



# Impact of the Epithelial Lining Fluid Milieu on Amikacin Pharmacodynamics Against *Pseudomonas aeruginosa*

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## Abstract

**Background** Even though nebulised administration of amikacin can achieve high epithelial lining fluid concentrations, this has not translated into improved patient outcomes in clinical trials. One possible reason is that the cellular and chemical composition of the epithelial lining fluid may inhibit amikacin-mediated bacterial killing.

**Objective** The objective of this study was to identify whether the epithelial lining fluid components inhibit amikacin-mediated bacterial killing.

**Methods** Two amikacin-susceptible (minimum inhibitory concentrations of 2 and 8 mg/L) *Pseudomonas aeruginosa* isolates were exposed in vitro to amikacin concentrations up to 976 mg/L in the presence of an acidic pH, mucin and/or surfactant as a means of simulating the epithelial lining fluid, the site of bacterial infection in pneumonia. Pharmacodynamic modelling was used to describe associations between amikacin concentrations, bacterial killing and emergence of resistance.

**Results** In the presence of broth alone, there was rapid and extensive ( $> 6 - \log_{10}$ ) bacterial killing, with emergence of resistance identified in amikacin concentrations  $< 976$  mg/L. In contrast, the rate and extent of bacterial killing was reduced ( $\leq 5 - \log_{10}$ ) when exposed to an acidic pH and mucin. Surfactant did not appreciably impact the bacterial killing or resistance emergence when compared with broth alone for either isolate. The combination of mucin and an acidic pH further reduced the rate of bacterial killing, with the maximal bacterial killing occurring 24 h following initial exposure compared with approximately 4–8 h for either mucin or an acidic pH alone.

**Conclusions** Our findings indicate that simulating the epithelial lining fluid antagonises amikacin-mediated killing of *P. aeruginosa*, even at the high concentrations achieved following nebulised administration.

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## Key Points

The epithelial lining fluid inhibits amikacin-mediated bacterial killing and amplifies the emergence of resistance, despite the high concentrations attained in the epithelial lining fluid following nebulisation

Future dosing formulation and design should account for the negative impact of the epithelial lining fluid to enhance bacterial killing and mitigate against the emergence of resistance

## 1 Introduction

Ventilator-associated pneumonia (VAP) affects up to 38% of mechanically ventilated patients, with an attributable mortality of 13% [1, 2]. Patient outcomes are enhanced by receiving antibiotics promptly that are active against the causative pathogen [1, 2]. Outcomes could be further improved by optimising the free antibiotic concentrations in epithelial lining fluid (ELF), the principal site of infection in VAP [3, 4].

Current guidelines recommend that patients with VAP receive intravenous antibiotics [5]. However, attaining target antibiotic exposures in the ELF is limited by the blood–alveolar barrier, which inhibits antibiotics diffusing into the site of infection [6]. The ELF penetration of anti-pseudomonal aminoglycosides, such as amikacin, is only about 8% of the concurrent plasma concentration [7]. As patient mortality is minimised when the aminoglycoside maximum concentration ( $C_{\max}$ ) relative to the pathogen minimum inhibitory concentration (MIC) exceeds eightfold, the impaired diffusion into the ELF following intravenous administration may be a key determinant of reduced clinical response rates for patients with VAP when receiving intravenous aminoglycosides [5, 8, 9]. Given the reduced amikacin ELF penetration, there has been renewed interest in nebulised therapy for the treatment of *Pseudomonas aeruginosa*, a causative pathogen in 16% of patients with VAP, and where amikacin retains activity for > 90% of multi-drug-resistant isolates [10, 11].

Inhaled delivery methods bypass the blood–alveolar barrier, leading to aminoglycoside concentrations within the ELF that are at least 35-fold greater than those achieved with intravenous administration. Such high antibiotic concentrations correspond to a  $C_{\max}/\text{MIC} > 8$  for susceptible pathogens [7, 12–14]. Despite high amikacin concentrations in ELF following inhalation, no mortality benefit was demonstrated for adjunct inhaled amikacin in two recently published, randomised controlled clinical trials [15–17]. In addition to methodological issues, such as poorly optimised ventilator settings, nebuliser selection and choice of

patient cohorts, possible pharmacokinetic/pharmacodynamic reasons why a clinical benefit was not demonstrated may include heterogeneity in antibiotic lung deposition [18–20], and an inhibitory effect of the ELF, which contains a complex mixture of mucins [21, 22] and surfactant [23] and has an acidic pH [24].

Mucins are glycoproteins that comprise the main macromolecular components of the mucous gel forming the ELF [25]. They have a major protective barrier and clearance function against pathogens. Mucins can also bind amikacin reducing the unbound, pharmacologically active amikacin concentration by up to 60% [21]. Similarly, surfactant, a charged phospholipoprotein produced by type II alveolar cells, has been shown to reduce the unbound fraction of aminoglycosides as much as 17% [23]. The decreased antibiotic free fraction is one potential mechanism mediating impaired aminoglycoside activity in vivo. Indeed, a recent study reported the MIC of *P. aeruginosa* increased 64-fold following the addition of a physiologically relevant concentration of mucin [22]. Moreover, acidification within the bronchi of patients with pneumonia ( $\text{pH } 6.48 \pm 0.12$ ; mean  $\pm$  standard deviation) reduces intracellular aminoglycoside uptake and bacterial killing [24, 26]. From these data, it is apparent that surfactant, an acidic pH and the presence of mucin likely contribute to altered aminoglycoside pharmacodynamics; however, it is unclear how these factors together, simulating the ELF milieu, may influence bacterial killing. Hence, this in vitro study aimed to describe the impact of mucins, surfactant and an acidic pH, alone and in combination, on amikacin-mediated *P. aeruginosa* bacterial killing and the emergence of amikacin resistance.

## 2 Materials and Methods

### 2.1 Antimicrobial Agents

Analytical reference standards of amikacin (LRAA5755; Sigma-Aldrich, Darmstadt, Germany) were used for in vitro susceptibility testing, preparing amikacin-containing cation-adjusted Mueller-Hinton (Ca-MH) agar for quantification of a less-susceptible bacterial population and static time-kill studies. Stock solutions were diluted in sterile water and stored at  $-80^\circ\text{C}$ .

### 2.2 Bacterial Isolates

The clinical *P. aeruginosa* isolates used in this study (#CTAP23 and #CTAP40) were sourced from tertiary hospitals in Brisbane, QLD, Australia, from critically ill patients. A *P. aeruginosa* (ATCC 27853) control isolate was used for MIC testing. Isolates were stored in Ca-MH broth with 20% glycerol v/v at  $-80^\circ\text{C}$ . Prior to susceptibility testing

and static time-kill study experiments, isolates were grown overnight on Ca-MH agar at 37 °C. A McFarland 0.5 standard was prepared using colonies of a similar morphology and further diluted in Ca-MH broth for in vitro susceptibility testing at the desired inoculum. For static time-kill studies, the resultant bacterial suspension was grown at 37 °C with constant agitation until a bacterial inoculum of  $\sim 1 \times 10^8$  colony-forming units (CFU)/mL was obtained based on a prior growth curve analysis.

### 2.3 In Vitro Susceptibility Testing

Minimum inhibitory concentration testing was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing broth microdilution guidelines [27, 28]. Briefly, serial two-fold dilutions of amikacin were prepared in Ca-MH broth in round-bottom and flat-bottom 96-well plates for CLSI and European Committee on Antimicrobial Susceptibility Testing methods, respectively. Bacterial colonies were added to sterile water to an equivalent turbidity of McFarland 0.5 standard. Sufficient volume of this bacterial suspension was added to Ca-MH broth to attain a final well bacterial concentration of  $\sim 5.5 \times 10^6$  CFU/mL. The inoculated plates were incubated for 16–20 h at 37 °C. In accordance with the CLSI guidelines, the round-bottom 96-well plates were visually inspected; the lowest amikacin concentration with no visible growth was defined as the MIC. A Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Ratastie, Finland) was used to determine the optical density of each well in the flat-bottom 96-well plate. The MIC was taken as the concentration of antibiotic resulting in an optical density  $< 0.1$  that of the growth control. Minimum inhibitory concentration tests using both CLSI and European Committee on Antimicrobial Susceptibility Testing methods were performed on two separate occasions with four replicates for each isolate and method. The MIC was the modal value for each method.

### 2.4 Static Time-Kill Assay

Static time-kill experiments were performed to assess the bacterial killing and emergence of amikacin resistance in the simulated ELF. A bacterial suspension was grown in Ca-MH broth to achieve the desired inoculum of  $\sim 1 \times 10^8$  CFU/mL, a bacterial density identified in over 26% of patients with VAP [29]. The bacterial suspension was centrifuged for 10 min at 4400g. The resulting pellet was re-suspended in the desired growth media for each combination of ELF variables (Ca-MH broth alone, mucin, surfactant and pH adjustment) as outlined below. After 24 h, the bacterial suspension was centrifuged and re-suspended in fresh antibiotic media and the associated environmental condition(s).

To simulate and maintain the acidic pH of the ELF during an acute infection, a sodium phosphate buffer was prepared. In brief, a sterile solution of sodium phosphate monobasic (ChemSupply, Gillman, Australia, batch number 268025) and sodium phosphate dibasic (Sigma-Aldrich, Darmstadt, Germany, batch number K46477180512) reagent was diluted in Ca-MH broth to achieve a final concentration of 0.1 M for both compounds. The pH was subsequently adjusted to 6.48 and 7.40 for the ELF milieu simulation and control, respectively, using sterile hydrochloric acid [24]. This was shown previously to maintain the desired pH within 0.20 of the target after 24 h. Previous growth controls for both isolates did not show a difference in bacterial growth between isolates exposed to the buffer or in Ca-MH broth alone. Porcine mucin (Sigma Aldrich, batch number SLBV4979) was added to the appropriate experimental arm to a final concentration of 5% w/v to simulate sputum [30]. A surfactant preparation used in clinical practice (Poractant alfa; Emerge Health, Hawthorn East, Australia, batch number 1063838) at a concentration of 0.044 mg/mL was employed as this approximated a concentration found in the ELF of patients with sepsis [31].

Amikacin concentrations of 976.1 mg/L and 410.3 mg/L were adopted for all experiments, reflecting the median and lower quartile maximum amikacin ELF concentrations, respectively, in a sample of mechanically ventilated patients receiving nebulised amikacin [14]. Amikacin concentrations of 488.1 mg/L and 205.2 mg/L were used in Ca-MH broth alone as a control experiment assuming 50% mucin binding. A growth control was included for each isolate and a combination of ELF milieu variables. Samples were taken at 0, 4, 8, 24, 32 and 48 hours and 0, 24 and 48 hours for quantification on Ca-MH agar and amikacin-supplemented Ca-MH agar, respectively, at a concentration four-fold the baseline isolate MIC. Samples were washed twice in phosphate-buffered saline to minimize antibiotic carry-over, appropriately diluted and plated for quantification. The detection limit was  $2 - \log_{10}$  CFU/mL. One replicate was performed for each combination of isolate, amikacin concentration and milieu condition.

### 2.5 Pharmacodynamic Modelling

The pharmacokinetic/pharmacodynamic data of both bacterial isolates were co-modelled using Pmetrics for R Version 1.5.2 based on previously described models of bacterial growth and resistance formation [32, 33]. Model parameter abbreviations are in Tables 1 and 2. Model diagnostics including the Akaike information criterion, Bayesian information criterion and coefficient of determination ( $R^2$ ) from the linear observed vs expected plots were used to evaluate and compare models.

Table 1 #CTAP23; combined parameter estimates

Parameter	Abbreviation	Ca-MH broth	pH	Surfactant	Mucin	Mucin + surfactant	Mucin + pH	Surfactant + pH	Mucin + pH + surfactant
		Mean (SD)							
Susceptible growth rate constant ( $\log_{10}$ CFU/mL/h)	$Kgmax_s$	1.88 (0.45)	1.71 (0.56)	2.15 (0.72)	2.35 (1.04)	2.29 (0.99)	2.12 (1.27)	1.89 (0.86)	2.95 (0.78)
Intermediate growth rate constant ( $\log_{10}$ CFU/mL/h)	$Kgmax_i$	0.11 (0.08)	0.13 (0.07)	0.12 (0.09)	0.13 (0.08)	0.13 (0.08)	0.27 (0.16)	0.16 (0.08)	0.31 (0.18)
Resistant growth rate constant ( $\log_{10}$ CFU/mL/h)	$Kgmax_r$	0.28 (0.11)	0.32 (0.1)	0.24 (0.14)	0.36 (0.09)	0.34 (0.1)	0.38 (0.11)	0.26 (0.15)	0.4 (0.13)
Maximum bacterial population (CFU/mL)	POPMAX	$6.1 \times 10^{12}$ ( $2.9 \times 10^{12}$ )	$5.4 \times 10^{12}$ ( $3.0 \times 10^{12}$ )	$4.7 \times 10^{12}$ ( $2.3 \times 10^{12}$ )	$5.8 \times 10^{12}$ ( $2.7 \times 10^{12}$ )	$5.6 \times 10^{12}$ ( $2.5 \times 10^{12}$ )	$3.8 \times 10^{12}$ ( $1.8 \times 10^{12}$ )	$4.3 \times 10^{12}$ ( $2.2 \times 10^{12}$ )	$4.5 \times 10^{12}$ ( $2.2 \times 10^{12}$ )
Susceptible killing rate constant ( $\log_{10}$ CFU/mL/h)	Kkillmax <sub>s</sub>	8.64 (3.1)	8.49 (3.89)	6.98 (2.82)	4.61 (2.63)	4.78 (2.73)	3.62 (2.27)	4.27 (2.04)	5.26 (2.35)
Intermediate killing rate constant ( $\log_{10}$ CFU/mL/h)	Kkillmax <sub>i</sub>	16.39 (9.26)	10.46 (9.97)	19.13 (7.7)	21.22 (6.13)	20.77 (6.46)	24.04 (6.24)	22.86 (6.39)	22.86 (7.47)
Amikacin concentration causing 50% Emax (mg/L)	EC50 <sub>s</sub>	141.28 (86.96)	136.65 (76.82)	124.98 (58.73)	162.6 (67.8)	155.68 (68.93)	98.78 (57.29)	126.98 (58.15)	109.9 (66.4)
Amikacin concentration causing 50% Emax <sub>i</sub> (mg/L)	EC50 <sub>i</sub>	2085.95 (584.73)	–	1753.71 (548.38)	1553.95 (633.82)	1319.28 (654.12)	–	–	–
Amikacin concentration causing 50% Emax <sub>i</sub> (mg/L) acidic pH	EC50 <sub>i,pH</sub>	–	6240.98 (2088.56)	–	–	–	3703.68 (1547.13)	4127.66 (1400.7)	3928.98 (1667.71)
Susceptible Hill coefficient	Hs	7.14 (3.28)	8.51 (3.76)	5.77 (2.32)	6.19 (3.51)	6.58 (2.61)	7.76 (2.06)	7.01 (2.28)	5.9 (2.46)
Intermediate Hill coefficient	Hi	12.75 (5.62)	10.19 (6.77)	14.21 (6.1)	10.28 (5.94)	12.17 (5.58)	10.15 (7.48)	14.25 (6.59)	5.99 (5.26)

Table 1 (continued)

Parameter	Abbreviation	Ca-MH broth		pH		Surfactant		Mucin		Mucin + sur- factant		Mucin + pH		Surfactant + pH		Mucin + pH + surfactant	
		Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD	Mean (SD)	SD
Resistant lag time (h)	RLag	29 (5.03)		30.18 (5.68)		31.27 (7.05)		30.48 (7.7)		31.75 (8.64)		28.97 (11.66)		32.29 (7.99)		22.05 (6.91)	
Quiescence transfer constant (h <sup>-1</sup> )	Q <sub>i</sub>	7.36 (2.93)		8.49 (1.69)		8.54 (2.41)		8.23 (1.76)		8.44 (1.71)		8.46 (1.58)		8.54 (2.02)		8.52 (1.75)	
Resistance transfer constant (h <sup>-1</sup> )	Q <sub>r</sub>	4.67 (2.98)		4.06 (3.71)		5.25 (2.73)		2.94 (1.74)		3.28 (1.82)		2.07 (2.07)		4.41 (2.42)		1.91 (2.41)	
Resistance threshold	Thresh	0.51 (0.05)		0.51 (0.06)		0.51 (0.04)		–		–		0.18 (0.02)		0.51 (0.06)		–	
Resistance threshold with mucin	Thresh <sub>Muc</sub>	–		–		–		0.17 (0.02)		0.17 (0.02)		0.18 (0.02)		–		0.18 (0.02)	
Quiescent bacterial population initial exposure	Quies	362.42 (118.94)		293.19 (107.46)		284.7 (149.44)		263.03 (110.77)		238.16 (126.26)		131.3 (101.08)		261.22 (127.55)		166.54 (143.06)	

Ca-MH cation-adjusted Mueller-Hinton, CFU colony-forming units, SD standard deviation

### 3 Results

#### 3.1 In Vitro Susceptibility Studies

The modal amikacin MIC was 2 mg/L and 8 mg/L for #CTAP23 and #CTAP40, respectively.

#### 3.2 Static Time-Kill Studies

For all amikacin concentrations against isolates exposed to standard Ca-MH broth, there was complete eradication of viable bacterial growth within 24 h, which was sustained for the 48-h experiment duration for the 976.1-mg/L exposure (Fig. 1; Table 1). Lower amikacin concentrations resulted in some bacterial regrowth from a nadir of no detected viable bacteria at 24 h to approximate 4-logs of bacterial regrowth, which was also observed on amikacin-containing Ca-MH agar (Fig. 2). For both isolates, the addition of an acidic pH reduced the extent and rate of bacterial killing, with a maximum 7-logs of bacterial killing occurring by 24 h for both isolates exposed to 976.1 mg/L and a maximum 3-logs of bacterial killing for both isolates exposed to 410.3 mg/L (Fig. 1; Table 3). Furthermore, the addition of mucin and an acidic pH also reduced the rate of bacterial killing for both isolates. After 8 h, there was approximately 7-logs and 2-logs of bacterial killing when exposed to either an acidic pH or mucin and the combination of the two, respectively, for both isolates in 976.1 mg/L of amikacin. The bacterial killing was approximately half in the same milieu (approximately 3- to 4-logs of bacterial killing for either an acidic pH or mucin alone and an approximately 1-log reduction when exposed to both an acidic pH and mucin) for both isolates in an amikacin concentration of 410.3 mg/L. After 24 h, rapid bacterial re-growth was observed with a corresponding increased growth of bacteria on amikacin-containing Ca-MH agar (Fig. 2). However, the rate and extent of regrowth differed between study isolates, with increased regrowth demonstrated by #CTAP23 (Table 3). Similarly, the combination of mucin and an acidic pH environment reduced the maximum bacterial killing to 3-logs with a slower rate when compared with the addition of either mucin or an acidic pH. After the total bacterial nadir at approximately 24 h, there was a rapid emergence of bacterial growth on both standard and amikacin-containing Ca-MH agar. The addition of surfactant did not appreciably impact bacterial killing when compared with Ca-MH broth alone. No appreciable difference was observed between the growth curves of isolates exposed to mucin, surfactant or an acidic pH compared with Ca-MH broth alone (Fig. 3).

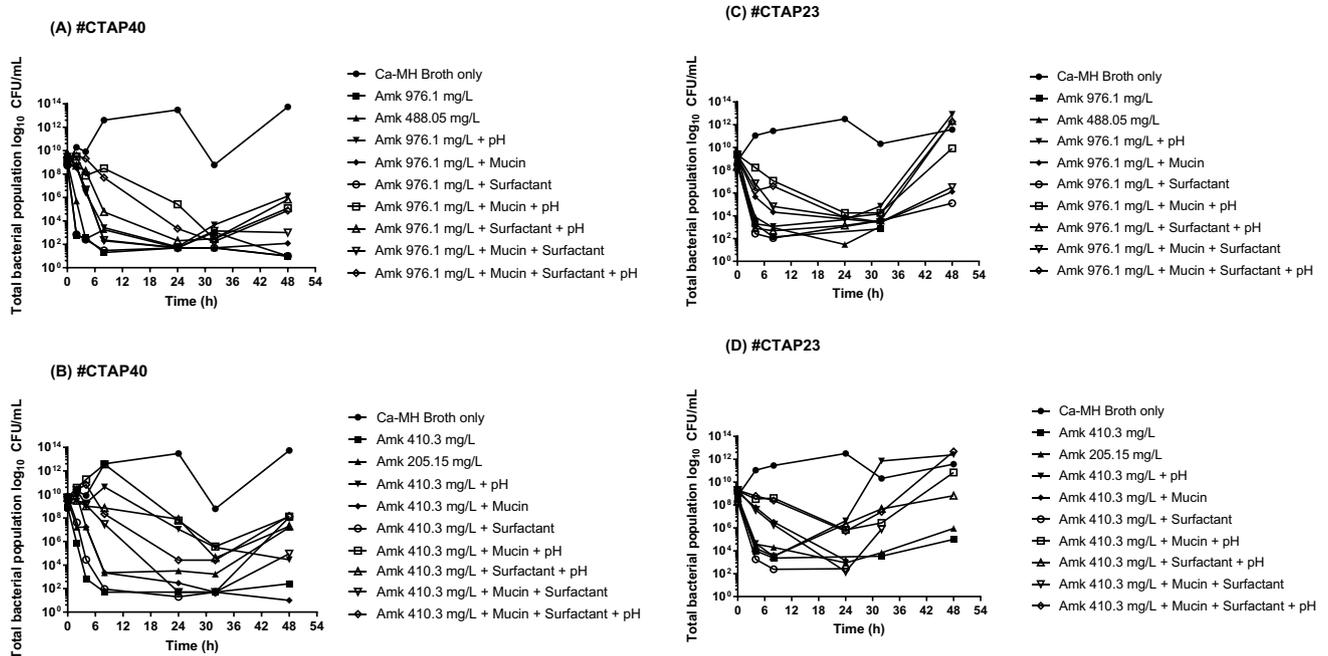
**Table 2** #CTAP40; combined parameter estimates

Parameter	Abbreviation	Ca-MH broth Mean (SD)	pH Mean (SD)	Surfactant Mean (SD)	Mucin Mean (SD)	Mucin + surfactant Mean (SD)	Mucin + pH Mean (SD)	Surfactant + pH Mean (SD)	Mucin + pH + surfactant Mean (SD)
Susceptible growth rate constant ( $\log_{10}$ CFU/mL/h)	Kgmax <sub>s</sub>	2.12 (0.88)	2.07 (1.12)	1.62 (0.87)	1.7 (0.7)	2.25 (1.05)	2.42 (1.2)	2.33 (1.27)	2.4 (1.29)
Intermediate growth rate constant ( $\log_{10}$ CFU/mL/h)	Kgmax <sub>i</sub>	0.05 (0.07)	0.16 (0.11)	0.07 (0.09)	0.21 (0.07)	0.2 (0.1)	0.29 (0.14)	0.16 (0.07)	0.13 (0.09)
Resistant growth rate constant ( $\log_{10}$ CFU/mL/h)	Kgmax <sub>r</sub>	0.26 (0.16)	0.22 (0.18)	0.35 (0.13)	0.12 (0.15)	0.26 (0.16)	0.41 (0.09)	0.37 (0.13)	0.37 (0.07)
Maximum bacterial population (CFU/mL)	POPMAX	$4.4 \times 10^{12}$ (2.5)	$4.0 \times 10^{12}$ ( $3.9 \times 10^{12}$ )	$4.3 \times 10^{12}$ ( $3.6 \times 10^{12}$ )	$4.9 \times 10^{12}$ ( $1.6 \times 10^{12}$ )	$4.0 \times 10^{12}$ ( $3.3 \times 10^{12}$ )	$3.1 \times 10^{12}$ ( $2.7 \times 10^{12}$ )	$5.0 \times 10^{12}$ ( $3.5 \times 10^{12}$ )	$5.3 \times 10^{12}$ ( $4.1 \times 10^{12}$ )
Susceptible killing rate constant ( $\log_{10}$ CFU/mL/h)	Kkillmax <sub>s</sub>	6.03 (2.59)	3.43 (1.85)	5.19 (3.31)	4.23 (2.2)	4.33 (2.23)	3.63 (2.2)	3.9 (1.73)	4.21 (2.45)
Intermediate killing rate constant ( $\log_{10}$ CFU/mL/h)	Kkillmax <sub>i</sub>	20.58 (4.68)	15.28 (9.07)	20.96 (5.98)	12.33 (6.85)	21.33 (6.15)	26.14 (5.72)	21.45 (6.31)	23.33 (5.89)
Amikacin concentration causing 50% Emax (mg/L)	EC50 <sub>s</sub>	126.81 (61.38)	165.45 (61.83)	136.23 (59.12)	178.13 (64.06)	134.31 (84.72)	129.39 (69.48)	180.63 (73.51)	169.63 (63.13)
Amikacin concentration causing 50% Emax <sub>i</sub> (mg/L)	EC50 <sub>i</sub>	1940.22 (602.8)	–	1728.19 (708.66)	1353.81 (408.55)	1562.25 (435.98)	–	–	–
Amikacin concentration causing 50% Emax <sub>i</sub> (mg/L) acidic pH	EC50 <sub>i_pH</sub>	–	3818.05 (1480.55)	–	–	–	4220.81 (1329.18)	4228.27 (1288.75)	4513 (1266.74)
Susceptible Hill coefficient	Hs	5.32 (2.04)	7.13 (3.43)	7.24 (2.34)	9.09 (2.52)	7.66 (3.14)	7.7 (2.48)	6.22 (3.61)	6.78 (4.56)

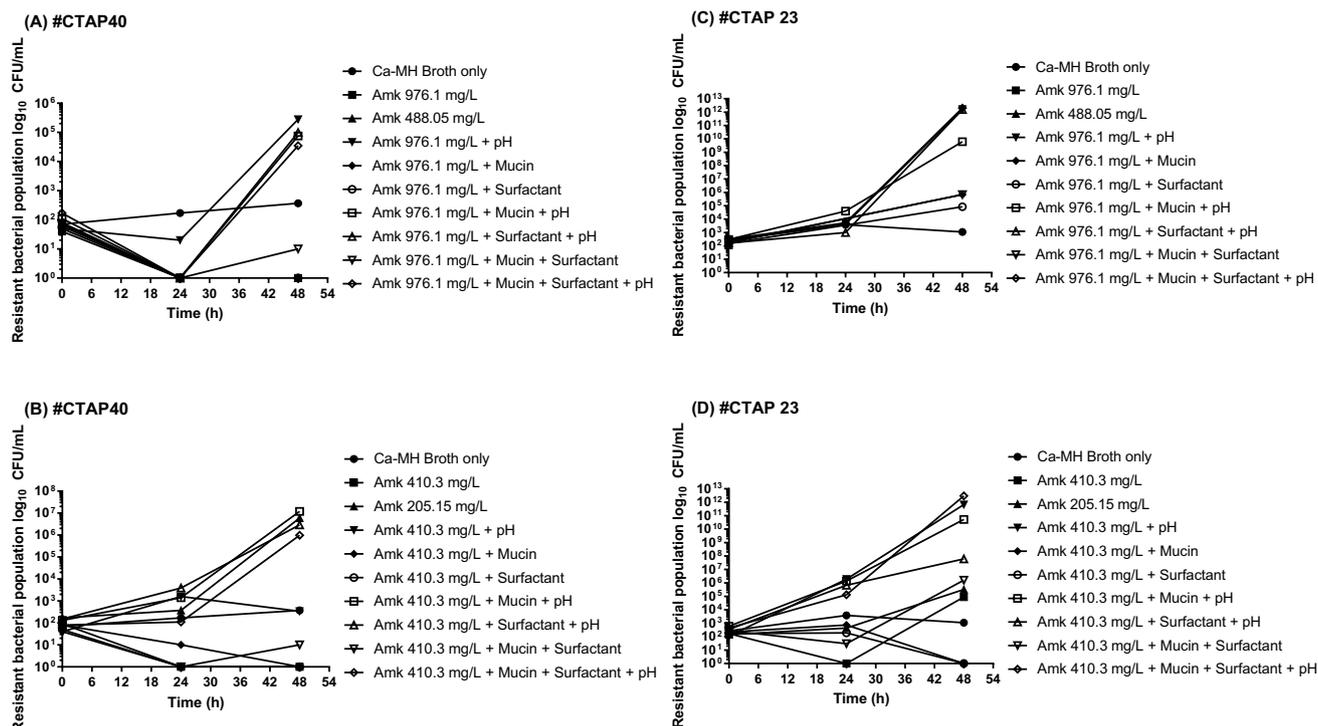
**Table 2** (continued)

Parameter	Abbreviation	Ca-MH broth Mean (SD)	pH Mean (SD)	Surfactant Mean (SD)	Mucin Mean (SD)	Mucin + surfactant Mean (SD)	Mucin + pH Mean (SD)	Surfactant + pH Mean (SD)	Mucin + pH + surfactant Mean (SD)
Intermediate Hill coefficient	Hi	14.95 (4.61)	4.91 (3.81)	8.18 (2.59)	5.48 (4.14)	6.69 (4.05)	4.05 (3.25)	6.41 (3.37)	5.26 (3.39)
Resistant lag time (h)	RLag	32.8 (6.52)	24.2 (6.7)	36.34 (5.19)	22.92 (5.18)	24.06 (10.19)	25.72 (8.3)	28.62 (6.86)	28.81 (5.92)
Quiescence transfer constant (h <sup>-1</sup> )	Q <sub>i</sub>	8.04 (2.5)	5.33 (3.57)	8.03 (1.95)	3.89 (3.42)	7.71 (2.02)	9.05 (1.33)	8.32 (2.41)	7.21 (1.66)
Resistance transfer constant (h <sup>-1</sup> )	Q <sub>r</sub>	4.01 (1.77)	2.88 (1.46)	4.67 (2.63)	6.79 (2.66)	3.29 (1.46)	1.34 (1.26)	2.49 (1.54)	2.22 (1.12)
Resistance threshold	Thresh	0.51 (0.05)	0.52 (0.07)	0.54 (0.09)	–	–	–	0.55 (0.1)	–
Resistance threshold with mucin	Thresh <sub>Muc</sub>	–	–	–	0.17 (0.01)	0.18 (0.02)	0.19 (0.02)	–	0.17 (0.03)
Quiescent bacterial population initial exposure	Quies	199.54 (168.71)	295.35 (69.93)	246.68 (196.69)	133.44 (123.66)	267.8 (87.22)	223.05 (123.94)	269.98 (72.37)	312.32 (76.81)

CFU colony-forming units, Ca-MH cation-adjusted Mueller-Hinton, SD standard deviation



**Fig. 1** Total bacterial killing curves for amikacin (Amk) in simulated epithelial lining fluid milieu. **a** #CTAP40 976.1 mg/L; **b** #CTAP40 410.3 mg/L; **c** #CTAP23 976.1 mg/L; **d** #CTAP23 410.3 mg/L. CFU colony-forming units



**Fig. 2** Resistant bacterial killing curves for amikacin (Amk) in simulated epithelial lining fluid milieu. **a** #CTAP40 976.1 mg/L; **b** #CTAP40 410.3 mg/L; **c** #CTAP23 976.1 mg/L, **d** #CTAP23 410.3 mg/L. CFU colony-forming units

**Table 3** Change in bacterial kill ( $\log_{10}$  CFU/mL) for both isolates exposed to amikacin at a concentration of 976.1 and 410.3 mg/L

Condition	Amikacin 976.1 mg/L				Amikacin 410.3 mg/L			
	#CTAP40		#CTAP23		#CTAP40		#CTAP23	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Broth only	-7.16	-7.86	-8.61	-8.61	-7.19	-6.48	-8.66	-3.66
Broth + pH	-7.19	-2.85	-5.80	3.46	-2.08	-4.67	-2.09	3.67
Broth + mucin	-7.11	-6.73	-9.17	-3.05	-7.29	-8.75	-5.96	-9.11
Broth + surfactant	-7.03	-7.73	-8.51	-3.40	-8.49	-1.72	-5.87	-8.30
Broth + mucin + pH	-3.70	-4.03	-5.10	0.57	-1.95	-1.62	-3.42	1.59
Broth + surfactant + pH	-7.43	-3.91	-5.25	3.93	-1.85	-2.45	-1.98	0.49
Broth + mucin + surfactant	-7.76	-6.46	-9.33	-2.82	-7.83	-4.56	-7.14	-9.32
Broth + mucin + surfactant + pH	-6.10	-4.57	-5.36	3.07	-5.00	-1.24	-3.58	3.39

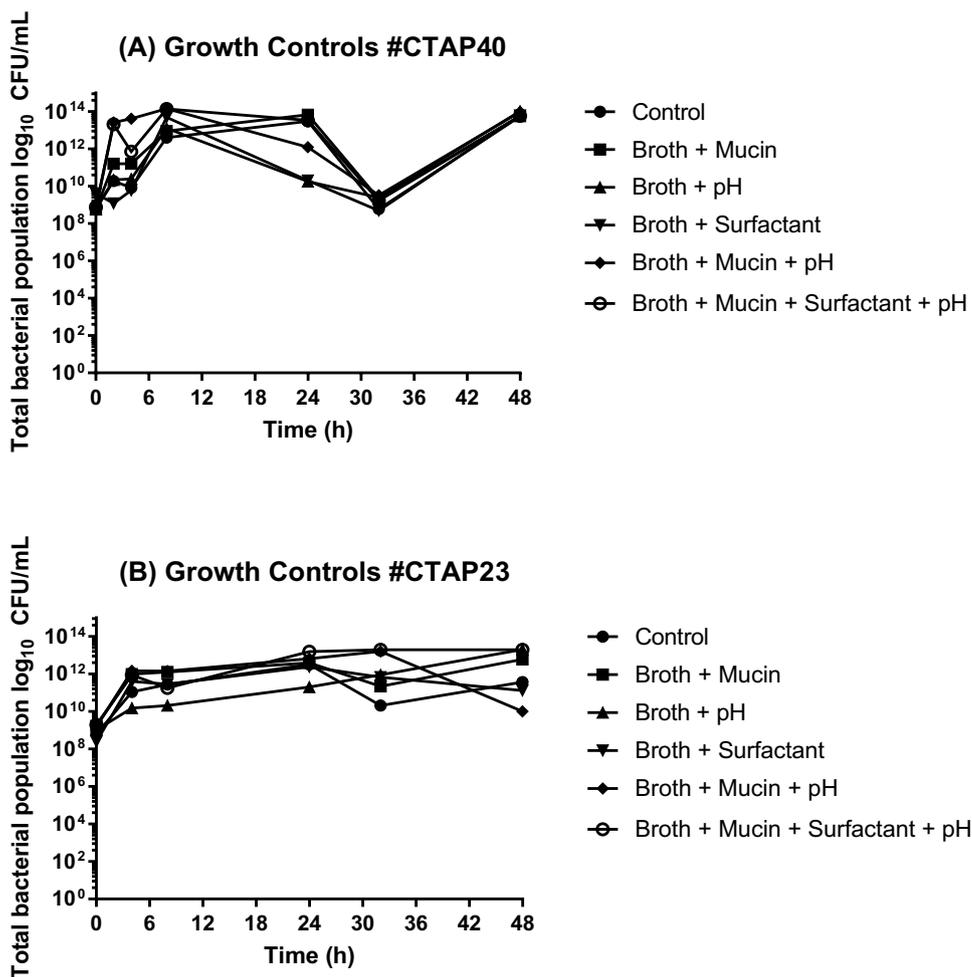
CFU colony-forming units, *h* hours

### 3.3 Pharmacodynamic Modelling

Our pharmacodynamic model is represented by Eqs. (1–4) that detail the growth and killing characteristics of the susceptible, intermediate and resistant bacterial populations, as well as the change in the quiescent bacterial population. Susceptible and intermediate bacterial populations were best described by incorporating amikacin-mediated bacterial killing and de novo emergence of resistance from a quiescent bacterial population. The formation of a quiescent

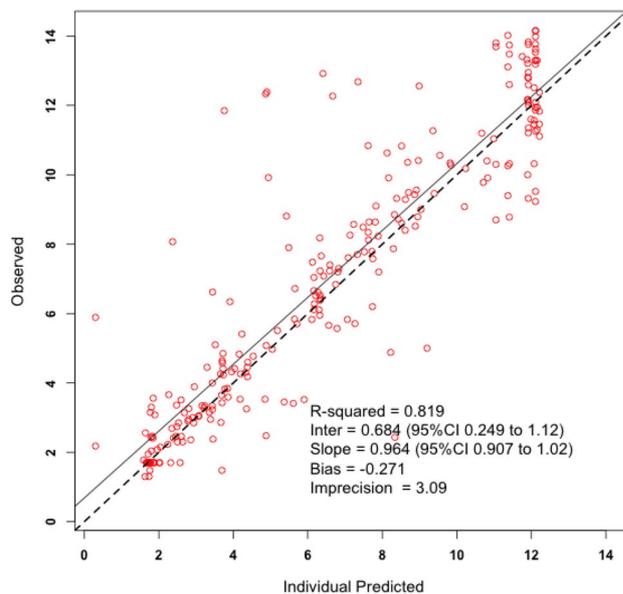
population was facilitated by a transfer from the susceptible and intermediate subpopulations ( $Q_i$ ) when a threshold bacterial density was reached, which was modelled as a parameter (Thresh) multiplied by the population maximum. There was no transfer from the susceptible or intermediate bacterial compartments to the quiescent state before the threshold bacterial density was reached. This value was separated into two variables, one derived from experimental conditions with mucin and another including experimental conditions excluding mucin. The initial bacterial density of the

**Fig. 3** Bacterial growth curves in different simulated epithelial lining fluid environments for #CTAP40 (a) and #CTAP23 (b). CFU colony-forming units

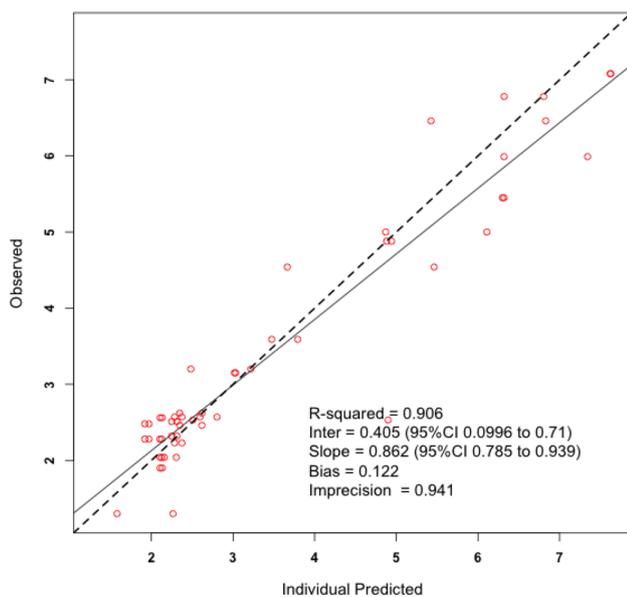


quiescent (Quies; Tables 1 and 2) population was estimated. The resistant lag time (RLag; Tables 1 and 2) represents the estimated lead time taken before bacteria are able to transfer from the quiescent population state to the resistant state. The amikacin concentration achieving 50% of the maximal bacterial killing for the intermediate bacterial subpopulation (EC50i) was separated into two variables, one derived from the presence of an acidic pH and another derived from experimental conditions without an acidic pH.

The model was able to describe the bacterial killing and emergence of amikacin resistance in the presence of mucin, surfactant and an acidic pH. Posterior  $R^2$  estimates were 0.819 and 0.906 for the total bacterial population and the combination of resistant and intermediate susceptibility isolates, respectively (Figs. 4 and 5 for the total bacterial population and intermediate/resistant population, respectively). Incorporating the pH and mucin as covariates improved the Akaike information criterion by 5.6 and improved the observed vs predicted plots. Conditions including an acidic pH and mucin had an appreciable reduction in the onset of resistance emergence and a reduced threshold for the onset of bacterial transfer to a quiescent state (Tables 1 and 2).



**Fig. 4** Observed vs predicted plot of the posterior model estimates for the total bacterial population. CI confidence interval



**Fig. 5** Observed vs predicted plot of the posterior model estimates for the intermediate and resistant bacterial subpopulations. *CI* confidence interval

Moreover, an acidic pH appreciably increased the  $EC_{50i}$ , reducing the bacterial killing of the intermediate subpopulation. The final model visual predictive checks are available in Figs. 1 (total bacterial population) and 2 (resistant and intermediate bacterial population) of the Electronic Supplementary Material.

$$\begin{aligned} \frac{dCFU_s}{dt} = & K_{gmax,s} \times CFU_s \times \left( 1 - \frac{CFU_s + CFU_i + CFU_r + CFU_q}{POP_{MAX}} \right) \\ & - CFU_s \times K_{killmax,s} \times \left( \frac{AMK^{H_s}}{EC_{50_s}^{H_s} + AMK^{H_s}} \right) - Q_i \times CFU_s \end{aligned} \quad (1)$$

$$\begin{aligned} \frac{dCFU_i}{dt} = & K_{gmax,i} \times CFU_i \times \left( 1 - \frac{CFU_s + CFU_i + CFU_r + CFU_q}{POP_{MAX}} \right) \\ & - CFU_i \times K_{killmax,i} \times \left( \frac{AMK^{H_i}}{EC_{50_i}^{H_i} + AMK^{H_i}} \right) - Q_i \times CFU_i \end{aligned} \quad (2)$$

$$\begin{aligned} \frac{dCFU_r}{dt} = & K_{gmax,r} \times CFU_r \\ & \times \left( 1 - \frac{CFU_s + CFU_i + CFU_r + CFU_q}{POP_{MAX}} \right) + Q_r \times CFU_q \end{aligned} \quad (3)$$

$$\frac{dCFU_q}{dt} = Q_i \times (CFU_s + CFU_i) - Q_r \times CFU_q \quad (4)$$

where  $CFU_i$  are  $\log_{10}$  colony-forming units/mL of the intermediate bacterial population,  $CFU_q$  are the  $\log_{10}$  colony-forming units/mL of the quiescent bacterial subpopulation,  $CFU_r$  are  $\log_{10}$  colony-forming units/mL of the resistant bacterial subpopulation,  $CFU_s$  are  $\log_{10}$  colony-forming

units/mL of the susceptible bacterial population,  $EC_{50i}$  is the amikacin concentration causing 50%  $E_{maxi}$  (mg/L),  $EC_{50s}$  is the amikacin concentration causing 50%  $E_{maxs}$  (mg/L),  $H_i$  is the Hill constant for the intermediate bacterial population,  $H_s$  is the Hill constant for the susceptible bacterial population,  $K_{gmax,i}$  is the growth rate constant of the intermediate bacterial population  $\log_{10}$  CFU/mL/h,  $K_{gmax,r}$  is the growth rate constant of the resistant bacterial population  $\log_{10}$  CFU/mL/h,  $K_{gmax,s}$  is the growth rate constant of the susceptible bacterial population  $\log_{10}$  CFU/mL/h,  $K_{killmax,s}$  is the maximum amikacin bacterial killing rate constant for the susceptible bacterial population  $\log_{10}$  CFU/mL/h,  $K_{killmax,i}$  is the maximum amikacin bacterial killing rate constant for the intermediate population  $\log_{10}$  CFU/mL/h,  $POP_{MAX}$  is the maximum bacterial population CFU/mL,  $Q_i$  is the rate transfer constant from the susceptible and intermediate bacterial populations to the quiescent bacterial population that was activated after a bacterial threshold was reached, which was altered by the presence of mucin  $h^{-1}$ , and  $Q_r$  is the rate transfer constant from the quiescent bacterial population to the resistant bacterial population  $h^{-1}$ .

## 4 Discussion

Our data support the hypothesis that the ELF antagonises bacterial killing of amikacin and amplifies emerging *P. aeruginosa* resistance. Importantly, this antagonism is observed at the high ELF amikacin concentrations that are achieved following inhalational delivery.

Our study results are consistent with the published data. A previous in vitro study investigated the impact of mucin on the tobramycin MIC against several bacterial species. [22]. Consistently, the addition of porcine mucin 2% increased the MIC up to 64-fold, which was attributed to the reduction in the free unbound tobramycin concentrations resulting from mucin binding [22]. Similarly, we identified an appreciable reduction in the rate, but not extent of bacterial killing by amikacin at a concentration of either 976.1 mg/L or 410.3 mg/L when mucin was added, compared with 488.1 mg/L or 205.2 mg/L in Ca-MH broth alone. A potential reason for the lack of a difference in the total bacterial killing over 24 h is the high amikacin concentrations used in our study, relative to the lower concentrations used in the mucin MIC experiments with tobramycin [22]. These factors, in addition to the lack of an identified difference in bacterial killing model parameters, would suggest that the effect of mucin may be multifactorial by promoting *P. aeruginosa* biofilm formation, thereby contributing to the emergence of amikacin resistance and reducing the rate of bacterial killing [34, 35]. It is likely that the effect of mucin extends beyond reducing the unbound fraction, given the difference in resistance emergence and bacterial killing and the similar

bacterial killing curves for a 976.1-mg/L and 488.05-mg/L amikacin concentration when exposed to broth alone. Additionally, bacterial killing was most impaired when mucin and an acidic milieu were combined, potentially suggesting an interaction effect whereby a reduction in pH induces a mucin state change. Another possible mechanism of mucin is inducing adaptive resistance, which was not identified in our study [36]. Additionally, the bacterial regrowth appeared to be increased at 48 h for #CTAP23 (MIC 2 mg/L) compared with #CTAP40 (MIC 8 mg/L), which may highlight a differential response between isolates to the selective pressure of amikacin and the ELF milieu.

The acidic ELF environment, in combination with mucin, further reduces the rate and extent of bacterial killing. However, this effect may be reversible. Introducing an alkaline media by adding L-arginine can restore aminoglycoside bacterial killing and inhibit biofilm formation, which warrants further examination [37]. Similar to mucin, studies have identified that surfactant at a concentration of 25 mg/mL can also antagonise tobramycin [23]. This contrasts with our study, which used a lower, physiologically appropriate surfactant concentration of 44 µg/mL, and we failed to identify an appreciable difference in bacterial killing or emerging resistance compared with CA-MH broth alone [31].

Patients prescribed aminoglycosides as monotherapy for pneumonia have reduced clinical cure rates compared with other sites of infection, such as the urinary tract [38, 39]. In addition to the reduced ELF: plasma penetration of amikacin following intravenous administration, the ELF inhibits aminoglycoside-mediated bacterial killing and suppresses neutrophil-mediated phagocytosis and bacterial eradication [40]. Therefore, the ability of intravenous aminoglycoside monotherapy to reduce the bacterial burden to below  $1 \times 10^6$  CFU/mL, a threshold associated with maximal granulocyte bacterial clearance, is likely low and may only be possible in patients achieving the maximal possible aminoglycoside exposure following nebulised therapy [29]. Furthermore, amplifying less-susceptible bacterial subpopulations identified in this study when bacteria were exposed to an acidic pH and to mucins highlights the importance of the ELF contributing to antibiotic resistance developing in patients with *P. aeruginosa* VAP [41]. In a small study (314 patients), 18% of those with recurrent VAP had amikacin-resistant isolates when this antibiotic was used to treat the original infection [41]. Taken together, it is likely that inhibition of both amikacin- and granulocyte-mediated bacterial killing by the ELF substantially contributes to the failure of previous clinical trials to demonstrate a clinical benefit from adding inhaled amikacin to standard VAP antibiotic management.

Nevertheless, our study has several limitations. First, a range of mucin concentrations has been used previously in similar studies; however, no study has yet reported the actual

mucin concentration range in patients with VAP [21, 22, 30]. Therefore, it is likely that other mucin concentrations may exist in vivo and indeed in different lung anatomical compartments. Second, we did not simulate the full amikacin concentration–time curve that may occur following nebulised administration and the inter-patient variability in the concentration–time curve within the ELF following inhalation [14]. However, we simulated the expected  $C_{max}$ , which would likely provide the maximum bacterial killing, but may also alter the propensity for antibiotic resistance emergence relative to the short duration of time the  $C_{max}$  would be achieved in vivo [42]. Third, we did not replicate the dynamic movement of mucin secretion and clearance as would occur in vivo. Fourth, porcine mucin was used that may have increased sulfation in composition to that found in human patients leading to an increased negative charge [43]; however, the core mucin structures are shared between porcine and human mucin [44, 45]. Fifth, we did not examine the effect of positively charged extra-cellular DNA in ELF upon amikacin activity, although prior studies suggest this would be minimal [30]. Sixth, we did not account for the effect of the host immune response for bacterial killing. Seventh, porcine surfactant was used to simulate the ELF environment, which differs in composition to human surfactant with less neutral lipids and reduced concentrations of surfactant proteins A-D, most notably A and D [46]. Last, we only assessed the effect of the ELF on two *P. aeruginosa* isolates with a single set of experiments. This key limitation should be addressed with future studies with a representative sample of MICs and clinical isolates. Moreover, a lack of replicates may be a cause for the lack of an appreciable difference in some mean model parameter estimates given the significant heterogeneity that exists between the ELF milieu parameters and isolates used.

## 5 Conclusions

We found that simulated ELF conditions inhibit the bacterial killing of even susceptible *P. aeruginosa* isolates and amplify antibiotic resistance developing to aminoglycosides, such as amikacin. Although based on a small number of clinical isolates, and additional studies are required, these results provide an insight into the difficulties faced when relying upon aminoglycosides to treat VAP caused by both susceptible and multi-drug resistant Gram-negative bacilli. They also support calls for the development of new antibiotic formulations to optimise bacterial killing and prevent the emergence of antibiotic resistance in pathogens responsible for this severe and difficult-to-treat hospital-acquired infection.

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## Declarations

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**Consent to Participate** Not applicable.

**Consent for Publication** No human data was used for this publication. All authors consent to publication of this manuscript.

**Availability of Data and Material** Not available.

**Code Availability** Available on reasonable request.

**Authors' Contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AH, FS, SN, SMSL and JR. The first draft was prepared by AH. All authors made significant contributions to the manuscript development and approved the final manuscript.

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