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Effect of High-Dose Antimicrobials on Biofilm Growth of *Achromobacter* Species Isolated from Cystic Fibrosis Patients

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MICs and biofilm inhibitory concentrations (BICs) were measured for 68 cystic fibrosis (CF) *Achromobacter* isolates for amikacin, aztreonam, colistin, levofloxacin, and tobramycin. With the exception of colistin and levofloxacin, the remaining antibiotics had MIC_{90}s, BICs at which 50% of the isolates were susceptible (BIC_{50}s), and BICs at which 90% of the isolates were susceptible (BIC_{90}s) equal to or above the highest concentrations tested. In a biofilm model, tobramycin was able to significantly increase killing of bacterial cells compared to controls, for intermediate-resistant strains only, at concentrations of 1,000 and 2,000 μg/ml.

Achromobacter species, previously known as *Alcaligenes* species, are multidrug-resistant Gram-negative bacteria that have increasingly been isolated from the sputum from cystic fibrosis (CF) patients worldwide (1). Case-control studies have shown increased decline in lung function following pulmonary infection with *Achromobacter xylosoxidans* (2, 3). As with *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia*, *A. xylosoxidans* species are intrinsically resistant to several classes of antibiotics (4–7). Currently, there are no recommendations for chronic suppressive aerosolized antimicrobial therapies to treat these infections in CF patients. The objectives of this study were thus to examine the effects of antibiotics available for aerosolization against a range of CF *Achromobacter* species grown planktonically and as biofilms, which are important in CF pulmonary infections.

TABLE 1 Antibiotic MICs and BICs for *Achromobacter* CF isolates measured by planktonic and biofilm susceptibility testing

MIC/BIC by antibiotic	Results for *Achromobacter* species		
	All species ($n = 68$)	*A. xylosoxidans* ($n = 50$)	Other species ($n = 18$)[a]
Amikacin			
MIC_{50}	512	1,024	256
BIC_{50}	>4,096	>4,096	>4,096
MIC_{90}	>4,096	>4,096	>4,096
BIC_{90}	>4,096	>4,096	>4,096
Aztreonam			
MIC_{50}	256	512	512
BIC_{50}	>2,048	>2,048	>2,048
MIC_{90}	2,048	2,048	2,048
BIC_{90}	>2,048	>2,048	>2,048
Colistin			
MIC_{50}	4	8	4
BIC_{50}	>256	>256	>256
MIC_{90}	64	64	64
BIC_{90}	>256	>256	>256
Levofloxacin			
MIC_{50}	20	20	20
BIC_{50}	5,120	5,120	5,120
MIC_{90}	20	20	20
BIC_{90}	>5,120	>5,120	>5,120
Tobramycin			
MIC_{50}	200	400	200
BIC_{50}	3,200	3,200	3,200
MIC_{90}	>3,200	>3,200	>3,200
BIC_{90}	>3,200	>3,200	>3,200

[a] "Other species" includes *A. denitrificans* ($n = 3$), *A. dolens* ($n = 5$), *A. insolitus* ($n = 5$), and *A. ruhlandii* ($n = 5$).

Sixty-eight *Achromobacter* isolates were collected from CF patients at The Hospital for Sick Children, Toronto, Ontario, Canada ($n = 15$), and the CF Foundation *B. cepacia* Research Laboratory and Repository, Ann Arbor, MI ($n = 53$). The collection of *Achromobacter* CF isolates included five species: *A. xylosoxidans* ($n = 50$), *A. denitrificans* ($n = 3$), *A. dolens* ($n = 5$), *A. insolitus* ($n = 5$), and *A. ruhlandii* ($n = 5$). Antimicrobial susceptibility testing was performed on isolates grown planktonically and as biofilms for amikacin, aztreonam, colistin, levofloxacin, and tobramycin, as previously described (8, 9). Five *A. xylosoxidans* CF isolates with intermediate biofilm inhibitory concentrations (BICs) (defined as ≤800 μg/ml tobramycin, representing the mean peak sputum concentration of aerosolized tobramycin [10]) and another 5 isolates from the same species but with high BICs (>800 μg/ml tobramycin) were selected for further study in the biofilm slide chamber model. After 48 h of growth, biofilms were treated with various concentrations of tobramycin (0, 8, 400,

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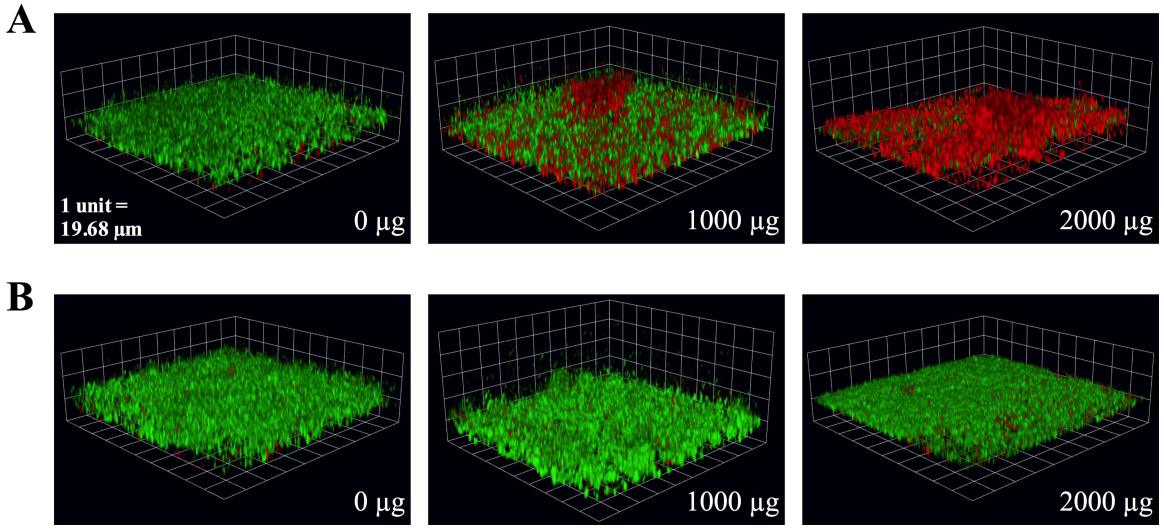

FIG 1 Confocal microscopy of live and dead (green and red, respectively) intermediately resistant (A) and highly resistant (B) *A. xylosoxidans* CF isolates treated with 1,000 and 2,000 μg/ml tobramycin in the biofilm slide chamber model.

1,000, and 2,000 μg/ml) for 24 h, stained using the FilmTracer LIVE/DEAD biofilm viability kit, and visualized by confocal microscopy (see Methods in the supplemental material).

Planktonic and biofilm susceptibility testing were performed on a total of 68 *Achromobacter* isolates. To describe the overall susceptibility results, the MICs and BICs at which 50% and 90% of the isolates were susceptible are presented in Table 1. For all *Achromobacter* species, the MIC_{50} of amikacin (512 μg/ml), aztreonam (256 μg/ml), colistin (4 μg/ml), levofloxacin (20 μg/ml), and tobramycin (200 μg/ml) were determined. With the exception of the MIC_{90}s of colistin (64 μg/ml) and levofloxacin (20 μg/ml), the remaining three antibiotics had MIC_{90}s, BIC_{50}s, and BIC_{90}s that were equal to or above the highest concentrations tested. The susceptibility results were varied among the isolates, and the distributions of the MICs and BICs are presented in Fig. S1 in the supplemental material. From the distributions of the MICs and BICs of all *Achromobacter* CF isolates, the five antibiotics tested had right-skewed MICs and left-skewed BICs. Correlations between MICs and BICs were calculated for each antibiotic using the Spearman correlation coefficient and were found to be statistically significant for amikacin ($r = 0.271$, $P \leq 0.05$) and tobramycin ($r = 0.261$, $P \leq 0.05$) only. To determine the effect of tobramycin, one of the most commonly used and available inhaled antibiotics in CF, on *A. xylosoxidans*, the most prevalent *Achromobacter* species in CF, biofilms grown in a slide chamber model were treated with various concentrations of antibiotic and imaged using confocal microscopy. Upon visualization of the intermediate-resistant strains, there appeared to be an increase in bacterial killing at concentrations of 1,000 and 2,000 μg/ml tobramycin, which was not observed with the highly resistant strains (Fig. 1). In addition, there was a statistically significant increase in the percentage of dead cells at concentrations of 400, 1,000, and 2,000 μg/ml tobramycin that showed a dose-response effect, but no change in thickness was observed in a comparison of intermediate-resistant biofilms to the untreated controls (Fig. 2). With the highly resistant strains, there was no statistically significant change in thickness or killing at any of the concentrations of tobramycin tested.

This study examined the antibiotic susceptibility of a large collection of *Achromobacter* isolates from CF patients and included the most common *Achromobacter* species encountered in this

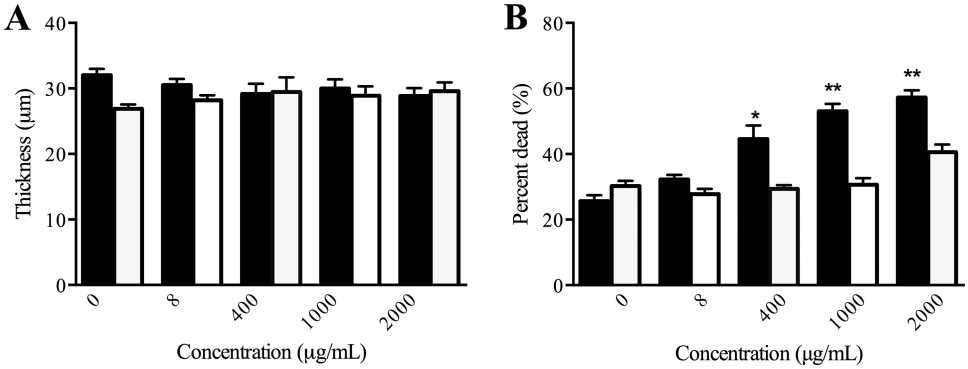

FIG 2 Mean (with standard error of the mean) thickness (A) and percent dead (B) of intermediately (black bars, $n = 5$) and highly (white bars, $n = 5$) resistant *Achromobacter xylosoxidans* CF isolates treated with increasing concentrations of tobramycin in the biofilm slide chamber model. *, $P \leq 0.05$; **, $P \leq 0.01$ compared to the untreated control.

population (11, 12). These *in vitro* results highlight their high degree of resistance to multiple classes of antibiotics in the context of both planktonic and biofilm growth. Several studies have noted that resistance among strains isolated from patients with CF is quite common; however, none have studied antibiotic susceptibility of *Achromobacter* spp. grown as biofilms (13–15). One of the strengths of this study was the investigation of antimicrobial activity against biofilm structures of *Achromobacter* using two models of biofilm growth, namely, the Calgary Biofilm Device and a slide chamber model with visualization using confocal microscopy. The results confirmed that when grown as biofilms, these bacteria, like others, exhibited higher tolerance to antibiotics (9, 16). The availability of aerosolized formulations of antibiotics, however, allows for higher pulmonary concentrations to be achieved in patients (10). All of the antibiotics tested in our study are either commercially available or in phase III study for aerosolization in CF patients, and the concentrations tested represent those achievable in the lungs after aerosolization. While the majority of isolates still had BICs above the achievable aerosolized concentration, levofloxacin and tobramycin showed the greatest efficacy against *Achromobacter* spp. overall, with the highest percentages of isolates with MICs and BICs below the achievable mean sputum drug concentrations (10). As we have previously shown (9), there was a significant correlation between the MICs and BICs for aminoglycosides but not for other antimicrobial classes tested, suggesting potential efficacy against both planktonically grown and biofilm-grown organisms. Data using the biofilm slide chamber model coupled with confocal microscopy confirmed the BIC data in this study. Strains deemed to be intermediate resistant via the Calgary Biofilm Device showed more killing with increasing doses of tobramycin. In contrast, strains with high resistance to tobramycin showed little increase in killing compared to that with the control. Inhaled levofloxacin and tobramycin thus represent the most promising treatment options, with effects that go beyond merely inhibiting *Achromobacter* growth to actually killing bacterial cells embedded in biofilms. Clinical trials, however, are needed to demonstrate true *in vivo* efficacy.

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