

Post-antibiotic Effect of Various Antibiotics against *Achromobacter xylosoxidans* Strains Isolated from Patients with Cystic Fibrosis

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ABSTRACT

Objectives: *Achromobacter xylosoxidans* is an emerging pathogen in cystic fibrosis (CF) disease, although relatively limited is known about its clinical impact and optimal management. Pharmacodynamics such as post-antibiotic effect (PAE) are increasingly being applied to the design of antibiotic dosing regimens. In the present study, meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol were tested for their PAE.

Materials and Methods: The in vitro activities of meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol were determined by the microbroth dilution technique. To determine the PAEs, *A. xylosoxidans* strains in the logarithmic phase of growth were exposed for 1 h to antibiotics. Recovery periods of test cultures were evaluated using viable counting after centrifugation.

Results: The mean values of in vitro PAEs at 1xMIC and 4xMIC concentrations were 3.21 ± 0.66 and 3.58 ± 0.68 hours for colistin, 1.54 ± 0.46 and 2.27 ± 0.41 hours for levofloxacin, 2.34 ± 0.48 and 3.47 ± 0.64 hours for tobramycin, 1.72 ± 0.47 and 2.24 ± 0.41 hours for tigecycline, 1.91 ± 0.14 and 2.13 ± 0.13 hours for chloramphenicol, respectively. The carbapenem antibiotics exhibited weak PAEs, as expected.

Conclusions: This study's findings could have important implications for the timing of doses during antimicrobial therapy with tested antibiotics.

Keywords: pharmacodynamics, antibiotic, non-fermentative, post-antibiotic effect, *Achromobacter xylosoxidans*

INTRODUCTION

Achromobacter xylosoxidans, which is an aerobic, non-fermentative gram-negative bacillus, is widely distributed in aquatic environments and has been discovered in the human ear canal, as well as in the gastrointestinal and respiratory tracts [1]. The bacterium can cause a variety of infections, including meningitis, pneumonia, bacteremia, nosocomial or urinary tract infections, infection from contaminated environmental springs such as dialysis fluids, medications, nebulizers, disinfectants [2-4]. In particular, the frequency of diagnosis of colonization/infection of the airways with *A. xylosoxidans* has raised in cystic fibrosis (CF)

patients [5-7]. The prevalence of *Achromobacter* infections in the group of patients has been observed to differ considerably widely between populations, with estimations of between 16.3 and 23.9% [8,9]. Besides movement tools such as improved swarming capability, which is a flagella-dependent kind of bacterial motility in low-viscosity fluid and aqueous conditions, the main virulence factor of the organism is its capacity to build and then survive within the biofilms [10]. Biofilm product is linked with resistance to environmental agents by improving intimate attachment to surfaces, resistance to phagocytic motion, and other host immune agents, shielding from antimicrobial activity, and improved spread

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across surfaces through bacterial motility. Because *A. xylosoxidans* can assist as a reservoir of horizontal genetic transfer elements usually involved in disseminating antibiotic resistance, the biofilm mode of growth is possible to support the development of multidrug resistance in CF patients [11,12].

The treatment of *Achromobacter* infections can be challenging, and the effectiveness of empirical therapy for CF-related infections with commonly available antimicrobials is threatened by the development of microbial resistance [13-16]. Besides the new studies on the urgent need to investigate new antimicrobials against multi-resistant bacteria, especially for CF patients [17,18], enhancing management strategies with existing treatments has played a crucial part in identifying dosage schedules for new or old antimicrobials. Therefore, the minimal inhibitory concentration (MIC) and post-antibiotic effect (PAE), which is the delayed regrowth of bacteria following exposure to an antibiotic, were investigated against *A. xylosoxidans* strains isolated from CF patients. In the present study, although antibiotics recommended as first or second or combinations antibiotics category for the treatment of *A. xylosoxidans* were chosen, without PAE antibiotics like beta-lactam antibiotics other than carbapenems and trimethoprim-sulbactam have not been included in this study [19,20].

MATERIALS AND METHODS

Bacterial Isolates: Four non-epidemiological-related clinical isolates of *A. xylosoxidans* strains isolated from CF patients and one type strain (LMG 1863^T) were obtained from the Department of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark. Identification, *nrda* allele and PFGE profile of clinical strains have been reported (AX-1, CF28; AX-2, CF38; AX-3, CF-29; AX-4, CF43) [21]. The *Pseudomonas aeruginosa* ATCC 27853 reference strain was used to verify that MIC values were within the accuracy range stated by The Clinical and Laboratory Standards Institute guidelines (CLSI) [22].

Antibiotics: Meropenem trihydrate (AstraZeneca Pharmaceuticals, UK), doripenem (Shionogi & Co. Ltd., Japan), colistin sulfate (Sigma-Aldrich, USA), levofloxacin (Sanofi Pharmaceuticals Inc, Turkey), tigecycline (Wyeth Pharmaceuticals, Collegeville, Pa., USA) tobramycin (Novartis Pharmaceuticals Corp., Turkey) and chloramphenicol (Deva Pharmaceuticals, Turkey) were used. Stock solutions of tested antibiotics were prepared in a solution of 0.85% to 0.9% NaCl (doripenem and tigecycline) or in water for injection (meropenem, levofloxacin, tobramycin, colistin, and chloramphenicol) according to manufacturer's recommendation at a concentration of 5120 mg/L. The stock solution of colistin, levofloxacin, and tobramycin were stored frozen at -80°C and were used within six months. Meropenem, doripenem, tigecycline, and chloramphenicol solutions were prepared on the day of use.

Media: Cation adjusted Mueller Hinton Broth (CAMHB) was prepared daily by adding 25 mg calcium per liter to liquid and 12.5 mg magnesium per liter to Mueller Hinton Broth (Oxoid Ltd., Basingstoke, UK). This medium was used for MIC and PAE. Tryptic soy agar (Oxoid Ltd.) was used for colony counts.

Determination of MIC: MICs were determined by the microbroth dilution technique described by the CLSI guidelines [22]. Serial two-fold dilutions ranging from 32 to 0.03 mg/L for meropenem, doripenem, and colistin, from 256 to 0.25 for levofloxacin, tigecycline tobramycin, and chloramphenicol were prepared in fresh CAMHB 96-well microtiter plates. The inoculum was made with a 4 to 6 h broth culture. Each bacterial strain was spectrophotometrically adjusted to OD₆₀₀=0.12-0.13 (corresponding to approximately 1x10⁸ CFU/mL), and further diluted in CAMHB to obtain a final concentration of 5x10⁵ CFU/mL in the test tray. The trays were placed in plastic bags to avoid evaporation, incubated at 37°C for 18-20 hours, and visually inspected for growth. CLSI interpretative criteria for susceptibility of *P. aeruginosa* ATCC 27853 were used.

Determination of PAEs: PAEs were determined by a standard viable counting method [23, 24]. Strain-antibiotic mixtures were incubated for one h to avoid prolonged antibiotic exposure and the consequent complete eradication of the organism. At time zero, 1 ml bacterial inoculum (OD=0.036) was added to tubes containing 29 ml CAMHB with and without (control) test antibiotics producing approximately 10⁶ CFU/ml as a final concentration of inoculum in the test tubes. The bacteria were exposed to meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol at 1xMIC or 4xMIC. After incubation for one h in a 37°C-calibrated shaking water bath, cells were pelleted by centrifugation at 5000 rpm for 10 min. The supernatant was poured off, and the pellets were washed twice in buffered sterile saline (0.9 % NaCl) before being resuspended in 30 ml of prewarmed CAMHB. Bacterial counts of the tube contents were determined at time 0, immediately before and after centrifugation, and every hour after centrifugation for 8 hours by spreading on pour plates using 10-fold dilutions in saline as needed. Antimicrobial carryover was checked by the inhibition of colonial growth at the site of the initial streak according to NCCLS guidelines [25]. Plates were examined after incubation for 18-24 hour at 37 °C. The PAE was determined by the equation $PAE = T - C$, at where *T* is the time (in hour) required for the viability count in the test strain to increase one log₁₀ above the count observed immediately after removal of the antimicrobial agent, and *C* is the corresponding time for the control strain to grow 1 log₁₀. Experiments were conducted in triplicate. The mean PAE value for each strain was calculated by averaging three study values.

Post-antibiotic effect of various antibiotics against *Achromobacter xylosoxidans* strains

Table 1. The MICs of tested antibiotics against five *A. xylosoxidans* strains

Organisms	MIC (mg/L)						
	MER	DOR	CS	LVX	^a TGC	TOB	CHL
AX-1	0.125	1	4	2	4	64	16
AX-2	0.125	0.25	0.5	2	4	32	16
AX-3	0.06	0.25	8	2	2	128	16
AX-4	0.125	0.25	4	1	4	32	32
AX-5 (LMG 1863 ^T)	0.5	1	2	8	8	128	32
PA	0.5	0.5	1	1	2	1	128

MER, Meropenem; DOR, doripenem; CS, colistin sulfate; LVX, levofloxacin; TGC, tigecycline; TOB, tobramycin; CHL, chloramphenicol; PA, *P. aeruginosa* ATCC 27853 (reference strain); AX: *Achromobacter xylosoxidans*. Bold was used for emphasizing the resistance or intermediated susceptible.

ote: CLSI breakpoints for *P. aeruginosa* for susceptibility and resistance to meropenem are ≤ 2 mg/L and ≥ 8 mg/L, for doripenem ≤ 2 mg/L and ≥ 8 mg/L, for colistin are ≤ 2 mg/L and ≥ 8 mg/L, for levofloxacin are ≤ 2 mg/L and ≥ 8 mg/L, for tobramycin are ≤ 4 mg/L and ≥ 16 mg/L and for chloramphenicol (for other Non-Enterobacteriaceae) are ≤ 8 mg/L and ≥ 32 mg/L.

^aSince no tigecycline CLSI MIC breakpoints exist for tigecycline to non-fermenting Gram-negative bacteria; we use a susceptibility breakpoint of ≤ 2 mg/L [54].

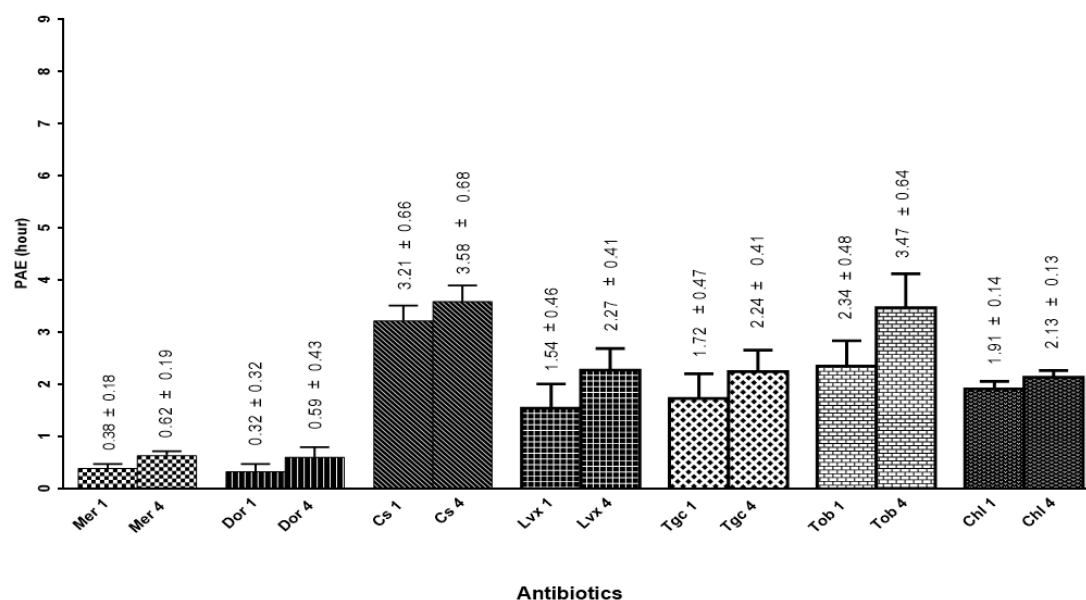


Figure 1. The mean PAE values of tested antibiotics against 5 *A. xylosoxidans* strains

Statistical Analysis: Statistical analysis of the study was performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA test was used to determine the change in PAE values of each antibiotic concentration. $p < 0.05$ was considered statistically significant.

RESULTS

The MICs of meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol for the five strains of *A. xylosoxidans* are shown in **Table 1**. According to the CLSI breakpoint [22], the results revealed that all clinical strains (AX-1, AX-2, AX-3, and AX-4) were susceptible to carbapenems (doripenem and meropenem). AX-2 was susceptible to colistin, whereas AX-3 were resistant. AX-1, AX-2, and AX-4 were resistant to tigecycline, whereas AX-3 was susceptible. The AX-5 strain (LMG 1863^T) was susceptible to carbapenems and colistin,

whereas it was resistant to tigecycline, tobramycin, and levofloxacin. All strain tested were resistant or intermediately resistant to chloramphenicol.

The mean PAE values for 5 *A. xylosoxidans* after one hour of exposure to antibiotics are displayed in **Figure 1**, and induction of PAE by meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol against each *A. xylosoxidans* strain is demonstrated in **Figure 2**. Colistin sulfate produced the longest PAE, ranging from 3.21 to 3.58 hours, although the difference between 1xMIC and 4xMIC was less than that observed for levofloxacin and tobramycin. Also, remarkable PAEs were induced by tobramycin (ranging from 2.34 to 3.47 hours) ($p < 0.001$), levofloxacin (ranging from 1.54 to 2.27 hours) ($p < 0.01$), tigecycline (ranging from 1.72 to 2.24 hours) ($p < 0.01$), and chloramphenicol (ranging from 1.91 to 2.13 hours) ($p < 0.01$). On the other hand, carbapenems (meropenem and

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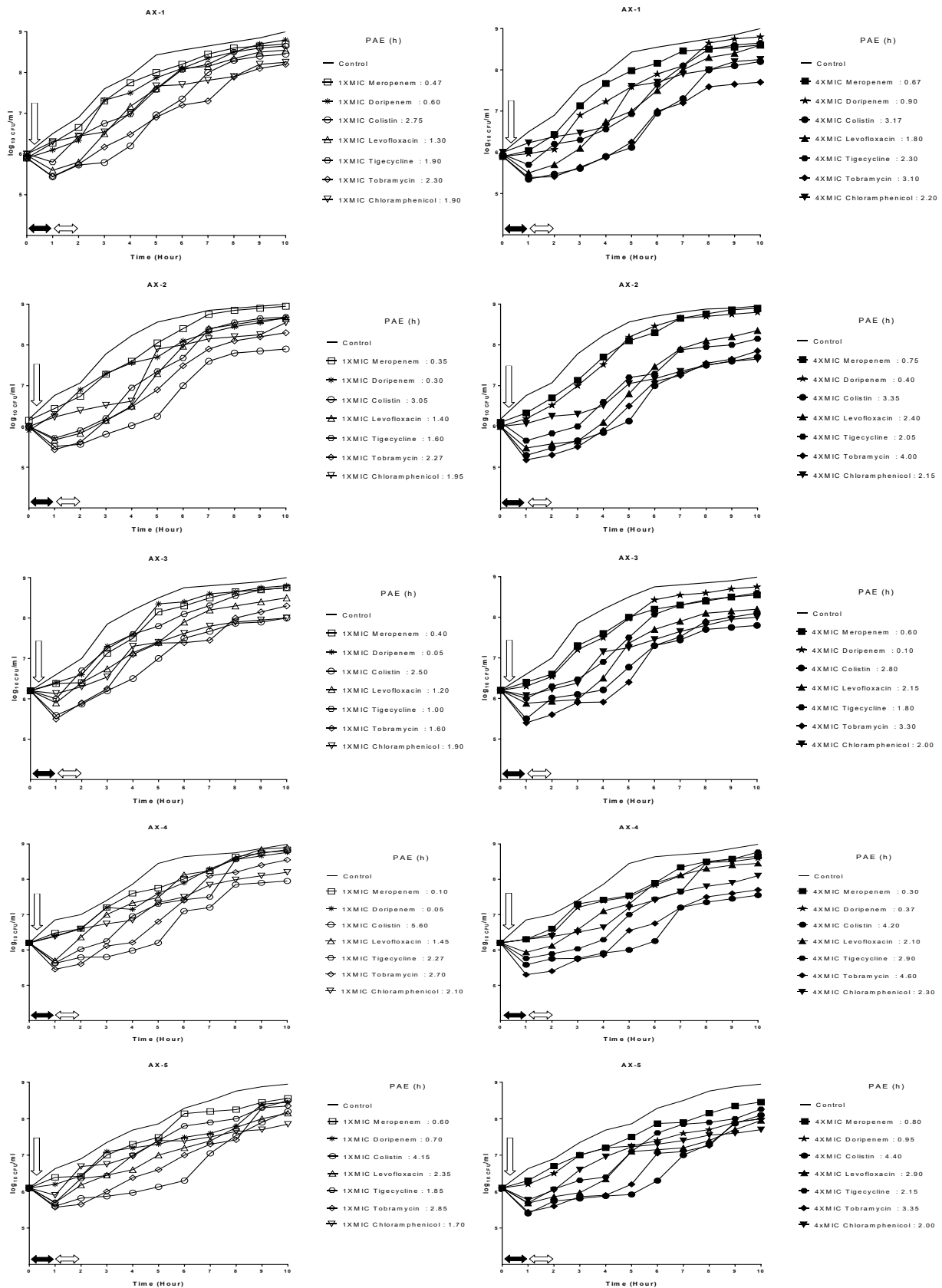


Figure 2. Induction of PAE by meropenem, doripenem, colistin, levofloxacin, tigecycline, tobramycin, and chloramphenicol against *A. xylosoxidans* strains (control, open circles; 1 x MIC, filled circles; 4 x MIC). The duration (in hours) of the PAE of each test culture is noted. Timings of the addition and removal of antibiotics and the length of exposure are indicated by the vertical white arrow, the horizontal white arrows, and the horizontal black arrows, respectively

doripenem) showed modest PAEs (ranging from 0.38 to 0.62 hours and 0.32 to 0.59 hours) against all strains ($p < 0.05$).

DISCUSSION

PAE is the persistent suppression of bacterial growth after short antimicrobial exposure of antimicrobial agents, and it is increasingly being used to select the most appropriate treatment and dosage schedules [23,25]. A long PAE provides the potential for administering the antimicrobial agent with longer intervals between doses. The PAE appears to be a feature of most antimicrobial agents and has been documented with a variety of common bacterial pathogens [26-28], but not for *A. xylosoxidans* yet. Several factors influence the presence or duration of the PAE, including the type of organism, type of antimicrobial, concentration of antimicrobial, duration of antimicrobial exposure, and antimicrobial combinations. In vitro methods used to determine the PAE include colony counts, optical density, and measurement of adenosine triphosphate in bacteria. Animal studies reveal in vivo PAEs by PAEs obtained in vitro for most organism/antimicrobial combinations [23,24]. The clinical relevance of the PAE is probably most important when designing dosage regimens. The presence of a long PAE allows aminoglycosides to be dosed infrequently; the lack of an in vivo PAE suggests that beta-lactam antimicrobials require frequent or continuous dosing [23].

Well-defined PAE pharmacodynamic parameters have not yet been firmly established for *A. xylosoxidans*. In our study, meropenem, which is the first choice carbapenem for *A. xylosoxidans* antibiotic therapy [19,20,29], was investigated for its PAE. Doripenem, which is unique owing to a sulphamoylaminoethyl-pyrrolidinylthio group at position two resulting in enhanced anti-gram-negative activity [30], offers potentially enhanced carbapenem activity for use in CF, and also might have diminished potential for the emergence of resistance [31], was tested for its PAE. Additionally, previous studies showed that doripenem has similar efficacy to meropenem for patients with pneumonia caused by *A. xylosoxidans*, and it showed strain-dependent activity with MIC₅₀ and MIC₉₀ of 0.5 and 8 mg/L [32-34]. To enhance antibiotics diversity, we performed the in vitro tests, whether doripenem could be a choice for CF patients with *A. xylosoxidans* pneumonia. Besides, carbapenems are the only β -lactams with a significant PAE against gram-negative organisms [35-37]. A PAE was observed by Nadler et al. [38] with meropenem when tested against four strains of *P. aeruginosa* (0.8-2 hours) and two strains of *Staphylococcus aureus* (0.7 and 1.7 hours). Also, a PAE of 1.8 (in vitro) to 4.3 (in vivo) hours for *P. aeruginosa* was recorded for doripenem [34]. Similar to previous studies of other bacteria, modest PAE values were imposed on *A. xylosoxidans* after 1 hour of exposure to meropenem or doripenem, both at 1xMIC and 4xMIC.

Colistin sulfate, which has rapid bactericidal activity with a detergent-like mechanism, is a second-line antibiotic in *A. xylosoxidans* therapy [19,20,29]. According to our study, it has significant PAEs against the tested *A. xylosoxidans* strains. It is in agreement with the results on other gram-negative non-fermentative bacteria, such as *Acinetobacter baumannii* or *P. aeruginosa* and *Enterobacteriaceae* such as *Klebsiella pneumoniae* [27-29]. This antibiotic at 4xMIC alone exhibited more PAEs than 1xMIC. A study showed that patients who received a higher average daily dose of colistin had a lower mortality [39], so the benefit of this prolonged PAE value of colistin sulfate might lead to prolonged dose interval without reduced efficacy, and possibly a lower frequency of adverse events. Clinically, there is not a universally accepted dosing recommendation for colistin. However, the interest in the use of colistin has led to some further understanding of its pharmacokinetics and dosing [40]. Additional studies on pharmacodynamics such as PAE are needed to establish the best colistin therapy protocol for daily dosing.

Tigecycline, owing to its enhanced spectrum of activity and favorable pharmacokinetics, has great potential in the management of *Burkholderia cepacia* complex (BCC) and several emerging pathogens in CF, namely *Stenotrophomonas maltophilia*, *A. xylosoxidans*, and *Mycobacterium abscessus* [19,20,31]. In vitro studies of tigecycline have demonstrated a prolonged PAE of 8.9 hours for *Streptococcus pneumoniae* and 4.9 hours for *Escherichia coli* [41]. Pankuch and Appelbaum [42], measured tigecycline PAEs against 2 *A. baumannii* strains and, in another previous study [27], reported a PAE value ranging from 0.05 to 1.40 hours against 6 *A. xylosoxidans*. In the present study, we determined that the PAE of tigecycline lasted 1.72 to 2.24 hours for tested strains of *A. xylosoxidans*. These data indicate the potential for tigecycline to exert a significant PAE at tested concentrations; thus, a long PAE might have an impact on dosing schedules because long PAEs can allow the antibiotic concentration to fall below MIC for significant periods without risk of regrowth.

Levofloxacin is recommended by CLSI as a second-line reporting antibiotic in the therapy of *A. xylosoxidans* [19,20,29]. Also, Sader reported that the use of newer fluoroquinolones is preferred when used in combination, given the greater in vitro activity. However, intrinsic resistance and poor activity are widely reported across the class [43]. Furthermore, fluoroquinolones generally produce significant in vitro PAEs against gram-negative strains [44-46]. Consistent with previous studies for other gram-negative bacteria, levofloxacin also possesses potent PAEs against *A. xylosoxidans*, and PAEs were prolonged in a concentration-dependent manner. The presence of a long PAE might allow levofloxacin to be dosed infrequently, perhaps once daily. However, additional studies are needed

to establish a levofloxacin therapy protocol for once-daily dosing.

Resistance to tobramycin is very high, especially with the intrinsic resistance by enzymatic modification to aminoglycosides, which occurs mainly by aminoglycosides modifying enzymes AAC(6')-Ib and aadA1 [47]. Although this antibiotic is not an option for *A. xylosoxidans* therapy, previous studies showed synergistic or additive effects for the combination of an aminoglycoside and a β -lactam against *A. xylosoxidans* strains [48, 49]. Moreover, antimicrobials that inhibit RNA or protein synthesis produce an in vitro PAE against gram-positive cocci and gram-negative bacilli [23,24]. Our data show that tobramycin has a significant PAE against *A. xylosoxidans*. These are in agreement with results obtained for other gram-negative and gram-positive bacteria [23,27,28]. According to our data, tobramycin PAEs were prolonged in a concentration-dependent manner, and at 4xMIC, tobramycin PAEs was prolonged by approximately 50% more than when the antibiotic was used at 1xMIC. The very high MIC of this drug plus worrisome side effects will likely prohibit the use of elevated dosing for intravenous administration; however, inhalation of antimicrobials is commonly administered to CF patients. The MICs should be viewed in light of peak sputum concentrations of approximately 1200 $\mu\text{g/g}$ after inhalation of 300 mg tobramycin [50]. The benefit of the prolonged PAE value of tobramycin could justify the use of inhaled tobramycin when intravenous administration is useless.

Chloramphenicol is the first antibiotic to be marketed as the product of chemical synthesis, and it has been produced this way exclusively since 1950. Chloramphenicol It is a broad-spectrum intravenous (IV) antibiotic [51]. In CF, chloramphenicol is used to treat lung infections caused by specific bacteria - for example, *P. aeruginosa* [52]. In a previous study by Athamna et al. [53] showed that chloramphenicol exhibited a PAEs of 1-2 h for 2 *Bacillus anthracis* strains at tested MICs. Our in vitro studies of chloramphenicol demonstrated similar PAEs against *A. xylosoxidans*, and PAEs were prolonged in a concentration-dependent manner.

In conclusion, this study shows that the single exposure of colistin, tobramycin, levofloxacin, or tigecycline produces a significant PAE on *A. xylosoxidans*, which might have implications for the difficult treatment of chronic *A. xylosoxidans* infections. As far as we know, this study is the first investigation of PAEs of various antibiotics that have been used in therapy for infections caused by *A. xylosoxidans* in CF patients.

Declaration of interest: The authors report no conflicts of interest.

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