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

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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.

Materials and Methods

Media, antibiotics, chemosensitisers and dyes

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

Bacterial strains

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

Antibiotic Susceptibility Testing

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

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

Determination of Minimum Inhibitory Concentrations (MIC)

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

Assessment of respiration in the presence of chemosensitisers

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

PCR amplification and sequencing of quinolone resistance-determining region of target genes

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

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

PCR for cat and cml genes

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

Reversal of antibiotic resistance

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

Assessment of permeability/efflux activity

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

Modulation of the efflux dynamics - Determination of kinetic parameters

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

Statistical analysis

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

Results and Discussion

Antibiotic susceptibility testing

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

MIC for selected chemosensitisers

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

Assessment of respiration in the presence of chemosensitisers

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

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

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

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

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

Selection of antibiotic resistant strains and synergy testing

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

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

Modulation of antibiotic resistance

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

In the presence of CPZ, CPZ and PAßN, isolate EAPOL13200 had an MIC for CHL of 16 mg/L (>10× reduction in the original MIC >512 mg/L). In the presence of NMP, decrease of resistance to CHL was also obtained with an 8× reduction in the original MIC (from >512 mg/L to 32 mg/L). Isolate EAART15737 showed a reversal of resistance to CHL (MIC from >512 mg/L to 16 mg/L) in the presence of CPZ, PAßN and NMP.

The isolate EA137454 exhibited a reduction of 4× in the MIC of CIP in the presence of CPZ. In this same isolate, a total reversal of resistance to CHL was observed in the presence of the chemosensitisers. The MIC of CHL was strongly reduced when CPZ, PAßN and NMP were present (reduction > 16×). Since there was a total reversal of resistance to CIP and CHL in the presence of CPZ, it is possible that this isolate has intrinsic mechanisms of resistance that are susceptible to this compound.

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

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

Target gene mutations

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

Assessment of efflux activity

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

PABN was unable to induce a significant increased accumulation of Hoechst-dye. In the presence of 40 mg/L PABN, *E. aerogenes* ATCC13048 and EA149399 were the only isolates that showed a small rate increase in dye accumulation (**Figure 6C**). This observation is in line with data obtained from the reversal assays wherein in the presence of PABN, the majority of the isolates did not show any increase in susceptibility to the antimicrobials tested. At the highest concentration of NMP (250 mg/L) there was a noted interference/possible quenching effect on the fluorescence of Hoechst-dye and this effect was more marked with glucose (**Figure 6B**).

Modulation of the efflux dynamics - determination of kinetic parameters

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

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

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

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

Conclusion

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

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

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

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

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

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Declarations

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Ethical Approval: Not required

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503

Figure legends

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

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

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. 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 PA β N. Assays were prepared in 96-well plates in media containing varying concentrations of PA β N 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. 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. 6A. An initial assay was conducted to evaluate the accumulation of Hoechst-dye by the *E. aerogenes* isolates. **6B.** Accumulation of Hoechst-dye by *E. aerogenes* ATCC13048 in the presence of different concentrations of Thioridazine (TZ); Chlorpromazine (CPZ); Phenylalanine arginine β -naphthylamide (PAβN) 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).

<i>E. aerogenes</i>	Strain ID	Biological	Hospital of	Year of	FQ resistance genes			
		sample	origin	isolation	<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
Reference strain	ATCC13048	Urine	-	-	(-)	(-)	(-)	(-)
Clinical isolates (n=19)	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
	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

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

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	64	958	⁴⁴
<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

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

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	+ PAßN	+ NMP	-	+ CPZ	+ PAßN	+ 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.

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

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* (PAβN) 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 (CCCCP) 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* (PAN) 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).