL) | Number of isolates | Number of isolates (%) | | | |
|---|---|---|---|---|---|
| | | AD | Pho | Pho24 | RCA |
| ≤ 0.5 | 16 | 16 (100) | 16 (100) | 16 (100) | 16 (100) |
| 1 | 59 | 59 (100) | 59 (100) | 59 (100) | 59 (100) |
| 2 | 65 | 65 (100) | 65 (100) | 65 (100) | 65 (100) |
| 4 | 6 | 0 | 0 | 0 | 2 |
| 8 | 2 | 0 | 0 | 0 | 2 |
| 16 | 4 | 4 | 3 | 4 | 4 |
| 32 | 9 | 9 | 5 | 9 | 9 |
| ≥ 64 | 4 | 4 | 4 | 4 | 4 |

AD, agar dilution; Pho, Phoenix NMIC-406; Pho24, Phoenix NMIC-406 manual reading at 24 hrs of incubation; RCA, rapid colistin *Acinetobacter* spp. method; BMD, broth microdilution

strands using an ABIPrism 3100 BioAnalyzer and Taq FS Terminator Chemistry (Taq FS, Perkin–Elmer). Sequences were examined and assembled with BioEdit software (version 7.0.5) and BLAST (version 2.6.0) software.

## 3. Results and Discussion

The 165 clinical isolates were identified as *A. baumannii* by MALDI-TOF and *rpoB* gene sequencing. Twenty-five of these were COL resistant, as determined by the BMD method. The $MIC_{50}$ and $MIC_{90}$ values of the susceptible population were 1 mg/L and 2 mg/L, respectively. A total of 46.7% of the susceptible isolates presented an MIC of 2 mg/L. Fifteen COL-susceptible isolates were categorised as HR; 13 of 15 showed an MIC of 2 mg/L; 2 of 15 an MIC of 1 mg/L; and 0 of 15 an MIC < 1 mg/L.

The correlations between the different methodologies and the reference method are shown in Table 1. In COL-susceptible isolates, the agreement between the different methods was 100%. False resistance was not detected in HR isolates in any of the performed tests; this was possibly due to the fact that resistant subpopulations are only able to grow after 24–48 h of incubation. However, in the 25 COL-resistant isolates, the performance varied according to the COL MIC value. The number of errors in each method in the resistant isolates was as follows: RCA four of 25; ADM eight of 25; Pho24 eight of 25, and Pho 13 of 25. The distribution of very major errors according to the MIC value is shown in Table 2.

In order to determine the genetic basis of colistin resistance in *A. baumannii*, 17 randomly selected isolates were amplified and sequenced with different COL MICs. Nine different *Lpx*A amino acid changes over five mutant isolates, 10 different *Lpx*C amino acid changes over five mutant isolates, and 15 different *Lpx*D amino acid changes over nine mutant isolates were observed (Table 2). Three isolates harboured *Lpx*A mutation at 175 amino acid position. Those three COL-resistant isolates contained the same amino acid change (L175K). Isolates presented an amino acid change over 176 position. Two COL-resistant isolates contained the same mutation at 176 position (I176S). Two COL-resistant isolates presented L175K and I176S amino acid changes with the same COL MIC. The presence of L175K and I176S mutations in the same isolate has no additive effect in the COL-resistant phenotype (Supplementary Table S1). All of the *Lpx*C mutant isolates had N287D amino acid change. The mutation H264N was found in four COL-resistant isolates. The Y260* mutation was found in two COL-resistant isolates. This mutation could have contributed to the increase in colistin MIC in *A. baumannii*. Other mutations in *Lpx*C were found only in COL-resistant isolates (Supplementary Table S1). Eight isolates had F311L amino acid changes to *Lpx*D. Moreover, seven isolates contained E312K amino acid changes to *Lpx*D, being COL-resistant, and two with an MIC value close to the breakpoint (Supplementary Table S1). Five isolates contained a new mutation in the *pmr*B gene. The most prevalent mutation was T232I. Interestingly, all mutations were found in COL-resistant isolates (Supplementary Table S1).

In isolates with COL MIC ≥ 16 mg/L and with a high proportion of resistant subpopulations, two groups were found: one (n = 12)

**Table 2**
Epidemiological, genomic and susceptibility data for *A. baumannii* colistin-resistant isolates.

| Isolate | City | Year | MIC (μg/mL) | | | | | | Chromosomal mutations | | | | Accession number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | BMD | AD | Pho | Pho24 | RAC | HR | LpxA | LpxC | LpxD | PmrB | |
| 87 | BA | 2004 | 64 | 64 | 4 | 4 | P | $2 \times 10^{1}$ | | | *X* | | MK533759 |
| 65027 | BA | 2005 | 4 | **1** | **≤ 1** | **≤ 1** | N | $1 \times 10^{-5}$ | | | *X* | | MK533760 |
| 214 | BA | 2013 | 4 | **1** | **≤ 1** | **≤ 1** | N | $3 \times 10^{-5}$ | | | *X* | | MK533761 |
| 220 | BA | 2013 | 4 | **2** | **≤ 1** | **≤ 1** | N | $2 \times 10^{-5}$ | | | *X* | | MK561297 |
| 259 | BA | 2014 | 16 | 16 | 4 | 4 | P | $2 \times 10^{1}$ | ND | ND | ND | ND | |
| 406 | BA | 2015 | 64 | 64 | 4 | 4 | P | $2 \times 10^{1}$ | ND | ND | ND | ND | |
| 86800 | BA | 2015 | 16 | 16 | 4 | 4 | P | $2 \times 10^{1}$ | ND | ND | ND | ND | |
| 4840 | Mendoza | 2016 | 32 | 32 | **2** | 4 | P | $4 \times 10^{1}$ | *X* | | | *X* | MK561298/MK561299 |
| 3662 | Mendoza | 2016 | 16 | 16 | **2** | 4 | P | $2 \times 10^{1}$ | | | | *X* | MK561300 |
| 2858 | Mendoza | 2016 | 32 | 32 | 4 | 4 | P | $1 \times 10^{1}$ | *X* | | *X* | | MK621809/MK62810 |
| 4888 | Mendoza | 2016 | 16 | 16 | 4 | 4 | P | $2 \times 10^{1}$ | | *X* | *X* | | MK621811/MK621812 |
| 8058 | Mendoza | 2016 | 32 | 32 | **2** | 4 | P | $3 \times 10^{1}$ | | | | *X* | MK621813 |
| 1993 | Mendoza | 2016 | 32 | 32 | 4 | 4 | P | $1 \times 10^{1}$ | *X* | | | | MK621814 |
| 2320 | Mendoza | 2016 | 32 | 32 | 4 | 4 | P | $2 \times 10^{1}$ | *X* | | | | MK621815 |
| 2315 | Mendoza | 2016 | 32 | 32 | 4 | 4 | P | $1 \times 10^{1}$ | ND | ND | ND | ND | |
| 4395 | Mendoza | 2016 | 32 | 32 | **2** | 4 | P | $1 \times 10^{1}$ | ND | ND | ND | ND | |
| 6218 | Mendoza | 2016 | 32 | 32 | 4 | 4 | P | $2 \times 10^{1}$ | | *X* | *X* | | MK621816/MK621817 |
| 2521 | Mendoza | 2016 | 32 | 32 | **2** | 4 | P | $3 \times 10^{1}$ | *X* | *X* | *X* | *X* | MK621818/MK621819 |
| 52SJ | San Juan | 2016 | 8 | **2** | **≤ 1** | **≤ 1** | P | $2 \times 10^{-3}$ | | *X* | | | MK533762 |
| 53SJ | San Juan | 2016 | 4 | **2** | **≤ 1** | **≤ 1** | P | $5 \times 10^{-4}$ | ND | ND | ND | ND | |
| 85 | Chaco | 2016 | 8 | **0.5** | **≤ 1** | **≤ 1** | P | $5 \times 10^{-5}$ | | | | *X* | MK860815 |
| 98726 | BA | 2017 | 4 | **2** | **≤ 1** | **≤ 1** | P | $4 \times 10^{-4}$ | | | *X* | | MK860813 |
| 269 | BA | 2017 | 4 | **1** | **≤ 1** | **≤ 1** | N | $8 \times 10^{-4}$ | | *X* | | | MK860814 |
| 47 | BA | 2018 | 64 | 32 | 4 | 4 | P | $1 \times 10^{1}$ | ND | ND | ND | ND | |
| 270 | BA | 2018 | 64 | 32 | 4 | 4 | P | $2 \times 10^{1}$ | ND | ND | ND | ND | |

AD, agar dilution; Pho, Phoenix NMIC-406; Pho24, Phoenix NMIC-406 manual reading at 24 h of incubation; RCA, rapid colistin *Acinetobacter* spp. method; HR, frequency of resistant subpopulations; BA, Buenos Aires city; P, positive; N, negative; ND, not determined
Bold type, very major errors

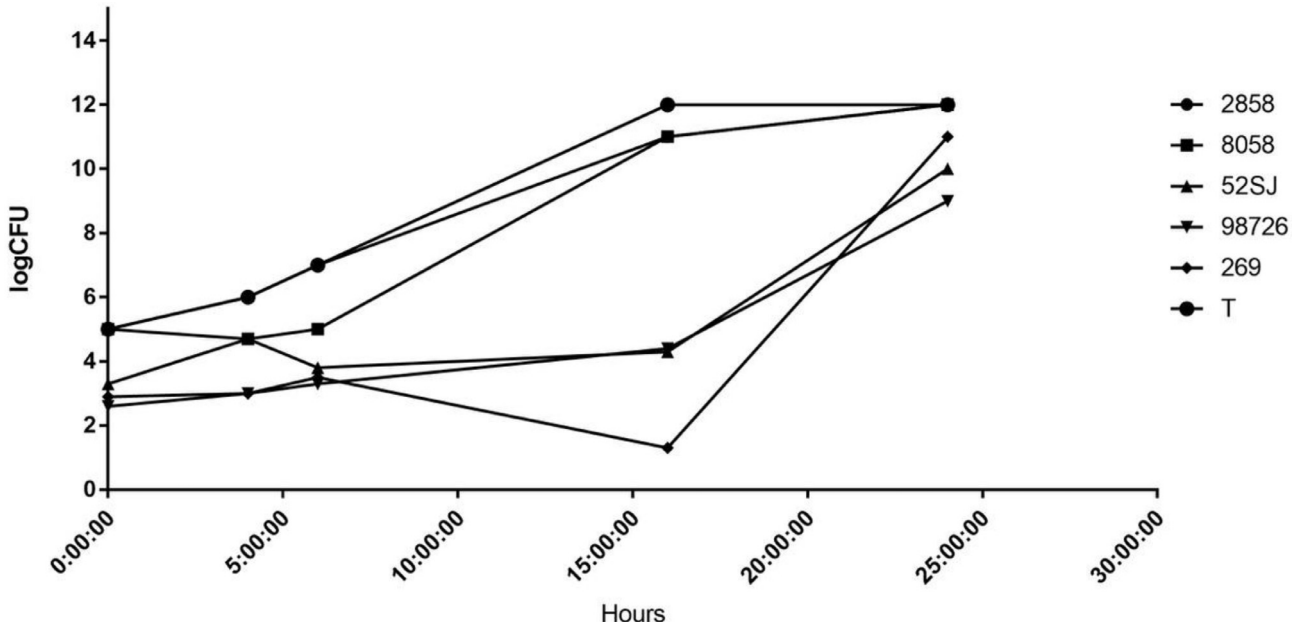


**Fig. 1.** Time-kill studies performed on five colistin-resistant *Acinetobacter baumannii* isolates.

was correctly categorised by Pho and Pho24, and the other (n = 5) was wrongly categorised by Pho, but was corrected when read at 24 h (Pho24). The isolates in the first group harboured heterogeneous mutations in *lpxA* and *lpxC* but not in *pmrB*. As observed in Fig. 1, the isolate 2858 (representative of the first group), which was correctly categorised by Pho, showed a killing curve similar to the T curve. Conversely, the isolates in the second group harboured mutations in the *pmrB*; the resistant CFUs showed a slow growth between 4 and 16 h. This delay in the growth of resistant subpopulations could be the reason why the Phoenix System is unable to detect them, as its final reading is performed at 10–12 h of incubation. The fact that all the isolates belonging to this group were correctly categorised by Pho24 reinforces the idea that the delay in growth speed is the main source of error. No differences were found when comparing the reference method and ADM in isolates with an MIC ≥ 16 mg/L.

There was no agreement between Pho and BMD in isolates with an MIC range of 4–8 mg/L and reading the panel at 24 h did not improve detection in these cases. In these isolates (52SJ, 98726 and 269), growth was slower and there was an increase in the resistant CFUs after 16 h of incubation. Most of these isolates (seven of eight) carried mutations in *lpxD*. Automated systems may also fail to detect these COL-resistant isolates due the low proportion of resistant subpopulations ($1 \times 10^{-4}$ –$1 \times 10^{-5}$). This low frequency would result in 25 COL-R CFU encoding to the inoculum introduced in the panel under the conditions described by the manufacturer; this CFU is 100 000 times lower compared with an homogeneously resistant isolate. Previous reports have shown similar results between BDM and ADM [23]. The current study observed discrepancies in eight borderline isolates. Similar results were described by Dafopoulou [4]. The low proportion of resistant subpopulations (between 20–0.2 CFU/mL at 4 mg /L) in agar dilution spot ($10^4$) could lead to the misdetection of these isolates. Six of seven isolates showed mutations in *lpx* genes.

In the present study, RCA showed the best performance. Discrepancies were observed in four of six isolates with a COL MIC 4 mg/L; this was an expected result as the concentration used in the test was very close to the MIC value. RCA is an in-house rapid colorimetric method for detecting colistin resistance in *Acinetobacter* spp., which is easier to perform than other tests that have been recently described [10].

*Acinetobacter baumannii* COL-dependent isolates have been described in the literature but their presence in clinical isolates is sporadic. The mutant isolate with COL MIC > 64 mg/L included in the current study did not show growth in the automatised system, and in the RCA test it was only able to grow in the tube containing COL. As previously mentioned, major errors were not detected and the growth of resistant subpopulations in the HR isolates was prevented by the early reading. As previously described by other authors [4,6], most of the errors were observed in isolates with MIC values close to the breakpoint.

## 4. Conclusion

There are multiple reasons to explain the failures in detecting COL resistance. The isolates that were correctly categorised showed high MIC values (> 16 mg/L), a high proportion of resistant subpopulations, and a growth speed that did not vary in the presence or absence of COL. While there are less of these characteristics present in COL-resistant isolates, it is more difficult to accurately detect them. The slow growth in isolates carrying mutations in *lpx*C is notorious. This phenomenon is also observed, to a lesser extent, in isolates with mutations in the *pmr*B gene. Heteroresistant subpopulations would also play a vital role in the misdetection and difficulties in observing the final reading point. Other factors that should also be mentioned are the proximity between the breakpoint and the wild type median MIC value.

## Declarations

### Declaration of Competing Interest

There are no conflicts of interest.

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## Ethical Approval

Not required.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.08.010.

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