

1 **Comparative evaluation of methods testing *in vitro* sensitivity to azithromycin in multi-**
2 **drug resistant *Pseudomonas aeruginosa* isolated from cystic fibrosis patients**

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

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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 \geq 512
34 µg/ml when measured in RPMI/FCS. In contrast, MICs were \geq 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

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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 5x10⁵ 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 5×10^5 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 (STATA Corp, 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.

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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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$. In contrast, *in 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 $\mu\text{g/ml}$ 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.

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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

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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

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328

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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 PAO1 was 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	
<i>P. aeruginosa</i> pos.	292	
<i>P. aeruginosa</i> MDR pos.*	49 (=16.8 % of <i>P. aeruginosa</i> pos.)	
MDR <i>P. aeruginosa</i>, 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
fosfomicin	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