1   ***In vitro* activity of ceftazidime, ciprofloxacin, meropenem,**

2   **minocycline, tobramycin and trimethoprim-sulfamethoxazole**

3   **against planktonic and sessile *Burkholderia cepacia* complex**

4   **bacteria**

5

6   **Elke Peeters, Hans J. Nelis and Tom Coenye***

7

8   Laboratory of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences,

9   Ghent University, Ghent, Belgium

10

11   * Correspondence address: Laboratory of Pharmaceutical Microbiology, Faculty of

12   Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent,

13   Belgium. Phone +32 92648141. Fax: +32 92648195. E-mail: Tom.Coenye@Ugent.be

14

18

19

20

21

22

23

24

Synopsis

Objectives: The goal of the present study was to obtain a comprehensive overview of the bacteriostatic and bactericidal effects of six commonly used antibiotics on planktonic as well as on sessile *Burkholderia cepacia* complex cells.

Methods: The bacteriostatic and bactericidal activities of ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and trimethoprim-sulfamethoxazole were determined on 38 *B. cepacia* complex strains. MICs and minimal biofilm inhibitory concentrations (MBICs) were determined using a traditional broth microdilution method and a novel resazurin-based viability staining, respectively. The bactericidal effects of the investigated antibiotics (using antibiotic concentrations corresponding to 10 x MIC; except for tobramycin, for which a final concentration of 4 x MIC was tested) on stationary phase planktonic cultures and on 24 h old biofilms were evaluated using conventional plate count methods.

Results: Our results confirm the innate resistance of *B. cepacia* complex organisms against six first-line antibiotics used to treat infected CF patients. All antibiotics showed similar bacteriostatic activities against exponentially growing *B. cepacia* complex planktonic cells and freshly adhered sessile cells (4 h). In addition, most of the antibiotics showed similar bactericidal effects on stationary phase planktonic cultures and on young and older biofilms.

Conclusions: Despite the general assumption that sessile cells show a decreased susceptibility to antibiotics, our data indicate similar bacteriostatic and bactericidal activity of six selected antibiotics against planktonic and sessile *B. cepacia* complex bacteria.

Introduction

Burkholderia cepacia complex bacteria are opportunistic pathogens that can cause severe infections in patients with cystic fibrosis (CF) or chronic granulomatous disease (CGD) and in immunocompromised individuals. [1] The taxonomy of the genus *Burkholderia* has undergone several major revisions over the last decades. In the mid-1990s, "*Burkholderia cepacia*" strains were demonstrated to belong to at least five distinct species, which were collectively referred to as the *B. cepacia* complex. [2] Further taxonomic analyses revealed that even more species were present within the *B. cepacia* complex and currently 17 *B. cepacia* complex species have been described. [2-5] Except for *Burkholderia ubonensis*, all of these species have been isolated from sputum of CF patients, [4,5] with *Burkholderia cenocepacia* and *Burkholderia multivorans* being predominant. [6]

Infections with *B. cepacia* complex bacteria in CF patients are often correlated with increased morbidity and mortality and the innate resistance of these organisms to a broad range of antibiotics complicates the treatment of infected patients. [7,8] This panresistance is caused by various mechanisms, including limited permeability, changes in lipopolysaccharide structure and the presence of several multidrug efflux pumps, inducible chromosomal beta-lactamases and altered penicillin-binding proteins. [9] In addition, *in vitro* biofilm formation has been described for multiple *B. cepacia* complex strains and this may contribute to their ability to survive in the CF lung environment by providing additional protection from antibiotics. [1,10,11]

Treatment of *B. cepacia* complex infected patients should preferably be based on the results of susceptibility tests and often includes combination therapy with two or three antibiotics showing synergistic activity. [7,12,13] *In vitro* susceptibility studies on *B. cepacia* complex strains show that breakpoint concentrations of ceftazidime,

75 ciprofloxacin, meropenem, tetracyclines (doxycycline or minocycline) or high doses of

76 tobramycin have a bacteriostatic activity against a considerable fraction of these

77 strains. [8,12,14] Consequently, these antibiotics are often used to treat *B. cepacia*

78 complex infected CF patients. In addition, co-trimoxazole (i.e. a combination of

79 trimethoprim and sulfamethoxazole) is still frequently used in the treatment of chronic

80 *B. cepacia* complex infections, although susceptibility testing of these complementary

81 antibiotics revealed a poor activity against many *B. cepacia* complex strains. [13,15]

82 Often, antibiotics showing a good *in vitro* activity fail *in vivo*. This failure is partly due

83 to differences between the *in vitro* test conditions and the actual *in vivo* challenge.

84 For example, in conventional susceptibility testing and multiple combination

85 bactericidal testing (MCBT), planktonic cultures with actively-multiplying cells are

86 used, [12,16] but these may poorly represent susceptibility of stationary phase or slow-

87 growing cultures. [17,18] In addition, bacterial cells may reside in biofilms. These

88 consortia of microbial cells are embedded in a matrix of self-produced extracellular

89 components and they are considered to exhibit an increased resistance compared to

90 their free-floating planktonic counterparts. [19] Finally, inactivation of antibiotics in

91 sputum or insufficient antibiotic concentrations in sputum, might also contribute to a

92 poor *in vivo* activity despite a satisfactory *in vitro* activity. [20]

93 The first objective of the present study was to evaluate the growth inhibitory effect of

94 six antibiotics (ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and

95 trimethoprim-sulfamethoxazole) on planktonic as well as on freshly adhered sessile

96 cells (4 h) of all *B. cepacia* complex species. The second objective was to examine

97 the bactericidal effect of these antibiotics on stationary phase planktonic cultures and

98 to compare this to the bactericidal effect observed for biofilms.

99

Materials and methods

Strains and culture conditions

The following strains were used: *Burkholderia cepacia* LMG 1222^T and LMG 18821; *B. multivorans* LMG 18822, LMG 18825, LMG 13010^T and LMG 17588; *B. cenocepacia* LMG 16656^T, LMG 18828, LMG 18829 and LMG 18830; *Burkholderia stabilis* LMG 14294^T and LMG 14086; *Burkholderia vietnamiensis* LMG 18835 and LMG 10929^T; *Burkholderia dolosa* LMG 18941 and LMG 18943^T; *Burkholderia ambifaria* LMG 19182^T and LMG 19467; *Burkholderia anthina* LMG 20980^T and LMG 20983; *Burkholderia pyrrocinia* LMG 14191^T and LMG 21824; *B. ubonensis* LMG 20358^T and LMG 24263; *Burkholderia latens* LMG 24064^T and R-11768; *Burkholderia diffusa* LMG 24065^T and LMG 24266; *Burkholderia arboris* LMG 24066^T and R-132; *Burkholderia seminalis* LMG 24067^T and LMG 24272; *Burkholderia metallica* LMG 24068^T and R-2712; *Burkholderia lata* LMG 6992 and R-9940; *Burkholderia contaminans* LMG 16227 and R-12710. All strains were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were kindly provided by Dr. P. Vandamme (Ghent University, Belgium). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were included as control strains and were obtained from the ATCC collection (Manassas, VA, USA). Cells were stored at -80°C using Microbank vials (Prolab Diagnostics, Richmond Hill, ON, Canada) and were subcultured twice on Mueller Hinton Agar (MHA; Oxoid, Hampshire, UK) before they were used in any experiment. All cultures were incubated aerobically at 37°C.

Antibiotics

Ceftazidime, ciprofloxacin, tobramycin and sulfamethoxazole were obtained from Sigma-Aldrich (St. Louis, MO, USA). Minocycline and trimethoprim were obtained

from Certa (Braine-l'Alleud, Belgium) and meropenem was obtained from AstraZeneca (London, UK).

Determination of the MIC

MICs were determined *in duplo* according to the EUCAST broth microdilution protocol using flat-bottomed 96-well microtitre plates (TPP, Trasadingen, Switzerland).[21] The range of antibiotic concentrations was from 0.25 mg/L to 128 mg/L for ceftazidime, ciprofloxacin, meropenem and minocycline; for tobramycin, higher concentrations were tested (between 2 mg/L and 1024 mg/L). Trimethoprim-sulfamethoxazole concentrations tested were between 0.25-4.75 mg/L and 128-2432 mg/L. The inoculum was standardized at appr. 5×10^5 cfu/mL. The plates were incubated at 37°C for 20 h and the optical density was determined at 590 nm using a multilabel microtitre plate reader (Victor[2], Perkin Elmer LAS, Waltham, MA, USA). The lowest concentration of antibiotic for which a similar optical density was observed in the inoculated and blank wells was recorded as the MIC. The quality of the test results was monitored using two control strains (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922). CLSI-interpretative criteria for MIC testing of non-Enterobacteriaceae were used to evaluate the MIC results.[16] An adapted breakpoint MIC of 256 mg/L was also included for tobramycin.[14]

Determination of the minimal biofilm inhibitory concentration (MBIC)

In order to determine the growth inhibitory effects of the antibiotics on freshly adhered sessile cells, a novel non-standard method using a resazurin-based viability staining was applied.[22] The MBIC was defined as the minimum concentration of antibiotic necessary to prevent biofilm growth and maturation (i.e. the lowest

concentration that resulted in no further increase in biofilm biomass after 4 h of adhesion).

First, an overnight culture was diluted in Mueller Hinton Broth (MHB, Oxoid) to prepare an inoculum suspension containing appr. 10^8 cfu/mL. This suspension was added to the wells of a round-bottomed 96-well microtitre plate (TPP). Following 4 h of adhesion, the supernatant (containing non-adhered cells) was removed from all wells and the plates were rinsed with physiological saline (PS; 0.9% NaCl). Plate counts performed in preliminary experiments confirmed that following this 4 h period appr. 10^5 adhered cells are present in each well. Subsequently, 200 µL of antibiotic-containing MHB (using identical antibiotic concentrations as in the MIC experiments) was added and plates were further incubated at 37°C. After 20 h of treatment, wells were again rinsed and finally 170 µL PS and 34 µL of a commercially available resazurin solution (CellTiter-Blue, CTB, Promega, Maddison, WI, USA) were added to all wells. Fluorescence was measured after 1 h incubation using a multilabel microtitre plate reader (λ_{ex}: 560 nm and λ_{em}: 590 nm). All MBIC experiments were performed *in duplo*.

Determination of the bactericidal effect of antibiotics on biofilms

For all strains, the bactericidal effect of the antibiotics on cells present in biofilms, which were grown for 24 h (4 h of adhesion and 20 h of biofilm formation), was determined using antibiotic concentrations corresponding to 10 x MIC, except for tobramycin, for which a final concentration of 4 x MIC was tested. In addition, the bactericidal effect of tobramycin on sessile cells in older biofilms (grown for 76 h [4 h adhesion, 72 h growth] or 100 h [4 h adhesion, 96 h growth]) was also evaluated.

Biofilms were grown on silicone discs (Q7-4735; Dow Corning, Midland, MI, USA) placed in the wells of a 24-well microtitre plate (TPP). Three discs were included per tested antibiotic and three untreated discs served as controls. Previous (unpublished) data from our own research group indicated that the number of cells present in the *B. cepacia* complex biofilms increased exponentially during appr. the first 16 h of biofilm development and remained constant afterwards.

Starting from an overnight culture, an inoculum suspension containing appr. 10^8 cfu/mL in MHB was prepared. Subsequently, 1 mL of this suspension was added to the wells. After 4 h of adhesion, all wells were rinsed three times using PS. Then, fresh sterile MHB was added and the biofilms were allowed to grow for an additional 20 h period. After 4 h adhesion and 20 h biofilm formation, all discs were rinsed once and subsequently transferred to the wells of a microtitre plate containing antibiotics (in PS). After a 20 h treatment period, all discs were again rinsed and transferred to 10 mL MHB. Sessile cells were removed from the discs by 3 cycles of vortexing (30s) and sonication (30s; Branson 3510, Branson Ultrasonics Corp, Danbury, CT, USA) and the number of cells was determined using conventional plate count methods.[23] Older biofilms (76 h and 100 h) were grown and treated similarly; but an additional refreshment of medium was performed every 24 h.

Determination of the bactericidal effect of antibiotics on stationary phase planktonic cultures

The bactericidal effect of all antibiotics on stationary phase planktonic cells was determined for each strain using antibiotic concentrations corresponding to 10 x MIC (or 4 x MIC for tobramycin).

198 Starting from an overnight culture, an inoculum suspension containing appr. 10^8

199 cfu/mL in MHB was prepared. This inoculum suspension was grown aerobically for

200 24 h (stationary phase planktonic cultures) in a shaking warm water bath at 37°C.

201 Subsequently, these cells were harvested by centrifugation, washed three times and

202 diluted in PS until a suspension was obtained containing (per mL) twice the number

203 of cfu present in the corresponding untreated biofilms. 500 μL of the latter

204 suspension was added to 500 μL of double-concentrated antibiotic solutions (in PS).

205 After 20 h of exposure to the antibiotics, the number of surviving cells in the treated

206 and untreated planktonic cultures was determined using conventional plate count

207 methods.

208

209 **Confocal laser scanning microscopy (CLSM)**

210 The effects of all antibiotics on *B. cenocepacia* LMG 16656 biofilm morfology were

211 visualized using CLSM. To this end, 1 μl of Syto9 (λ_{exc}: 480 nm; λ_{em}: 500 nm;

212 3.34mM in DMSO) and 1 μl of propidium jodide (λ_{exc}: 490 nm; λ_{em}: 635 nm; 20 mM in

213 DMSO) (LIVE/DEAD BacLight bacterial viability kit L7012; Invitrogen, Carlsbad, CA,

214 USA) were added to the biofilm supernatant. After a 15 min incubation period at room

215 temperature, the biofilms were visualized with a Nikon C1 confocal laser scanning

216 module attached to a motorized Nikon TE2000-E inverted microscope (Nikon

217 Benelux, Brussels, Belgium) equipped with a Plan Apochromat 60.0x/1.20/0.22 water

218 immersion objective.

219

220 # Results

221 **MIC and MBIC experiments**

222 The results of the broth microdilution MIC tests are shown in Table 1. In general, the
223 MICs observed for ciprofloxacin, tobramycin and trimethoprim-sulfamethoxazole
224 varied widely, with MICs ranging from <0.25 mg/L to 128 mg/L, from 2 mg/L to 1024
225 mg/L and from 0.25-4.75 mg/L to >128-2432 mg/L, respectively. The MICs for
226 meropenem were between 0.5 mg/L and 32 mg/L, among the tested antibiotics
227 representing the narrowest range. Meropenem, minocycline and ceftazidime were
228 the most active antibiotics as only 39.5%, 15.8% and 39.5% of the tested strains,
229 respectively, showed no growth-inhibition at breakpoint antibiotic concentrations.
230 Although low concentrations of tobramycin (≤ 4 mg/L) were only able to inhibit growth
231 of *B. contaminans* R-12710, high concentrations of tobramycin (> 256 mg/L) were
232 active against 34 out of the 38 strains tested (89.5%). Among the antibiotics tested,
233 ciprofloxacin and trimethoprim-sulfamethoxazole had the lowest activity, as 47.4%
234 and 76.3% of the tested strains continued growing in the presence of breakpoint
235 concentrations of these antibiotics.

236 In general, the MICs observed for planktonic cells and the MBICs observed for
237 freshly adhered (4 h) sessile cells were highly similar; only in 3.9% of the cases the
238 MBICs showed more than a four fold difference compared to the corresponding MICs
239 (data not shown). A scatter plot illustrating the high similarity between the minimal
240 concentrations of ciprofloxacin necessary to prevent growth of planktonic and freshly
241 adhered sessile cells is presented in Figure 1. Similar scatter plots were obtained for
242 all other antibiotics (data not shown).

243

244 **Determination of the bactericidal effect of antibiotics on stationary phase**
245 **planktonic cultures and on biofilms**

The bactericidal effect of all antibiotics was determined on stationary phase planktonic cultures, which were grown for 24 h, as well as on 24 h old biofilms. In addition, the bactericidal effect of tobramycin on older planktonic cultures and biofilms (grown for 76 h or for 100 h) was also determined for three selected strains (*B. multivorans* LMG 18825, *B. cenocepacia* LMG 16656 and *B. dolosa* LMG 18943). In order to allow a comparison under similar conditions, the number of planktonic cells was adjusted to be equal to the number of sessile cells present in the corresponding biofilms.

In general, the bactericidal effect of most antibiotics was comparable for all tested strains. Box plots representing an overview of the number of cfu/ (disc or mL) in the untreated controls and in the treated planktonic cultures and biofilms are shown in Figure 2. On average, less than a 90% reduction in the number of surviving cells was observed after treating planktonic cultures and biofilms with trimethoprim-sulfamethoxazole, minocycline or ceftazidime. Among the tested antibiotics, tobramycin showed the highest bactericidal activity against planktonic *B. cepacia* complex cells, despite the fact that the tested concentrations were only four times higher than the corresponding MICs. In some cases, treatment with tobramycin even resulted in a total eradication of all planktonic cells. For 36 of the tested strains (94.7%), tobramycin had the highest bactericidal activity of the tested antibiotics against sessile cells. CLSM images of untreated and ceftazidime-, ciprofloxacine-, meropenem-, minocycline- and trimethoprim-sulfamethoxazole-treated *B. cenocepacia* LMG 16656 biofilms revealed that no changes in biofilm morphology were induced by the latter treatments (Figure 3A and 3B and data not shown). The large reductions in cells numbers following a treatment with tobramycin were also confirmed (Figure 3C).

271   For the majority of antibiotics tested, the fraction of surviving planktonic and sessile

272   cells was similar (representative results for two strains are shown in Figure 4); after

273   treatment with ciprofloxacin, minocycline or trimethoprim-sulfamethoxazole, the

274   fraction of surviving sessile and planktonic cells showed less than a 10-fold

275   difference for all strains. Treatment with ceftazidime or meropenem led to more than

276   a 10-fold difference in reduction between planktonic cultures and biofilms for 3 (7.9%)

277   and 6 (15.8%) strains, respectively. For 24 of the strains tested (63.2%), the fraction

278   of sessile cells surviving a tobramycin treatment showed more than a 10-fold

279   difference compared to the fraction of surviving planktonic cells.

280   Treatments with tobramycin on older biofilms and on older planktonic cultures

281   resulted in similar reductions as seen for the biofilms and the stationary phase

282   planktonic cultures that were grown for only 24 h (Figure 5).

283

Discussion

285   Antibiotic resistance is considered an important virulence factor of *B. cepacia*

286   complex organisms. [1] Although therapy is usually guided by antimicrobial

287   susceptibility testing, eradication of *B. cepacia* complex organisms is rarely achieved.

288   [24] Multiple hypotheses have been formulated to explain this failure, including

289   inadequate antibiotic concentrations or inactivation of the antibiotic in sputum,

290   impaired host defences in CF patients, biofilm formation, 'inoculum' effect and *in vivo*

291   growth rate of these organisms. [18] In the present study, we have focussed on the

292   growth inhibitory and bactericidal effects of ceftazidime, ciprofloxacin, meropenem,

293   minocycline, tobramycin and trimethoprim-sulfamethoxazole on 38 *B. cepacia*

294   complex strains belonging to 17 species.

295

Growth inhibitory concentration of antibiotics for exponentially growing planktonic cultures and for freshly adhered sessile cells

In general, our results confirm the previously reported high intrinsic resistance of *B. cepacia* complex strains against a broad variety of antibiotics. [8,12,13] Meropenem, minocycline and ceftazidime showed the best growth inhibitory activity at clinically relevant concentrations. Although *B. cepacia* complex organisms are typically resistant against aminoglycosides, [9] high doses of tobramycin inhibited the majority of tested strains. Nebulized tobramycin yielding high peak concentrations in sputum, are increasingly used for treating CF patients. [17,25,26] Consequently, these higher concentrations should be taken into account when evaluating the usefulness of this antibiotic. Several reports confirm that nebulized tobramycin shows great promise in the treatment of *B. cepacia* complex infected CF patients: for example, Middleton *et al*. recently described the complete eradication of *B. cepacia* complex organisms from the lungs of CF patients by using a combination of nebulized tobramycin and amiloride. [24] In addition, a combination therapy with nebulized and intravenous meropenem and tobramycin also resulted in the successful treatment of a female CF-patient suffering from cepacia syndrome, although the sputum samples of the latter patient remained positive for *B. cenocepacia*. [27]

Multiple studies have reported a decreased susceptibility to certain antibiotics (e.g. meropenem, ceftazidime) of *P. aeruginosa* and *B. cepacia* complex isolates grown as biofilms. [28-31] However, the results of the present study indicate that the growth inhibitory concentrations for exponentially growing *B. cepacia* complex planktonic cells and for freshly adhered sessile cells are similar. This discrepancy between our results and those from previous studies may be due to pronounced differences in experimental approach. For example, some studies compared the bacteriostatic

activity of antibiotics against planktonic and dispersed cells with the bactericidal activity against sessile cells (Minimal Biofilm Eradication Concentration; MBEC). [30,31] Other studies compared the minimal growth inhibitory concentrations of antibiotics on actively growing planktonic cultures with biofilm inhibitory concentrations (BICs) obtained after treating 20 h-old biofilms. [28,29] Yet, in order to allow a correct comparison between the susceptibility of planktonic and sessile *B. cepacia* complex cells, experimental conditions (including growth phase) should be identical. In fact, previous research on a *B. cepacia* strain revealed a dramatic decrease in susceptibility for ceftazidime and ciprofloxacin during the progression of the exponential growth phase. An increase of the resistance was observed for both planktonic cultures and biofilms of this strain during the later stages of the exponential growth phase, compared to the earlier stages of exponential growth. [18] Consequently, in the present study, we aimed to compare the growth inhibitory effects of antibiotics against planktonic and sessile *B. cepacia* complex cells under similar experimental conditions.

Bactericidal effect of antibiotics on stationary phase planktonic cultures and on biofilms

The bactericidal effect of all antibiotics, at a concentration of 10 x MIC (4 x MIC for tobramycin), was evaluated for planktonic cultures grown for 24 h as well as for biofilms obtained after 24 h (4 h of adhesion and 20 h of biofilm formation). Trimethoprim-sulfamethoxazole and minocycline, both bacteriostatic agents, yielded the lowest reductions in cell numbers under these test conditions. Tobramycin had the highest bactericidal effect among the antibiotics tested: on average reductions of

345   more than 99.999% and 99.98% were observed when treating planktonic cultures

346   and biofilms, respectively.

347   For the majority of strains, treatment with ceftazidime, ciprofloxacin, meropenem,

348   minocycline or trimethoprim-sulfamethoxazole yielded similar reductions in the

349   number of planktonic and sessile cells. In addition, CLSM images revealed that no

350   changes in biofilm morphology were induced by the latter treatments. Treatment with

351   tobramycin mostly resulted in a higher reduction in the number of planktonic cells

352   compared to that observed in biofilms; yet, for some strains, including *B. dolosa* and

353   *B. anthina* strains, similar reductions were observed under both conditions.

354   Previously, Spoering and Lewis reported similar bactericidal activity of carbenicillin,

355   ofloxacin and tobramycin on sessile and planktonic *P. aeruginosa* PAO1 cells. [17]

356   They concluded that the general assumption about sessile cells showing an

357   increased tolerance against antibiotics relative to stationary-phase planktonic

358   cultures is unwarranted. The minor differences in reductions in the antibiotic-treated

359   planktonic cultures and biofilms observed for the large majority of *B. cepacia* complex

360   strains in the present study are in agreement with their observations. Yet, unlike their

361   observations for *P. aeruginosa* PAO1, we did observe a decreased susceptibility of

362   sessile cells towards tobramycin for multiple *B. cepacia* complex strains. This

363   decreased susceptibility could be due to binding of the cationic tobramycin to biofilm

364   components, resulting in retarded penetration. Yet, other biofilm specific factors may

365   also play a role. [32]

366   In order to evaluate a possible increase in resistance towards tobramycin in older

367   biofilms and planktonic cultures compared to their younger counterparts, we also

368   determined the bactericidal effect of tobramycin on biofilms and planktonic cultures

369   grown for 76 h and 100 h. In previous studies on the susceptibility of *P. aeruginosa*

biofilms, an increased resistance of older biofilms against this antibiotic was reported.[33] Yet, in the present study no meaningful differences in susceptibility between the older biofilms and planktonic cultures, on the one hand, and their younger counterparts, on the other hand, were observed (Figure 5). A possible explanation for this unexpected result may lie in the differences of the used growth conditions and consequently, in the differences in observed growth patterns. Unlike in some previous studies, cell numbers did not increase further in the older *B. cepacia* complex biofilms compared to the younger biofilms (grown after 24 h). [33, 34] In fact, the impact of these variations in growth pattern, which are the result of differences in nutritional limitations, can influence greatly the susceptibility to antibiotics. [35]

In conclusion, under the conditions used in the present study, our results show similar bacteriostatic activities of the antibiotics tested against exponentially growing planktonic *B. cepacia* complex cells and freshly adhered sessile cells. In addition, similar bactericidal activities were observed against planktonic cultures and biofilms for the majority of antibiotics tested. The results of the present study support the hypothesis that the selection of antibiotics for the treatment of *B. cepacia* complex infected CF patients should not only be based on conventional culturing techniques for planktonic cells. In fact, the lack of correlation between the conventional *in vitro* susceptibility tests and the clinical response caused by these antibiotics *in vivo,* suggests that other methods focussing on the bactericidal effect of antibiotics against stationary phase planktonic cells or biofilms may provide a better alternative for clinicians to select the best possible treatment. [29,36,37]

Acknowledgements

Funding

Transparency declaration

None to declare

References

1. Mahenthiralingam E, Urban TA, Goldberg JB. The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* 2005; **3**: 144-56.

2. Vandamme P, Holmes B, Vancanneyt M *et al*. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* 1997; **47**: 1188-200.

3. Coenye T, Vandamme P, Govan JRW *et al*. Taxonomy and identification of the *Burkholderia cepacia* complex. *J Clin Microbiol* 2001; **39**: 3427-36.

4. Vanlaere E, LiPuma JJ, Baldwin A *et al. Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp.nov., novel species within the *Burkholderia cepacia* complex. *Int J Syst Evol Microbiol* 2008; **58**: 1580-90.

418 5. Vanlaere E, Baldwin A, Gevers D *et al.* Taxon K, a complex within the

419 *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia*

420 *contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int J Syst Evol Microbiol* 2009;

421 **59**: 102-11.

422 6. Mahenthiralingham E, Baldwin A, Vandamme P. *Burkholderia cepacia* complex

423 infection in patients with cystic fibrosis. *J Med Microbiol* 2002; **51**: 533-8.

424 7. Speert DP. Advances in *Burkholderia cepacia* complex. *Paediatr Respir Rev* 2002;

425 **3**: 230-5.

426 8. Nzula S, Vandamme P, Govan JRW. Influence of taxonomic status on the *in vitro*

427 antimicrobial susceptibility of the *Burkholderia cepacia* complex. *J Antimicrob*

428 *Chemother* 2002; **50**, 265-269.

429 9. Burns JL. Antibiotic resistance of *Burkholderia* spp. In: Coenye T, Vandamme P,

430 eds. *Burkholderia: Molecular microbiology and genomics*. Norfolk: Horizon

431 Bioscience, 2007; 81-91.

432 10. Conway BAD, Venu V, Speert DP. Biofilms formation and acyl homoserine

433 lactone production in the *Burkholderia cepacia* complex. *J Bacteriol* 2002; **184**: 5678-

434 85.

435 11. Tomlin KL, Clark SRD, Ceri H. Green and red fluorescent protein vectors for use

436 in biofilm studies of the intrinsically resistant *Burkholderia cepacia* complex. *J*

437 *Microbiol Methods* 2004; **57**: 95-106.

438 12. Aaron SD, Ferris W, Henry DA *et al.* Multiple combination bactericidal antibiotic

439 testing for patients with cystic fibrosis infected with *Burkholderia cepacia. Am J*

440 *Respir Crit Care Med* 2000; **161**: 1206-12.

441 13. Zhou J, Chen Y, Tabibi S *et al.* Antimicrobial susceptibility and synergy studies of

442 *Burkholderia cepacia* complex isolated from patients with cystic fibrosis. *Antimicrob*

443 *Agents Chemother* 2007; **51**: 1085-8.

444 14. Aaron SD, Vandemheen KL, Ferris W *et al.* Combination antibiotic susceptibility

445 testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria:

446 a randomised, double-blind, controlled clinical trial *Lancet* 2005; **366**: 463-71.

447 15. Cystic Fibrosis Trust. *Antibiotic treatment for cystic fibrosis, Report of the UK*

448 *cystic fibrosis trust antibiotic group, second edition.* Cystic Fibrosis Trust, Bromley,

449 Kent, UK, 2002.

450 16. Clinical and Laboratory Standards Institute. *Performance Standards for*

451 *Antimicrobial Susceptibility Testing; Fourteenth Informational Supplement M100-S14.*

452 CLSI, Wayne, PA, USA, 2004.

453 17. Spoering AL, Lewis K. Biofilms and planktonic cells of *Pseudomonas aeruginosa*

454 have similar resistance to killing by antimicrobials. *J Bacteriol* 2001; **183**: 6746-51.

455 18. Desai M, Bühler T, Weller PH *et al.* Increasing resistance of planktonic and

456 biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during

457 exponential growth. *J Antimicrob Chemother* 1998; **42**: 153-160.

458 19. Dunne WM. Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev*

459 2002; **15**: 155-66.

460 20. Moriarty TF, McElnay JC, Elborn JS *et al.* Sputum antibiotic concentrations:

461 Implications for treatment of cystic fibrosis lung infection. *Pediatr Pulmonol* 2007; **42**:

462 1008-17.

463 21. Hasselmann C. Determination of minimum inhibitory concentrations (MICs) of

464 antibacterial agents by broth dilution. *Clin Microbiol Infect* 2003; **9**: ix-xv.

465   22. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification

466   of microbial biofilms grown in microtiter plates. *J Microbiol Methods* 2008; **72**: 157-65.

467   23. Heersink J. Basic biofilm analytical methods. In: Hamilton M, Heersink J,

468   Buckingham-Meyer K, Goeres D, Eds. *The biofilms laboratory: Step-by-step*

469   *protocols for experimental design, analysis, and data interpretation.* Bozeman:

470   Cytergy Publishing, 2003; 16-23.

471   24. Middleton PG, Kidd TJ, Williams B. Combination aerosol therapy to treat

472   *Burkholderia cepacia* complex. *Eur Respir J* 2005; **26**: 305-8.

473   25. Eisenberg J, Pepe M, Williams-Warren J *et al.* A comparison of peak sputum

474   tobramycin concentration in patients with cystic fibrosis using jet and ultrasonic

475   nebulizer systems. *Chest* 1997; **111**: 955-62.

476   26. Geller DE, Rosenfeld M, Waltz DA *et al.* Efficiency of pulmonary administration of

477   tobramycin solution for inhalation in cystic fibrosis using an improved drug delivery

478   system. *Chest* 2003; **123**: 28-36.

479   27. Weidmann A, Webb AK, Dodd ME *et al.* Successful treatment of cepacia

480   syndrome with combination nebulised and intravenous antibiotic therapy. *J Cyst*

481   *Fibros* 2008; **7**: 409-11.

482   28. Caraher E, Reynolds G, Murphy P *et al.* Comparison of antibiotic susceptibility of

483   *Burkholderia cepacia* complex organisms when grown planktonically or as biofilm *in*

484   *vitro*. *Eur J Clin Microbiol Infect Dis* 2007; **26**: 213-6.

485   29. Moskowitz SM, Foster JM, Emerson J *et al.* Clinically feasible biofilm

486   susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic

487   fibrosis. *J Clin Microbiol* 2004; **42**: 1915-22.

488   30. Ceri H, Olson ME, Stremick C *et al.* The Calgary biofilm device: new technology

489   for rapid determination of antibiotic susceptibilities. *J Clin Microbiol* 1999; **37**: 1771-6.

490   31. Hill D, Rose B, Pajkos A *et al.* Antibiotic susceptibilities of *Pseudomonas*

491   *aeruginosa* isolates derived from patients with cystic fibrosis under aerobic,

492   anaerobic and biofilm conditions. *J Clin Microbiol* 2005; **43**: 5085-90.

493   32. Walters MC, Roe F, Bugnicourt A *et al.* Contributions of antibiotic penetration,

494   oxygen limitation and low metabolic activity to tolerance of *Pseudomonas aeruginosa*

495   biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 2002; **47**:

496   317-23.

497   33 Anwar H, Costerton JW. Enhanced activity of combination of tobramycin and

498   piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa.*

499   *Antimicrob Agents Chemother* 1990; **34**: 1666-71

500   34. Sauer K, Camper AK, Ehrlich GD *et al. Pseudomonas aeruginosa* displays

501   multiple phenotypes during development as a biofilm. *J Bacteriol* 2002; **184**: 1140-54.

502   35. Brown MRW, Collier PJ, Gilbert P. Influence of growth rate on susceptibility to

503   antimicrobial agents: modification of the cell envelope and batch and continuous

504   culture studies. *Antimicrob Agents Chemother* 1990; **34**: 1623-8.

505   36. Anwar H, Strap JL, Chen K *et al.* Dynamic interactions of biofilms of mucoid

506   *Pseudomonas aeruginosa* with tobramycin and piperacillin. *Antimicrob Agents*

507   *Chemother* 1992; **36**: 1208-14.

508   37. Keays T, Ferris W, Vandemheen KL *et al.* A retrospective analysis of biofilm

509   antibiotic susceptibility testing: a better predictor of clinical response in cystic fibrosis

510   exacerbations. *J Cyst Fibros* 2009; **8**: 122-7.

511

512   Table1: MIC of six antibiotics for 38 *B. cepacia* complex strains and two control

513   strains.

Strain	MIC (mg/L)					
	CAZ (8[a])	CIP (1[a])	MEM (4[a])	MIN (4[a])	TOB (4[a] or 256[b])	SXT (2-38[a])
B. cepacia LMG 18821	64	16	16	16	512	8-152
B. cepacia LMG 1222[T]	32	1	8	2	32	8-152
B. multivorans LMG 18822	32	4	16	4	128	16-304
B. multivorans LMG 18825	4	4	8	1	128	8-152
B. multivorans LMG 13010[T]	4	4	4	2	64	4-76
B. multivorans LMG 17588	4	1	4	2	64	8-152
B. cenocepacia LMG 16656[T]	128	8	32	16	256	>128-2432
B. cenocepacia LMG 18828	32	128	8	4	256	128-2432
B. cenocepacia LMG 18829	8	4	4	8	128	32-608
B. cenocepacia LMG 18830	8	16	4	1	1024	8-152
B. stabilis LMG 14294[T]	8	32	4	1	128	>128-2432
B. stabilis LMG 14086	4	0,5	2	1	32	2-38
B. vietnamiensis LMG 18835	4	1	2	2	64	8-152
B. vietnamiensis LMG 10929[T]	4	1	1	1	16	8-152
B. dolosa LMG 18943[T]	>128	64	32	4	128	32-608
B. dolosa LMG 18941	32	64	8	4	256	16-304
B. ambifaria LMG 19182[T]	4	<0.25	2	1	16	2-38
B. ambifaria LMG 19467	2	2	2	2	128	4-76
B. anthina LMG 20980[T]	2	<0.25	1	<0.25	16	4-76
B. anthina LMG 20983	4	<0.25	2	<0.25	32	1-19
B. pyrrocinia LMG 14191[T]	16	1	8	2	64	8-152
B. pyrrocinia LMG 21824	16	2	4	4	512	8-152
B. ubonensis LMG 20358[T]	4	1	8	2	64	4-76
B. ubonensis LMG 24263	8	1	16	2	64	2-38
B. latens LMG 24064[T]	4	4	2	4	32	8-152
B. latens R-11768	16	8	16	8	512	16-304
B. diffusa LMG 24065[T]	32	2	4	1	128	4-76
B. diffusa LMG 24266	32	2	4	1	64	4-76
B. arboris LMG 24066[T]	4	1	2	2	64	1-19
B. arboris R-132	8	<0.25	8	<0.25	128	1-19
B. seminalis LMG 24067[T]	8	2	4	16	128	4-76
B. seminalis LMG 24272	4	1	2	2	64	8-152
B. metallica LMG 24068[T]	32	0.5	8	4	64	4-76
B. metallica R-2712	16	0.5	8	2	64	2-38
B. lata LMG 6992	2	0.25	0.5	0.5	32	0.25-4.75
B. lata R-9940	2	0.25	1	1	16	2-38
B. contaminans LMG 16227	16	1	4	8	32	8-152
B. contaminans R-12710	8	0.25	2	1	2	4-76
P. aeruginosa ATCC 27853	2	0.5	0.5	-	0.5	16-304
E. coli ATCC 25922	0.25	0.008	0.016	0.5	0.5	≤0.5-9.5

514   [a] Breakpoint concentrations CLSI guidelines non-Enterobacteriaceae

515   ^b Breakpoint for high concentrations of tobramycin achieved by nebulization

516   CAZ: ceftazidime; CIP: ciprofloxacin; MEM: meropenem; MIN: minocycline; TOB:

517   tobramycin; SXT: trimethoprim-sulfamethoxazole (1-19)

518

519   Figure 1: Scatter plot of the minimal concentrations of ciprofloxacin necessary to

520   inhibit growth of planktonic cultures (MIC) and freshly adhered sessile cells (4 h,

521   MBIC) of the 38 tested *B. cepacia* complex strains.

522   Figure 2: Boxplot illustrating the distribution of the number of cfu/ (disc or mL)

523   recovered from the untreated and treated planktonic cultures (white) and biofilms

524   (grey) for all 38 tested *B. cepacia* complex strains.

525   UC: untreated control; CAZ: ceftazidime; CIP: ciprofloxacin; MEM: meropenem; MIN:

526   minocycline; TOB: tobramycin; SXT: trimethoprim-sulfamethoxazole (1-19)

527   Figure 3: Representative images of 24 h old *B. cenocepacia* LMG 16656 biofilms,

528   which were treated with saline (A), ciprofloxacin (80 mg/L; B) or tobramycin (1024

529   mg/L; C) during 20 h. The scale bar represents 20 μm.

530   Figure 4: Average numbers of cells (log) present in treated and untreated *B.*

531   *multivorans* LMG 18825 and *B. cenocepacia* LMG 16656 planktonic cultures (white

532   bars) and biofilms (grey bars). Error bars represent standard deviations.

533   UC: untreated control; CAZ: ceftazidime; CIP: ciprofloxacin; MEM: meropenem; MIN:

534   minocycline; TOB: tobramycin; SXT: trimethoprim-sulfamethoxazole (1-19)

535   Figure 5: Average numbers of cells (log) present in untreated and tobramycin treated

536   *B. multivorans* LMG 18825, *B. cenocepacia* LMG 16656 and *B. dolosa* LMG 18943

537   planktonic cultures (untreated: black bars; treated: white bars) and biofilms

538   (untreated: shaded bars; treated: grey bars) which were first grown for 24 h, 76 h or

539   100 h. Error bars represent standard deviations.