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1 **Modulation of antibiotic resistance in clinical isolates of *Enterobacter***
2 ***aerogenes* – a strategy combining antibiotics and chemosensitisers**

3

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27

28 **Running title:** Modulating antibiotic resistance in *E. aerogenes* clinical isolates

29

30

31 **Abstract**

32 **Purpose:** The main focus of this study was to evaluate the antimicrobial susceptibility
33 profiles of a selected number of human clinical isolates of *Enterobacter aerogenes* and
34 explore the effects of selected chemosensitisers on the reversal of the resistant phenotype of
35 these isolates.

36 **Methodology:** This study design was accomplished by: (i) characterising several multidrug
37 resistant (MDR) *E. aerogenes* clinical isolates; (ii) evaluating the contribution of target gene
38 mutations to the resistance phenotype focusing on fluoroquinolones and chloramphenicol
39 only; (iii) evaluating the contribution of membrane permeability and efflux to the MDR
40 phenotype; (iv) assessing the combined action of selected antimicrobials and
41 chemosensitisers (to identify combinations with synergistic effects and able to reduce the
42 MIC); (v) understand how these combinations can modulate the permeability or efflux on
43 these isolates.

44 **Results:** Resistance to ciprofloxacin couldn't be totally reversed due to pre-existing mutations
45 in target genes. Chloramphenicol susceptibility was efficiently restored by the addition of the
46 selected chemosensitisers. From the modulation kinetics it was clear that phenothiazines were
47 able to increase the accumulation of the Hoescht dye.

48 **Conclusions:** Modulations of permeability and efflux in the presence of chemosensitisers
49 help us to propose more appropriate chemotherapeutic combinations that can set the model to
50 be used in the treatment of these and other MDR-infections.

51

52 **Keywords:** Chemosensitisers; efflux pump; fluoroquinolones; multidrug resistance;
53 permeability; combination therapy.

54

55

56 **Introduction**

57 Acute hospital-acquired urinary tract infections are mainly associated with *Escherichia coli*
58 [1]. However, pathogens such as *Enterobacter*, *Klebsiella*, some *Proteus* species and
59 enterococci can also be involved in cases of cystitis and pyelonephritis [2]. *Enterobacter*
60 represents with *Klebsiella* the prominent enterobacterial members of the ESKAPE group that
61 encompasses the main MDRs described in the most worrying/complicated infections in
62 hospitals [3].

63

64 In 1999, *Enterobacter aerogenes* represented 48.7% of the extended-spectrum beta-
65 lactamases strains, due to the dissemination of TEM-24 [4]. In 2007, the numbers decreased,
66 being replaced by *E. coli* CTX-M producers [5, 6]. It is known that during therapy, strains
67 acquire resistance to β -lactams [4-7]. At present, *E. aerogenes* is the fourth most frequently
68 isolated bacteria in the clinical setting. This prevalence has increased greatly due to the
69 introduction of extended-spectrum cephalosporins and carbapenems [7-9]. This has
70 contributed to the emergence of pandrug *E. aerogenes* isolates resistant to last-line antibiotics.
71 Moreover, clinical strains exhibiting efflux activity are resistant to β -lactams, quinolones,
72 tetracyclines and chloramphenicol [10-13]. Approximately 40% of MDR clinical strains have
73 an active efflux [14].

74

75 Clinical isolates of *E. aerogenes* that overexpress efflux pumps in combination with loss of
76 porins have been reported in numerous studies [10, 15]. The study conducted by Doumith *et*
77 *al.* also showed that carbapenem resistance in *Klebsiella* and *Enterobacter* was exclusively
78 due to combinations of β -lactamases with impermeability caused by loss of outer membrane
79 proteins, where efflux was not implicated [16]. Taken these studies into account, reduction of
80 membrane permeability through the loss of porins together with increased expression of

81 efflux pumps can constitute a survival response involved in bacterial adaptation under stress
82 [17-18]. This is frequently linked with alteration of antibiotics or modification of the drug
83 targets in multidrug resistant (MDR) isolates [19]. In *E. coli*, *E. aerogenes*, *K. pneumoniae*
84 and *Salmonella enterica* several genes and external factors are involved in the emergence of
85 multidrug resistance [18-20]. The combined action of these mechanisms during an infection
86 confers a decrease in bacterial susceptibility ensuring dissemination and colonisation of the
87 patient and favoring the acquisition of additional mechanisms of resistance [15-18].

88

89 New antibiotics and novel approaches to treat MDR bacteria have been pursued [21]. Among
90 these, the development of chemosensitisers to return the bacteria to its susceptibility status is
91 to consider [22]. These compounds are usually of synthetic or natural origin, however a new
92 “trend” to modify existing compounds has gained strength in the last years [22-25].

93

94 The present study is focused on the modulation of efflux-mediated resistance to
95 fluoroquinolones or chloramphenicol, in clinical isolates of *E. aerogenes*. The strategy used
96 combined antibiotics and chemosensitisers to assess possible reversal of resistance to the
97 selected antibiotics. Efflux activity was assessed by a real-time fluorometric assay in the
98 presence of these compounds to evaluate their action on the efflux mechanism of resistance.

99

100

101

102 **Materials and Methods**

103 *Media, antibiotics, chemosensitisers and dyes*

104 Mueller-Hinton (MH) broth, MH agar (MHA), Phosphate Buffered Saline (PBS) solution,
105 ciprofloxacin (CIP), chloramphenicol (CHL), cefotaxime (CTX), thioridazine (TZ),
106 chlorpromazine (CPZ), ouabain (OUB), verapamil (VER), phenylalanine-Arginine- β -
107 naphthylamide (PA β N), Omeprazole (OME), esomeprazole (ESO), 1-(1-naphthylmethyl)-
108 piperazine (NMP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and Hoescht dye
109 solution (stock 20 mM). They were all purchased from Sigma (Ireland).

110

111 *Bacterial strains*

112 A random analysis using nineteen *E. aerogenes* isolates was performed in this study. These
113 bacterial isolates were obtained from several hospital origins from the south of France in
114 order to avoid bias. Nineteen *E. aerogenes* clinical isolates were identified as follows:
115 EACRO, EA9AB32, EA17AB48, EA29AB151, EA26UR132, EA22SP84, EA132263,
116 EA149399, EA171450, EAPOL13200, EAART15737, EA178LAV, EABAL959, EA23214,
117 EA108418, EA118259, EA122554, EA131538, EA137454 (**Table 1**). The antibiotic
118 susceptible *E. aerogenes* ATCC13048 was used as reference strain.

119

120 *Antibiotic Susceptibility Testing*

121 Antibiotic susceptibility testing was initially determined using the VITEK-2 platform and
122 sensititre platesTM GNX-2F (TREK Diagnostic Systems, LTD; East Grinstead, West Sussex,
123 UK) as per the manufacturers recommendation. Disc susceptibility testing was also performed
124 and resistance reported according to the European Committee on Antimicrobial Susceptibility
125 Testing (http://www.eucast.org/antimicrobial_susceptibility_testing/breakpoints/) validated
126 for *E. coli* were followed, only for comparison terms [26]. For POL, the breakpoints used

127 were the ones established by the Societe Francaise de Microbiologie, 2007. For COL the
128 results were analysed in comparison with the reference strain and other available literature
129 [27-28]. Isolates resistant to three or more different classes of antibiotics were considered
130 MDR. Isolates demonstrating mono or multiple resistances to CIP and CHL were selected for
131 further studies.

132

133 ***Determination of Minimum Inhibitory Concentrations (MIC)***

134 The MIC of antibiotics and chemosensitisers were determined by broth microdilution method
135 [26]. All experiments were carried out in triplicate and in three separate occasions.

136

137 ***Assessment of respiration in the presence of chemosensitisers***

138 To evaluate the effect of the chemosensitisers on the respiration of the isolates, assays were
139 prepared in 96-well plates as for MIC determination with media containing a tetrazolium
140 redox dye. Plates were placed in an OmniLog incubator-reader at 37°C for 24 hours. The
141 reduction of the dye due to respiration was monitored and the results recorded as Omnilog
142 units/time.

143

144 ***PCR amplification and sequencing of quinolone resistance-determining region of target***

145 ***genes***

146 Primers were designed using *E. aerogenes* ATCC13048 (KCTC2190) (GenBank:
147 CP002824.1) as reference. For isolate 178LAV primer Ec_parE_Rev was used to amplify the
148 QRDR of *parE* (**Table 2**). Genomic DNA was extracted using a DNeasy Blood & Tissue kit
149 (QIAGEN). PCR assays were performed as described [37]. PCR products were digested with
150 1 U *DpnI* (New England Biolabs) at 37°C for 1 hour before being purified using the Wizard®

151 SV Gel and PCR Clean-Up System (Promega) and sequenced (Source BioScience
152 LifeSciences).

153

154 ***PCR for cat and cml genes***

155 To verify if the resistance to CHL was due to the presence of *cat* and/or *cmlA* genes, a PCR
156 assay was performed [29]. The mixture was initially denaturated (4 min at 94°C), followed
157 by 35 cycles of amplification (30 seconds of denaturation at 94°C, 30 seconds of annealing at
158 55°C for *cat* and 59°C for *cml*, 1 minute of elongation at 72°C) and a final elongation step (5
159 min at 72°C). Amplifications were carried out on a G-Storm GS1 Thermal Cycler, UK.

160

161 ***Reversal of antibiotic resistance***

162 Antibiotics tested were CIP and CHL, which are concerned by efflux-mediated resistance [30-
163 1]. Briefly, 100 µl of MH broth was distributed into each well of the 96-wells microplate,
164 except for column 12, that contained 200 µl of the highest concentration of antimicrobials.
165 Serial twofold dilutions were performed. The chemosensitisers, TZ, CPZ and NMP were
166 added at 0.5x MIC and PABN at 40 mg/L. 5 µl of adjusted bacterial inoculum were inoculated
167 into the corresponding wells. Plates were incubated at 37°C for 18 hours. All assays were
168 conducted in triplicate in separate occasions.

169

170 ***Assessment of permeability/efflux activity***

171 A real-time assessment of the efflux activity was conducted using a modified 96-well
172 microplate method and fluorescence readings of hoechst dye monitored with a FluoroSkan
173 Ascent Fl (Thermo Scientific, Hampshire) [32]. Efflux assays were performed by adding 20
174 µl PABN or the other chemosensitisers (at three different concentrations) to the cultures
175 monitored previously. Assays were repeated 4 times per isolate.

176 *Modulation of the efflux dynamics - Determination of kinetic parameters*

177 To analyse the kinetics of accumulation promoted by the chemosensitisers (1/5 MIC), the rate
178 of accumulation of Hoechst dye and the time necessary to reach a plateau (designated PI)
179 were determined. A regression model was applied to the accumulation of Hoechst-dye curve
180 using a logarithmic or semi-logarithmic regression (case dependent). The regression equation
181 was used to calculate the rate of fluorescence.

182

183 *Statistical analysis*

184 All data were analysed using Microsoft Excel and SPSS Statistics version 20.0. A dendrogram
185 representing the antimicrobial susceptibility data was generated using software from Applied
186 Maths (Bionumerics, Version 7.1, Applied Math, Austin, TX).

187

188

189

190

191 **Results and Discussion**

192 *Antibiotic susceptibility testing*

193 The majority of the isolates were resistant to ceftazidime and ticarcillin-clavulanic acid
194 (n=13), followed by resistance to CTX (n=12) (**Figure 1**). Twelve isolates were also resistant
195 to azetronam. Resistance to CIP (n=11) and levofloxacin (n=10) was also observed.
196 Resistance to tobramycin and trimetoprim/sulfamethoxazole was recorded in 10 of the
197 isolates. Lastly, nine of the isolates were resistant to CHL. EAPOL13200 and EA131538
198 exhibited a *quasi*-pan resistant phenotype with susceptibility to POL.

199

200 *MIC for selected chemosensitisers*

201 The chemosensitisers, TZ and CPZ were the ones that showed higher inhibitory activity (MIC
202 = 50 to >200mg/L) (**Table 3**). PAβN and NMP showed lower inhibitory activity, with MIC
203 values between 250 and 2,000 mg/L and 500 and 1,000 mg/L, respectively. The
204 chemosensitisers, OUB, VER, OME and ESO exhibited lower antibacterial activity (MIC >
205 2,000 mg/L).

206

207 *Assessment of respiration in the presence of chemosensitisers*

208 Growth curves of all isolates were performed in the presence of varying concentrations of TZ,
209 CPZ, PAβN and NMP to ensure the chemosensitisers did not exert any effect on bacterial
210 respiration. In the presence of 200 and 400 mg/L of the chemosensitisers, there was a clear
211 impact on the respiration of *E. aerogenes* ATCC13048, over the course of a *quasi*-growth
212 curve (**Figure 2**). Similar effect was obtained with the other compounds. In the presence of
213 100 mg/L TZ and 50 mg/L CPZ there was an extension on the lag phase up to 6-7 hours
214 (**Figure 2**). At the highest concentrations of PAβN (400, 200 and 100 mg/L), there was a
215 notable reduction in respiration, with similar effects being observed in the presence of varying

216 concentrations of NMP (**Figure 2**). Importantly, concentrations of these same
217 chemosensitisers used for the reversal assays, did not exert any influence on the respiration of
218 *E. aerogenes* ATCC13048 (**Figure 2**).

219

220 In the presence of 200 mg/L TZ, isolates EA178LAV, EA122554, EA22SP84, and EA23214
221 exhibited a delay in the lag phase. After several hours, the respiration was re-established
222 (**Figure 3**). Similar results were previously reported for *Salmonella*, suggesting a process of
223 adaptation occurring among specific isolates [33].

224

225 A clear inhibition of bacterial respiration was obtained for the majority of the isolates at 320
226 and 160 mg/L PABN (**Figure 4**). 80 mg/L PABN elicit a delay in the lag phase of EACRO,
227 EAART15737, EA178LAV, EA108418, EAPOL13200, EA23214 and EA118259 (**Figure 4**
228 **A-E, I, J and K**). After 5 hours or more of exposure to 40 mg/L PABN a more pronounced
229 effect on the respiration of EACRO, EA108418, EA122554, EAPOL13200 and EA131538
230 (**Figure 4 A, D, E, H, L**) was obtained. The same was obtained with 20 mg/L PABN for
231 isolates EA137454, EA149399, EAPOL13200 and EA131538 (**Figure 4 F, G, H, L**).

232 When NMP was added at the highest concentrations there was a clear inhibition on the
233 respiration of all isolates (**Figure 5**). At a concentration of 250 mg/L only EAART15737
234 seemed to be affected (**Figure 5B**). Isolates EA149399, EAPOL13200, EA23214, EA118259
235 and EA131538 showed a delay on the lag phase when incubated in the presence of 125 mg/L
236 NMP (**Figure 5 G, H, J, K, L**).

237

238 *Selection of antibiotic resistant strains and synergy testing*

239 Based on the resistance profiles and considering the MIC values obtained for CIP and CHL,
240 twelve isolates were selected for further study (**Table 1**). Isolate EA149399 was only tested

241 for reversal of resistance to CHL due to its susceptibility to the fluoroquinolones tested. CIP
242 and CHL were chosen to assess the possible contribution of efflux to the resistant profile of
243 the isolates [14]. All isolates (with the exception of EA149399) had MIC values of CIP
244 between 4 and 64 mg/L. Similarly, for CHL values were between 128 and >512 mg/L when
245 compared with the reference strain (4 mg/L).

246

247 *Modulation of antibiotic resistance*

248 The effect on resistance level to CIP and CHL was assessed in the presence of CPZ, PA β N
249 and NMP (**Table 4**).

250

251 In the presence of CPZ, CPZ and PA β N, isolate EAPOL13200 had an MIC for CHL of 16
252 mg/L (>10 \times reduction in the original MIC >512 mg/L). In the presence of NMP, decrease of
253 resistance to CHL was also obtained with an 8 \times reduction in the original MIC (from >512
254 mg/L to 32 mg/L). Isolate EAART15737 showed a reversal of resistance to CHL (MIC from
255 >512 mg/L to 16 mg/L) in the presence of CPZ, PA β N and NMP.

256

257 The isolate EA137454 exhibited a reduction of 4 \times in the MIC of CIP in the presence of CPZ.
258 In this same isolate, a total reversal of resistance to CHL was observed in the presence of the
259 chemosensitisers. The MIC of CHL was strongly reduced when CPZ, PA β N and NMP were
260 present (reduction > 16 \times). Since there was a total reversal of resistance to CIP and CHL in the
261 presence of CPZ, it is possible that this isolate has intrinsic mechanisms of resistance that are
262 susceptible to this compound.

263

264 For the remaining isolates no significant reductions in the MIC of CHL (\geq 512 mg/L) were
265 observed (**Table 4**). PCR assays to detect *cat* [encoding a chloramphenicol acetyltransferase]

266 and *cmlA* [encoding a phenicol-specific efflux pump] were negative, failing to yield an
267 amplicon from any of the corresponding resistant isolates. Therefore, we can rule out a direct
268 contribution of both CAT and CML enzymes in this resistance. The differences in values
269 obtained for the susceptible restoration levels for EACRO, EA22SP84, EA178LAV *versus*
270 those obtained for EAPOL13200, EA137454 and others are likely due to other factors. The
271 divergence between the level of susceptibility restored for CHL compared to the level
272 obtained for fluoroquinolones can be associated to ability of chemosensitizers to restore
273 antibiotics susceptibility. This effect depends of the antibiotic chemical structure, their
274 respective affinity to AcrB pockets and the relative affinity of chemosensitizers for AcrB
275 pockets [34-35]. These findings highlight the differences between the isolates since not all of
276 them responded in the same way to the combinations tested suggesting difference in the
277 expression level of AcrB pump, expression of other efflux pumps, possible mutations inside
278 AcrB changing the ligand affinity, etc.

279

280 ***Target gene mutations***

281 Comparative analyses were done using *E. aerogenes* ATCC13048 (KCTC2190) as reference.
282 Amino acid substitutions were identified in a number of target genes, associated with
283 resistance to fluoroquinolones (**Table 1**). Eleven isolates contained single or multiple
284 substitutions within the GyrA subunit along with either single or double substitutions in
285 GyrB. The most common amino acid substitution identified in 10 of the 11 resistant isolates,
286 was located within the *gyrA*-encoding subunit and was denoted as T83I (**Table 1**). In GyrB,
287 the most common amino acid substitution found was E466D (eight of the isolates). These
288 mutations can explain the failure of susceptibility restoration by chemosensitisers when used
289 in combination.

290

291 *Assessment of efflux activity*

292 All isolates were assessed for their efflux-associated MDR phenotype and ability to
293 accumulate hoechst dye (**Figure 6A**). In general, all isolates demonstrated the same
294 fluorescence level as the reference strain. From the four tested chemosensitisers the most
295 efficient compound promoting the accumulation of the dye was TZ, followed by NMP and
296 lastly CPZ. In the presence of glucose, there was a lower fluorescence signal detected,
297 reflecting a reduced accumulation of the dye due to the energy provided to fuel the efflux
298 machinery. Similarly, from the 3 concentrations tested for each chemosensitiser, the highest
299 (TZ 200 mg/L; CPZ 100 mg/L and NMP 250 mg/L) were those that resulted in an increased
300 accumulation of the dye. This response was dose dependent. At these concentrations, addition
301 of glucose had no effect, suggesting that the efflux systems were working at saturation and
302 that respiration was impaired.

303

304 PA β N was unable to induce a significant increased accumulation of Hoechst-dye. In the
305 presence of 40 mg/L PA β N, *E. aerogenes* ATCC13048 and EA149399 were the only isolates
306 that showed a small rate increase in dye accumulation (**Figure 6C**). This observation is in line
307 with data obtained from the reversal assays wherein in the presence of PA β N, the majority of
308 the isolates did not show any increase in susceptibility to the antimicrobials tested.

309 At the highest concentration of NMP (250 mg/L) there was a noted interference/possible
310 quenching effect on the fluorescence of Hoechst-dye and this effect was more marked with
311 glucose (**Figure 6B**).

312

313 *Modulation of the efflux dynamics - determination of kinetic parameters*

314 CCCP is able to dissipate the proton motive force which is the energy driving force of AcrAB
315 efflux pump when the other like PA β N or NMP can block or saturate the AcrB transport for

316 some antibiotics [36]. An effect of CCCP, PA β N and NMP on the accumulation of the dye
317 was noted with isolates EA149399, EAPOL13200, EAART15737, EA137454 and EA118259
318 (**Table 5**). Interestingly, the maximal restoring of CHL susceptibility was obtained with
319 PA β N-NMP on isolates EAPOL13200, EAART15737 and EA137454. This observation
320 suggests that the activity of efflux pump as transporter of CHL and dye is efficiently targeted
321 by the chemosensitisers in these isolates.

322

323 Regarding the mechanism and taking into account the effect of glucose, this becomes more
324 complex. Comparing the effect of PA β N alone *versus* PA β N+glucose (or NMP alone *versus*
325 NMP+glucose) with the effect of CCCP alone *versus* CCCP+glucose, it is clear that CCCP is
326 a real efflux energy un-coupler and its uncoupling impact can be partly restore in the presence
327 of glucose-. The results obtained with PA β N, NMP and CCCP were of the same order of
328 magnitude supporting some additional effect for CPZ under these conditions. Regarding CPZ,
329 the permeabilizing effect reported for phenothiazines on biological membranes can boost the
330 observed accumulation in association with the chemical properties of Hoechst-dye [37].

331 Regarding CPZ, an effect is observed with strain EA122554. Taken together, these data
332 illustrates the impact that chemosensitisers can have on the accumulation of dyes, which can
333 be different from the effect on antibiotic activity.

334

335

336 **Conclusion**

337 Overall, we explored the potential utility of a series of chemosensitisers in combination with
338 antimicrobials to revert antimicrobial resistance in clinical isolates of *E. aerogenes*. The
339 isolates studied were also characterized with target gene mutations identified. These
340 genotypes most likely underpin our inability to reverse the resistant phenotypes in the
341 presence of chemosensitisers. For some of the isolates, permeability differences were noted
342 that could contribute to their resistant profile.

343

344 Interestingly, the presence of an energy-dependent efflux of Hoescht-dye, CCCP sensitive and
345 activated by glucose, is demonstrated in strains EACRO, EA149399, EAPOL13200,
346 EAART15737, EA118259, EA137454. In strains EAPOL13200 and EA137454, PA β N can
347 impair this efflux when NMP is able to alter its activity in strains EAPOL13200 and
348 EA149399. Regarding these effects, we may hypothesize that PA β N and NMP can have same
349 activity behavior in strain EA149399 and exhibit different action in strains EAPOL13200 and
350 EA137454. These differences in the inhibitor activity may be due to different affinities of
351 Hoescht-dye, PA β N and NMP for the binding sites located inside efflux pump or by the steric
352 hindrances caused by the fixation in the AcrB pockets [38-39].

353

354 The more important point is the correlation between accumulation studies and restoration of
355 antibiotic activity. For fluoroquinolone, it is clear that the presence of target mutations in the
356 various isolates drastically impair the recovery of ciprofloxacin susceptibility whatever the
357 chemosensitizers used. About CHL, the resistance is efficiently reversed by the three
358 chemosensitizers in three strains for which an effect of CCCP on Hoescht accumulation has
359 been evidenced. This indicates that CPZ, PA β N and NMP are able to block the CHL efflux in
360 these strains and restore a partial or complete susceptible phenotype. Interestingly, no

361 restoration of CHL susceptibility is obtained for the strain EA149399 for which an efflux
362 CCCP, PABN and NMP sensitive is observed with Hoescht. The divergence observed
363 between the susceptibility restoration and the dye accumulation is probably due to the
364 difference of affinity and binding constant for the pump, and/or the presence of the antibiotic
365 target which initiates the bacterial death when the dye assay is only based to DNA or
366 membrane association.

367

368 This divergence dye-antibiotic must be taken into consideration when assessing the
369 contribution of efflux in clinical isolates and the evaluation of new adjuvants or
370 chemosensitizers. In this way, the use of the recent concept "structure intracellular
371 concentration activity relationship", or SICAR will be more efficient to study the correlation
372 accumulation-activity of clinically-used or new antibiotics and the potential of new
373 adjuvants/chemosensitizers [40].

374

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378

379

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503

504 **Figure legends**

505 **Figure 1: A heat-map showing the antimicrobial susceptibility profiles for *E. aerogenes***
506 **ATCC13048 along with 19 clinical isolates (see also Table 1).** The antimicrobial
507 compounds clusters are grouped according to their class.

508

509 **Figure 2: Bacterial respiration trace profiles for *E. aerogenes* ATCC13048 alone and in**
510 **the presence of various concentrations of four chemosensitisers.**

511 Respiration were assessed in the presence of varying concentrations of thioridazine (TZ),
512 chlorpromazine (CPZ), phenylalanine arginine β -naphthylamide (PA β N) and (D) 1-(1-
513 naphthylmethyl)-piperazine (NMP) using an Omnilog incubator-reader for 24 hours.

514

515 **Figure 3: Bacterial respiration trace profiles for four clinical *E. aerogenes* measured in**
516 **the presence of various concentrations of TZ.** Assays were prepared in 96-well plates in

517 media containing varying concentrations of TZ and also a redox dye. After inoculation with
518 the bacterial suspension, the plates were placed in the OmniLog incubator-reader at 37°C for
519 24 hours and the reduction of the dye monitored over time. This was done for *E. aerogenes*
520 clinical isolates EA178LAV; EA122554; EA22SP84; EA23214 in the presence of varying
521 concentrations of TZ.

522

523 **Figure 4: Bacterial respiration trace profiles for clinical *E. aerogenes* measured in the**
524 **presence of various concentrations of PA β N.** Assays were prepared in 96-well plates in

525 media containing varying concentrations of PA β N and also a redox dye. After inoculation
526 with the bacterial suspension, the plates were placed in the OmniLog incubator-reader at 37°C
527 for 24 hours and the reduction of the dye monitored over time.

528 **Figure 5: Bacterial respiration trace profiles for clinical *E. aerogenes* measured in the**
529 **presence of various concentrations of NMP.** Assays were prepared in 96-well plates in
530 media containing varying concentrations of NMP and also a redox dye. After bacterial
531 inoculation, the plates were incubated in the OmniLog incubator-reader at 37°C for 24 hours
532 and the reduction of the dye monitored over time.

533

534 **Figure 6: Accumulation of Hoechst-dye by: (A) the *E. aerogenes* isolates tested ; (B) *E.***
535 ***aerogenes* ATCC13048 in the presence of selected chemosensitisers; (C) *E. aerogenes***
536 **ATCC13048 and clinical isolate EA149399 in the presence of different concentrations of**
537 **PABN. 6A.** An initial assay was conducted to evaluate the accumulation of Hoechst-dye by
538 the *E. aerogenes* isolates. **6B.** Accumulation of Hoechst-dye by *E. aerogenes* ATCC13048 in
539 the presence of different concentrations of Thioridazine (TZ); Chlorpromazine (CPZ);
540 Phenylalanine arginine β -naphthylamide (PABN) and 1-(1-naphthylmethyl)-piperazine
541 (NMP). Cyanide-m-chlorophenylhydrazone (CCCP) was used as an internal control. Parallel
542 samples containing 0.4% glucose (G) were also performed. The accumulation assay in the
543 presence of the chemosensitisers was run for 30 minutes (mins).

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<i>E. aerogenes</i>	Strain ID	Biological sample	Hospital of origin	Year of isolation	FQ resistance genes			
					<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
Reference strain	ATCC13048	Urine	-	-	(-)	(-)	(-)	(-)
	EA23214	Catheter	NIMES	2005	T83I	S464F	S80I	N477D
	EA178LAV	Urine	MAR	1999	T83I	E466D	S80I	N477D
	EA108418	-	MAR	2003	T83I	E466D	S80I	N477D
	EA137454	-	MAR	2003	A67S, T83I	L451F, Q465R	S80I	N477D
	EA122554	-	MAR	2003	T83I	E466D	S80I	N477D
	EA131538	-	MAR	2003	T83I	E466D	S80I	N477D
	EA118259	-	MAR	2003	T83I	E466D	S80I	N477D
Clinical isolates (n=19)	EABAL959	Urine	AUB	2002	nd	nd	nd	nd
	EAPOL13200	Sputum	AUB	2002	T83I	E466D	S80I	N477D
					T83Y, D87H,			
	EAART15737	Rectal swab	AUB	2002	M127L, S128A, N200D	R387K	S80I	N477D
	EACRO	Urine	AUB	2002	T83I	E466D	S80I	N477D
	EA9AB32	Sputum	MAR	1994	nd	nd	nd	nd
	EA22SP84	Suppuration	MAR	1995	T83I	E466D	S80I	N477D
	EA17AB48	Sputum	MAR	1995	nd	nd	nd	nd
	EA29AB151	Sputum	MAR	1995	nd	nd	nd	nd
	EA26UR132	Urine	MAR	1995	nd	nd	nd	nd
	EA171450	Sputum	MAR	2002	nd	nd	nd	nd
	EA132263	Sputum	MAR	2002	nd	nd	nd	nd
	EA149399	Blood culture	MAR	2002	nd	nd	nd	nd

552 **Table 1. Study collection including *E. aerogenes* ATCC13048 and 19 clinical isolates and**
553 **showing their clinical origin; geographical location, year of isolation and mutations in**
554 **target genes.**

555 **Legend.** MAR – Marseille, AUB – Aubagne, NIMES – Nîmes, nd –not determined.

Target	Primer	Sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
<i>gyrA</i>	Ea_gyrA_Fwd1	GCTCGTATCTGGATTATGCG	64	637	This work
	Ea_gyrA_Rev1	CTCTTCAATACCGCGACG			
<i>gyrB</i>	Ea_gyrB_Fwd	TACCAACAACATTCCGCAGC	64	831	This work
	Ea_gyrB_Rev	ATGTACTGTTCTGCTTGCC			
<i>parC</i>	Ea_parC_Fwd	ATGCCTACCTGAACTACTCC	64	701	This work
	Ea_parC_Rev	GTCTTCTTTCGTCCACACGG			
<i>parE</i>	Ea_parE_Fwd	GACCGAAAGCTACGTGAACC	64	875	This work
	Ea_parE_Rev	TCTTACGCTTCAACTGCTCC			
	Ec_parE_Rev	GTTCGGATCAAGCGTGGTTT			
<i>cat</i>	Ec_cat_Fwd	AGT TGC TCA ATG TAC CTA TAA CC			45
	Ec_cat-Rev	TTG TAA TTC ATT AAG CAT TCT GCC			
	Ec_cml_Fwd	CCG CCA CGG TGT TGT TGT TAT C			
<i>cmlA</i>	Ec_cml_Rev	CAC CTT GCC TGC CCA TCA TTA G			46

557 **Table 2. Target genes, amplification primers and PCR reaction conditions used for**
558 **characterisation of the isolates.**

559 **Note:** Fwd, forward; Rev, revers

<i>E. aerogenes</i> strains	Compounds class / MIC (mg/L)			
	Phenothiazines		Peptidomimetic	Piperazine
	TZ	CPZ	PABN	NMP
ATCC13048	>200	100	2000	1000
EACRO	200	100	1000	1000
EA9AB32	>200	100	1000	500
EA17AB48	200	100	1000	1000
EA29AB151	200	100	1000	1000
EA26UR132	200	100	1000	500
EA22SP84	>200	>200	1000	500
EA132263	200	100	500	500
EA149399	100	100	250	500
EA171450	200	50	1000	500
EAPOL13200	200	100	1000	500
EAART15737	>200	100	1000	500
EA178 LAV	200	100	1000	500
EABAL959	200	100	1000	500
EA23214	>200	100	1000	500
EA108418	200	100	1000	500
EA118259	200	100	1000	500
EA122554	200	100	1000	500
EA131538	200	100	1000	500
EA137454	200	100	1000	500

Table 3. MIC values for selected chemosensitisers. Legend. Thioridazine (TZ); Chlorpromazine (CPZ); 1-(1-naphthylmethyl)-piperazine (NMP); and Phe-Arg- β -naphthylamide (PABN). Minimum Inhibitory Concentration (MIC). Based on their resistance to CIP, NAL and CHL, strains highlighted in bold were selected for further synergism testing.

Table 4: MIC values measured in the absence or in the presence of selected chemosensitisers along with mutations in target genes.

<i>E. aerogenes</i> strains	CIP				CHL				FQ resistance genes (mutation)			
	-	+ CPZ	+ PABN	+ NMP	-	+ CPZ	+ PABN	+ NMP	<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
ATCC13048	1	(-)	(-)	(-)	4	(-)	(-)	(-)	(-)	(-)	(-)	(-)
EACRO	16	8 (2x)	16	8 (2x)	>512	512	512	512	T83I	E466D	S80I	N477D
EA22SP84	32	32	64 (2x)	16 (2x)	>512	512	512	>512	T83I	E466D	S80I	N477D
EA149399	1	(-)	(-)	(-)	128	128	128	128	NA	NA	NA	NA
EAPOL13200	32	16 (2x)	32	16 (2x)	>512	16 (10x)	16 (10x)	32 (8x)	T83I	E466D	S80I	N477D
EAART15737	64	32 (2x)	32 (2x)	32 (2x)	>512	16 (10x)	16 (10x)	16 (10x)	T83Y, D87H, M127L, S128A, N200D	R387K	S80I	N477D
EA178LAV	16	8 (2x)	16	16	>512	512	512	512	T83I	E466D	S80I	N477D
EA23214	32	16 (2x)	32	16 (2x)	>512	512	512	512	T83I	S464F	S80I	N477D
EA108418	32	16 (2x)	32	16 (2x)	>512	512	512	512	T83I	E466D	S80I	N477D
EA118259	32	16 (2x)	32	16 (2x)	>512	256 (>2x)	512	512	T83I	E466D	S80I	N477D
EA122554	16	8 (2x)	16	8 (2x)	>512	512	512	512	T83I	E466D	S80I	N477D
EA131538	16	16	32	16	>512	512	512	512	T83I	E466D	S80I	N477D
EA137454	4	1 (4x)	8	4	>512	1 (>18x)	2 (16x)	2 (16x)	A67S, T83I	L451F, Q465R	S80I	N477D

Legend. Ciprofloxacin (CIP); Chloramphenicol (CHL); (-) not determined since the isolate is susceptible to the antibiotics. NA - not applicable.

Significant differences in the MIC (considered as > 4-fold) when in the presence of the chemosensitisers are marked (**bold**). Isolates highlighted in bold were selected for the checkerboard assay.

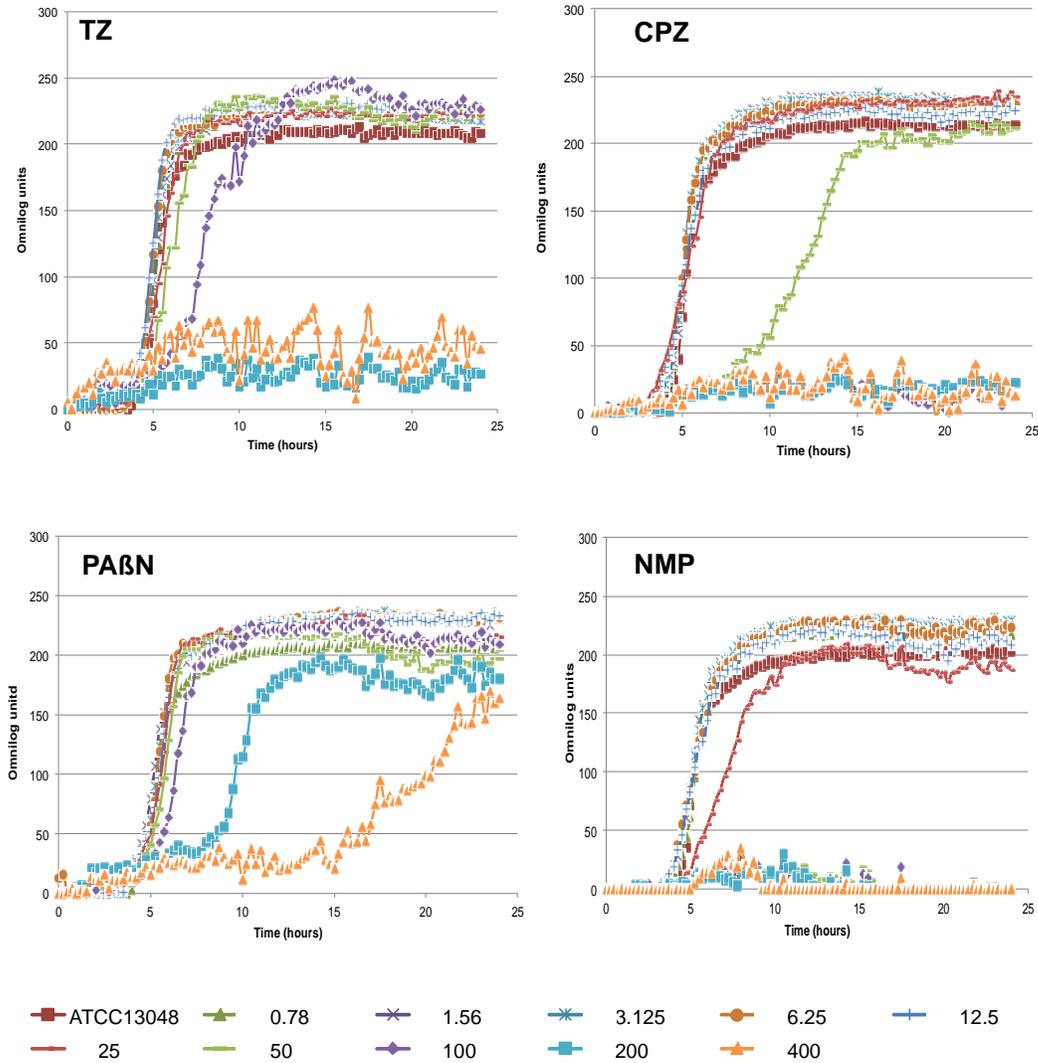
Table 5. Accumulation rates measured in a Hoescht-dye uptake assay and shown as a heat-map.

<i>Enterobacter</i> strains	Chemosensitisers							
	CPZ		PABN		NMP		CCCP	
	wo/Gluc	w/Gluc	wo/Gluc	w/Gluc	wo/Gluc	w/Gluc	wo/Gluc	w/Gluc
ATCC13048	0.7216	0.4996	0.085	0.0832	0.1689	0.153	0.0645	0.1622
EACRO	0.4966	0.378	0.0411	0.0275	0.0544	0.0204	0.145	0.0058
EA22SP84	0.5748	0.3964	0.0845	0.0887	0.1047	0.1042	0.0427	0.0833
EA149399	0.8903	0.643	0.0897	0.0207	0.113	0.0103	0.0969	-0.0009
EAPOL13200	0.5009	0.3579	0.0065	0.0078	0.0844	0.0206	0.1128	0.0023
EAART15737	0.6169	0.3714	0.0149	0.0152	-0.2135	-0.1291	0.0667	0.0024
EA178LAV	0.5871	0.4078	0.0311	0.0248	0.0561	0.0686	0.006	0.0719
EA23214	0.6285	0.2984	0.0093	0.014	0.018	-0.0026	-0.0035	0.0293
EA108418	0.7383	0.3999	0.0384	0.0606	0.1035	0.0737	0.0082	0.1094
EA118259	0.6424	0.3975	0.0804	0.0413	0.1018	0.169	0.0839	0.0134
EA122554	0.6755	0.1347	0.0834	0.0855	0.1274	0.1007	0.035	0.0832
EA131538	0.6887	0.4519	0.0326	0.0155	0.1191	0.0766	0.0072	0.0966
EA137454	0.7409	0.5719	0.1212	0.0172	0.1633	0.1546	0.1753	0.0123

Legend. wo/Gluc – without glucose; w/Gluc – with glucose; Thioridazine (TZ); Chlorpromazine (CPZ); Phenylalanine arginine β -naphthylamide (PA β N); 1-(1-naphthylmethyl)-piperazine (NMP); cyanide-m-chlorophenylhydrazine (CCCP). Values are representative of the rate of accumulation of Hoescht dye in the presence of the chemosensitisers and when in the presence or absence of glucose. The grey color scheme indicates a significant effect (ratio without/with glucose >4) of energy (glucose) on the accumulation rate.

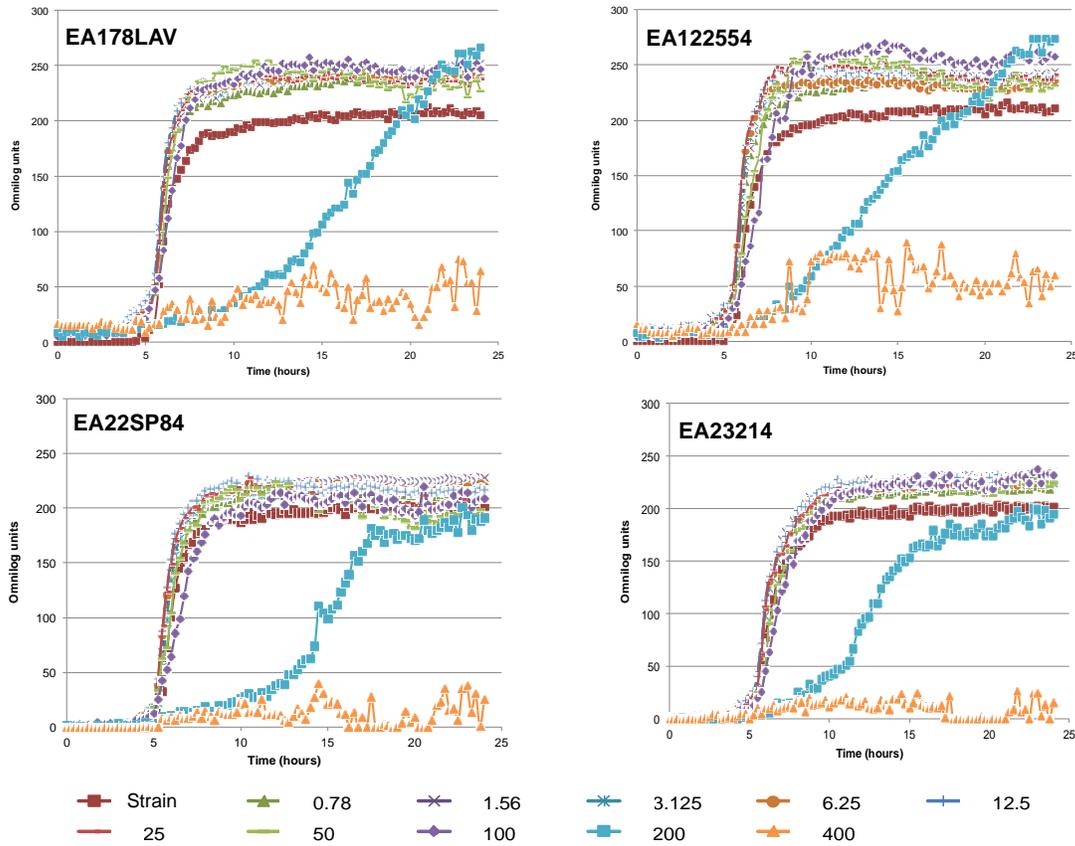
Legend of Figure 1. The dendrogram was produced with software from Applied Maths (Bionumerics, Version 7.1, Applied Math, Austin, TX). Resistance profiles of the isolates are color-coded: resistant (red); intermediate (yellow/orange); susceptible (green). Penicillin G was included in the assay and analysis as an internal control (data not shown). *E. coli* ATCC25922 was included as quality control.

Figure 2. Bacterial respiration trace profiles for *E. aerogenes* ATCC13048 alone and in the presence of various concentrations of four chemosensitisers.



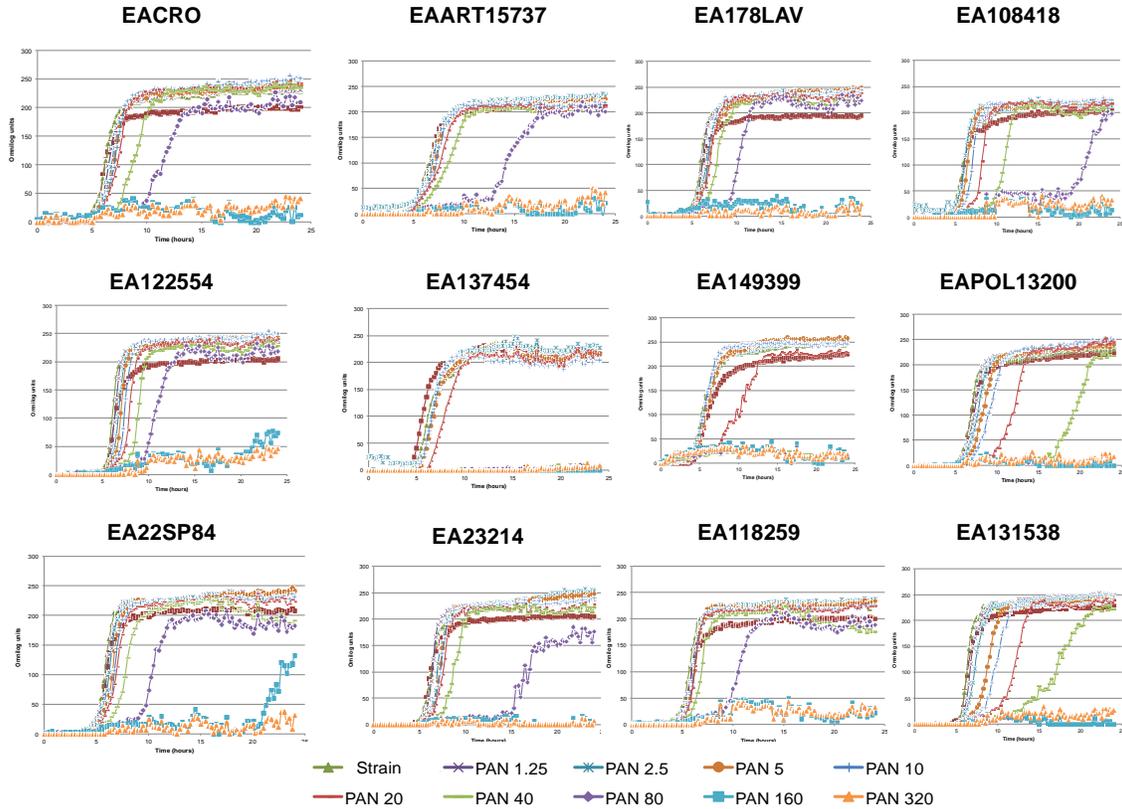
Legend of Figure 2. Respiration were assessed in the presence of varying concentrations of thioridazine (TZ), chlorpromazine (CPZ), *phenylalanine arginine β -naphthylamide* (PA β N) and (D) *1-(1-naphthylmethyl)-piperazine* (NMP) using an Omnilog incubator-reader for 24 hours.

Figure 3. Bacterial respiration trace profiles for four clinical *E. aerogenes* measured in the presence of various concentrations of TZ.



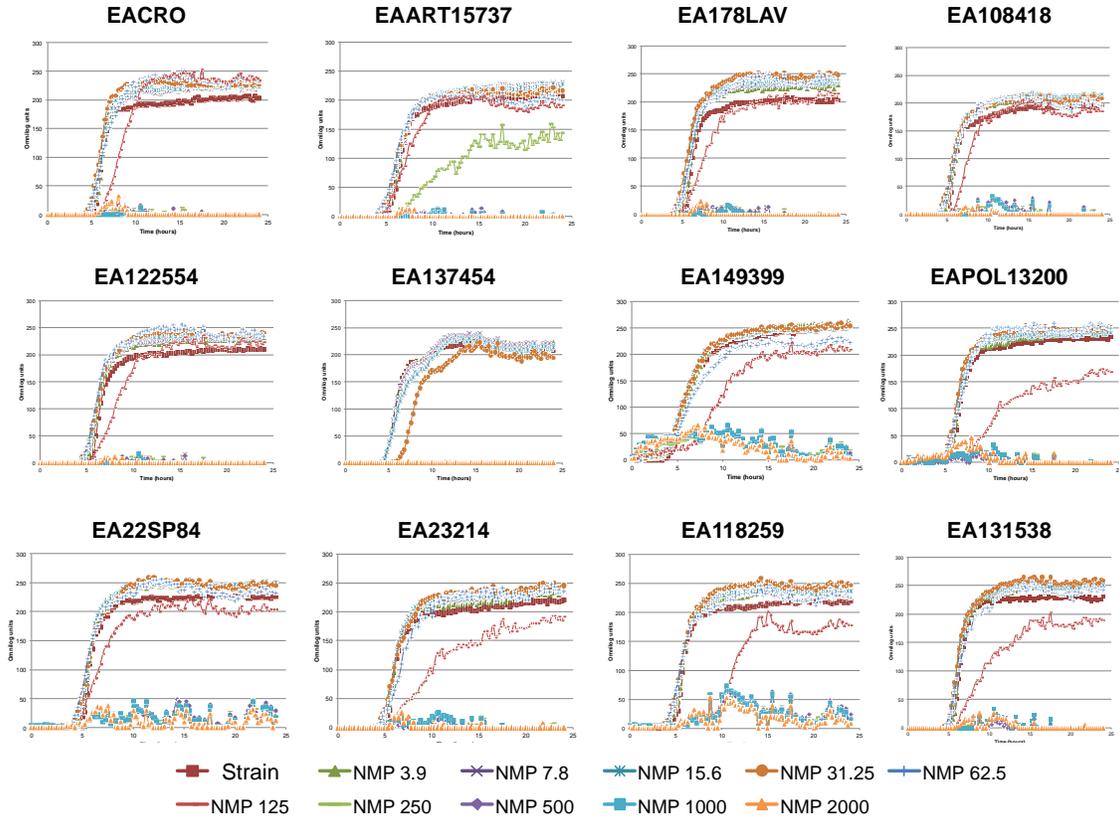
Legend of Figure 3. Assays were prepared in 96-well plates in media containing varying concentrations of TZ and also a redox dye. After inoculation with the bacterial suspension, the plates were placed in the OmniLog incubator-reader at 37°C for 24 hours and the reduction of the dye monitored over time. This was done for *E. aerogenes* clinical isolates EA178LAV; EA122554; EA22SP84; EA23214 in the presence of varying concentrations of TZ.

Figure 4. Bacterial respiration trace profiles for clinical *E. aerogenes* measured in the presence of various concentrations of of PABN.



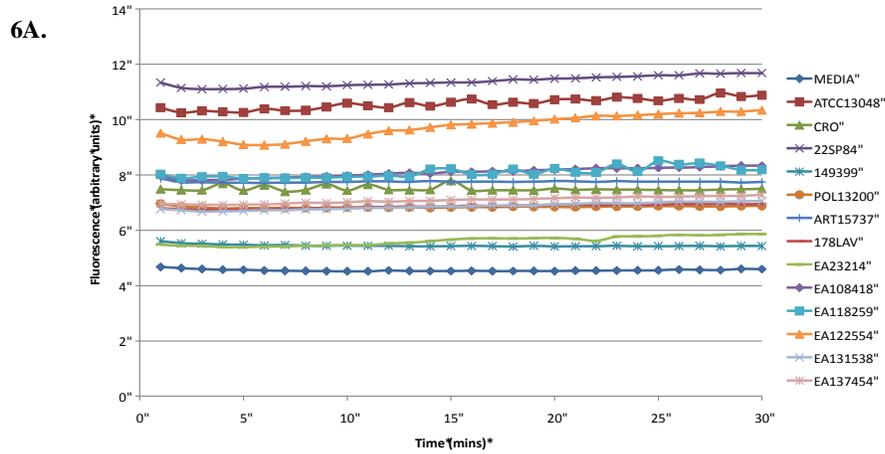
Legend of Figure 4. Assays were prepared in 96-well plates in media containing varying concentrations of **PABN** and also a redox dye. After inoculation with the bacterial suspension, the plates were placed in the OmniLog incubator-reader at 37°C for 24 hours and the reduction of the dye monitored over time.

Figure 5. Bacterial respiration trace profiles for clinical *E. aerogenes* measured in the presence of various concentrations of NMP.



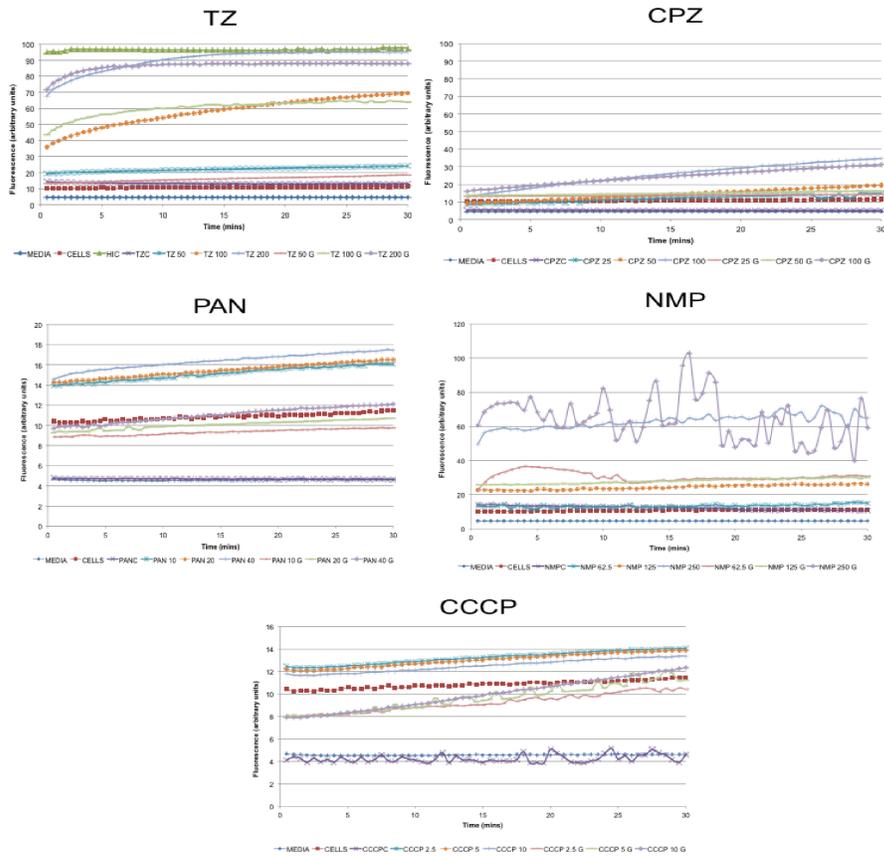
Legend of Figure 5. Assays were prepared in 96-well plates in media containing varying concentrations of NMP and also a redox dye. After bacterial inoculation, the plates were incubated in the OmniLog incubator-reader at 37°C for 24 hours and the reduction of the dye monitored over time.

Figure 6 : Accumulation of Hoechst-dye by : (A) the *E. aerogenes* isolates tested ; (B) *E. aerogenes* ATCC13048 in the presence of selected chemosensitisers; (C) *E. aerogenes* ATCC13048 and clinical isolate EA149399 in the presence of different concentrations of PAβN.



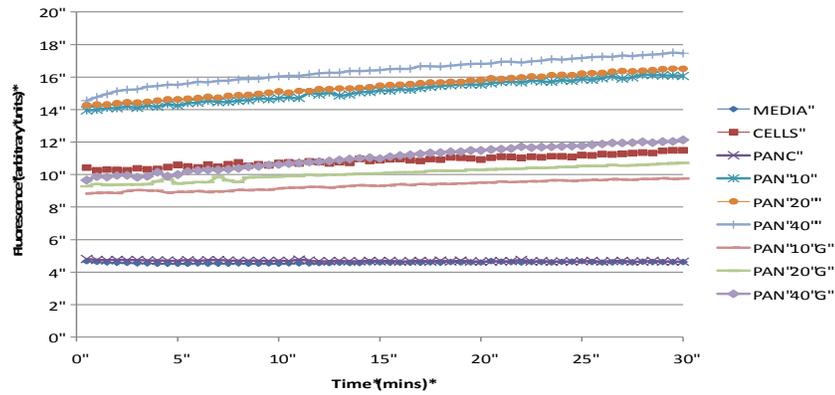
Legend of Figure 6A. An initial assay was conducted to evaluate the accumulation of Hoechst-dye by the *E. aerogenes* isolates.

6B.

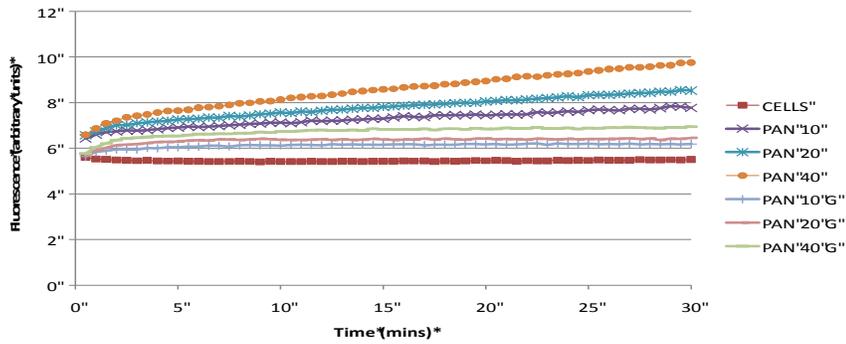


Legend of Figure 6B. Accumulation of Hoechst-dye by *E. aerogenes* ATCC13048 in the presence of different concentrations of Thioridazine (TZ); Chlorpromazine (CPZ); **Phenylalanine arginine β -naphthylamide (PAN)** and 1-(1-naphthylmethyl)-piperazine (NMP). Cyanide-m-chlorophenylhydrazone (CCCP) was used as an internal control. Parallel samples containing 0.4% glucose (G) were also performed. The accumulation assay in the presence of the chemosensitisers was run for 30 minutes (mins).

6C. a



b



Legend of Figure 6C. Accumulation of Hoechst-dye by *E. aerogenes* ATCC13048 (a) and clinical isolate EA149399 (b) when in the presence of *Phenylalanine arginine β -naphthylamide* (PABN) Aliquots containing 0.4% glucose (G) were also performed in parallel. The accumulation assay in the presence of the chemosensitisers was run for 30 minutes (mins).