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1 ***In vitro* evaluation of novel bi- or tri-antibiotic combination against clinical isolates of**
2 ***Mycobacterium abscessus***

3

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20 **Abstract**

21 *Mycobacterium abscessus* is an emerging pathogen, intrinsically resistance to many
22 antimycobacterial drugs. The recommended treatment is limited to combination of intravenous
23 amikacin (AMK), and ceftiofloxacin (FOX) with oral clarithromycin (CLR). However, recent
24 reports demonstrate intrinsic resistance to CLR in *M. abscessus* clinical isolates.
25 Fluoroquinolones, rifamycins, linezolid (LZD) or clofazimine (CLO) can be added when
26 standard therapy is ineffective. This study aims to evaluate the *in vitro* efficacy of several
27 combinations against clinical isolates of *M. abscessus* including FOX and AMK and replacing
28 CLR to avoid the induced resistance. Minimum inhibitory concentration (MICs) were
29 determined for ciprofloxacin (CIP), moxifloxacin (MXF), rifampicin (RIF), rifabutin (RFB),
30 CLO, LZD and CLR according to CLSI guidelines. Then, these antibiotics were investigated in
31 tri-combinations with FOX plus AMK to compare their efficacy against *M. abscessus* reference
32 strain CIP104536, and two clinical isolates Ma1611 and T28 using time-kill kinetic assays.
33 Efficacy of several bi-combinations was evaluated against T28. Clinical isolate T28 was
34 resistant to all antibiotics, CIP104536 and Ma1611 were susceptible to intermediate against all
35 tested antibiotics. Tri-combinations including FOX plus AMK in presence of LZD, MXF, RIF
36 or RFB were active against CIP104536 and Ma1611. Tri-combinations including CLO or CIP
37 were also active against CIP104536 but inactive against Ma1611. All tested triple combinations
38 were inactive against T28. Since T28 was highly resistant to AMK and FOX alone
39 demonstrated only initial killing followed by regrowth, FOX was used in bi-combinations.
40 Hereafter, bi-combinations of FOX with LZD, RIF and RFB were effective and prevented the
41 regrowth observed with FOX alone. Bi-combination FOX with RFB was the most active against
42 strain T28. Tri-combinations were highly efficient against *M. abscessus* reference strain and
43 intermediate to susceptible clinical isolate Ma1611 but not against multidrug-resistant isolate
44 T28. The synergy between FOX and rifamycins suggests a potent role of this combinations that

45 may warrant further optimization of treatment regimen for the treatment of *M. abscessus*
46 pulmonary infections.

47

48 **Introduction**

49 Over past few years, *Mycobacterium abscessus*, a rapidly growing mycobacteria, has emerged
50 as an opportunistic pathogen responsible for wide spectrum of infections specially, pulmonary
51 infections in cystic fibrosis (CF) patients, leading to rapid decline in respiratory function (1).
52 *M. abscessus* is naturally resistant to most of the antibiotics including anti-tuberculous agents
53 (2), meaning that there are no effective therapeutic drug regimens to eradicate *M. abscessus*
54 pulmonary infections, as stated by the American Thoracic Society (ATS) (3). The
55 recommended treatment consists of a combination of an oral clarithromycin (CLR) with
56 intravenous (IV) amikacin (AMK) and cefoxitin (FOX), is highly associated with poor
57 prognosis and mortality (4). The induced macrolide resistance in more than 80% cases and
58 inadequate antibiotic concentration after IV administration may explain this treatment failure
59 (5). Since macrolide resistance compromises the treatment efficacy, there is an urgent need to
60 find a replacement and identify better therapeutic options. This issue can be resolved by
61 repurposing existing efficient antimycobacterial agents i.e. FOX and AMK (6–10) by
62 nebulization (NEB) (11, 12). NEB could achieve high lung drug concentration with
63 concomitant low systemic absorption, low serum drug concentration, and consequently reduced
64 toxicity (13). Furthermore, several studies of combinations have shown additive, synergistic or
65 antagonistic effect in different cases against this infection. For example, combinations of
66 clofazimine (CLO) with AMK or CLR (14, 15), AMK with linezolid (LZD) (16), rifampicin
67 (RIF) with carbapenem (17), CLR with LZD or tigecycline (TGC) or vancomycin (16, 18, 19)
68 have shown synergistic activity in various *in vitro* or *in vivo* studies. Despite of showing
69 synergistic activity, these bi-combinations are most of the time associated with the development

70 of resistance and poor outcomes. In such cases, triple combinations could allow to better
71 eradicate *M. abscessus* infections, but the data related triple combinations are quite limited.
72 Hence, to evaluate the *in vitro* bacterial sterility over time, following the exposure to combined
73 antibiotics, several *in vitro* experiments containing two and three antibiotics in combination
74 were performed on different strains of *M. abscessus*. Then, the activity of bi- or tri-
75 combinations were compared with different *M. abscessus* strains.

76

77 **Results**

78 *Antimicrobial susceptibility testing:* The minimal inhibitory concentrations (MIC) determined
79 for each antibiotic has been shown in Table 1. The clinical isolate T28 was fully resistant to
80 almost all antibiotics tested and intermediate for FOX (20). Isolate T28 was highly resistant to
81 AMK with MIC > 1024 mg/L alike LZD, RIF and CLR (MIC \geq 256 mg/L). Both *M. abscessus*
82 CIP104536 and Ma1611 strains demonstrated almost similar profile except CLO and CLR. All
83 antibiotics fall under the breakpoints for susceptible to intermediate, except RIF. CIP104536
84 was susceptible to CLR (MIC = 1 mg/L) while MIC value of CLR was over the resistant
85 breakpoint for Ma1611 (MIC = 16 mg/L). The clinical breakpoints for RFB and CLO against
86 *M. abscessus* are not determined yet, however MIC value for CLO was higher against Ma1611
87 (MIC = 16 mg/L) than CIP104536 (MIC = 2 mg/L) and T28 (MIC = 4 mg/L). MIC value for
88 RFB was 8 times higher for T28 (MIC = 16 mg/L) than CIP104536 and Ma1611 (MIC = 2
89 mg/L). Furthermore, all three strains were resistant to RIF.

90 *Time kill curves of antibiotics alone and in combinations against reference strain: M. abscessus*
91 CIP104536 was exposed to several antibiotics including FOX, AMK, CLR, CLO, CIP, MXF,
92 LZD, RIF and RFB, at MICs value that were close to the concentrations achievable in humans
93 (21), as shown on Figure 1A. None of the antibiotic was active alone, showing rapid regrowth
94 from day 2. The time kill curves of triple-combinations containing AMK, FOX and a third

95 molecule against CIP104536 are presented on Figure 1B. All triple combinations were efficient
96 showing rapid bacterial killing to reach detection limit from day 4 for combinations with RIF,
97 RFB, CIP, MXF and CLO, and from day 6 in case of combination with LZD. The combination
98 including CLR was the least active and could not reach detection limit within 8 days.

99 *Time kill curves of triple combinations against clinical isolates:* We then compared the activity
100 of these triple combinations against the clinical isolates. All triple combinations were tested
101 using the similar concentration used for reference strain. The activity of the triple combinations
102 was expressed as a change in log CFU at day 4 and day 8 from respective bacterial CFU count
103 at time 0 (Figure 2). First, the bacterial growth in control without antibiotic was lower for
104 multidrug resistant T28 isolate (2-log bacterial growth at day 8) compared to the other strains
105 (around 4-log bacterial growth at day 8). As presented in Figure 1B and Figure 2A, all
106 combinations were effective against reference strain showing bacterial decay up to 4-log (below
107 the limit of detection) at day 8. The effect of triple combinations containing RIF, RFB, LZD,
108 MXF and CIP against Ma1611 (Figure 2B) was comparable to the reference strain (Figure 2A)
109 but regrowth was observed for triple combinations containing CLR from day 4. The
110 combination with CLO also showed bacterial killing with 2-log CFU decrease against Ma1611,
111 but regrowth was observed at day 8 (Figure 2B). However, none of the triple combinations were
112 active against *M. abscessus* T28 (Figure 2C), showing only bacteriostatic activity for most of
113 the combinations except for the combination with LZD, which achieved 2-log decrease in
114 bacterial density from day 4 with no regrowth observed.

115 *Time kill curves of bi-combinations against M. abscessus multidrug resistant T28 isolate:* To
116 find an active combination against T28, we decided to evaluate the combination using
117 comparatively higher concentration of FOX corresponding to its own MIC (i.e. 64 mg/L). Other
118 antibiotics were used at the MICs of reference strain corresponding to human C_{max} (21). For *M.*
119 *abscessus* T28, all antibiotics tested alone were not active (Figure 3A) except FOX and RFB.

120 Both FOX and RFB have shown initial bacterial killing (2-log CFU decrease, until day 4)
121 followed by regrowth. Hereafter, we evaluated the efficacy of bi-combinations (Figure 3B and
122 3C). Bi-combination of FOX with RFB was the most active showing complete bacterial killing
123 (CFU count below the limit of detection) after day 4. The combination of FOX with RIF or
124 LZD showed slow decrease in CFU and reached 3 to 4-log decrease in CFU at day 8. Also, the
125 combinations with RFB, RIF and LZD have completely prevented the bacterial regrowth
126 observed with FOX alone. Bi-combinations of FOX with CIP, MXF and CLO have initially
127 shown a bacterial load decrease up to 4-log, but regrowth was observed after day 6 (Figure 3C).
128 The combination with CLR have also shown initial bacterial killing but a rapid regrowth was
129 observed after day 4 and the combination was as active as FOX alone.

130

131 **Discussion**

132 Starting from the recommended antibiotic combination treatment including AMK, FOX and
133 CLR, we conducted our studies to find a replacement for CLR, as CLR has been reported to be
134 responsible for resistance development and treatment failure in most cases. In present study,
135 the recommended combination was the least active that was consistent with previous *in vitro*
136 studies. Ferro *et al.* (9) reported only a small decrease in bacterial population size when exposed
137 to AMK, FOX or CLR. This combination also failed in hollow fiber system because of observed
138 bacterial regrowth (22).

139 As mentioned previously (23, 24), FOX is an unstable cephalosporin antibiotic with an *in vitro*
140 degradation half-life of 1.5 days (12). In such cases, Schoutrop *et al.* (24) suggested for daily
141 addition of unstable antibiotic to obtain useful test results, also as already been applied to
142 imipenem. Hence, in this study, 50% FOX amount was added each 24 h in order to compensate
143 the FOX degradation.

144 In previous studies, Park *et al.* (25) reported moderate activity of CIP and MXF against several
145 *M. abscessus* isolates, but also suggested to use these antibiotics in combination to avoid
146 mutational resistance. Ferro *et al.* (26) observed no activity of MXF in hollow fiber system and
147 also suggested to use MXF only in combination. Maurer *et al.* (27) reported no *in vitro* activity
148 of MXF up to 24h. Consistently, combinations with fluoroquinolones (CIP and MOX) have
149 shown efficient bacterial killing against reference strain and the clinical isolate Ma1611, but
150 were poorly efficient against T28.

151 As Wallace *et al.* (28) reported excellent potential of LZD against rapid growing mycobacteria,
152 we tested the triple combination with LZD that was efficient against reference strain and
153 Ma1611 and was the most active of the tested triple combinations against T28. But, LZD in
154 combination with AMK, MXF, TGC and FOX were shown rarely synergistic by Zhang *et al.*
155 (16). A possible explanation for this discrepancy could be that we used triple combinations
156 including FOX and AMK, instead of double combinations in the previous study.

157 Several studies reported CLO as a potent antibiotic for treatment against NTM infection (14,
158 15). Also in previous study, CLO showed synergistic effect with AMK (14) and prevented
159 bacterial regrowth in presence of AMK against the reference strain (15). Consistently, our tested
160 triple combination with CLO has also shown good activity against reference strain. However,
161 an initial bacterial killing followed by a regrowth was observed in both clinical isolates
162 suggesting limited use of this antibiotic against *M. abscessus* infections.

163 Anti-tuberculous agents rifamycins are not generally used in clinical practice for the treatment
164 of pulmonary infections caused by *M. abscessus* due to poor *in vitro* activity and development
165 of resistance (29). However, our triple combinations containing RIF and RFB showed bacterial
166 killing against reference strain and Ma1611, and bacteriostatic activity against T28. These
167 results are consistent with a recent study, where RFB exhibited potential *in vitro* activity against
168 the reference strain and CLR-resistant of *M. abscessus* (29). However, the concentration used

169 for RFB was 2 to 3 times higher than achievable peak serum concentration post-oral
170 administration (21), which means that this combination may be unreasonable in clinical setting.
171 Also, the susceptibility breakpoints for CLO and RFB are not determined yet, but MICs values
172 and achievable peak serum concentration (21) indicates that it might be difficult to use these
173 antibiotics against *M. abscessus* infections. Although TGC showed the best activity and has
174 also shown synergistic effect with AMK (8, 18), we decided to not use TGC, as MIC of TGC
175 for reference strain (30) (MIC = 4 mg/L) was comparatively higher than achievable peak serum
176 concentration ($C_{\max} = 1.5$ mg/L after 0.1g IV).

177 The clinical isolate T28 was found to be highly resistant to AMK (MIC > 1024 mg/L) due to
178 presence of acquired resistance to aminoglycosides by A1408G mutation of the *rrs* gene
179 encoding rRNA 16S and intermediate to FOX (MIC = 64 mg/L). Consequently, we decided to
180 remove AMK and to evaluate the activity of bi-combinations including FOX (at its
181 corresponding MIC) and a second antibiotic. FOX alone has shown initial bacterial killing
182 followed by regrowth, which were prevented by the combinations with LZD and rifamycins i.e.
183 RIF and RFB.

184 As explained above, in many cases even though being the antibiotics of choice, FOX and AMK
185 can only be used at high concentration. In addition, in several cases systemic administration of
186 these antibiotics is not tolerable because of toxicity (i.e. ototoxicity and nephrotoxicity by AMK
187 and neutropenia and thrombocytopenia by FOX). Therefore, an approach of replacing systemic
188 administration of FOX (12) and AMK (11) by NEB can be a good option to achieve high lung
189 drug concentration with less systemic side effects.

190 Furthermore, in case of *M. abscessus* pulmonary infection, bacteria can swiftly grow and
191 survive in extracellular airway mucus as well as intracellularly within macrophages (31). CLR,
192 LZD and RFB accumulate in lung tissues at concentrations above their susceptible MICs values
193 (20). However, CLR and LZD cannot be the first choice of antibiotics because of their

194 associated resistance (2, 16), in contrast to RFB. Indeed, RFB demonstrates intracellular activity
195 in combination against *M. abscessus* within this niche (32). On other hand, AMK acts
196 extracellularly due to its limited permeability in macrophages and FOX penetration within lung
197 tissue is not documented. However, using nebulized antibiotics (AMK and FOX), very high
198 local concentrations can be achieved, also a very small part could penetrate inside cells and
199 reach intracellular forms of *M. abscessus*. Including all these factors, triple combination of
200 nebulized AMK and FOX with an oral RFB (bi-combination in case of AMK and CLR
201 resistance), can be an auspicious treatment option against intracellular *M. abscessus* pulmonary
202 infections.

203 This experimental study, carried out over 8 days, which is comparatively longer than other
204 reported *in vitro* studies for antibiotics alone or in combination (9, 15, 17, 27, 32–34), has one
205 limitation. The objective of the study was to compare the activity of tri-combinations at C_{max},
206 so antimicrobial activity in bi- or tri-antibiotic combinations at various different concentrations
207 was not investigated. Hence, a follow up study to optimize the most potent combination
208 including RFB could an interesting option, especially against multidrug resistant *M. abscessus*
209 isolates like T28.

210 In conclusion, time-kill assays revealed that combinations including FOX, AMK and
211 antimicrobials screened from LZD, RIF, RFB, CIP, MXF and CLR may provide an alternative
212 treatment for *M. abscessus* pulmonary infections. The efficient activity of these combinations
213 may warrant a “renaissance” of treatment against *M. abscessus*. The addition of third antibiotic
214 remains controversial as susceptibility to antibiotics depends on the geographical diversity.
215 However, the combination of FOX with RFB, RIF or LZD may act as an effective treatment
216 approach even if *M. abscessus* isolates are resistant to AMK.

217

218 **Materials and method**

219 *Bacterial strain and suspension preparation: Mycobacterium abscessus subspecies abscessus,*
220 reference strain CIP104536, was obtained from Institute Pasteur (Paris, France), clinical isolate
221 Ma1611 was isolated from lung expectoration (CHU of Poitiers, France) and T28 was isolated
222 from bronchial aspiration in patients with cystic fibrosis (Lariboisière Hospital, Paris, France).
223 Stock vials were conserved at -80°C. For each experiment, the mycobacterial inoculum was
224 prepared according to CLSI guidelines (20).

225 *Antimicrobial susceptibility testing (AST):* MICs were determined by broth microdilution
226 method using Cation-adjusted Mueller–Hinton broth 2 (CAMHB II; Sigma-Aldrich, Saint-
227 Quentin-Fallavier France) supplemented with 10% oleic acid/bovine albumin/dextrose/catalase
228 (OADC) growth supplement (BD, BBL™, Sparks, MD, USA) and 0.5% glycerol (Carl Roth
229 GmbH + Co. KG, Karlsruhe, Germany) for each antibiotic. The antibiotics were serially diluted
230 to the desired final concentration in a 96-well plate. The bacterial suspension (1×10^6 CFU/mL)
231 was added to each well, with the final antibiotic concentrations ranging from 0.125 to 1024
232 mg/L. A positive control, only with bacterial suspension and without antibiotic was included
233 for each strain. The plates were incubated at 30°C for 3 days (20). MICs were visually
234 determined. All experiments were performed in duplicate.

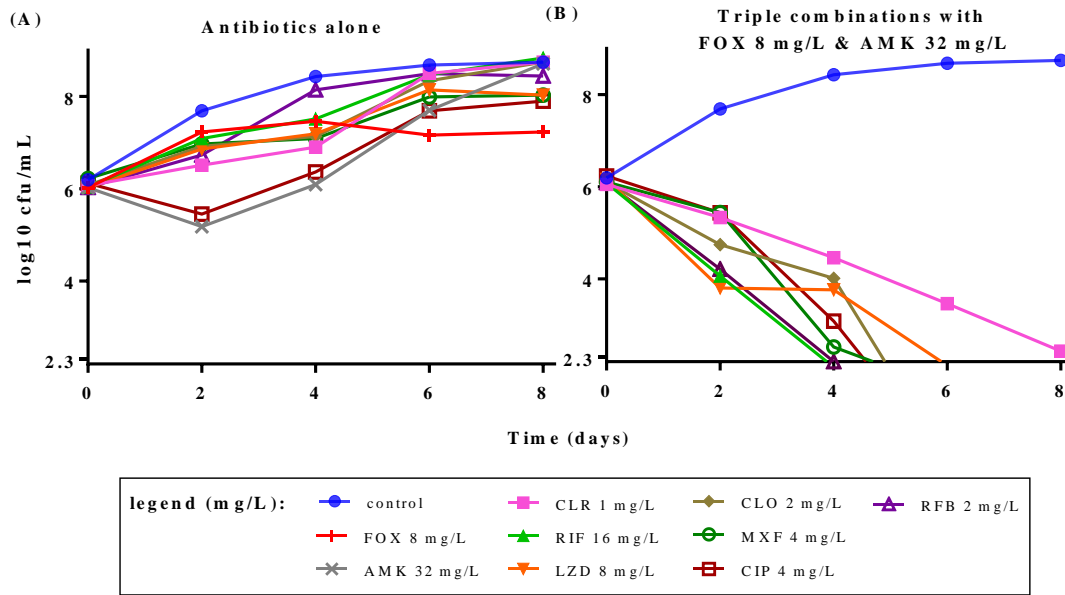
235 *Time-kill kinetics assay. For single antibiotics:* Individual tubes of 20 mL of middlebrook 7H9
236 broth (BD, BBL™, Sparks, MD, USA) with 10% oleic acid/bovine albumin/dextrose/catalase
237 (OADC) growth supplement (BD, BBL™, Sparks, MD, USA) and 0.5% glycerol containing
238 FOX, AMK, CIP, MXF, LZD, CLO, CLR, RIF and RFB at their MICs values for reference
239 strain CIP104536, were inoculated with the bacterial suspension ($\sim 1 \times 10^6$ CFU/mL) and
240 incubated at $35^\circ \pm 2^\circ\text{C}$, under shaking conditions (150 rpm) up to 8 days. Bacteria were
241 quantified at 0, 2, 4, 6 and 8 days by plating serial dilutions prepared with sterile phosphate
242 buffer saline (PBS pH 7.4, Gibco™, by life technologies, France) on middlebrook 7H11 agar
243 plates (BD, BBL™, Sparks, MD, USA) with 10% OADC and 0.5% glycerol (20). CFUs were

244 enumerated after 3-5 days of incubation at $35 \pm 2^\circ\text{C}$. The theoretical detection limit was set to
245 200 CFU/mL i.e. $2.3 \log_{10}$ CFU/mL. *For antibiotic combination:* Following the same procedure
246 as described above, time-kill assays for triple combinations containing FOX and AMK in
247 presence of 3rd antibiotic were tested against CIP104536, Ma1611 and T28 at concentrations
248 detailed in Table 2. Bi-combinations of FOX with CIP, MXF, LZD, CLO, RFB, RIF and CLR
249 were performed only for T28 isolate using FOX at the T28 MIC concentration.

250 *Antibiotics:* The following antibiotics were obtained as microbiological standards from their
251 manufacturers: FOX (Panpharma, Luitré, France), AMK (Acros, Illkirch, France), LZD (Ark
252 Pharma, Neuilly-sur-Seine, France). RIF, RFB, CLR, CIP, MXF and CLO were purchased from
253 Sigma (Saint-Quentin-Fallavier, France). Stock solutions were prepared using appropriate
254 solvents and stored at -80°C .

255 *Curve fittings:* Curve fittings were performed with GraphPad Prism (version 7.04) software for
256 Windows (GraphPad Prism Software, San Diego, CA).

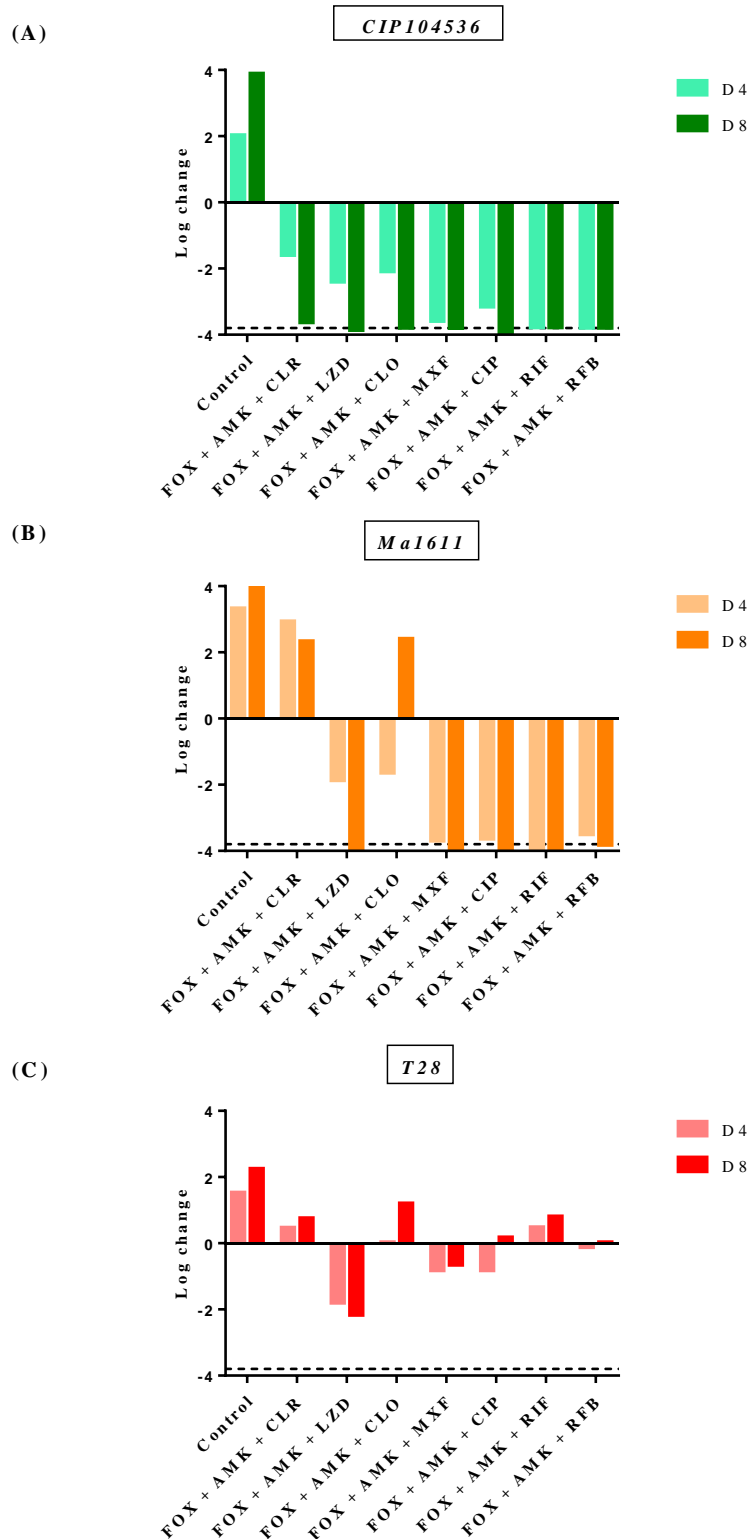
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259 **Figure 1:** Effect of various combinations on *M. abscessus* CIP 104536. (A) Activity of
 260 several screened antibiotics were tested alone and (B) then tested in combination with
 261 ceftiofur and amikacin based on the obtained MICs values as shown in Table 1. 50% amount
 262 of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant
 263 throughout the experiment. CFUs were determined at the interval of 2 days. The limit of
 264 quantification is 200 CFU/mL (2.3 in log₁₀).

265



266

267 **Figure 2:** *In vitro* activity of various triple combinations against *M. abscessus* (A) CIP104536
 268 (B) Ma1611 and (C) T28. Concentrations for each antibiotic are as mentioned on Figure 1.
 269 Log change was calculated using respective positive control data. For Comparison purpose,
 270 results are compared at day 4 and day 8. The dashed line represents the limit of quantification.

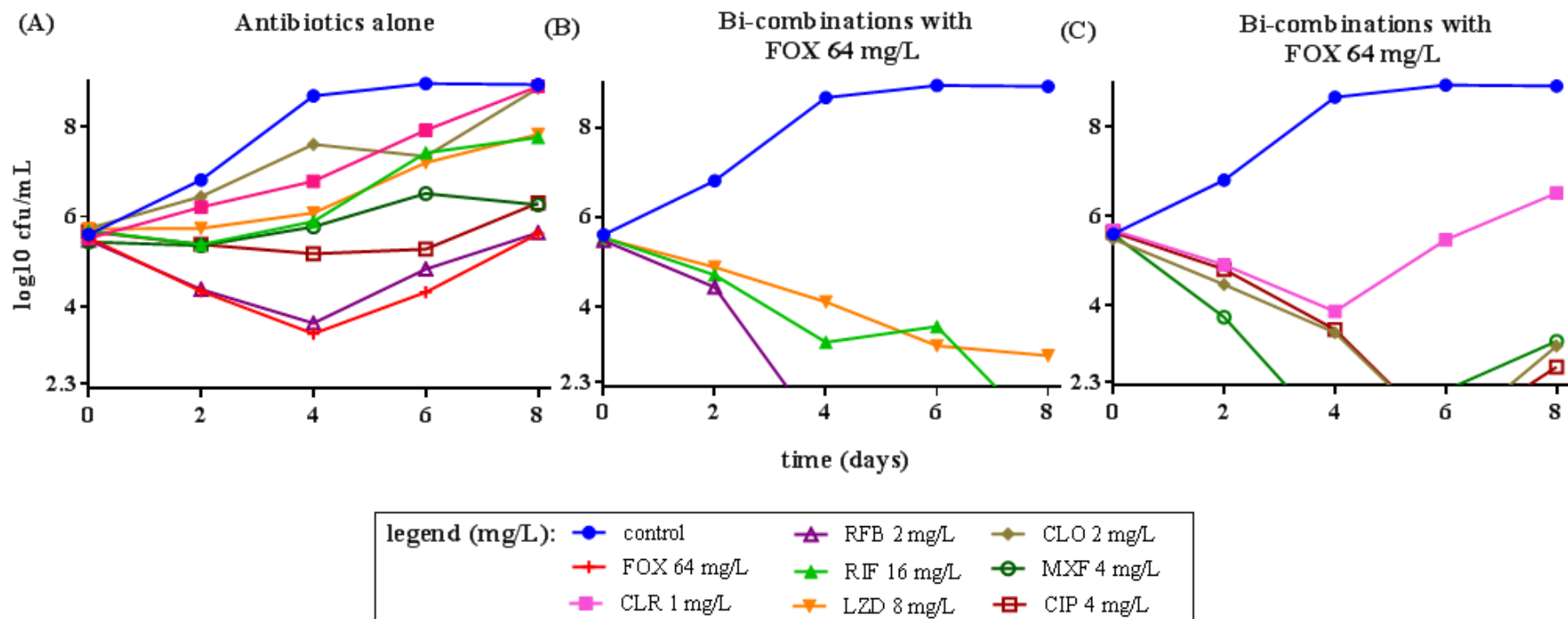


Figure 3: Effect of various combinations on *M. abscessus* T28. (A) Activity of several screened antibiotics alone, (B) ceftiofur in combination with linezolid, rifampicin and rifabutin, and (C) ceftiofur in combination with clarithromycin, clofazimine, moxifloxacin and ciprofloxacin. 50% amount of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant throughout the experiment. CFUs were determined at the interval of 2 days. The dashed line represents the limit of quantification.

Antibiotics	MIC breakpoints			MICs (mg/L)		
	S	I	R	CIP 104536	Ma1611	T28
	FOX	<16	32	>128	8	8
AMK	<16	32	>64	32	16	>1024
CIP	<1	2	>4	4	4	8
MXF	<1	2	>4	4	4	16
LZD	<8	16	>32	8	8	256
RIF			>1	16	16	256
CLR	<2	4	>8	1	16	>256
CLO	ND			2	16	4
RFB				2	2	16

FOX, cefoxitin ; AMK, amikacin; CIP, ciprofloxacin; MXF, moxifloxacin; LZD, linezolid; CLO, clofazimine; RFB, rifabutin; RIF, rifampicin; CLR, clarithromycin; S, susceptible; I, intermediate; R, resistance; ND, not determined.

	3 rd antibiotic	Concentrations used for 3 rd antibiotic
FOX*† + AMK*	+ CIP	4 mg/L
	+ MXF	4 mg/L
	+ LZD	8 mg/L
	+ CLO	2 mg/L
	+ RFB	2 mg/L
	+ RIF	16 mg/L
	+ CLR	1 mg/L

* FOX and AMK concentrations were 8 mg/L and 32 mg/L respectively
† 50% amount of FOX was compensated each 24h up to 8 days to maintain the FOX amount constant throughout the experiment
FOX, cefoxitin; AMK, amikacin; CIP, ciprofloxacin; MXF, moxifloxacin; LZD, linezolid; CLO, clofazimine; RFB, rifabutin; RIF, rifampicin; CLR, clarithromycin

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