

1 **Title:** LONGITUDINAL SURVEILLANCE AND COMBINATION ANTIMICROBIAL
2 SUSCEPTIBILITY TESTING OF MULTIDRUG-RESISTANT *ACHROMOBACTER*
3 SPP. FROM CYSTIC FIBROSIS PATIENTS

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15 **Running title:** Synergy Testing and diversity of *Achromobacter* spp. recovered from
16 Cystic Fibrosis patients.

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20 **Abstract**

21 **Background:** *Achromobacter* spp. are recognized as an emerging pathogen in
22 patients with Cystic Fibrosis (CF). Though recent works have established species-
23 level identification using *nrdA* sequencing, there is a dearth in knowledge relating to
24 species-level antimicrobial susceptibility patterns and antimicrobial combinations
25 which hampers the use of optimal antimicrobial combinations for the treatment of
26 chronic infections. The aims of this study were i) to identify at species-level referred
27 *Achromobacter* isolates ii) to describe species-level antimicrobial susceptibility
28 profiles iii) to determine the most promising antimicrobial combination for chronic
29 *Achromobacter* infections.

30 **Methods:** A total of 112 multidrug-resistant (MDR) *Achromobacter* spp. isolates from
31 39 patients were identified using *nrdA* sequencing. Antimicrobial susceptibility and
32 combination testing were carried out using the Etest method.

33 **Results:** We detected six species of *Achromobacter* and found that *A. xylosoxidans*
34 was the most prevalent species. Interestingly, sequence analysis showed it was
35 responsible for persistent infection (18/28 patients) followed by *A. ruhlandii* (2/3
36 patients). Piperacillin-tazobactam (70.27%) and cotrimoxazole (69.72%) were the
37 most active antimicrobials. Differences were observed in species-level susceptibility
38 to ceftazidime, carbapenems, ticarcillin-clavulanate, and tetracycline. Antimicrobial
39 combinations with cotrimoxazole or tobramycin demonstrate the best synergy while
40 cotrimoxazole gave the best susceptibility breakpoint Index values.

41 **Conclusions:** This study enriches the understanding of MDR *Achromobacter* spp.
42 epidemiology, confirms prevalence and chronic colonization of *A. xylosoxidans* in CF

43 lungs. It presents *in vitro* data to support the efficacy of new combinations for use in
44 the treatment of chronic *Achromobacter* infections.

45 **Keywords:** *Achromobacter* spp.; *A. xylosoxidans*; Cystic Fibrosis; Antimicrobial
46 susceptibility testing; Synergy testing; Etest

47 **1.0 Introduction**

48 Several pathogens have been reported as causing chronic infections but
49 *Achromobacter* spp. have increasingly been implicated as causal agents of infection
50 and colonisation in CF individuals (1-7). National CF registries have reported slight
51 increasing rates of *Achromobacter* spp. colonisation/infection in individuals (8)
52 varying between 2 and 17 % (9).

53 *Achromobacter* spp. are aerobic, Gram-negative, catalase- and oxidase-positive,
54 non-fermenting bacilli that are phenotypically similar but genetically distinct and are
55 widely distributed in the environment (5). Innate and readily acquired adaptive
56 resistance with antimicrobial exposure thereby altering expression of certain genes
57 promote chronic infection and this has been extensively described in literature (3).
58 This intrinsic resistance to multiple antimicrobials limits the therapeutic options for
59 *Achromobacter* spp. infections (5, 6).

60 The clinical relevance of isolation of *Achromobacter* spp. in the sputum of CF
61 patients is unclear. Some studies have proposed a link between decline of lung
62 function and chronic infection by *Achromobacter xylosoxidans* especially in patients
63 with chronic *Pseudomonas aeruginosa* infections (10). Others on the other hand
64 postulate that the biofilm-forming ability of *Achromobacter xylosoxidans* correlates
65 with poor lung function (11).

66 *A. xylosoxidans* is the most frequently isolated species from clinical samples but
67 there are known difficulties associated with the species identification of
68 *Achromobacter* isolates (9, 12). Conventional identification methods such as

69 biochemical test, or indeed mass spectrometry, were shown to lack the optimal
70 discriminatory power needed to characterise *Achromobacter* isolates at species-level
71 (1, 13, 14). However, Spilker et al. (15, 16) reported characterization of new
72 *Achromobacter* species through sequencing of the *nrdA* housekeeping gene.
73 Characterisation of *Achromobacter* clinical isolates at the species-level and its
74 relationship with antimicrobial resistance profiles is expected to increase our
75 understanding of the clinical relevance of colonisation by this seemingly CF-related
76 bacterium (1).

77 The aims of this present study were:

78 i) To identify at species-level all *Achromobacter* spp. isolates referred to the Scottish
79 CF antimicrobial susceptibility centre using *nrdA* sequencing.

80 ii) To describe species-level antimicrobial susceptibility profiles of *Achromobacter*
81 spp.

82 iii) To determine most promising antimicrobial combination treatment for multidrug-
83 resistant *Achromobacter* spp.

84

85 **2.0 Results**

86 **2.1 Study population and geographic metadata**

87 A total of 112 presumptive multidrug-resistant *Achromobacter* spp. were referred for
88 extended antimicrobial susceptibility testing. Isolates were collected over an 18 year
89 period (24 Sep 2001 to 09 Oct 2019) from 9 hospitals: 8 in Scotland and one in
90 Belfast (North Ireland). Edinburgh had the highest patient population (43.59%) and

91 submitted 60/112 samples. Glasgow and Aberdeen with patient proportions of
92 17.95% and 12.82% submitted 10 and 17/112 samples respectively. Patient age
93 ranged from 11 to 78 years with a median age at first referral of 28 years. Study
94 population comprised of 17 (43.59%) and 22 (56.41%) male and female patients
95 respectively. Of this population, 18 individuals had single isolate referrals whilst the
96 remaining 21 individuals had multiple referrals (2-9 isolates per individual) with
97 colonization periods of 1-10 years (Table 1).

98 **2.2 Species prevalence**

99 *nrdA* sequencing differentiated the 112 referred isolates into 6 different species. We
100 observed that *A. xylosoxidans* (n=88/112, 78.57%) was the most prevalent species
101 amongst our patients (n=28/39, 71.79%) while *A. insuavis* was the second most
102 prevalent *Achromobacter* spp. (10%). To illustrate the degree of sustained
103 colonisation at patient level we constructed a neighbour joining tree (Fig 1) which
104 demonstrates that repeat isolates from patients (>2 submissions) were
105 representatives of the patients' first referred isolates for 19 out of the 21 patients
106 (90.48%).

107 **2.3 Antimicrobial susceptibility testing**

108 Using 18 antimicrobials susceptibility testing were performed on the 112
109 *Achromobacter* isolates. Though unequal sampling was carried out in our study, Fig
110 2 shows that most of the *Achromobacter* isolates were resistant ($\geq 93.33\%$) to the
111 aminoglycosides; amikacin, gentamicin, and tobramycin as well as the
112 fluoroquinolones; ciprofloxacin and levofloxacin (83.04 - 91.96%). The most active

113 antimicrobials were piperacillin-tazobactam (70.27%), followed by co-trimoxazole
114 (69.72%) and minocycline (62.39%). Aztreonam had no activity against
115 *Achromobacter* spp. (For detailed information, see supplementary data). Thereafter,
116 we grouped the first-referred study isolates and determined if differences existed in
117 the antimicrobial susceptibility patterns (Fig 2). Statistical analysis showed that
118 susceptibility differences exist for amikacin ($p=0.032$), gentamicin ($p=0.002$),
119 tobramycin ($p=0.007$), ciprofloxacin ($p=0.001$), levofloxacin ($p<0.001$) doxycycline
120 ($p<0.001$), minocycline ($p=0.005$) and ticarcillin/clavulanate ($p=0.043$). There was no
121 statistical difference in the susceptibility of both *Achromobacter* groups to aztreonam,
122 chloramphenicol, colistin, co-trimoxazole, ceftazidime or piperacillin-tazobactam.

123 **2.4 Antimicrobial synergy testing**

124 Antimicrobial synergy testing was conducted using the direct overlay Etest method
125 and findings classified using the FICI and SBPI criteria. In summary, a total of 738
126 antimicrobial combinations were tested with a mean of 6.6 combinations per isolate.

127 **2.4.1 FICI**

128 Using FICI criteria, the rates of synergy and antagonism between pairs of
129 antimicrobials were 10.57% and 2.30% respectively. Species grouping showed 2-
130 fold higher rate of synergy (11.75%) in *A. xylosoxidans* (n=604) when compared with
131 non-*xylosoxidans* (n=138) cumulatively (5.22 % synergy).

132 The rates of antimicrobial antagonism in *A. xylosoxidans* were 2.32% while in non-
133 *xylosoxidans* 2.24% was observed. A summary of the most synergistic combinations
134 for *Achromobacter* spp. were ceftazidime + imipenem (n=14, 50% synergy),

135 tobramycin + ceftazidime (n=23, 34.78% synergy) and tobramycin + imipenem
136 (n=32, 21.88% synergy). The highest rate of antagonism was observed with
137 ceftazidime + co-trimoxazole (n=24, 12.50 % antagonism) and minocycline +
138 ticarcillin-clavulanate (n= 18, 11.11% antagonism). However when grouped as
139 species; for *A. xylosoxidans* ceftazidime + imipenem combinations (n=13, 53.85%)
140 as well as tobramycin with ceftazidime (n=18, 38.89%)/imipenem (n=26, 23.08%)
141 were the most synergistic combinations. While for non-*xylosoxidans* the antimicrobial
142 combination of tobramycin and ceftazidime (n=5/ 20%) was the most synergistic
143 combination. Interestingly, for *A. xylosoxidans* (Table 2), cotrimoxazole combinations
144 followed by tobramycin was the most prevalent synergistic combinations. While in
145 non-*xylosoxidans* (Table 3) tobramycin combinations were the most prevalent
146 synergistic combinations. This suggests that to achieve synergy, antimicrobial
147 combinations with cotrimoxazole and tobramycin should be explored.

148 **2.4.2 SBPI**

149 But in the laboratory, there is uncertainty about which combinations might be
150 synergistic *in vitro*, therefore our laboratory previously proposed use of the SBPI
151 method. For the 738 antimicrobial combinations tested in all study *Achromobacter*
152 spp., the median SBPI was 4.67 while the mean SBPI was 15.20. By species the
153 median and mean values of 4.00 and 15.79 were observed for *A. xylosoxidans* while
154 higher values (10.00 and 23.34) were observed for non- *xylosoxidans*.

155 The highest median SBPI for *Achromobacter* spp. was combinations of levofloxacin
156 with piperacillin-tazobactam (12.00) or co-trimoxazole (9.63). Tobramycin when
157 combined with either ceftazidime or imipenem gave the lowest median values. Table

158 2 demonstrates that highest median SBPI for *A. xylosoxidans* was combinations of
159 cotrimoxazole with Ticarcillin-clavulanate (11.33) and imipenem (10.00). Similarly, for
160 non- xylosoxidans (Table 3) a high SBPI value was obtained for co-trimoxazole
161 combinations with levofloxacin (31.28) or ceftazidime (SBPI 22.44).

162 **3.0 Discussion**

163 Our study focused on *Achromobacter* spp. which has been reported as one of the
164 emerging pathogens found in cystic fibrosis patients (4, 17, 18). As the Scottish CF
165 antimicrobial reference laboratory we receive only multi and extensively drug
166 resistant isolates from Scottish hospitals as well as Belfast for antimicrobial synergy
167 testing. It is therefore difficult to show that our study is a representative picture of the
168 Scottish CF population. However, like other studies (3-5, 19) our study reiterates the
169 dominance of *A. xylosoxidans* (28 out of 39 patients) amongst the CF population.
170 Also, with an *A. xylosoxidans* prevalence of 78.57% our study agrees with the
171 estimated UK prevalence of 78.4% (3). Amoureux et al. (1) suggested that its
172 dominant role might be due to either a higher natural abundance or the presence of
173 favourable selective factors for example the possession of innate resistance to
174 disinfectants such as quaternary ammonium compounds which ensures its ability to
175 thrive in clinical samples. Also, *A. insuavis* was the second most patient carried
176 *Achromobacter* spp. but the persistent colonization of *A. ruhlandii* in our CF
177 population meant that the latter was the second most isolated species. Previous
178 studies have demonstrated that persistent CF infections are mainly attributed to *A.*
179 *xylosoxidans*, *A. insuavis* and *A. dolens* (5). This was also observed in our study,
180 however longitudinal analysis of our data agrees with Gade *et al.* (6) that *A. ruhlandii*

181 is also capable of persisting in the CF airways. The mechanisms of persistence has
182 not been fully established with several hypotheses postulated. Gade *et al.* (6) reports
183 that inter-patient transmission might be possible while Edwards *et al.* (5) showed that
184 *Achromobacter* spp. is patient specific and there was clearance in all but one patient
185 when treated with oral cotrimoxazole. Also, Dupont *et al.* (4) reported that the
186 environmental habitat might not play a role in the reseeded of isolates for patients
187 with persistent infections. We hypothesize that these isolates may not be entirely
188 eradicated from the CF airways during treatment and on the development of
189 favourable conditions multiply and cause pulmonary exacerbations. This is because
190 analysis of the antimicrobial susceptibility patterns of isolates from patients with
191 repeated submissions show similar antimicrobial patterns while results from our *nrdA*
192 sequencing demonstrate persistent infection. We also did not observe any evidence
193 of potential transmission between individuals or shared geographical location.
194 However, further epidemiological studies on these isolates is necessary to enrich our
195 knowledge on the mechanisms of persistence.

196 Coward *et al.* (3) reported that there are no established guidelines for managing CF
197 patients who persistently harbour *Achromobacter* spp. with antimicrobial
198 susceptibility pattern/testing less defined. Indeed, there is a dearth in the knowledge
199 of species-level antimicrobial susceptibility patterns as well as the most promising
200 synergistic combinations. Similar to other studies (3, 19-21) the most active
201 antimicrobial was piperacillin-tazobactam, and cotrimoxazole at 70% while
202 minocycline was third at 62%. As expected, we observed a high resistance of our
203 isolates to the aminoglycosides. This resistance reported by Bador *et al.* (22) is due

204 to the possession of AxyXY-OprZ efflux system which confer resistance to
205 aminoglycoside in *Achromobacter* spp. especially *A. xylosoxidans*, *A. ruhlandii* and
206 *A. insuavis* which make up 96% of this study. Similarly as observed by Amoureux *et*
207 *al.* (19), *Achromobacter* spp. was more susceptible to imipenem (47%) compared to
208 meropenem (37%) although our values (given that our samples were MDR and XDR
209 strains) had a lower susceptibility percentage. Also, mirrored in our observation as
210 described in most CF and environmental isolates were high ciprofloxacin (92%) and
211 aztreonam (100%) resistance while for the newer β -lactam combinations such as
212 ceftolozane-tazobactam (100%) and ceftazidime-avibactam (78%) our isolates were
213 resistant to these drugs. This observation was also made by Coward *et al.* (3). But,
214 few CF studies have described species antimicrobial susceptibility patterns of
215 *Achromobacter* spp. We demonstrate that in *Achromobacter* spp. differences exist in
216 the susceptibility of non-*xylosoxidans* compared with *A. xylosoxidans* to the
217 carbapenems, cephalosporins or tetracyclines. The presence of resistance-
218 nodulation-cell-division-type pumps in *A. xylosoxidans* such as AxyABM, AxyXY-
219 OprZ and TetA confers the cell with the ability to pump cephalosporins,
220 fluoroquinolones, aztreonam, chloramphenicol, carbapenems and tetracycline out of
221 the cell (23, 24). Papalia *et al.* (25) reported that a homologue of AxyABM was
222 present in *A. ruhlandii*, therefore conferring it with the ability to expel
223 chloramphenicol. Further research is needed to determine the presence of efflux
224 pumps and characterize if present in non-*xylosoxidans*.

225 Irrespective of isolate susceptibility, antibiotic exposure gives rise to the emergence
226 of multi drug and extensively drug resistant strains causing limited therapeutic

227 options in patient management. Therefore to reduce toxicity and improve efficacy
228 while preventing the emergence of drug resistance, multiple antibiotics thought to be
229 effective as single agents are typically often prescribed in the clinic (26). But there is
230 limited information on synergistic combination for the treatment of *Achromobacter*
231 spp. infections. To the best of our knowledge this is the first time antimicrobial
232 synergy results are described for *Achromobacter* spp. to species level. Analysis of
233 our data showed that there were differences in the synergy observed in both
234 *Achromobacter* groups when there was availability of the interpretative guidelines.
235 For all and first- referred isolates, there was ~50% increase in synergy for *A.*
236 *xylosoxidans* compared to non-*xylosoxidans* when two CLSI interpretative guidelines
237 were known. Though not a remit of this study, more research would enhance
238 knowledge on how the more resistant *A. xylosoxidans* is able to demonstrate more
239 synergistic combinations than non-*xylosoxidans*.

240 At a genus level, our results demonstrate that for *Achromobacter* spp. combinations
241 of ceftazidime + imipenem (50%) was the most synergistic combinations. This is in
242 contrast with observations made by Saiman et al. (27) which stated that ciprofloxacin
243 + meropenem combinations (9%) were the most synergistic combinations. It is worth
244 noting that not all the combinations were tested at the same frequency and
245 differences existed in the rates observed for both studies. But, Gómara et al. (26)
246 reported that due to lack of standardization, differences exist in synergy reported
247 using different methods. Indeed, Saiman used the checkerboard while our lab used
248 the direct overlay E-test method. It might also be due to our cut off which analysed
249 only combinations which had been tested more than 5 times. The unpredictability of

250 synergy and the non-correlation of synergy and clinical efficacy was the reason our
251 lab had earlier proposed the use of SBPI (28) as a useful parameter for comparing *in*
252 *vitro* effectiveness of combinations thereby ranking them. Our results suggest that
253 combinations of cotrimoxazole with several antimicrobials are able to give a high
254 SBPI values although these values do not predict a synergistic FICI. A major
255 limitation of our data is the lack of information on the clinical outcomes of our
256 combinations. Therefore, further investigation is required to assess if there is a
257 correlation of SPBI values and clinical efficacy. In clinical practice however, there is
258 a growing evidence showing the lack of effectiveness seen when guiding
259 antimicrobial selection based on in vitro synergy testing. Indeed, results from a
260 randomized, double-blind controlled trial demonstrated that there was no difference
261 between groups and proposed synergy testing in CF patients should be stopped
262 (29). However, this study was carried out using multiple-combination bactericidal test
263 method and with various degrees of agreement in synergy methods, it is possible
264 that others might be more clinically relevant. Finally, the selected study of multidrug-
265 resistant strains may have overestimated our observation of persistence. It would be
266 interesting to how the use of random selection would impact on our results.

267 In summary, the *Achromobacter* spp. remains a key emerging pathogen in CF
268 individuals and has been implicated in pulmonary exacerbations. This research
269 reiterates the prevalent MDR species that make up the *Achromobacter* genus and
270 highlights their susceptibility profiles to several antimicrobials. It also attempts to give
271 antimicrobial combinations which might be used in the treatment of chronic
272 *Achromobacter* infections. With inconsistency reported in clinical outcomes of these

273 patients, accurate identification of *Achromobacter* spp. will undeniably play a vital
274 role in approach taken during therapeutic management of CF patients.

275

276 **4.0 Materials and method**

277 **4.1 Study Isolates**

278 A total of 112 presumptive *Achromobacter* spp. identified by 8 Scottish and 1
279 Northern Ireland laboratories were collected over an 18 year period (24th September
280 2001 – 9th October 2019) when they were sent for extended susceptibility testing.
281 Isolates were stored in the bacterial preservation system MICROBANK™ (PRO-LAB
282 DIAGNOSTICS Ontario, Canada) at -80°C.

283 Isolates were plated on receipt onto Mueller-Hinton agar (MH), MacConkey agar,
284 *Pseudomonas* Cetrimide agar and *Burkholderia cepacia* selective agar plates (All
285 agar plates manufactured by Oxoid Ltd., Basingstoke, UK). Following 18-24 hr
286 incubation at 35 °C in ambient air, plates were examined for purity and thereafter
287 incubated a further 24 hr to confirm for purity. Oxidase testing (Oxoid Ltd.,
288 Basingstoke, UK) was performed as a confirmatory test on 18-24 hr colonies.
289 Oxidase positive and non-lactose fermenting isolates were accepted as
290 *Achromobacter* spp

291 **4.2 *nrdA* Sequencing**

292 Species identification of isolates was carried out by *nrdA* sequencing according the
293 method described by Spilker et al. (15). Briefly, a single colony of *Achromobacter*
294 spp. was suspended in 20 µl of lysis buffer composed of 0.25 % (v/v) sodium

295 dodecyl sulfate (Sigma-Aldrich, Irvine, UK) and 0.05 N NaOH (Sigma-Aldrich, Irvine,
296 UK). On heating for 15 mins at 95 °C, 180 µl of high-pressure-liquid-chromatography
297 grade water (Sigma-Aldrich, Irvine, UK) was added to the suspension. The solution
298 was centrifuged at 13,300 rpm for 5 mins and the supernatants stored at -20 °C. Full
299 length *nrdA* amplification and sequencing was carried out using the *nrdA*-specific
300 forward (GAACTGGATTCCCGACCTGTTC) and reverse
301 (TTCGATTTGACGTACAAGTTCTGG) primers as previously published (15).
302 Amplified PCR products were sequenced by Eurofins genomics (GATC Biotech AG,
303 Konstanz, Germany) and sequence chromatograms were visualized and edited
304 using the SeqMan Pro (DNASStar, Madison, WI, USA). Allele numbers were assigned
305 to each isolate using the *Achromobacter* MLST database
306 (<http://pubmlst.org/org/achromobacter/>). Trimmed sequences were aligned using
307 MegAlign Pro (DNASStar, Madison, WI, USA). Clustal W in MegAlign Pro was used to
308 generate a neighbour joining tree with 1,000 bootstrap replications using default
309 parameters. Study isolates were grouped as either *A. xylosoxidans* or non-
310 *xylosoxidans* following *nrdA* identification.

311 **4.3 Minimum Inhibitory Concentration (MIC) testing**

312 MIC testing was performed on MH Agar using the Etest methodology according to
313 manufacturer's instructions (Liofilchem, Abruzzi, Italy and BioMerieux, Basingstoke,
314 UK). The antimicrobials tested were amikacin, gentamicin, tobramycin, ciprofloxacin,
315 levofloxacin, aztreonam, ceftazidime, piperacillin/tazobactam, imipenem,
316 meropenem, colistin, chloramphenicol, minocycline and co-trimoxazole. Data relating
317 to susceptibility to ticarcillin/clavulanate which the service had stopped testing were

318 included in the analyses up to its stop date (2018). While susceptibility data of
319 antimicrobials introduced by the service later than 2001, namely doxycycline
320 (October 2003), Ceftazidime-avibactam (January 2018) and ceftolozane-tazobactam
321 (January 2018) were included in the analyses from the time of introduction.

322 In this study, MIC values between the standard doubling dilution scale were rounded
323 up to the next doubling dilution (e.g. 0.75 = 1.0 mg/L). The MICs for amikacin,
324 gentamicin, tobramycin, ciprofloxacin, levofloxacin, aztreonam, ceftazidime,
325 piperacillin/tazobactam, imipenem, meropenem, ticarcillin/clavulanate, doxycycline,
326 chloramphenicol, minocycline and co-trimoxazole were interpreted as susceptible
327 (S), intermediate (I) or resistant (R) according to the Clinical and Laboratories
328 Standards Institute (CLSI) approved interpretive standards for non-
329 enterobacteriaceae (30). Ceftazidime/avibactam, ceftolozane/tazobactam and
330 colistin were interpreted as per CLSI standards for *Pseudomonas aeruginosa* (30). In
331 this study, multidrug-resistance (MDR) was defined as acquired non-susceptibility to
332 at least one agent in ≥ 3 antimicrobial groups (31).

333 **4.4 Combination testing**

334 Combination testing was performed using a minimum of six pairs of antimicrobials (A
335 + B), as described previously (28). Briefly, MH agar plates were inoculated with two
336 Etest strips (A and B) placed top to tail according to the manufacturer's instructions.
337 After 1 hr to allow antimicrobial migration into the agar, each strip is removed and
338 fresh Etest is placed in opposite orientation on the imprint (Etest A strip replaced with
339 fresh Etest B strip and vice versa). Plates were further incubated for 22-24 hrs in
340 ambient air at 35 °C.

341 **4.4.1 Fractional inhibitory concentration index (FICI)**

342 Indices derived from the combination MIC results were calculated using the MIC
343 value read off the Etest strip and interpreted as described below.

344
$$FICI = (MIC A_{\text{combination}} / MIC A_{\text{single}}) + (MIC B_{\text{combination}} / MIC B_{\text{single}}).$$

345 Where an MIC was found to be greater than the antimicrobial range tested, the next
346 doubling dilution above the highest value of the range tested was used to calculate
347 the FICI (e.g. if an MIC of >256mg/L was found then the FICI was calculated using
348 512mg/L) (32). The indices were interpreted as: synergy - $FICI \leq 0.5$, no interaction -
349 $FICI > 0.5$ and ≤ 4.0 and antagonism - $FICI > 4.0$ (33).

350 Analyses of species susceptibility to double combinations of antimicrobials tested ≥ 5
351 times was carried out when CLSI breakpoints for non-enterobacteriaceae was
352 known.

353 **4.4.2 Susceptible breakpoint index (SBPI)**

354 $SBPI = (\text{Susceptible breakpoint of antimicrobial A} / MIC \text{ of antimicrobial A}_{\text{combination}}) +$
355 $(\text{Susceptible breakpoint of antimicrobial B} / MIC \text{ of antimicrobial B}_{\text{combination}})$ (28). The
356 combination results were graded and reported in rank order of their SBPI results
357 from highest to lowest SBPI which displays the effectiveness of the combination in
358 decreasing order. Any combination found to be antagonistic ($FICI > 4.0$), was not
359 ranked and was not recommended for therapy irrespective of the SBPI result.

360 **4.5 Statistical methods**

361 Descriptive statistics were derived using Microsoft Office Excel 2013 and IBM SPSS
362 statistics for windows, Version 24 (IBM Corp., Armonk, N.Y., USA). A two-tailed
363 Mann Whitney test was performed using GraphPad Prism, Version 8.4.0 (GraphPad
364 software, San Diego, California, USA).

365

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375

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519 **Figure Legends**

520 **Table 1. Characteristics of study population and demographics**

521 P00, anonymized participant number; F, female; M, male; -, data unavailable

522

523 **Fig 1. Neighbour joining tree illustrating the *nrdA* clustering of *Achromobacter***
524 ***spp.* isolates from patients with repeated submission.**

525 P, patient; 01-38 anonymized number, a-i; repeated samples submitted by patient.

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527 **Fig 2. *Achromobacter spp.* susceptibility patterns.** Percentage susceptibility of
528 all-referred *Achromobacter spp.*, first-referred isolates (*A. xylosoxidans* and non-
529 *xylosoxidans*) to several antimicrobials. AMK, amikacin; GEN, gentamicin; TOB,
530 tobramycin; CIP, ciprofloxacin; LVX, levofloxacin; ATM, aztreonam; CAZ, ceftazidime;
531 TZP, piperacillin-tazobactam; IPM, imipenem; MEM, meropenem; COL, colistin; TIM,
532 ticarcillin-clavulanate; CHL, chloramphenicol; DOX, doxycycline; MIN, minocycline;
533 SXT, co-trimoxazole

534 ^a CLSI-approved interpretative standards for non-enterobacteriaceae

535 ^b CLSI-approved interpretative standards for *P. aeruginosa*. Colistin resistance may
536 be over-estimated due to limitations of the diffusion method.

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539 **Table 2. Summary of antimicrobial combinations tested on *A. xylosoxidans***
540 **isolates**

541 TOB, tobramycin; LVX, levofloxacin; CAZ, ceftazidime; IPM, imipenem; MEM,
542 meropenem; TIM, ticarcillin-clavulanate; CHL, chloramphenicol; MIN, minocycline;
543 SXT, co-trimoxazole

544 ^a Percentage active when used as a single agent

545 ^b Number of times the combinations were tested

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549 **Table 3. Summary of antimicrobial combinations tested on *Non-xylooxidans***
550 **isolates**

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553 TOB, tobramycin; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; MIN,
554 minocycline, SXT, co-trimoxazole; CHL, chloramphenicol

555 ^a Percentage active when used as a single agent

556 ^b Number of times the combinations were tested

Table 1. Characteristics of study population and demographics

Participant number	Sex	Location	Age at first submission (yrs)	No of samples submitted	Period of colonization (yrs)
P01	F	Inverclyde	12	5	4
P02	F	Glasgow	16	1	-
P03	F	Edinburgh	13	6	9
P04	F	Edinburgh	20	3	1
P05	M	Kilmarnock	11	4	3
P06	M	Edinburgh	22	1	-
P07	F	Edinburgh	18	8	10
P08	F	Dumfries	25	5	5
P09	M	Dundee	24	5	8
P10	M	Aberdeen	20	7	10
P11	F	Aberdeen	16	1	-
P12	M	Edinburgh	24	7	8
P13	F	Edinburgh	18	6	6
P14	F	Edinburgh	54	1	-
P15	F	Edinburgh	25	1	-
P16	F	Edinburgh	19	9	6
P17	M	Edinburgh	23	4	4
P18	M	Glasgow	13	4	4
P19	M	Edinburgh	28	1	-
P20	F	Dundee	33	1	-
P21	M	Edinburgh	30	3	3
P22	M	Edinburgh	18	2	2
P23	F	Aberdeen	21	4	1
P24	M	Glasgow	20	1	-
P25	M	Aberdeen	48	3	4
P26	F	Glasgow	20	1	-
P27	F	Aberdeen	30	2	3
P28	F	Glasgow	71	1	-
P29	M	Edinburgh	32	1	-
P30	F	Belfast	69	1	-
P31	F	Belfast	44	1	-
P32	F	Belfast	-	1	-
P33	F	Belfast	-	1	-
P34	M	Glasgow	21	1	-
P35	F	Glasgow	24	1	-
P36	F	Edinburgh	23	3	2
P37	M	Edinburgh	78	2	2
P38	M	Edinburgh	16	2	2
P39	M	Belfast	-	1	-

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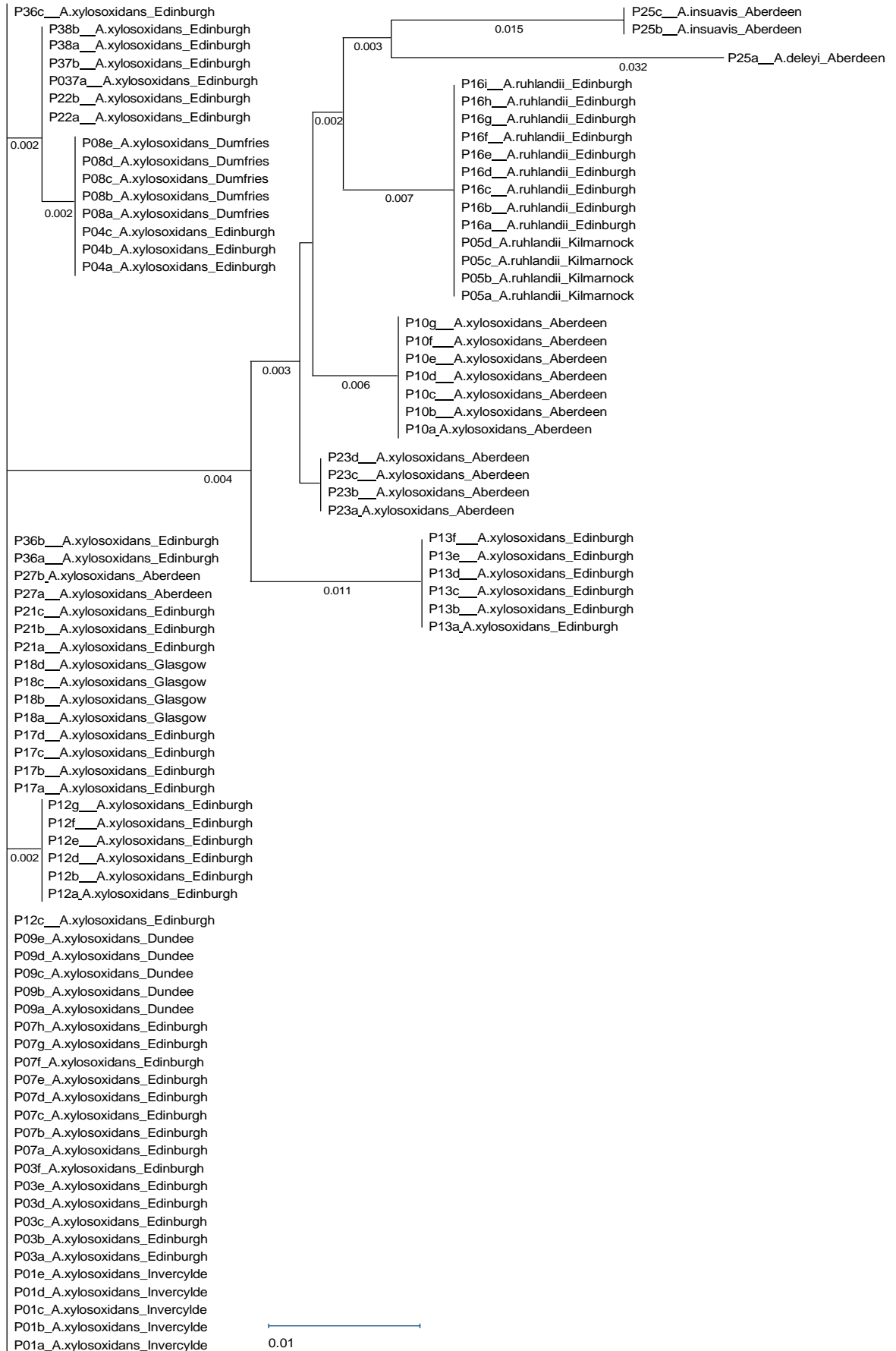


Fig 1. Neighbour joining tree illustrating the *nrdA* clustering of *Achromobacter* spp. isolates from patients with repeated submission.

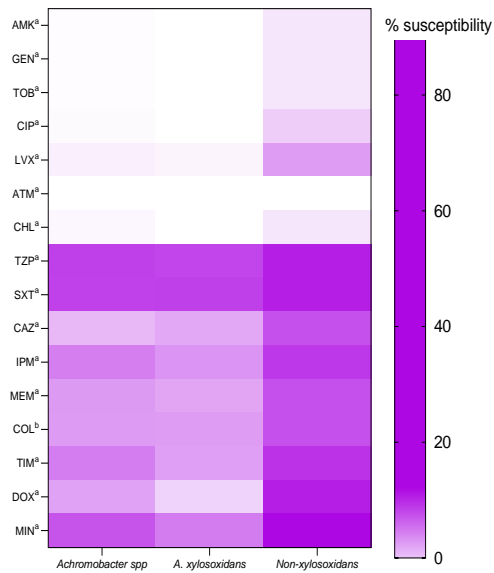


Fig 2. *Achromobacter* spp. susceptibility patterns.

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Table 2. Summary of antimicrobial combinations tested on *A. xylosoxidans* isolates

First (%S ^a)	Antimicrobial Second(%S ^a)	Number ^b	Synergy		Antagonistic		SBPI	
			%	Rank	%	Rank	Median	Rank
CAZ (23)	IPM (46.6)	13	53.85	1			5.17	5
TOB (0)	CAZ (23)	18	38.89	2			3.17	13
TOB (0)	IPM (46.6)	26	23.08	3			3.33	10
IPM (46.6)	SXT (64.7)	10	20.00	4			10.00	2
CAZ (23)	SXT (64.7)	15	20.00	4	13.33	1	8.50	4
MIN (54.1)	SXT (64.7)	15	13.33	6	6.67	5	4.67	6
TOB (0)	MEM (36.8)	15	13.33	6			2.79	16
TOB (0)	TIM (49.3)	15	13.33	6			2.75	18
SXT (64.7)	CHL (2.4)	16	12.50	9			9.13	3
TOB (0)	SXT (64.7)	10	10.00	10	10.00	3	4.23	8
SXT (64.7)	TIM (49.3)	10	10.00	10			11.33	1
LVX (1.1)	MIN (54.1)	16					3.33	10
LVX (1.1)	TIM (49.3)	10					4.46	7
MIN (54.1)	CHL (2.4)	24					3.08	14
MIN (54.1)	CAZ (23)	19					3.33	10
MIN (54.1)	TIM (49.3)	18			11.11	2	3.08	14
MIN (54.1)	IPM (46.6)	11					3.67	9
TOB (0)	MIN (54.1)	13			7.69	4	2.79	16

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Table 3. Summary of antimicrobial combinations tested on *Non- xylooxidans* isolates

Antimicrobial		Number ^b	Synergy		Antagonistic		SBPI	
First (%S ^a)	Second (%S ^a)		%	Rank	%	Rank	Median	Rank
CAZ (37.5)	TOB (4.2)	5	20.00	1			3.42	8
LVX (20.8)	MIN (91.7)	6	16.67	2			6.67	3
TOB (4.2)	IPM (50)	6	16.67	3			4.33	6
CAZ (37.5)	SXT (87.5)	9		4	11.11	1	22.44	2
LVX (20.8)	SXT (87.5)	6		5			31.28	1
MIN (91.7)	CHL (4.2)	5		7			6.67	3
TOB (4.2)	MEM (37.5)	5					4.33	6
CAZ (37.5)	MIN (91.7)	5					6.46	5