1 **Comparative evaluation of methods testing *in vitro* sensitivity to azithromycin in multi-**

2 **drug resistant *Pseudomonas aeruginosa* isolated from cystic fibrosis patients**

3

4

5 Michael Sörensen[1,2]*, Dennis Nurjadi[1], Bakhodur Khakimov[1], Sébastien Boutin[1], Alexander

6 H. Dalpke[1,3,]*, Tatjana Eigenbrod[1,]*

7

8 [1]Department of Infectious Diseases, Medical Microbiology and Hygiene, Heidelberg

9 University Hospital, 69120 Heidelberg, Germany

10 [2]Laboratory Enders and Partners, 70193 Stuttgart, Germany

11 [3]Institute of Medical Microbiology and Hygiene, Technical University Dresden, 01307

12 Dresden, Germany

13

14 Running title: Susceptibility testing of azithromycin for *Pseudomonas aeruginosa*

15

16

17 *Address for correspondence:

18 Prof. Dr. Alexander Dalpke, Prof. Dr. Alexander H. Dalpke, Institute of Medical Microbiology

19 and Hygiene, Medical Faculty, Technical University Dresden, Dresden, Germany,

20 Fetscherstraße 74, 01307 Dresden, Germany, Tel.: +49 351 4586550,

21 alexander.dalpke@ukdd.de

22

23

24 **Abstract**

25 Long-term treatment with azithromycin is a therapeutic option in Cystic Fibrosis (CF) patients
26 chronically infected with *P. aeruginosa*. It was recently shown that azithromycin has direct
27 antimicrobial activity when *P. aeruginosa* isolates are tested in Roswell Park Memorial Institute
28 medium supplemented with fetal calf serum (RPMI 1640/FCS) by broth microdilution. We now
29 investigated whether (i) azithromycin might also be active against multidrug resistant (MDR)
30 *P. aeruginosa* isolated from CF patients and (ii) how *in vitro* sensitivity assays perform in
31 synthetic cystic fibrosis sputum medium (SCFM), a medium that mimics the particular CF
32 airway environment. In 17 (59%) out of 29 MDR *P. aeruginosa* CF isolates MICs for
33 azithromycin ranged between 0.25 and 8 µg/ml and 12 isolates (41%) showed a MIC ≥512
34 µg/ml when measured in RPMI/FCS. In contrast, MICs were ≥256 µg/ml for all *P. aeruginosa*
35 MDR isolates when tested in either SCFM or in conventional cation-adjusted Mueller Hinton
36 Broth. High MIC values observed in CF adapted medium SCFM for both PAO1 and MDR *P.*
37 *aeruginosa* CF isolates, as opposed to findings in RPMI, argue against routine azithromycin
38 MIC testing of CF isolates.

39

40 **Keywords:** cystic fibrosis, *Pseudomonas aeruginosa*, multidrug resistance, broth
41 microdilution, azithromycin, SCFM

42

43

44 **Background**

45 Cystic fibrosis (CF) is a chronic disorder caused by autosomal-recessive mutations in the cystic
46 fibrosis transmembrane conductance regulator (CFTR) gene. Airway infections with
47 *Pseudomonas aeruginosa* (*P. aeruginosa*) are common in CF patients. They are associated with
48 a decline in lung function, thereby contributing to increased mortality (1). Current therapeutic
49 strategies aim at eradicating initial or first infection with *P. aeruginosa*. When eradication fails,
50 chronic infection can develop and then therapy tries to suppress *P. aeruginosa* load (2, 3).
51 Yet, antibiotic targeting of *P. aeruginosa* can be challenging as approximately 20 % of *P.*
52 *aeruginosa* positive patients have been reported by the North American CF registry to carry
53 multidrug resistant (MDR) strains, as defined by resistance to all routinely tested antibiotics in
54 two or more of the following classes: β-lactams, fluoroquinolones and aminoglycosides (4). In
55 these patients, inhaled antibiotics such as tobramycin, colistin and aztreonam have been
56 suggested as therapy of choice due to the high concentrations that can be achieved upon local
57 delivery (5).
58 Moreover, in patients chronically infected with *P. aeruginosa*, long-term treatment with
59 macrolides, especially azithromycin, is an accepted therapeutic option and is progressively
60 becoming standard of care (5-9). The positive effect of azithromycin on clinically relevant end
61 points, including increase in FEV1 and lower risk of pulmonary exacerbations (9), has primarily
62 been attributed to anti-inflammatory and anti-virulence activities of azithromycin. Indeed, sub-
63 inhibitory concentrations of azithromycin have been demonstrated to impair motility, quorum
64 sensing and virulence factor expression including protease activity in *P. aeruginosa* (10).
65 Although traditionally *P. aeruginosa* is considered to be intrinsically resistant to macrolides,
66 recent data indicate that macrolides may possess an *in vitro* antimicrobial activity against *P.*
67 *aeruginosa* depending on the medium used for susceptibility testing by broth microdilution
68 (BMD) (11, 12). Thus, minimal inhibitory concentrations (MICs) of azithromycin were
69 significantly lower in Roswell Park Memorial Institute medium (RPMI 1640), a medium

70 commonly used for culturing eukaryotic cells, in bronchoalveolar lavage fluid or in cation-
71 adjusted Mueller Hinton Broth (CA-MHB) supplemented with serum as compared to MICs
72 measured conventionally in CA-MHB alone (11, 12). Therefore, it was suggested that MIC
73 assessment of azithromycin in *P. aeruginosa* CF isolates using RPMI 1640 could be
74 implemented as routine diagnostic measurement in microbiology laboratories (12).
75 However, it has not been studied whether azithromycin also exhibits antimicrobial activity
76 against MDR *P. aeruginosa*, especially in the context of CF disease. Of note, previously used
77 test media like RPMI1640/FCS do not truly reflect the physiological airway environment
78 observed in CF patients which might affect the interpretation of antibiotic susceptibility. In the
79 present study, we therefore set out (i) to investigate the *in vitro* efficacy of azithromycin in
80 MDR *P. aeruginosa* isolates derived from respiratory specimen of CF patients by (ii) using
81 different media for BMD. Test media were CA-MHB, RPMI 1640 and synthetic cystic fibrosis
82 sputum medium (SCFM), a medium mimicking the nutritional composition of CF sputum that
83 was suggested to reflect physiological conditions (13).
84
85
86 **Methods**
87 **Study population and routine microbiological analysis of samples**
88 The study was done as a retrospective study on *P. aeruginosa* strains stored from Cystic fibrosis
89 patients who received in- or out-patient medical care at the University Hospital between January
90 2013 and December 2016. From those patients *P. aeruginosa* strains that were tested in the
91 routine microbiology laboratory had been stored in skim milk at -80°C. The surveillance of
92 multi-resistant organisms is performed in concordance to the German Infection Protection Act.
93 The local ethics advisory board of the Heidelberg University Hospital was consulted prior to
94 study begin for conformity with the current regulations (S-474/2018). Strain selection is
95 described in the results section in detail. Identification at the species level of isolates cultured

96 from respiratory samples was performed with MALDI-TOF (Bruker) and/or VITEK®2
97 (Biomerieux). Routine susceptibility testing was performed on the VITEK®2 system for fast
98 growing isolates (AST-N248), while agar diffusion was used for slowly growing isolates in
99 accordance with current German guidelines for microbiological laboratory standards (14).
100 Evaluation of colistin susceptibility was performed additionally within this study in
101 cryopreserved isolates by BMD in concordance with current EUCAST (European Committee
102 on Antimicrobial Susceptibility Testing) recommendations (15) using the commercially
103 available Micronaut-S MIC strips (Merlin Diagnostics, Germany). All data for antimicrobial
104 susceptibility testing were interpreted according to EUCAST clinical breakpoints.
105
106 **Determination of azithromycin MIC by BMD**
107 Azithromycin MICs were determined by BMD in 96-well microtiter plates in a concentration
108 range of 0.125 µg/ml to 1024 µg/ml according to current diagnostic standards (16, 17). Briefly,
109 *P. aeruginosa* clinical isolates or laboratory control strain PAO1 were grown overnight on
110 Columbia Blood Agar plates at 36 +/- 1°C and inoculated into CA-MHB, RPMI 1640 with
111 stable glutamine supplemented with 30% fetal calf serum (FCS) or synthetic cystic fibrosis
112 sputum medium (SCFM) (13) at a final concentration of $5x10^5$ CFU/ml. MICs were read as the
113 lowest concentration of azithromycin at which visible growth was inhibited. Two isolates failed
114 to grow in SCFM and were therefore excluded from analysis. Reference strain PAO1 has been
115 described previously (18) and was obtained from the Leibniz Institute DSMZ (German
116 Collection of Microorganisms and Cell Lines, #22644).
117
118 **Determination of *P. aeruginosa* growth curves**
119 Bacterial growth curves were evaluated by using the Cell Growth Quantifier system (CGQ,
120 Aquila Biolabs). CGQ is a technology for non-invasive real-time monitoring of biomass in
121 shake flasks which is based on the measurement of the amount of light scattered towards a

122 sensor as a function of the current biomass concentration inside the flask. To this end, *P.*
123 *aeruginosa* was inoculated at a final concentration of 5 x $10^5$ CFU/ml into the indicated culture
124 media, transferred into Erlenmeyer conical flasks and shaken in the dark in 5% CO2, endvolume
125 10 ml, at 36 +/-1 °C, 200 rpm. Backscattered light was continuously measured by CGQ over
126 24 h.

127

128 **Statistical analysis**

129 Data were analyzed using the STATA13 software (STATACorp, USA). Statistical analysis of
130 AZM MICs in different test media was performed by 2-way ANOVA using GraphPad Prism
131 Software. A p-value of $<0.05$ was considered statistically significant.

132

133

134 **Results**

135 **Study population**

136 Between January 2013 and December 2016, we received respiratory materials from 930 CF
137 patients. *P. aeruginosa* was identified in 292 patients out of which 49 (=16.8 %) carried MDR
138 *P. aeruginosa* (Table 1). MDR was defined according to the rules of the German Commission
139 for Hospital Hygiene and Infection Prevention (KRINKO) as combined resistance to
140 piperacillin, piperacillin/tazobactam, ceftazidime, imipenem, meropenem and ciprofloxacin.
141 This definition is also in line with the one of the North American CF registry (4, 5). Random
142 isolates from approximately two-thirds (n=30/49) of these MDR *P. aeruginosa* positive patients
143 had been cryopreserved in skim milk at -80°C. As two isolates were not cultivable and as one
144 sample contained two different MDR isolates, 29 isolates from 28 patients were finally included
145 in the present study. All patients were classified as chronic *P. aeruginosa* carriers with the
146 exception of one patient with an intermittent carriage status (19). Most patients in the study
147 cohort were aged between 21 and 40 years. The youngest patient who was tested positive for

148 MDR *P. aeruginosa* was 7 years old. Co-resistance to other antibiotics in MDR *P. aeruginosa*
149 was common: all isolates were resistant to aztreonam, 69% to fosfomycin and resistance to
150 aminoglycosides ranged from 62% (tobramycin) to 93% (gentamicin). Non-susceptibility to
151 colistin was observed in 17% of isolates (Table 1). For tobramycin and colistin, antimicrobial
152 susceptibility testing was interpreted for systemic administration as neither EUCAST nor CLSI
153 (Clinical & Laboratory Standards Institute) provide breakpoint values for local application of
154 these antibiotics via inhalation.
155

156 **Determination of azithromycin MIC by BMD in MDR *P. aeruginosa* using different test**
157 **media**

158 Previous studies suggested that the medium used for BMD critically influences the *in vitro*
159 susceptibility of *P. aeruginosa* towards azithromycin (11, 12). Yet it remains unclear if
160 azithromycin exerts direct antimicrobial effects also in MDR *P. aeruginosa* isolates and in CF
161 adapted test medium. We therefore determined azithromycin MICs in (i) MDR *P. aeruginosa*
162 CF clinical isolates using (ii) different media for BMD including CA-MHB (medium
163 commonly used for BMD), RPMI 1640 (eukaryotic cell culture medium used by (11, 12)) and
164 SCFM (medium mimicking CF airway milieu). Unlike previously described (11), *P.*
165 *aeruginosa* reference strain PAO1 failed to grow in RPMI 1640 alone but required the presence
166 of FCS (Fig. 1A). Yet, in line with the data of Buyck *et al.* (11), azithromycin MICs against
167 PAO1 were 1 µg/ml when measured in RPMI supplemented with 30% FCS and ranged between
168 128-256 µg/ml in CA-MHB in three independent experiments (Fig. 1B). Surprisingly, in
169 SCFM, azithromycin MIC of PAO1 was reproducibly determined with ≥1024 µg/ml and was
170 thus even slightly higher than in CA-MHB (Fig. 1B). Of note, in MDR *P. aeruginosa* clinical
171 isolates derived from CF patients, two distinct populations became evident when azithromycin
172 MICs were assessed in RPMI/FCS: In 17/29 MDR isolates (59%), MIC ranged between 0.25
173 and 8 µg/ml whereas 12/29 MDR isolates (41%) had a MIC of ≥512 µg/ml (Fig. 1C). However,

174 MICs were ≥256 μg/ml for all MDR isolates when measured either in CF adapted medium
175 SCFM or conventional CA-MHB (Fig. 1C).
176
177
178 **Discussion**
179 Several clinical studies have validated the beneficial effects of long-term treatment with
180 azithromycin in CF patients chronically infected with *Pseudomonas aeruginosa* (*P.*
181 *aeruginosa*) and its usage has progressively entered clinical guidelines (5-9). The efficiency of
182 azithromycin has been attributed to its anti-inflammatory and anti-virulence properties
183 including e.g. inhibition of motility, quorum sensing and protease activity (9, 10, 20, 21).
184 Although *P. aeruginosa* is considered naturally resistant to macrolides, *in vitro* susceptibility
185 was previously demonstrated upon testing in alternative media including eukaryotic cell
186 medium RPMI 1640 (supplemented or not with FCS) or serum-supplemented CA-MHB,
187 suggesting that macrolides might additionally exert direct antimicrobial activity on *P.*
188 *aeruginosa* (10,11). The differences observed in phenotypic susceptibility to azithromycin
189 depending on the test medium have been ascribed to increased outer-membrane permeability
190 and decreased expression of efflux pumps in the presence of RPMI 1640 or serum, leading to
191 enhanced azithromycin accumulation inside the bacteria (11). The authors therefore proposed
192 that azithromycin MIC testing of *P. aeruginosa* CF isolates in RPMI 1640 could routinely be
193 included in microbiological diagnostics (11).
194 Extending previous findings, we demonstrate here that in 17 out of 29 (59%) MDR *P.*
195 *aeruginosa* CF isolates, MIC values were low when tested in RPMI supplemented with FCS,
196 ranging from 0.25-8 μg/ml. In contrast, i*n vitro* resistance with high MICs to azithromycin even
197 in RPMI/FCS as found in 12 out of 29 MDR isolates in the present study might be explained
198 by mutations in the 23S rRNA which are frequently detected in CF isolates. Indeed, Mustafa *et*
199 *al.* observed mutations in domain V of 23S rRNA in 43% of CF *P. aeruginosa* isolates while

200 mutations were absent in 48 tested strains derived from patients suffering from hospital
201 acquired pneumonia (12). Thus, testing in RPMI/FCS might be an option to identify *P.*
202 *aeruginosa* resistance caused by 23S rRNA mutation.
203 However, although RPMI and CA-MHB supplemented with FCS have been suggested to more
204 closely resemble the eukaryotic environment and therefore to constitute the better test medium,
205 these media do not necessarily reflect the particular milieu in the airways of CF patients. It was
206 suggested that the physiological situation of CF airways might be better mimicked by SCFM
207 which imitates the specific nutritional composition and ion concentrations of CF sputum (13).
208 We therefore evaluated susceptibility of MDR *P. aeruginosa* in this medium. Of note,
209 azithromycin MICs were consistently ≥256 µg/ml in SCFM in all *P. aeruginosa* clinical isolates
210 as well as in reference strain PAO1, arguing against a direct antimicrobial effect of
211 azithromycin in the airways of CF patients. Macrolides are protonated in acidic environments
212 going along with reduced activity. SCFM was used with a pH of 6.8, which might interfere with
213 activity, yet, these conditions probably are those to be encountered in the CF airways. As a
214 conclusion, our data therefore do not support routine azithromycin MIC assessment in CF
215 clinical isolates using RPMI/FCS, as proposed previously (12). This study shows that for CF
216 isolates and macrolides *in vitro* testing is associated with a high level of uncertainty. SCFM,
217 sputum adapted medium, might be more appropriate for antimicrobial susceptibility testing than
218 conventional broth. This notion is also supported by a recent publication of Diaz Iglesias *et al*
219 who investigated antibiotic susceptibility, biofilm formation and metabolic activity using
220 different media (22).
221 Our results do not substantiate a direct antimicrobial effect of azithromycin on *P. aeruginosa*
222 when tested in SCFM, a medium that represents the CF environment at best. Our data therefore
223 do not support the implementation of azithromycin MIC assessment of *P. aeruginosa* CF
224 isolates in routine microbiological diagnostics as suggested previously (12). The results warrant

225 further assessment of the *in vivo* efficacy of azithromycin in the subgroup of MDR *P.*
226 *aeruginosa* infected CF patients in prospective clinical trials.
227
228
229
230 **Abbreviations**
231 CF, cystic fibrosis; *P. aeruginosa, Pseudomonas aeruginosa*; CFU, colony forming units;
232 SCFM, Synthetic Cystic Fibrosis Sputum Medium; CA-MBH, cation adjusted Mueller Hinton
233 Broth; RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum; BMD, broth
234 microdilution; MIC, minimal inhibitory concentration; MDR, multi-drug resistance; LIS,
235 laboratory information system; CGQ, Cell Growth Quantifier
236
237 **Declarations**

238 **Ethics approval and consent to participate**
239 The surveillance of multi-resistant organisms is performed in concordance to the German
240 Infection Protection Act. The local ethics advisory board of the Heidelberg University Hospital
241 was consulted prior to study begin for conformity with the current regulations (S-474/2018).
242
243 **Consent for publication**
244 not applicable
245
246 **Availability of data and material**
247 The datasets used and/or analysed during the current study are available from the corresponding
248 author on reasonable request.
249

250 **Competing interests**

251 The authors declare that they have no competing interests.

252

253 **Funding**

254 The authors received no specific grant from any funding agency.

255

256 **Authors' contributions**

257 MS and TE concepted and supervised the entire study. BK, MW and TE performed
258 experiments. MW, DN, SB and TE analyzed the data. AHD provided resources and critically
259 discussed the results. TE wrote the initial draft of the manuscript. All authors read and approved
260 the final manuscript.

261

262 **Acknowledgements**

263 We thank Suzan Leccese and Selina Hassel for excellent technical support.

264

265

266 **References**

267 1. Mall MA, Hartl D. CFTR: cystic fibrosis and beyond. Eur Respir J. 2014;44(4):1042-
268 54.
269 2. Lund-Palau H, Turnbull AR, Bush A, Bardin E, Cameron L, Soren O, et al.
270 Pseudomonas aeruginosa infection in cystic fibrosis: pathophysiological mechanisms and
271 therapeutic approaches. Expert Rev Respir Med. 2016;10(6):685-97.
272 3. Mayer-Hamblett N, Kloster M, Rosenfeld M, Gibson RL, Retsch-Bogart GZ, Emerson
273 J, et al. Impact of Sustained Eradication of New Pseudomonas aeruginosa Infection on Long-
274 term Outcomes in Cystic Fibrosis. Clin Infect Dis. 2015;61(5):707-15.
275 4. Report CFFPRAD. https://wwwcfforg/Research/Researcher-Resources/Patient-
276 Registry/2017-Patient-Registry-Annual-Data-Reportpdf Accessed 22 May 2019.
277 5. Stefani S, Campana S, Cariani L, Carnovale V, Colombo C, Lleo MM, et al. Relevance
278 of multidrug-resistant Pseudomonas aeruginosa infections in cystic fibrosis. Int J Med
279 Microbiol. 2017;307(6):353-62.
280 6. Chalmers JD. Macrolide resistance in Pseudomonas aeruginosa: implications for
281 practice. Eur Respir J. 2017;49(5).

282 7. Mogayzel PJ, Jr., Naureckas ET, Robinson KA, Mueller G, Hadjiliadis D, Hoag JB, et
283 al. Cystic fibrosis pulmonary guidelines. Chronic medications for maintenance of lung health.
284 Am J Respir Crit Care Med. 2013;187(7):680-9.
285 8. Principi N, Blasi F, Esposito S. Azithromycin use in patients with cystic fibrosis. Eur J
286 Clin Microbiol Infect Dis. 2015;34(6):1071-9.
287 9. Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, et al.
288 Azithromycin in patients with cystic fibrosis chronically infected with Pseudomonas
289 aeruginosa: a randomized controlled trial. JAMA. 2003;290(13):1749-56.
290 10. Imperi F, Leoni L, Visca P. Antivirulence activity of azithromycin in Pseudomonas
291 aeruginosa. Front Microbiol. 2014;5:178.
292 11. Buyck JM, Plesiat P, Traore H, Vanderbist F, Tulkens PM, Van Bambeke F. Increased
293 susceptibility of Pseudomonas aeruginosa to macrolides and ketolides in eukaryotic cell culture
294 media and biological fluids due to decreased expression of oprM and increased outer-membrane
295 permeability. Clin Infect Dis. 2012;55(4):534-42.
296 12. Mustafa MH, Khandekar S, Tunney MM, Elborn JS, Kahl BC, Denis O, et al. Acquired
297 resistance to macrolides in Pseudomonas aeruginosa from cystic fibrosis patients. Eur Respir J.
298 2017;49(5).
299 13. Palmer KL, Aye LM, Whiteley M. Nutritional cues control Pseudomonas aeruginosa
300 multicellular behavior in cystic fibrosis sputum. J Bacteriol. 2007;189(22):8079-87.
301 14. Hogardt MH, Susanne; Balke, Beate; Kahl, Barbara C.; Schmoldt, Sabine; Leitritz,
302 Lorenz; Jäger, Gundula; Kappler, Matthias; Suerbaum, Sebastian; Heesemann, Jürgen
303 Atemwegsinfektionen bei Mukoviszidose. Qualitätsstandards in der mikrobiologisch-
304 infektiologischen Diagnostik 2006;MiQ24.
305 15. Matuschek E, Ahman J, Webster C, Kahlmeter G. Antimicrobial susceptibility testing
306 of colistin - evaluation of seven commercial MIC products against standard broth microdilution
307 for Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp.
308 Clin Microbiol Infect. 2018;24(8):865-70.
309 16. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial
310 activity: A review. J Pharm Anal. 2016;6(2):71-9.
311 17. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the
312 minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc.
313 2008;3(2):163-75.
314 18. Oberhardt MA, Puchalka J, Fryer KE, Martins dos Santos VA, Papin JA. Genome-scale
315 metabolic network analysis of the opportunistic pathogen Pseudomonas aeruginosa PAO1. J
316 Bacteriol. 2008;190(8):2790-803.
317 19. Pressler T, Bohmova C, Conway S, Dumcius S, Hjelte L, Hoiby N, et al. Chronic
318 Pseudomonas aeruginosa infection definition: EuroCareCF Working Group report. J Cyst
319 Fibros. 2011;10 Suppl 2:S75-8.
320 20. Castellani C, Duff AJA, Bell SC, Heijerman HGM, Munck A, Ratjen F, et al. ECFS
321 best practice guidelines: the 2018 revision. J Cyst Fibros. 2018;17(2):153-78.
322 21. Molinari G, Guzman CA, Pesce A, Schito GC. Inhibition of Pseudomonas aeruginosa
323 virulence factors by subinhibitory concentrations of azithromycin and other macrolide
324 antibiotics. J Antimicrob Chemother. 1993;31(5):681-8.
325 22. Diaz Iglesias Y, Wilms T, Vanbever R, Van Bambeke F. Activity of antibiotics against
326 Staphylococcus aureus in an in vitro model of biofilms in the context of cystic fibrosis:
327 influence of the culture medium. Antimicrob Agents Chemother. 2019; 63(7):e00602-19
328

329

330

331 **Figure Legends**

332 **Fig. 1: Evaluation of azithromycin MICs against *P. aeruginosa* strain PAO1 and MDR *P.*
333 *aeruginosa* CF isolates in different media**

334 (A) *P. aeruginosa* strain PAO1was inoculated in the indicated media and increase in bacterial
335 growth was continuously evaluated over 24 h by measuring backscattered light intensity using
336 Cell Growth Quantifier system. CA-MHB: cation-adjusted Mueller Hinton Broth; RPMI:
337 Roswell Park Memorial Institute 1640 medium; FCS: fetal calf serum; SCFM: synthetic cystic
338 fibrosis sputum medium. Data indicate mean (solid lines) +/- SD (dotted lines) from three
339 independent experiments. (B, C) MICs of azithromycin were determined by broth microdilution
340 in *P. aeruginosa* strain PAO1 (B) and in multidrug resistant (MDR) clinical *P. aeruginosa*
341 isolates derived from cystic fibrosis patients (C) using CA-MHB, SCFM and RPMI 1640
342 supplemented with 30% FCS as test medium. Bars indicate mean +/- SD from three independent
343 experiments (B) or median values (C). For MDR isolates, n=29 for CA-MHB and RPMI/FCS
344 and n=27 for SCFM. Statistical analysis was performed by 2-way ANOVA. (**) $p<0.01$; (***)
345 $p<0.001$; ns: not significant

346

347 **Table 1**
348

| | CF patients | |
|---|---|---|
| | n | |
| **total** | 930 | |
| ***P. aeruginosa* pos.** | 292 | |
| ***P. aeruginosa* MDR pos.*** | 49 (=16.8 % of *P. aeruginosa* pos.) | |
| | **MDR *P. aeruginosa*, n=29** | |
| | n | % |
| **Age** | | |
| 0-10 | 4 | 14 |
| 11-20 | 2 | 7 |
| 21-30 | 10 | 34 |
| 31-40 | 7 | 24 |
| 41-50 | 2 | 7 |
| >51 | 4 | 14 |
| **Resistance** to** | | |
| colistin*** | 5 | 17 |
| fosfomycin | 20 | 69 |
| aztreonam | 29 | 100 |
| gentamicin | 27 | 93 |
| tobramycin*** | 18 | 62 |
| amikacin | 25 | 86 |
| **carriage status ******| | |
| intermittent | 1 | 3 |
| chronic | 28 | 97 |

* defined as combined resistance to piperacillin/tazobactame, ceftazidime, imipenem, meropenem and ciprofloxacin

** categorized as resistant if MIC interpreted as intermediate or resistant

*** i.v., no EUCAST breakpoints for inhalation

**** definitions
chronic: > 50% *P. aeruginosa* positive samples within 12 months
intermittent: < 50% *P. aeruginosa* positive samples within 12 months
negative: > 1 year *P. aeruginosa* negative

349