Supplementary

Materials and Methods

Isolate collection

Sixty-eight *Achromobacter* isolates were prospectively collected from CF patients between The Hospital for Sick Children, Toronto, Ontario (n = 15) and the CF Foundation *Burkholderia cepacia* Research Laboratory and Repository, Ann Arbor, Michigan (n = 53). The collection of *Achromobacter* CF isolates included five species: *Achromobacter xylosoxidans* (n = 50), *Achromobacter denitrificans* (n = 3), *Achromobacter dolens* (n = 5), *Achromobacter insolitus* (n = 5) and *Achromobacter ruhlandii* (n = 5).

Planktonic susceptibility testing

Conventional antimicrobial susceptibility testing was performed on isolates grown planktonically by broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines (1). Antibiotic panels containing various concentrations of antibiotics typically used via inhalation, that is, amikacin (0, 16, 32, 64, 128, 256, 512, 1024, 2048 and 4096 µg/mL), aztreonam (0, 32, 64, 128, 256, 512, 1024 and 2048 µg/mL), colistin (0, 4, 8, 16, 32, 64, 128 and 256 µg/mL), levofloxacin (0, 2, 20, 40, 80, 160, 320, 640, 1280, 2560 and 5120 µg/mL) and tobramycin (0, 10, 100, 200, 400, 800, 1600 and 3200 µg/mL) in cation-adjusted Mueller Hinton Broth (CAMHB) were prepared. Bacterial inoculum was prepared by diluting 1.5 mL of a 0.5 McFarland standard suspension into 25 mL of sterile distilled water. 10 µL of diluted bacteria was added to each well of the antibiotic panel using sterile 96-well plate inoculators (Nunc, Burlington, Ontario) to achieve an inoculum of approximately 5×10^5 colony forming

units (CFU)/mL. Panels were then incubated under aerobic conditions at 37°C for 24 hours. Purity of the inoculum was checked by streaking 1uL of the positive control well onto Columbia agar plates with 5% sheep blood (Oxoid, Nepean, Canada). Minimum inhibitory concentrations (MICs) were determined by visually assessing the turbidity of wells for indication of bacterial growth after the 24-hour incubation period. MICs for each isolate were repeated in duplicates and the higher MIC was recorded. If the MIC values were greater than 2 double dilutions, this was considered a major error and the experiment was repeated.

Biofilm susceptibility testing

Antimicrobial susceptibility testing was performed on isolates grown as biofilms by a modified Calgary biofilm method (2). Bacterial suspension for biofilm generation was prepared by diluting 300 µL of 0.5 McFarland standard into 19.7 mL of tryptic soy broth (TSB). 150 µL of diluted bacterial suspension was then added into each well of 96-well MBEC^{TM} Biofilm Inoculator plates (Innovotech, Edmonton, Canada). The plates were then incubated under aerobic conditions at 37°C on an orbital shaker (Labnet Orbit 1000, Woodbridge, New Jersey) to generate biofilm on the pegs of the inoculator lid. The OD_{650nm} of the bacterial suspension in each well containing the growing biofilm were measured using the MRX Microplate Reader (Dynex Technologies, Chantilly, Virginia). Once an OD_{650nm} of approximately 0.062 was achieved, corresponding to approximately 5×10^5 CFU/mL, the biofilm-laden peg lid was transferred into antibiotic panels. The panel contained antibiotics amikacin, aztreonam, colistin, levofloxacin and tobramycin in CAMHB prepared at identical concentrations described above. To confirm that the proper inoculum had been achieved, serial dilutions from a peg inoculated antibiotic-free well were plated onto Columbia agar plates with 5% sheep blood. Testing was

repeated for isolates where the inoculum did not fall between 10^4 to 10^6 CFU/mL. Antibiotic plates with the biofilm-laden peg lid were incubated under aerobic conditions at 37°C for 24 hours. After incubation, the peg lid was transferred into a 96-well plate with 200 µL of sterile distilled water for 1 minute to rinse off residual antibiotics from pegs. The peg lid was then placed into a recovery plate containing 100 µL CAMHB. The recovery plate was then incubated again under aerobic conditions at 37°C for 24 hours. Biofilm inhibitory concentrations (BICs) of each isolate were determined by visually assessing the turbidity of each well. BICs for each isolate were repeated in duplicates and the higher BIC was recorded.

Biofilm slide-chamber model and confocal microscopy

Five *A. xylosoxidans* CF isolates with intermediate BICs, defined as less than or equal to 800 µg/mL tobramycin, and another 5 isolates from the same species but with high BICs, defined as greater than 800 µg/mL tobramycin, were selected for further study in the biofilm slide-chamber model. A single bacterial colony of each isolate was inoculated from Columbia agar plates with 5% sheep blood into 4 mL of lysogeny broth (LB). Inoculated cultures were grown under aerobic conditions at 37°C for approximately 15 hours with shaking at 250 RPM. Cultures were then diluted 1:1000 and grown to an OD_{600nm} of 0.6 before being diluted again to an OD_{600nm} of 0.1. 250 µL of 0.1 OD_{600nm} cultures was then seeded into each chamber of an eight-chambered coverslip slide (LabTek II Chamber Slide on 1.5 Borosilicate, VMR, Missisauga). The chamber slide was then incubated under aerobic conditions at 37°C for 24 hours to allow static biofilm formation. After incubation, media was removed and replaced with fresh LB for an additional 24 hours at 37°C. Biofilms were then treated with various concentrations of tobramycin (0, 8, 400, 1000 and 2000 µg/mL) with LB in a total volume of 250 µL for 24 hours.

Treated biofilms were stained for 45 minutes using 200 µL of staining reagent from the FilmTracer® LIVE/DEAD Biofilm Viability Kit (Life Technologies, Burlington, Ontario). Staining reagent was then removed and replaced with 250 µL of fresh LB. Confocal images were acquired using a Quorum WaveFX spinning disk confocal system (Quorum Technologies Inc., Guelph, Canada). All images were acquired using a 25X water objective (total magnification of 250X) on a Zeiss AxioVert 200M Microscope. Spectral borealis lasers (Green: 491nm, Red: 561nm) were used for excitation. Emission filter sets of 515/40 and 624/40 where used to visualize the SYTO9 and propidium idodide stains respectively. Images in the Z-Stack were obtained at a distance of 0.8µm to obtain the depth of the biofilm. Volocity software (PerkinElmer, Guelph) was used for acquisition and analysis of images. For each strain tested, the percent dead was calculated as the (mean florescence of the red channel/ (mean fluorescence of green channel +mean fluorescence of red channel))*100% for three views of a chamber. This was done for three independent chambers for each strain in a group. The mean % dead for the intermediate resistant group (5 strains) and the high resistant group (5 strains) was then plotted and analyzed for statistical significance.

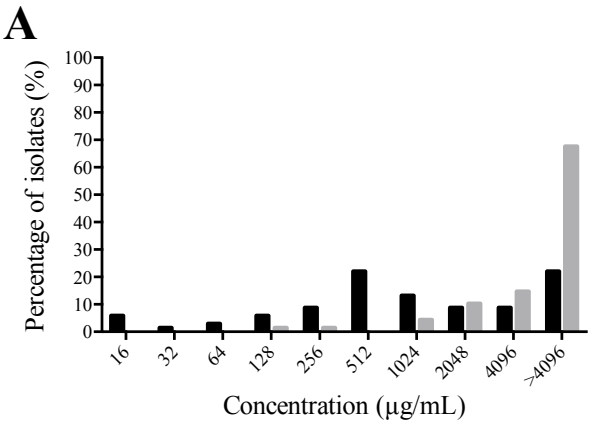
References

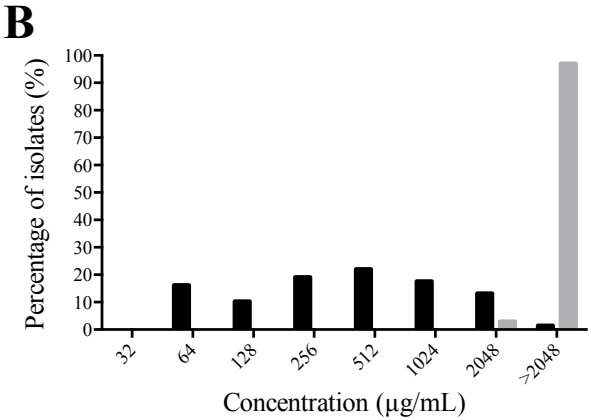
1. **Clinical and Laboratories Standards Institute.** 2012. Performance standards for 303 antimicrobial susceptibility testing: 22nd informational supplement M100-S22. CLSI, Wayne, PA.

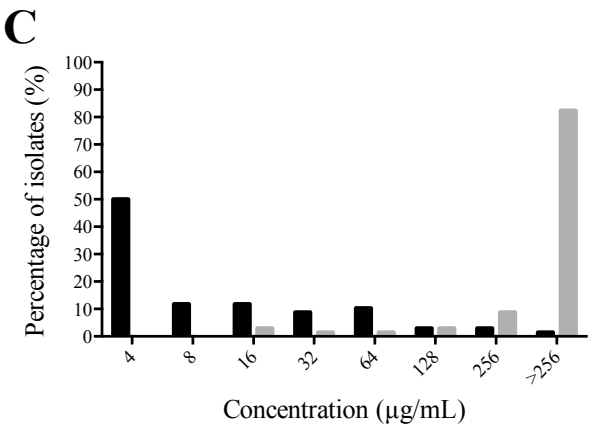
2. **Ratjen A, Yau Y, Wettlaufer J, Matukas L, Zlosnik JE, Speert DP, LiPuma JJ, Tullis E, Waters V.** 2015. In Vitro Efficacy of High-Dose Tobramycin against *Burkholderia cepacia* Complex and *Stenotrophomonas maltophilia* Isolates from Cystic Fibrosis Patients. Antimicrob Agents Chemother **59(1):**711-3.

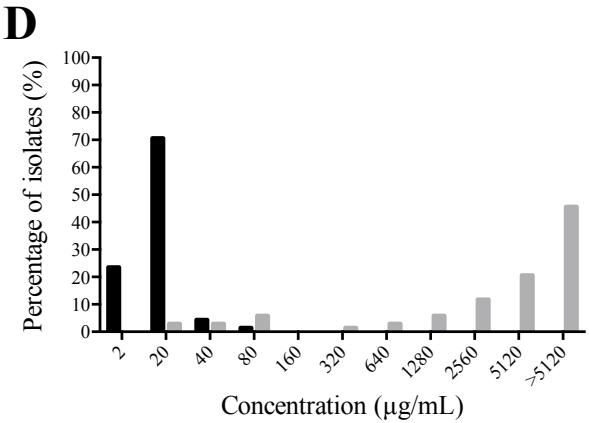
FIGURE LEGENDS

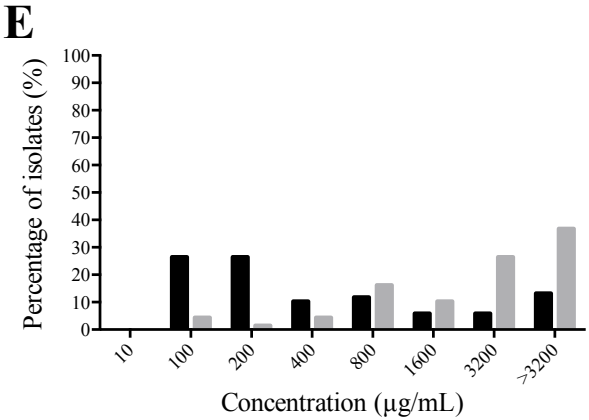
SUPPLEMENTAL FIG 1. Distribution of MICs (■) and BICs (■) for amikacin (**A**), aztreonam (**B**), colistin (**C**), levofloxacin (**D**) and tobramycin (**E**) measured by planktonic and biofilm susceptibility testing for *Achromobacter* CF isolates.











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