Materials and Methods

Bacterial isolates

A total of 125 CF *S. maltophilia* isolates from sputum and bronchoalveolar lavage specimens were prospectively collected from the microbiology laboratories at the Hospital for Sick Children (74 isolates from 51 CF patients; maximum of 2 isolates per patient)) and St. Michael's Hospital (51 isolates from 35 CF patients; maximum of 2 isolates per patient) in Toronto, Canada between January 2011 and July 2012. *S. maltophilia* isolates were stored at -80^{o}C in glycerol citrate. Upon retrieval from the freezer, each isolate was subcultured 3 times onto Columbia agar with 5% sheep blood (Oxoid, Nepean, Ontario, Canada) prior to susceptibility testing.

Antibiotic Panels

Antibiotic panels, in a 96-well microtiter plate format, were prepared with cation-adjusted Mueller Hinton broth (CAMHB; Becton Dickinson, Mississauga, Ontario, Canada) using 9 different antibiotics (ceftazidime, ticarcillin-clavulanate, tobramycin, levofloxacin, moxifloxacin, trimethoprim-sulfamethoxazole, doxycycline, colistin, azithromycin). All antibiotics were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for ticarcillin-clavulanate (Sigma, St Louis, MO, USA), ceftazidime (GlaxoSmithKline, Mississauga, Ontario, Canada) and moxifloxacin (Bayer, Toronto, Ontario, Canada). For ceftazidime, ticarcillin-clavulanate, moxifloxacin, trimethoprim-sulfamethoxazole and doxycycline, antibiotics were tested at 2 concentrations: minimum inhibitory concentration (MIC) at the susceptible breakpoint and MIC at the intermediate interpretative criteria

(correlating to serum achievable concentrations) for *S. maltophilia* and other non-*Enterobacteriaceae* according to the Clinical and Laboratory Standards Institute (CLSI) (1). For azithromycin, as there are no CLSI interpretative criteria for non-fermentative bacilli, concentrations of 0.4 mg/L and 0.8 mg/L were chosen based on previous studies done on *Burkholderia cepacia* and *P. aeruginosa* (15). Tobramycin (100 mg/L and 200 mg/L) (2) and colistin (100 mg/L and 200 mg/L) (3) were tested at concentrations achievable in CF sputum by aerosolization. Levofloxacin was tested at both high concentrations (50 mg/L and 100 mg/L- corresponding to achievable sputum levels by aerosolization) (4-5) as well as low concentrations (2 mg/L and 4 mg/L- corresponding to achievable serum levels). Each well contained 100 µl of CAMHB with the antibiotic(s), alone or in combinations, at these set concentrations. In total, there were 10 single drugs (9 different antibiotics including a low and high dose for levofloxacin) and 37 dual drug combinations tested for each isolate. Each plate had a well that was a sterility control (only broth, no antibiotics) and a well that was a growth control (broth plus bacterial inoculum but no antibiotics). Antibiotic panels were stored at -80^{o}C until use.

Planktonic Antimicrobial Susceptibility Testing

Planktonic susceptibility testing of *S. maltophilia* isolates was performed by broth-microdilution methods according to CLSI guidelines (1). Bacterial inoculum was prepared by diluting 1.5 ml of a 0.5 McFarland turbidity standard with 25 ml of distilled water. 10 µl of the bacterial inoculum was added to each well of the prepared antibiotic testing panel and incubated in aerobic conditions at 37˚C for 18 – 24 hours overnight. The plates were read the following day by assessing turbidity within the wells as signs of bacterial growth.

Biofilm Antimicrobial Susceptibility Testing

S. maltophilia isolates were grown as biofilms using the Calgary biofilm technique (6) with a few modifications. The bacterial inoculum was prepared by diluting 300 µl of a 0.5 McFarland turbidity standard in 19.7 ml of Trypticase-Soy Broth. 100 µl of the inoculum was added to each well of a 96-well microtitre plate with a peg lid (Innovotech, Manitoba, Canada). The plates were incubated in aerobic conditions at 37˚C on a shaker (Labnet Orbit 1000, Woodbridge, New Jersey) to allow for biofilm formation on the peg lid. An estimation of the inoculum was determined by measuring OD_{650nm} using the MRX Microplate Reader (Dynex Technologies, Chantilly, Virginia) of the broth in the well in which the biofilm peg was growing. Once an OD of approximately 0.062 was achieved (known to correspond to approximately 10^5 colony forming units (CFU)/ml), the biofilm-laden peg lid was placed into the antibiotic testing panel. To confirm that the proper inoculum had been achieved, an inoculum check was performed by serial dilutions and plating of the broth from the well onto solid media in which the biofilm peg was growing for each isolate tested. If the inoculum did not fall between 10^4-10^6 CFU/ml, testing was repeated. The antibiotic panel was then incubated for 18-24 hours under aerobic conditions at 37˚C. Each biofilm-laden peg was was placed in 200 µl of sterile distilled water to rinse out residual antibiotics, before being placed into 100 µl of CAMHB in the recovery plate. The plate was then incubated for 18-24 hours under aerobic conditions at 37˚C and assessed for turbidity the following day. Biofilm inhibitory concentrations were determined as per CLSI planktonic interpretative breakpoints for single drugs.

Interpretative criteria for dual antibiotic combinations

For dual drug combinations, the isolate was considered: resistant if resistant to both drugs; intermediate if intermediate to both drugs; and sensitive if sensitive to both drugs. For example, when testing doxycycline and ceftazidime in dual drug combinations, these drugs were combined in the following way: doxycycline at 4 mg/L (susceptible breakpoint) with ceftazidime at 8 mg/L (susceptible breakpoint) and doxycycline at 8 mg/L (intermediate interpretative criteria) with ceftazidime at 16 mg/L (intermediate interpretative criteria). If there was no growth at doxycycline 4 mg/L /ceftazidime 8 mg/L, the isolate was considered susceptible. If there was no growth at doxycycline 8 mg/L/ceftazidime 16 mg/L but growth at doxycycline 4 mg/L/ceftazidime 8 mg/L, the isolate was considered intermediate. If there was growth with both of these dual drug concentrations, the isolates was considered resistant.

Statistical Analysis

The proportion of isolates susceptible to different antibiotics by planktonic growth were compared to results by biofilm growth using Chi-square or Fisher's exact test where appropriate (GraphPad Prism version 5.04).

References

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Table 1.

A. Antibiotic combinations against planktonically-grown *S. maltophilia* isolates

Antibiotic combination	% (n) of susceptible isolates
Levofloxacin$_{100}$/azithromycin	99 (124)
Levofloxacin$_{100}$/trimethoprim-sulfamethoxazole	99 (124)
Levofloxacin$_{100}$/ticarcillin-clavulanate	99 (124)
Levofloxacin$_{100}$/colistin$_{200}$	99 (124)
Doxycycline/colistin$_{200}$	98 (123)
Levofloxacin$_{100}$/ceftazidime	98 (123)
Colistin$_{200}$/trimethoprim-sulfamethoxazole	98 (123)
Tobramycin/levofloxacin$_{100}$	98 (123)
Levofloxacin$_{4}$/colistin$_{200}$	98 (122)
Moxifloxacin/colistin$_{200}$	98 (122)
Ceftazidime/colistin$_{200}$	97 (121)
Doxycycline/ticarcillin-clavulanate	96 (120)
Doxycycline/levofloxacin$_{100}$	96 (120)
Doxycycline/moxifloxacin	96 (120)
Doxycycline/trimethoprim-sulfamethoxazole	95 (119)
Doxycycline/ceftazidime	94 (118)
Moxifloxacin/ticarcillin-clavulanate	94 (118)
Doxycycline/levofloxacin$_{4}$	94 (117)
Ticarcillin-clavulanate/colistin$_{200}$	92 (115)
Levofloxacin$_{4}$/ticarcillin-clavulanate	92 (115)
Moxifloxacin/trimethoprim-sulfamethoxazole	92 (115)
Moxifloxacin/ceftazidime	92 (115)
Doxycycline/azithromycin	90 (112)
Levofloxacin$_{4}$/trimethoprim-sulfamethoxazole	90 (112)
Moxifloxacin/tobramycin$_{200}$	86 (107)
Doxycycline/tobramycin$_{200}$	84 (105)
Moxifloxacin/azithromycin	84 (105)
Levofloxacin$_{4}$/ceftazidime	83 (104)
Tobramycin$_{200}$/trimethoprim-sulfamethoxazole	82 (102)
Ticarcillin-clavulanate/trimethoprim-sulfamethoxazole	80 (100)
Ceftazidime/trimethoprim-sulfamethoxazole	79 (99)
Tobramycin$_{200}$/ticarcillin-clavulanate	72 (90)
Levofloxacin$_{4}$/azithromycin	69 (86)
Tobramycin$_{200}$/ceftazidime	65 (81)
Tobramycin$_{200}$/azithromycin	61 (76)
Ticarcillin-clavulanate/azithromycin	34 (42)
Ceftazidime/azithromycin	21 (26)

B. Antibiotic combinations against biofilm-grown *S.maltophilia* isolates

Antibiotic combination	% (n) of susceptible isolates
Ceftazidime/colistin$_{200}$	65 (81)
Levofloxacin$_{100}$/ticarcillin-clavulanate	62 (78)
Colistin$_{200}$/trimethoprim-sulfamethoxazole	62 (78)
Moxifloxacin/colistin$_{200}$	61 (76)
Doxycycline/colistin$_{200}$	60 (75)
Levofloxacin$_{100}$/ceftazidime	59 (74)
Levofloxacin$_{100}$/azithromycin	58 (73)
Levofloxacin$_{100}$/colistin$_{200}$	58 (72)
Levofloxacin$_{4}$/colistin$_{200}$	58 (72)
Ticarcillin-clavulanate/colistin$_{200}$	58 (72)
Levofloxacin$_{100}$/trimethoprim-sulfamethoxazole	51 (64)
Doxycycline/levofloxacin$_{100}$	44 (55)
Moxifloxacin/ceftazidime	39 (49)
Tobramycin$_{200}$/levofloxacin$_{100}$	37 (46)
Moxifloxacin/trimethoprim-sulfamethoxazole	35 (44)
Moxifloxacin/ticarcillin-clavulanate	33 (41)
Moxifloxacin/azithromycin	30 (37)
Moxifloxacin/tobramycin$_{200}$	29 (36)
Doxycycline/tobramycin$_{200}$	24 (30)
Levofloxacin$_{4}$/trimethoprim-sulfamethoxazole	22 (28)
Levofloxacin$_{4}$/ceftazidime	22 (28)
Doxycycline/ticarcillin-clavulanate	21 (26)
Doxycycline/moxifloxacin	21 (26)
Levofloxacin$_{4}$/azithromycin	21 (26)
Tobramycin$_{200}$/trimethoprim-sulfamethoxazole	21 (26)
Doxycycline/levofloxacin$_{4}$	19 (24)
Doxycycline/ceftazidime	18 (22)
Levofloxacin$_{4}$/ticarcillin-clavulanate	18 (22)
Ceftazidime/trimethoprim-sulfamethoxazole	17 (21)
Tobramycin$_{200}$/trimethoprim-sulfamethoxazole	17 (21)
Doxycycline/azithromycin	16 (20)
Doxycycline/trimethoprim-sulfamethoxazole	14 (18)
Tobramycin$_{200}$/ceftazidime	14 (17)
Tobramycin$_{200}$/azithromycin	13 (16)
Ticarcillin-clavulanate/ trimethoprim-sulfamethoxazole	11 (14)
Ticarcillin-clavulanate/azithromycin	6 (8)
Ceftazidime/azithromycin	6 (8)