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## Evaluation of long-term co-administration of tobramycin and clarithromycin in a mature biofilm model of cystic fibrosis clinical isolates of

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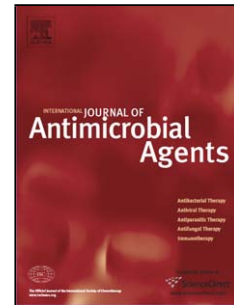
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## Accepted Manuscript

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1 **Evaluation of long term co-administration of tobramycin**  
2 **and clarithromycin in a mature biofilm model of cystic**  
3 **fibrosis clinical isolates of *Pseudomonas aeruginosa***

4  
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22

23 **Abstract**

24 *Pseudomonas aeruginosa* colonization and chronic lung infection, associated with biofilm  
25 formation, is the major cause of morbidity and mortality in CF patients. Therefore there is an  
26 urgent need to develop alternative ways to treat biofilm associated clinical infections.

27  
28 We performed a kinetic study over 28 days of a twice daily co-administration of the  
29 antibiotics tobramycin and clarithromycin on 12-day old, mature *P. aeruginosa* biofilms -  
30 formed on microplate pegs - for 23 clinical isolates of various phenotypes and genotypes to  
31 simulate the treatment of CF patients with inhaled tobramycin through aerosolisation  
32 (Tobi®). Drug activity was assessed by enumeration of persistent bacteria before, during and  
33 after treatment. We chose a mature (12-day old) biofilm model because a previous study  
34 suggested that such a biofilm was a more realistic *in vitro* model than 24-hour old biofilm.

35  
36 A synergistic activity of the drug combination was confirmed on biofilms of 9/23 isolates of  
37 *P. aeruginosa*. Of these 9 isolates, a total destruction of the biofilm was observed for 5 of  
38 them. The combination treatment was superior or equivalent to the treatment with tobramycin  
39 alone as an activity was observed on 47.8% of the isolates with the combination versus 26.1%  
40 with tobramycin alone. There was no correlation observed between drug susceptibility  
41 profiles and the phenotype or the genotype of the isolates.

42

43

44 **Keywords:** *Pseudomonas aeruginosa*, mature biofilm, synergistic activity, treatment  
45 simulation, cystic fibrosis

46

47

## 48 1. Introduction

49 The major cause of morbidity and mortality in CF is chronic lung disease caused by a vicious  
50 cycle of infection and inflammation that leads to progressive deterioration in pulmonary  
51 function, respiratory failure and death<sup>1</sup>. In particular, patients suffer from infection due to  
52 opportunistic *Pseudomonas aeruginosa*. *P. aeruginosa* overproduces an alginate  
53 exopolysaccharide within the CF lung, which appears to form a protective barrier around the  
54 bacterial cells and limits exposure to oxidative radicals, antibiotics and phagocytes<sup>2</sup>. Bacterial  
55 biofilms play a relevant role in persistent infections which are rarely eradicated with  
56 antimicrobial therapy.

57  
58 In a previous work we showed that an *in vitro* model of mature biofilm was more appropriate  
59 to mimic *in vivo* conditions in CF patients and we demonstrated a synergistic activity of a  
60 tobramycin/clarithromycin combination on biofilms of *P. aeruginosa* PAO1 and a clinical  
61 strain PYO2 using a kinetic study of a twice-daily co-administration over 9 days of  
62 antimicrobial agents on 12-day old biofilms<sup>3</sup>. In the present study, we tested 23 clinical  
63 isolates of various phenotypes and genotypes in order to confirm the synergistic activity of the  
64 combination tobramycin/clarithromycin on a broader sample of isolates. The possible  
65 correlation between susceptibility of the biofilms to the treatment and the phenotype and  
66 genotype of the isolates was also investigated.

67

## 68 2. Materials and Methods

### 69 2.1. Antimicrobial agents

70 We used micronized tobramycin (Teva, Petach Tikva, Israel) and clarithromycin (Teva).  
71 Stock solutions (10 mg/mL) of tobramycin were prepared in sterile water. For the preparation  
72 of clarithromycin stock solutions (2 mg/mL), the product was dissolved in methanol and  
73 completed with pH 6.5 phosphate buffer. Stock solutions were stored at -20°C until use. The  
74 tested concentrations of antibiotics were 4 µg/ml for tobramycin and 200 µg/ml for  
75 clarithromycin.

### 77 2.2. Bacterial strains

78 *P. aeruginosa* clinical isolates from sputum of 23 CF patients from diverse origins (Belgium,  
79 Germany, UK, the Netherlands) were tested. The isolates and epidemic strains<sup>4-6</sup> included six  
80 mucoid phenotypes (MC075-450457, MC086, MC093, MC099-450467, MC110 and MC278)  
81 and seventeen non-mucoid phenotypes (Clone C, LUH 7752, MC039, MC075-350649,  
82 MC075-450456, MC084, MC096, MC099-450524, MC116, MC142, MC161, MC178,  
83 MC305, MC325, PHLS 8916, PHLS 8959 and PHLS 8960) and their genotypes were  
84 established using AFLP<sup>7</sup>. Identification of the strains was confirmed by tDNA-PCR<sup>7</sup> and the  
85 API 20 NE system (bioMérieux, Marcy-l'Etoile, France).

86  
87 Bacterial suspensions were made from fresh sub-cultures and aliquots were stored at -20 °C in  
88 glycerol until use. Before use, bacterial suspensions were spread onto Mueller-Hinton solid  
89 medium and incubated at 35 °C for 24 hours.

90

91

92 2.3. *Genotyping*

93 Genotyping was carried out by means of selective restriction fragment amplification (AFLP)  
94 as described previously<sup>7</sup>. Using high resolution capillary electrophoresis the fingerprints were  
95 immediately digitized and cluster analysis was performed.

96

97 2.4. *Inoculum preparation for biofilm study*

98 The inoculum was prepared in CAMHB at a concentration of  $\sim 10^8$  colony-forming units  
99 (CFUs/mL) as described in a recent study<sup>3</sup>.

100

101 2.5. *Biofilm formation*

102 Biofilms were formed in 96-well microplates (Nunc Micro Wells Plates<sup>®</sup>) as described  
103 previously<sup>3</sup>. The plates were covered with lids presenting 96 pegs on which the biofilm can  
104 build up. The medium was changed daily from day 1 to day 12 in order to promote biofilm  
105 formation and maturation.

106

107 2.5. *Kinetic study of antimicrobial agents co-administration to 12-day old biofilms*

108 The kinetic study was performed as described previously<sup>3</sup>. The effect of antimicrobial drugs  
109 on preformed biofilm was studied on all 23 clinical isolates. After formation of mature  
110 biofilms, the pegs were removed, rinsed and put in contact twice daily during 28 days with  
111 tobramycin at 4  $\mu\text{g/mL}$  and clarithromycin at 200  $\mu\text{g/mL}$  alone or in combination in a new  
112 microplate. Enumeration of bacteria was also realised as described previously<sup>3</sup>. The  
113 logarithmic decrease of CFUs from antibiotic-treated biofilms was calculated using the  
114 following formula: logarithmic decrease =  $[\log(\text{CFUs}_{\text{positive control}}) - \log(\text{CFUs}_{\text{X}})]$ ; whereby x  
115 corresponds to the tested antimicrobial agent alone or in combination.

116

117 All tests were done in triplicate. All graphical evaluations were made using GraphPad Prism  
118 4.03 (GraphPad Software Inc. San Diego, CA, USA).

119

120

### 121 **3. Results**

#### 122 *3.1. Kinetic study of co-administration of antimicrobial agents to 12-day old biofilms*

123 Following the repeated co-administration of the two antimicrobial agents to the 12-day old  
124 biofilms, three different behaviours were observed, as illustrated in Table 1.

125

126 Firstly, a synergy between tobramycin and clarithromycin was recorded for 9/23 biofilms  
127 (group S), with a logarithmic bacterial decrease ranging between 1.8 and 7.5.

128

129 Figure 1A shows the synergy between tobramycin and clarithromycin for isolate MC161.

130 When tobramycin was used alone, we observed resistance during the whole treatment

131 whereas with the combination tobramycin/clarithromycin we observed an important

132 logarithmic decrease ( $\log_{5.3}$ ) of the number of viable bacteria present in the biofilm.

133 Secondly, for 8/23 biofilms (group TW), the activity of the combination

134 tobramycin/clarithromycin was similar to that of tobramycin alone (Figure 1B) and no

135 significant logarithmic decrease of the number of cfu/mL was recorded (0-1.5).

136

137 Thirdly for 6/23 biofilms (group TS) tobramycin alone or in combination with clarithromycin

138 eradicated all the biofilm, represented by a strong decrease of the number of cfu/mL ( $\log_6 -$

139  $\log_{6.5}$ ), such as for isolate MC099 (figure 1C). No bacteria could be cultured from the pegs

140 after 28 days of treatment, suggesting that in this group the kinetic model applied had a

141 bactericidal effect.



142 When considering the global response of the treatments the combination versus tobramycin  
143 alone showed a better antimicrobial activity (minimum 1 log reduction) for 10 strains and an  
144 identical activity for 13 other tested strains. Antimicrobial reduction higher than 4 logs during  
145 treatment was obtained for 11 strains with the combination compared to for 6 strains when  
146 tobramycin was administered alone. Overall the combination treatment was superior to the  
147 treatment with tobramycin alone as a marked activity was observed on 47.8% of the isolates  
148 with the combination treatment versus 26.1% with tobramycin alone (Table 1).

149

### 150 *3.2. Drug susceptibility versus phenotype and genotype*

151 Twenty-two of the 23 strains were genotyped using selective restriction fragment  
152 amplification as described previously<sup>7</sup>. The Dice algorithm was used to calculate a similarity  
153 matrix which was used to carry out cluster analysis by means of the Neighbour Joining  
154 algorithm. A graphic presentation of the relatedness among the strains according to AFLP-  
155 analysis is presented in Figure 4. Several highly similar fingerprints were observed, e.g. for  
156 the isolates Clone C, MC075-450456, MC075-450457 (muroid) and MC093 (muroid), but  
157 visual inspection of the fingerprints indicated several different peaks present, even between  
158 strains from the same patient, i.e. between the two MC075 isolates. These genotypically  
159 related isolates were both synergistically inhibited, unlike a third isolate of this patient  
160 (MC075-350649) with a completely different fingerprint, which was not inhibited by  
161 tobramycine alone or the combination. The two isolates from patient MC099, one muroid and  
162 one non-muroid, were indistinguishable from each other according to AFLP, but these strains  
163 showed a different behaviour with regard to their susceptibility to the antibiotics, i.e. MC099-  
164 450467 (muroid) was strongly inhibited by tobramycin alone whereas MC099-450524 (non-  
165 muroid) was synergistically inhibited.

166

#### 167 4. Discussion

168 Tobramycin is one of the most effective antibiotics against *P. aeruginosa* infections in CF,  
169 however eradication of the infection remains barely achievable.

170

171 A treatment able to eradicate chronic *P. aeruginosa* infections would increase the survival of  
172 CF patients significantly<sup>8</sup>.

173

174 Therefore, improving the efficiency of tobramycin treatment of biofilm grown *P. aeruginosa*  
175 was one of the objectives of this study.

176

177 During previous studies<sup>3,9</sup>, we developed a mature biofilm model formed on microplate pegs  
178 which seems more appropriate than planktonic and fresh biofilm models to simulate *in vivo*  
179 conditions as met in CF patients and we demonstrated that the combination of  
180 tobramycin/clarithromycin might offer a better treatment for CF patients. In this study, we  
181 developed a kinetic *in vitro* model of mature biofilm mimicking the treatment TOBI<sup>®10</sup>  
182 (tobramycin inhalation solution) of patients, during which TOBI<sup>®</sup> is inhaled twice a day  
183 during 28 days with intervals as close as possible to 12 hours apart,  
184 and no less than 6 hours apart. We tested concentrations of antibiotics in line with reported  
185 drug deposition<sup>11</sup> in the lungs for tobramycin and clarithromycin.

186

187 Biofilms were considered as mature from 12 days onwards referring to their formation  
188 process. Indeed from the 9th day onwards the last stage of maturation is reached. It was  
189 previously established that after 12 days of formation, the biofilm reaches full maturation<sup>12</sup>.

190 Moreover, we decided to follow a 28 days regimen for the antibiotics to mimic the therapeutic  
191 reality better than with a single dose antibiotic on a 'young' biofilm. This approach was

192 inspired by the standard treatment with TOBI<sup>®</sup> (Novartis) tobramycin inhalation solution  
193 USP<sup>10</sup>.

194

195 We confirmed an improvement in tobramycin activity by co-administration with  
196 clarithromycin for some biofilms. An activity with a 4-logarithmic reduction was observed on  
197 47.8% of the isolates with the combination versus 26.1 % with tobramycin alone.

198 Establishing a correlation between the genetic profile of isolates and their behavior with  
199 regard to antibiotic treatment would allow to predict the efficiency of a treatment combining  
200 tobramycin and clarithromycin. However, AFLP-analysis showed that there was little  
201 correlation between the genotype of isolates and their behavior with regard to antibiotics,  
202 especially because two isolates from the same patient (MC099), both with an identical AFLP  
203 fingerprint, but one mucoid and one non-mucoid, behaved differently with regard to antibiotic  
204 treatment.

205

206 Furthermore, there was no correlation between mucoidy and antibiotic resistance, although  
207 mucoid strains seemed to be more susceptible to tobramycin or combination therapy than  
208 non-mucoid strains, i.e. only one of the 7 mucoid strains (MC093) responded weakly to both  
209 tobramycin and combination therapy, whereas 7 of the 16 non-mucoid strains were difficult to  
210 treat by both antibiotic regimens.

211

212 Interestingly, the three UK epidemic strains seemed to be susceptible to tobramycin alone  
213 (PHLS 8916 and PHLS 8959) or to the combination therapy (PHLS 8960), whereas the  
214 continental epidemic strains (German clone C and Dutch LUH 7752) were resistant to both  
215 tobramycin and the combination therapy.

216 The synergistic effect observed between tobramycin and clarithromycin could be the  
217 consequence of the ability of clarithromycin to increase the permeability of the biofilm<sup>13</sup>.  
218 Moreover clarithromycin seems to have multifactorial activity. Mitsuya *et al.*<sup>14</sup> suggested that  
219 macrolides may have an inhibitory effect on the GDP-mannose dehydrogenase (GMD)  
220 production cycle, hence inhibiting the formation of biofilm. GMD is one of the constituents of  
221 bacterial biofilm that is known to be involved in the production of alginate. Other authors<sup>15</sup>  
222 also hypothesize that clarithromycin could inhibit the synthesis of alginate.

223  
224 To our knowledge, this kind of kinetic long-term treatment study based on mature biofilm has  
225 never been carried out before.

226  
227 The results confirm previous findings<sup>3</sup> that the coadministration of tobramycin/clarithromycin  
228 offers a considerable potential for the treatment of at least some biofilm forming *P.*  
229 *aeruginosa* strains in CF.

230  
231 In the future, animal or human models will be needed to corroborate these data with a  
232 measurable clinical outcome and to establish whether this mature biofilm kinetic treatment  
233 model can be used to predict the clinical outcome of different therapeutic strategies

234

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240

241 **Declarations**

242 **Funding:** This work was supported by Laboratoires S.M.B. SA, Belgium.

243 **Competing interests:** The authors declare no conflict of interest for this study.

244 **Ethical approval:** The clinical isolates tested were part of a routine taking realized at  
245 Hospital Gent, Belgium, for the characterization of CF infection strains by different studies  
246 including this one and do not require ethic clearance.

247

248

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289  
290  
291

292 **Legends**

293 Table legends:

294 Table 1: Effect of the antimicrobial agents tobramycin and clarithromycin, alone or in  
295 combination, on 23 clinical isolates of *P. aeruginosa*

296

297 Figure legends:

298 Figure 1: Kinetic study of the effect of administration of the antimicrobial agents tobramycin  
299 and clarithromycin, alone or in combination, on 12-day-old biofilms of (A) *P. aeruginosa*  
300 MC161-550367, (B) *P. aeruginosa* MC093-450507 and (C) *P. aeruginosa* MC099-450467

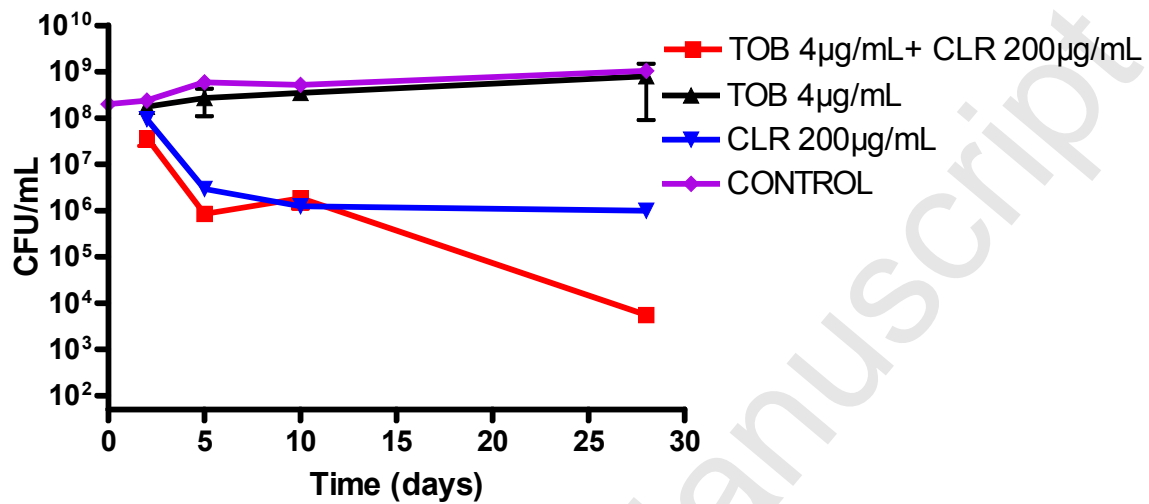
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302 Figure 2: Phylogenetic tree constructed on the basis of AFLP-fingerprints of the studied  
303 isolates

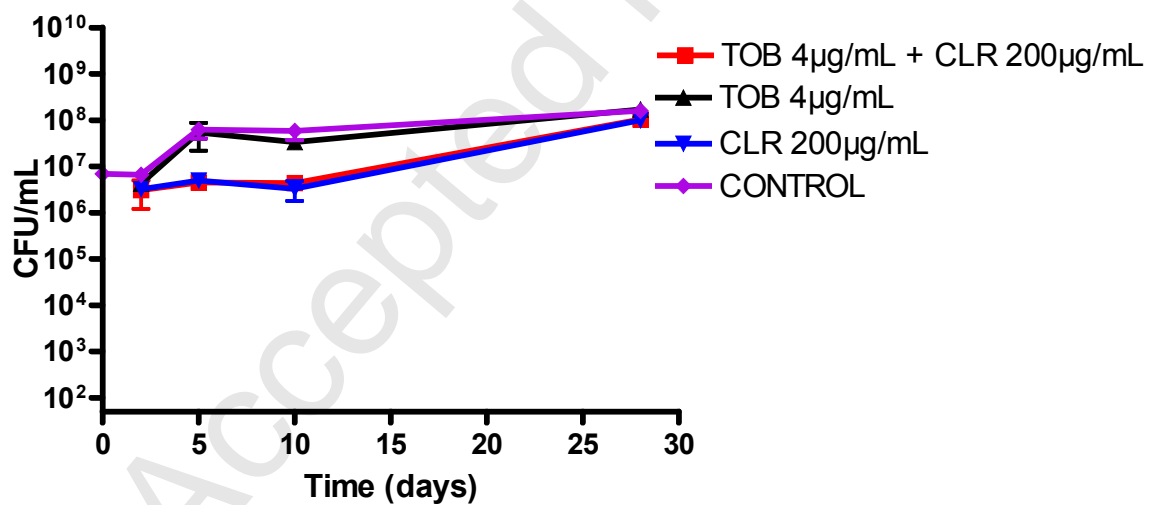


Figure 1: Kinetic study of the effect of administration of the antimicrobial agents tobramycin and clarithromycin, alone or in combination, on 12-day-old biofilms of (A) *P. aeruginosa* MC161, (B) *P. aeruginosa* MC093, and (C) *P. aeruginosa* MC099-450467

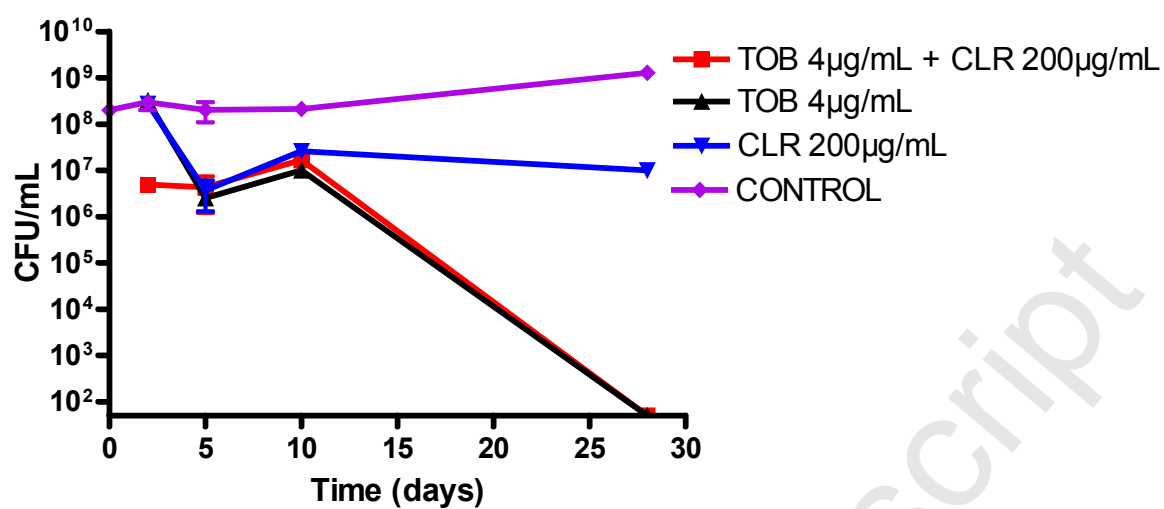
(A)



(B)



(C)



TOB: tobramycin

CLR: clarithromycin

CFU: colony-forming units

Table 1: Effect of antimicrobial agents alone and in combination on 23 clinical isolates of *P. aeruginosa*

Behaviour	Logarithmic decrease			Strain number	Phenotype	Patient's origin	Genotype cluster
	TOB	CLR	TOB+CLR				
S	+	+	++	MC075-450457 KL	Mucoid	SVZ-TH	(a)
S	-	-	+	MC086	Mucoid	RUG-JL	
S	-	-	+	MC110	Mucoid	ULB-COCL	
S	-	+	+	MC075-450456 BL	Non-mucoid	SVZ-TH	(a)
S	-	+	++	MC084	Non-mucoid	SVZ-JB	
S	-	-	+	MC099-450524	Non-mucoid	RUG-DS	b
S	+	+	+	MC116	Non-mucoid	ULB-DS	
S	-	+	++	MC161	Non-mucoid	KUL-KB	
S	-	+	++	PHLS 8960	Non-mucoid	Epidemic strain Manchester UK	
TS	++	+	++	MC099-450467	Mucoid	RUG-DS	b
TS	++	-	++	MC278	Mucoid	UCL-SF	NT
TS	++	-	++	MC096	Non-mucoid	KUL-LG	
TS	++	-	++	MC178	Non-mucoid	ULB-WAAL	
TS	++	-	++	PHLS 8916	Non-mucoid	Epidemic strain Midlands UK	
TS	++	+	++	PHLS 8959	Non-mucoid	Epidemic strain Liverpool UK	
TW	-	-	-	MC093	Mucoid	KUL-LM	(a)
TW	-	-	-	Clone C	Non-mucoid	Epidemic strain Germany	(a)
TW	-	-	+	MC039	Non-mucoid	RUG-BDW	
TW	-	-	-	MC075-350649 Mat	Non-mucoid	SVZ-TH	
TW	-	+	+	MC142	Non-mucoid	ULB-GS	
TW	-	-	+	MC305	Non-mucoid	RUG-AD	
TW	+	-	+	MC325	Non-mucoid	RUG-KDS	
TW	+	-	+	LUH 7752	Non-mucoid	Epidemic strain the Netherlands	

Legend:

- S synergy of tobramycin and clarithromycin  
 TS tobramycin alone, strong decrease  
 TW tobramycin alone, weak decrease  
 (a) very similar but not identical fingerprints  
 b strains belong to the same cluster, because of identical fingerprints  
 TOB tobramycin  
 CLR clarithromycin  
 + Logarithmic decrease of the number of cfu/mL  $\geq 1$   
 - Logarithmic decrease of the number of cfu/mL  $< 1$   
 ++ Logarithmic decrease of the number of cfu/mL  $> 4$

Figure 2: Phylogenetic tree constructed on the basis of AFLP-fingerprints of the studied isolates

