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Modulation of antimicrobial resistance in clinical isolates of *Enterobacter aerogenes*: A strategy combining antibiotics and chemosensitisers

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ABSTRACT

Objective: The main focus of this study was to evaluate the antimicrobial susceptibility profiles of a number of human clinical isolates of *Enterobacter aerogenes* isolates and to explore the effects of selected chemosensitisers on reversal of the resistant phenotype of these isolates.
Methods: This study design was accomplished by: (i) characterising several multidrug-resistant (MDR) *E. aerogenes* clinical isolates; (ii) evaluating the contribution of target gene mutations to the resistance phenotype, focusing on fluoroquinolones and chloramphenicol only; (iii) evaluating the contribution of membrane permeability and efflux to the MDR phenotype; (iv) assessing the combined action of selected antimicrobials and chemosensitisers in order to identify combinations with synergistic effects able to reduce the minimum inhibitory concentration (MIC); and (v) understanding how these combinations can modulate the permeability or efflux of these isolates.
Results: Resistance to ciprofloxacin could not be totally reversed owing to pre-existing mutations in target genes. Chloramphenicol susceptibility was efficiently restored by the addition of the selected chemosensitisers. From the modulation kinetics it was clear that phenothiazines were able to increase the accumulation of Hoechst dye.
Conclusions: Modulation of permeability and efflux in the presence of chemosensitisers can help us to propose more appropriate chemotherapeutic combinations that can set the model to be used in the treatment of these and other MDR infections.

1. Introduction

Acute hospital-acquired urinary tract infections are mainly associated with *Escherichia coli* [1]. However, pathogens such as *Enterobacter* spp., *Klebsiella* spp., some *Proteus* spp. and enterococci can also be involved in cases of cystitis and pyelonephritis [2]. Along with *Klebsiella*, *Enterobacter* represents the prominent enterobacterial members of the 'ESKAPE' group of pathogens that encompasses the main multidrug-resistant (MDR) species described in the most worrying/complicated infections in hospitals [3].

In 1999, *Enterobacter aerogenes* represented 48.7% of extended-spectrum β-lactamase (ESBL)-producing strains owing to the dissemination of TEM-24 [4]. In 2007 the number had decreased, being replaced by CTX-M-producing *E. coli* [5,6]. It is known that during therapy, strains acquire resistance to β-lactams [4–7]. At present, *E. aerogenes* is the fourth most frequently isolated bacterium in the clinical setting. This prevalence has increased greatly due to the introduction of extended-spectrum cephalosporins and carbapenems [7–9]. This has contributed to the emergence of pandrug-resistant *E. aerogenes* isolates that are

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Table 1
Study collection including the reference strain *Enterobacter aerogenes* ATCC 13048 and 19 clinical *E. aerogenes* isolates showing their clinical origin, geographical location, year of isolation, and mutations in fluoroquinolone (FQ) resistance-related genes.

E. aerogenes strain	Biological sample	Hospital of origin	Year of isolation	FQ resistance gene mutations			
				GyrA	GyrB	ParC	ParE
ATCC 13048	Urine	–	–	(–)	(–)	(–)	(–)
Clinical isolates							
EA23214	Catheter	Nîmes	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	N/D	N/D	N/D	N/D
EAPOL13200	Sputum	AUB	2002	T83I	E466D	S80I	N477D
EAART15737	Rectal swab	AUB	2002	T83Y, D87H, M127L, S128A, N200D	R387K	S80I	N477D
EACRO	Urine	AUB	2002	T83I	E466D	S80I	N477D
EA9AB32	Sputum	MAR	1994	N/D	N/D	N/D	N/D
EA22SP84	Suppuration	MAR	1995	T83I	E466D	S80I	N477D
EA17AB48	Sputum	MAR	1995	N/D	N/D	N/D	N/D
EA29AB151	Sputum	MAR	1995	N/D	N/D	N/D	N/D
EA26UR132	Urine	MAR	1995	N/D	N/D	N/D	N/D
EA171450	Sputum	MAR	2002	N/D	N/D	N/D	N/D
EA132263	Sputum	MAR	2002	N/D	N/D	N/D	N/D
EA149399	Blood culture	MAR	2002	N/D	N/D	N/D	N/D

MAR, Marseille; AUB, Aubagne; N/D, not determined.

resistant to last-line antibiotics. Moreover, clinical strains exhibiting efflux activity are resistant to β-lactams, quinolones, tetracyclines and chloramphenicol [10–13]. Approximately 40% of MDR clinical strains exhibit active efflux [14].

Clinical isolates of *E. aerogenes* that overexpress efflux pumps in combination with loss of porins have been reported in numerous studies [10,15]. A study conducted by Doumith et al. also showed that carbapenem resistance in *Klebsiella* and *Enterobacter* was exclusively due to combinations of β-lactamases with impermeability caused by loss of outer membrane proteins, whereas efflux was not implicated [16]. Taking these studies into account, reduction of membrane permeability through the loss of porins together with increased expression of efflux pumps can constitute a survival response involved in bacterial adaptation under stress [17,18]. This is frequently linked with alteration of antibiotics or modification of the drug targets in MDR isolates [19]. In *E. coli*, *E. aerogenes*, *Klebsiella pneumoniae* and *Salmonella enterica*, several genes and external factors are involved in the emergence of multidrug resistance [18–20]. The combined action of these mechanisms during an infection confers a decrease in bacterial susceptibility ensuring dissemination and colonisation of the patient and favouring the acquisition of additional mechanisms of resistance [15–18].

New antibiotics and novel approaches to treat MDR bacteria have been pursued [21]. Among these, the development of chemosensitisers to return bacteria to their susceptible status has been considered [22]. These compounds are usually of synthetic or natural origin, however a new trend to modify existing compounds has gained strength in the last years [22–25].

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

2. Materials and methods

2.1. Media, antibiotics, chemosensitisers and dyes

Mueller–Hinton broth, Mueller–Hinton agar, phosphate-buffered saline, ciprofloxacin (CIP), chloramphenicol (CHL), cefotaxime (CTX), thioridazine (TZ), chlorpromazine (CPZ), ouabain, verapamil, phenylalanine-arginine β-naphthylamide (PAβN), omeprazole, esomeprazole, 1-(1-naphthylmethyl)-piperazine (NMP), carbonyl

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

Target gene	Primer name	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
gyrA	Ea_gyrA_Fwd1	GCTCGTATCTGGATTATGCG	64	637	This work
	Ea_gyrA_Rev1	CTCTTCAATACCGCGACG			
gyrB	Ea_gyrB_Fwd	TACCAACAACATTCCGCAGC	64	831	This work
	Ea_gyrB_Rev	ATGTACTGTTCCTGCTTGCC			
parC	Ea_parC_Fwd	ATGCCTACCTGAACTACTCC	64	701	This work
	Ea_parC_Rev	GTCTTCTTTCGTCCACACGG			
parE	Ea_parE_Fwd	GACCGAAAGCTACGTGAACC	64	875	This work
	Ea_parE_Rev	TCTTACGCTTCAACTGCTCC			
	Ec_parE_Rev	GTTCGGATCAAGCGTGGTTT	64	958	[29]
cat	Ec_cat_Fwd	AGT TGC TCA ATG TAC CTA TAA CC			
	Ec_cat-Rev	TTG TAA TTC ATT AAG CAT TCT GCC			[30]
cmlA	Ec_cml_Fwd	CCG CCA CGG TGT TGT TGT TAT C			[31]
	Ec_cml_Rev	CAC CTT GCC TGC CCA TCA TTA G			

cyanide *m*-chlorophenyl hydrazone (CCCP) and Hoechst dye solution (stock 20 mM) were all purchased from Sigma (Arklow, Ireland).

2.2. Bacterial strains

A random analysis using 19 *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. Details of the 19 *E. aerogenes* clinical isolates are given in Table 1. Antimicrobial-susceptible *E. aerogenes* ATCC 13048 was used as a reference strain.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was initially performed using a VITEK®2 platform (bioMérieux, Marcy-l'Étoile, France) and Sensititre™ GNX-2F plates (TREK Diagnostic Systems Ltd., East Grinstead, UK) as per the manufacturer's recommendations. Disk diffusion susceptibility testing was also performed and resistance was reported according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/antimicrobial_susceptibility_testing/breakpoints/) validated for *E. coli* only for comparison terms [26]. For polymyxin B, the breakpoints used were those established by the Société Française de Microbiologie (SFM) [26]. For colistin, 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.

2.4. Determination of minimum inhibitory concentrations (MICs)

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

Table 3
Minimum inhibitory concentrations (MICs) for selected chemosensitisers against the *Enterobacter aerogenes* isolates.

E. aerogenes strain	MIC (mg/L)			
	Phenothiazines		Peptidomimetic	Piperazine
	TZ	CPZ	PAβN	NMP
ATCC 13048	>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
EA178LAV	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

TZ, thioridazine; CPZ, chlorpromazine; PAβN, phenylalanine-arginine β-naphthylamide; NMP, 1-(1-naphthylmethyl)-piperazine.
Based on their resistance to ciprofloxacin and chloramphenicol, strains highlighted in bold were selected for further synergism testing.

2.5. Assessment of respiration in the presence of chemosensitisers

To evaluate the effect of the chemosensitisers on 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 (Biolog Inc., Hayward, CA) at 37 °C for 24 h. Reduction of the dye due to respiration was monitored and the results were recorded as OmniLog units/time.

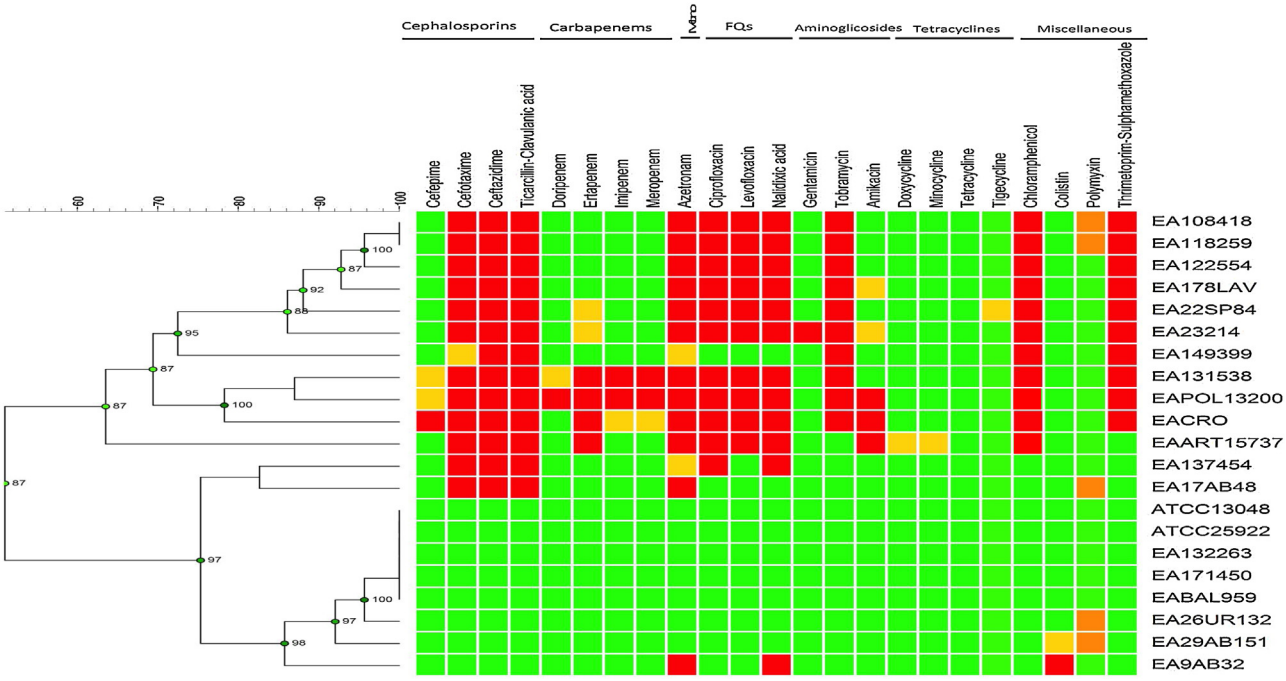

Fig. 1. Heat map showing the antimicrobial susceptibility profiles for *Enterobacter aerogenes* ATCC 13048 along with 19 clinical *E. aerogenes* isolates (see Table 1). The antimicrobial compound clusters are grouped according to their class. The dendrogram was produced with BioNumerics v.7.1 software (Applied Maths, Austin, TX). Resistance profiles of the isolates are colour-coded as follows: red, resistant; yellow/orange, intermediate; and green, susceptible. Penicillin G was included in the assay and analysis as an internal control (data not shown). *Escherichia coli* ATCC 25922 was included as quality control.

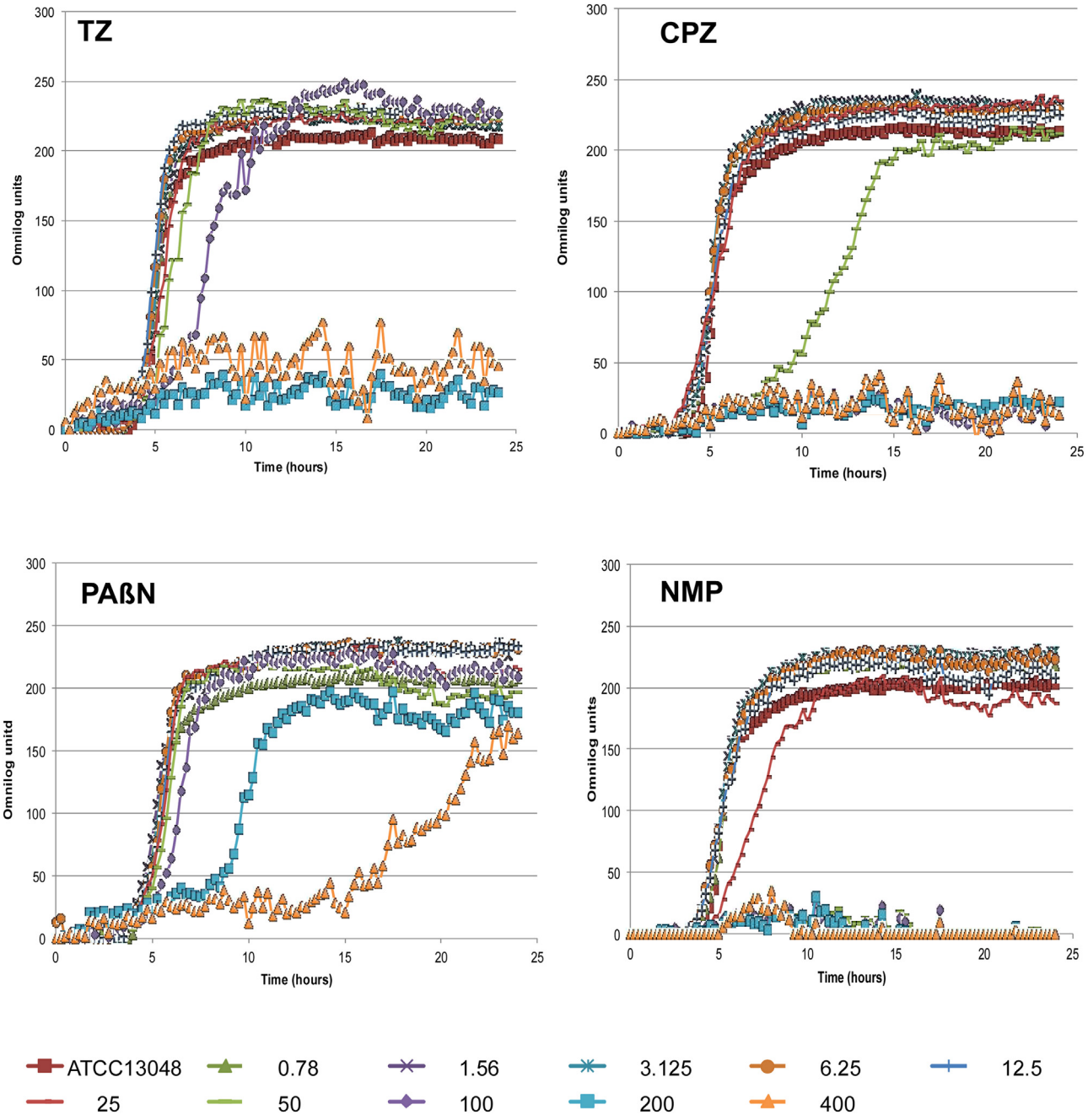

Fig. 2. Bacterial respiration trace profiles for *Enterobacter aerogenes* ATCC 13048 alone and in the presence of various concentrations of the four chemosensitisers thioridazine (TZ), chlorpromazine (CPZ), phenylalanine-arginine β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) assessed using an OmniLog incubator/reader for 24 h.

2.6. PCR amplification and sequencing of the quinolone resistance-determining region (QRDR) of target genes

Primers were designed using *E. aerogenes* ATCC 13048 (strain KCTC2190) (GenBank **CP002824.1**) as reference (Table 2). For isolate EA178LAV, the 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, Courtaboeuf, France). PCR assays were performed as described previously [32]. PCR products were digested with 1 U of *Dpn*I (New England Biolabs, Évry, France) at 37 °C for 1 h before being purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Charbonnières-les-Bains, France) and sequenced (Source BioScience, Nottingham, UK).

2.7. PCR for cat and cml genes

A PCR assay was performed to verify whether the resistance to CHL was due to the presence of *cat* and/or *cmlA* genes [33]. The mixture was initially denatured (4 min at 94 °C), followed by 35 cycles of amplification (30 s of denaturation at 94 °C, 30 s of annealing at 55 °C for *cat* and 59 °C for *cml*, and 1 min of elongation at 72 °C), and a final elongation step (5 min at 72 °C). Amplification

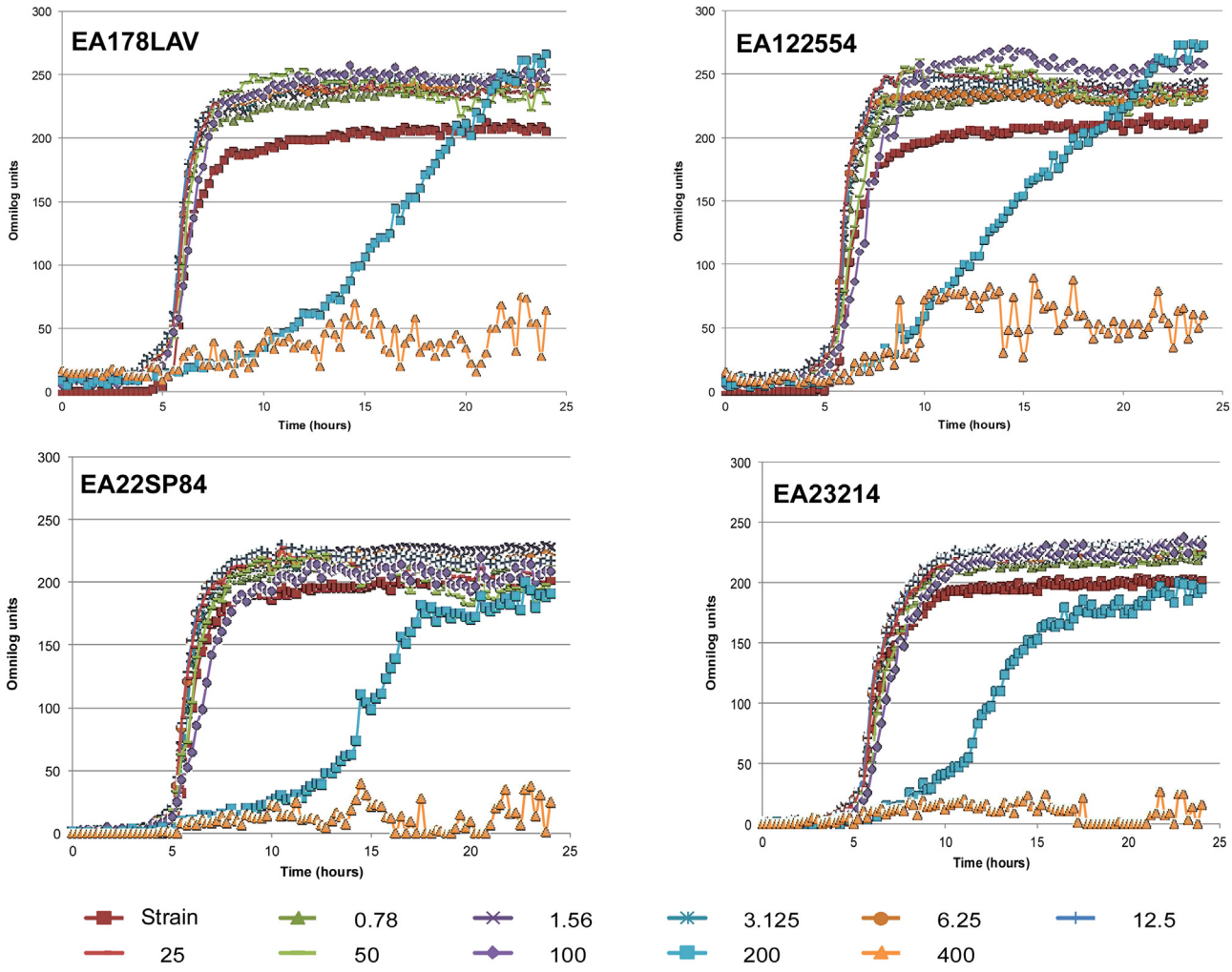

Fig. 3. Bacterial respiration trace profiles for four clinical *Enterobacter aerogenes* isolates (EA178LAV, EA122554, EA22SP84 and EA23214) measured in the presence of various concentrations of thioridazine (TZ). Assays were prepared in 96-well plates in media containing varying concentrations of TZ as well as a tetrazolium redox dye. Following inoculation with the bacterial suspension, the plates were placed in an OmniLog incubator/reader at 37 °C for 24 h and the reduction of the dye was monitored over time.

was carried out on a G-Storm GS1 Thermal Cycler (Akribis Scientific Ltd., Knutsford, UK).

2.8. Reversal of antimicrobial resistance

The antibiotics tested were CIP and CHL, which are affected by efflux-mediated resistance [34,35]. Briefly, 100 μL of Mueller–Hinton broth was distributed into each well of a 96-well microplate, except for column 12 that contained 200 μL of the highest concentration of antibiotic. Serial two-fold dilutions were then performed. The chemosensitisers TZ, CPZ and NMP were added at 0.5× MIC and PAβN at 40 mg/L. Then, 5 μL of adjusted bacterial inoculum was added to the corresponding wells and the plates were incubated at 37 °C for 18 h. All assays were conducted in triplicate on separate occasions.

2.9. Assessment of permeability/efflux activity

A real-time assessment of efflux activity was conducted using a modified 96-well microplate method and fluorescence readings of Hoechst dye monitored using a FluoroSkan Ascent™ FL (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) [36]. Efflux assays were performed by adding 20 μL of PAβN or other chemosensitisers (at three different concentrations) to the cultures monitored previously. Assays were repeated four times per isolate.

2.10. Modulation of efflux dynamics: determination of kinetic parameters

To analyse the kinetics of accumulation promoted by the chemosensitisers (0.2× MIC), the rate of accumulation of Hoechst dye and the time necessary to reach a plateau were determined. A regression model was applied to the accumulation of Hoechst dye curve using a logarithmic or semi-logarithmic regression (case dependent). A regression equation was used to calculate the rate of fluorescence.

2.11. Statistical analysis

All data were analysed using Microsoft Excel (Microsoft Corp., Redmond, WA) and IBM SPSS Statistics v.20.0 (IBM Corp., Armonk, NY). A dendrogram representing the antimicrobial susceptibility data was generated using BioNumerics v.7.1 software (Applied Maths, Austin, TX).

3. Results and discussion

3.1. Antimicrobial susceptibility testing

The majority of isolates were resistant to ceftazidime and ticarcillin/clavulanic acid ($n = 13$), followed by resistance to CTX ($n = 12$) (Fig. 1). Twelve isolates were also resistant to aztreonam.

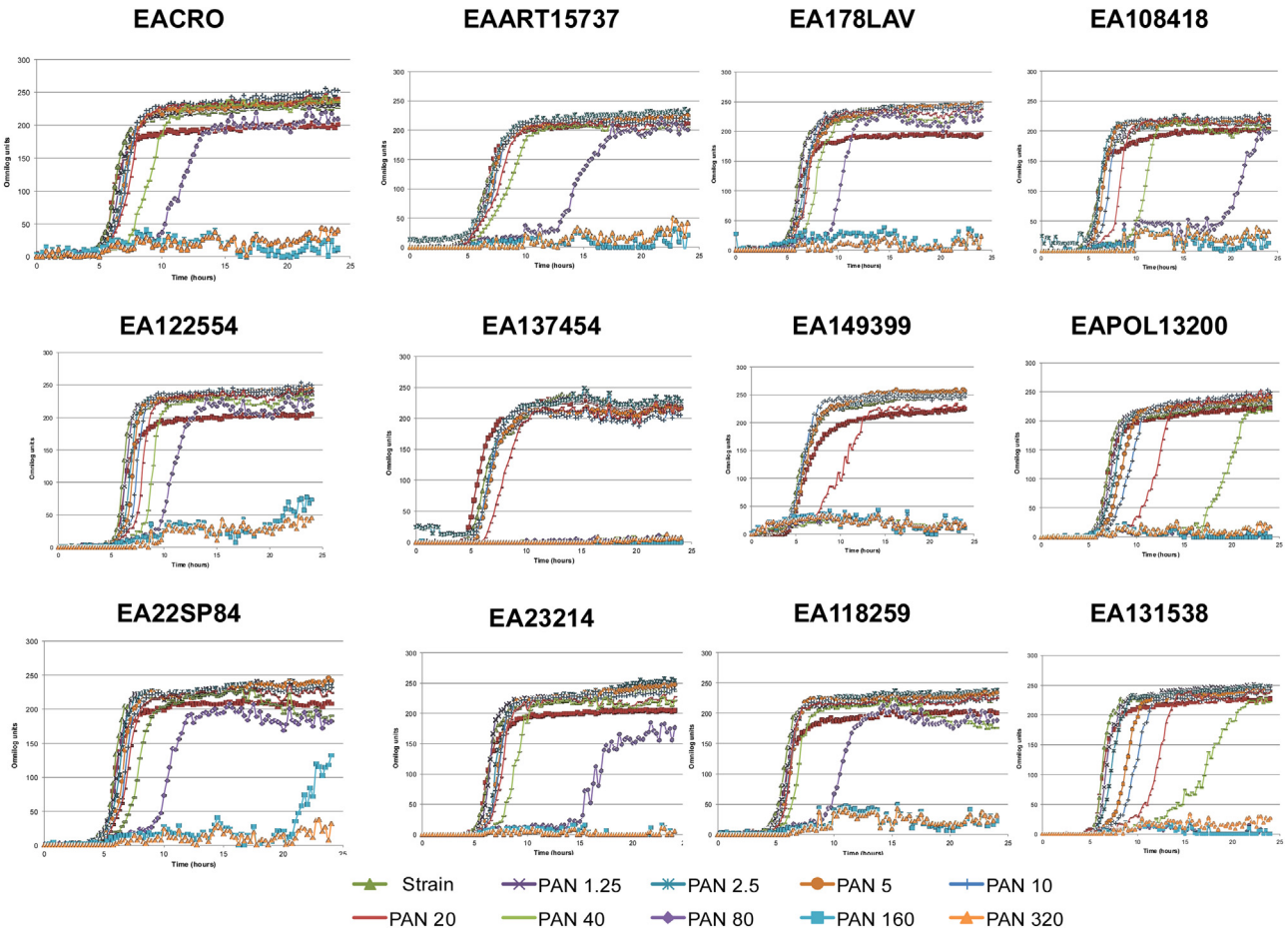

Fig. 4. Bacterial respiration trace profiles for clinical *Enterobacter aerogenes* isolates measured in the presence of various concentrations of phenylalanine-arginine β-naphthylamide (PAβN). Assays were prepared in 96-well plates in media containing varying concentrations of PAβN as well as a tetrazolium redox dye. Following inoculation with the bacterial suspension, the plates were placed in an OmniLog incubator/reader at 37 °C for 24 h and the reduction of the dye was monitored over time.

Resistance to CIP ($n = 11$) and levofloxacin ($n = 10$) was also observed. Resistance to tobramycin and trimethoprim/sulfamethoxazole was recorded in ten isolates each. Lastly, 11 of the isolates were resistant to CHL. EAPOL13200 and EA131538 exhibited a quasi-panresistant phenotype with susceptibility to polymyxin B.

3.2. Minimum inhibitory concentrations for selected chemosensitisers

The phenothiazine chemosensitisers TZ and CPZ showed the highest inhibitory activities (MICs of 50 mg/L to >200 mg/L) (Table 3). PAβN and NMP showed lower inhibitory activities with MICs between 250–2000 mg/L and 500–1000 mg/L, respectively. The chemosensitisers ouabain, verapamil, omeprazole and esomeprazole exhibited lower antibacterial activity (MICs > 2000 mg/L) (data not shown).

3.3. Assessment of respiration in the presence of chemosensitisers

Growth curves of all isolates were performed in the presence of varying concentrations of TZ, CPZ, PAβN and NMP to ensure that the chemosensitisers did not exert any effect on bacterial respiration. In the presence of 200 mg/L and 400 mg/L of the chemosensitisers, there was a clear impact on the respiration of *E. aerogenes* ATCC 13048 over the course of a quasi-growth curve (Fig. 2). A 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 h (Fig. 2). At the highest concentrations of PAβN (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 (Fig. 2). Importantly, concentrations of these same chemosensitisers used for the reversal assays did not exert any influence on the respiration of *E. aerogenes* ATCC 13048 (Fig. 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, respiration was re-established (Fig. 3). Similar results were previously reported for *Salmonella*, suggesting a process of adaptation occurring among specific isolates [32].

A clear inhibition of bacterial respiration was obtained for the majority of the isolates at 320 mg/L and 160 mg/L PAβN (Fig. 4). At 80 mg/L, PAβN elicited a delay in the lag phase of EACRO, EAART15737, EA178LAV, EA108418, EAPOL13200, EA23214 and EA118259. After ≥5 h of exposure to 40 mg/L PAβN a more pronounced effect on the respiration of EACRO, EA108418, EA122554, EAPOL13200 and EA131538 was observed. The same was observed with 20 mg/L PAβN for isolates EA137454, EA149399, EAPOL13200 and EA131538 (Fig. 4).

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

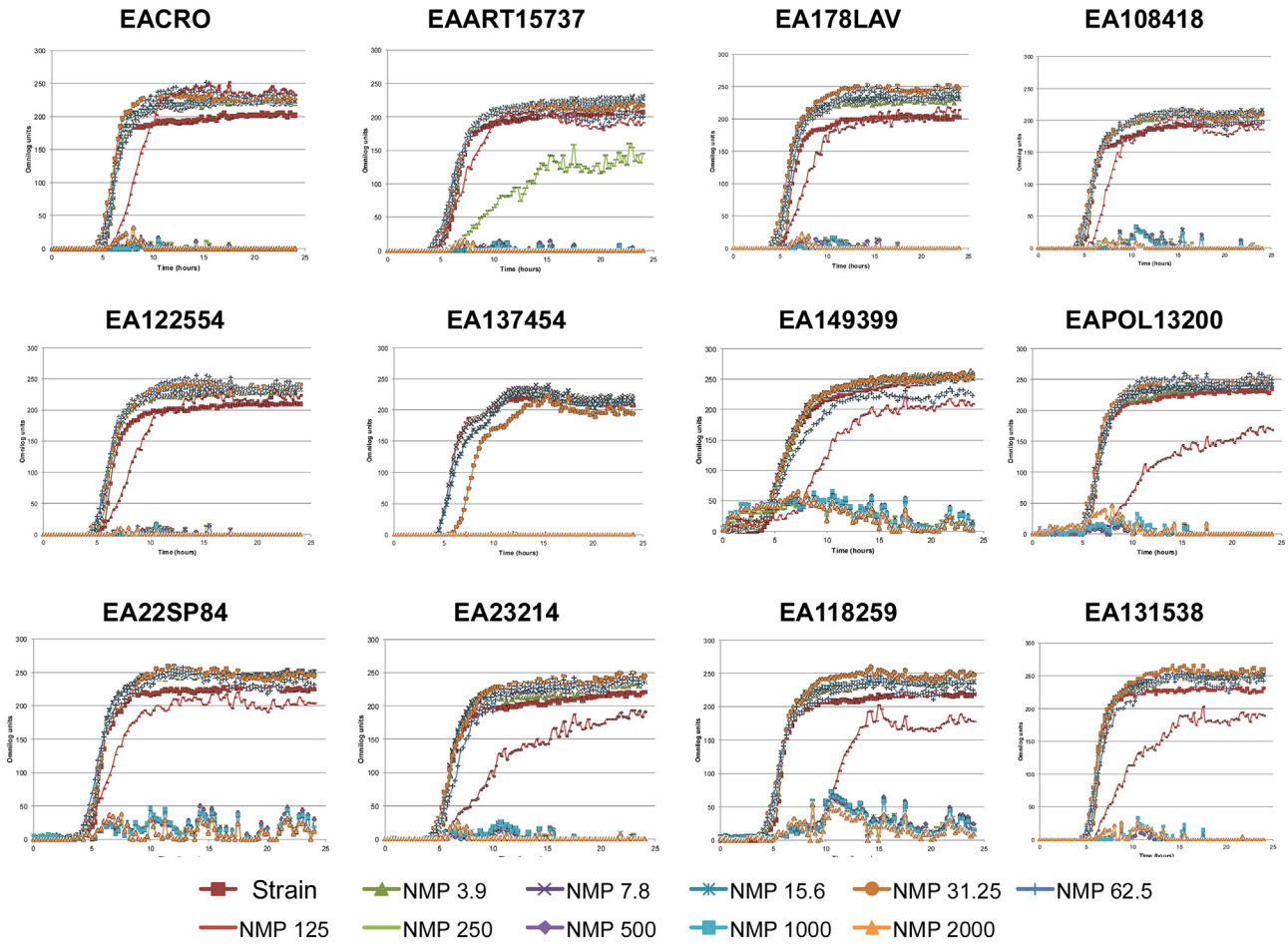

Fig. 5. Bacterial respiration trace profiles for clinical *Enterobacter aerogenes* isolates measured in the presence of various concentrations of 1-(1-naphthylmethyl)-piperazine (NMP). Assays were prepared in 96-well plates in media containing varying concentrations of NMP as well as a tetrazolium redox dye. Following bacterial inoculation, the plates were incubated in an OmniLog incubator/reader at 37 °C for 24 h and the reduction of the dye was monitored over time.

3.4. Selection of antimicrobial-resistant strains and synergy testing

Based on the resistance profiles and considering the MICs obtained for CIP and CHL, 12 isolates were selected for further study (Table 3). Isolate EA149399 was only tested for reversal of resistance to CHL owing 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 CIP MICs between 4–64 mg/L. Similarly, CHL MICs were between 128 mg/L and >512 mg/L compared with 4 mg/L for the reference strain ATCC 13048.

3.5. Modulation of antimicrobial resistance

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

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

Isolate EA137454 exhibited a reduction of 4× in the CIP MIC 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 CHL MIC was strongly reduced when CPZ, PAβN and NMP were present (reduction of ≥256×). 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 CHL MICs (≥128 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 susceptibility restoration levels for EACRO, EA22SP84 and 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 with the level obtained for fluoroquinolones can be associated with the ability of chemosensitisers to restore antimicrobial susceptibility. This effect depends of the antibiotic chemical structure, their respective affinity for AcrB pockets and the relative affinity of chemosensitisers for AcrB pockets [37,38]. 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 the AcrB pump, expression of other efflux pumps, possible mutations inside AcrB altering the ligand affinity, etc.

Table 4
Minimum inhibitory concentrations (MICs) measured in the absence or presence of selected chemosensitisers, along with mutations in fluoroquinolone (FQ) resistance-related genes.

E. aerogenes	Ciprofloxacin				Chloramphenicol				FQ resistance gene mutations			
strain	Alone	+CPZ	+PAβN	+NMP	Alone	+CPZ	+PAβN	+NMP	*gyrA*	*gyrB*	*parC*	*parE*
ATCC 13048	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 (32x)	16 (32x)	32 (16x)	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 (>512×)	2 (256x)	2 (256x)	A67S, T83I	L451F, Q465R	S80I	N477D

CPZ, chlorpromazine; PAβN, phenylalanine-arginine β-naphthylamide; NMP, 1-(1-naphthylmethyl)-piperazine.
(–) not determined as the isolate was susceptible to the antibiotic; NA, not applicable.
Significant differences in the MIC (considered as >4-fold) in the presence of the chemosensitisers are indicated by grey shading.

3.6. Target gene mutations

Comparative analyses were done using *E. aerogenes* ATCC 13048 (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 in GyrA, identified in 10 of the 11 resistant isolates, was 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 restoration of susceptibility by chemosensitisers when used in combination.

3.7. Assessment of efflux activity

All isolates were assessed for their efflux-associated MDR phenotype and their ability to accumulate Hoechst dye (Fig. 6A). In general, all isolates demonstrated the same fluorescence level as the reference strain. Among the four tested chemosensitisers, the most efficient compound promoting the accumulation of the dye was TZ, followed by NMP and lastly CPZ. A lower fluorescence signal was detected in the presence of glucose, reflecting reduced accumulation of the dye owing to the energy provided to fuel the efflux machinery. Similarly, among the three concentrations tested for each chemosensitiser, the highest (200 mg/L TZ, 100 mg/L CPZ and 250 mg/L NMP) 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.

PAβN was unable to induce a significant increase in accumulation of Hoechst dye. In the presence of 40 mg/L PAβN, *E. aerogenes* ATCC 13048 and EA149399 were the only isolates that showed a small rate increase in dye accumulation (Fig. 6C). This observation is in line with data obtained from the reversal assays wherein in the presence of PAβN 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 (Fig. 6B).

3.8. Modulation of efflux dynamics: determination of kinetic parameters

CCCP is able to dissipate the proton-motive force, which is the energy driving force of the AcrAB efflux pump, whilst the others compounds such as PAβN or NMP can block or saturate AcrB transport for some antibiotics [39]. An effect of CCCP, PAβN and NMP on accumulation of the dye was noted with isolates EA149399, EAPOL13200, EAART15737, EA137454 and EA118259 (Table 5). Interestingly, maximum restoration of CHL susceptibility was obtained in the presence of PAβN or 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 PAβN alone versus PAβN + 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 uncoupler and its uncoupling impact can be partly restored in the presence of glucose. The results obtained with PAβN, NMP and CCCP were of the same order of magnitude, supporting some additional effect for CPZ under these conditions. Regarding CPZ, the permeabilising effect reported for

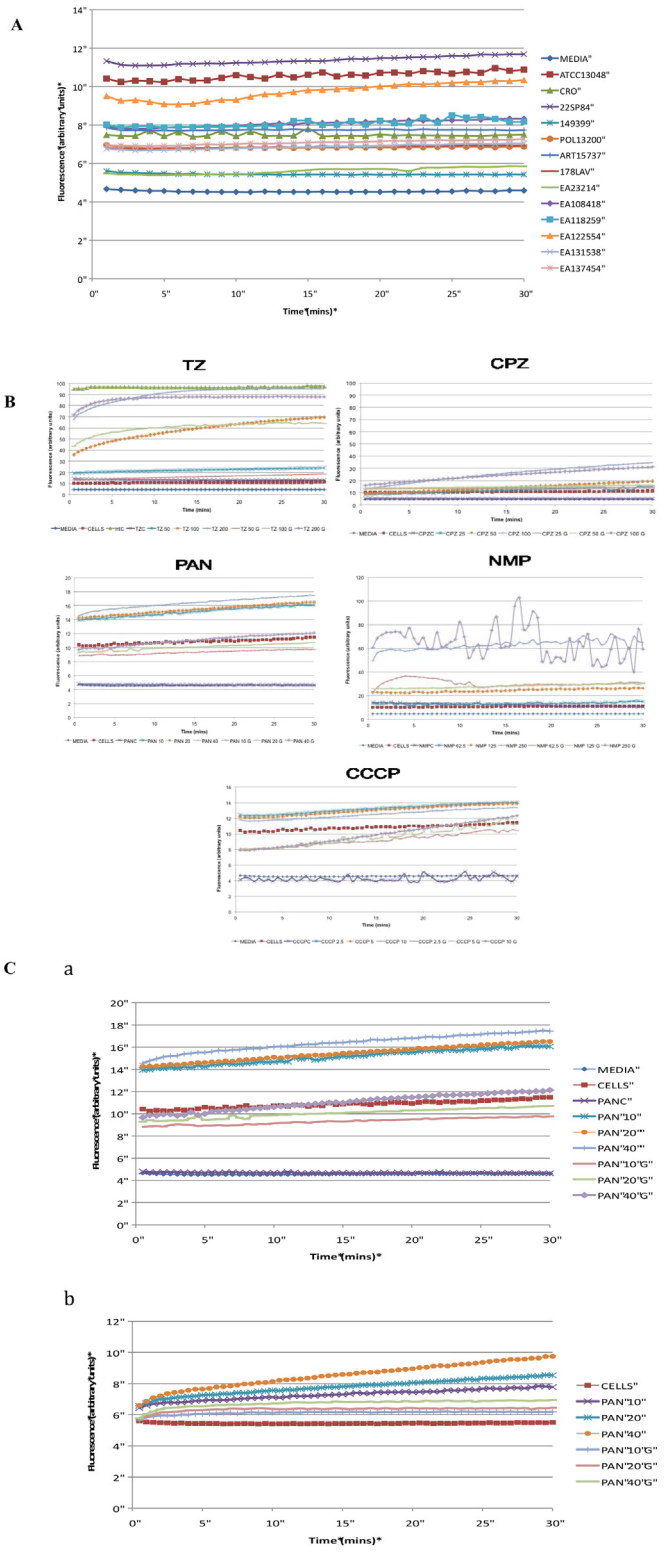

Fig. 6. Accumulation of Hoechst dye by (A) the *Enterobacter aerogenes* isolates tested, (B) *E. aerogenes* ATCC 13048 in the presence of selected chemosensitisers and (C) *E. aerogenes* ATCC 13048 and clinical isolate EA149399 in the presence of different concentrations of phenylalanine-arginine β-naphthylamide (PAN). (A) An initial assay was conducted to evaluate the accumulation of Hoechst dye by the *E. aerogenes* isolates. (B) Accumulation of Hoechst dye by *E. aerogenes* ATCC 13048 in the presence of different concentrations of thioridazine (TZ), chlorpromazine (CPZ), PAN and 1-(1-naphthylmethyl)-piperazine (NMP). Cyanide *m*-chlorophenyl hydrazone (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 min. (C) Accumulation of Hoechst dye by *E. aerogenes* ATCC 13048 (a) and clinical isolate EA149399 (b) in the presence of different concentrations of 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 min.

Table 5
Accumulation rates of *Enterobacter aerogenes* isolates measured in a Hoechst dye uptake assay.

E. aerogenes strain	Chemosensitiser							
	CPZ		PAβN		NMP		CCCP	
	–Gluc	+Gluc	–Gluc	+Gluc	–Gluc	+Gluc	–Gluc	+Gluc
ATCC 13048	0.7216	0.4996	0.0852	0.0832	0.1689	0.153	0.0645	0.1622
EACRO	0.4966	0.378	0.0411	0.0275	0.0544	0.0204	0.1458	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	0.67555	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

Gluc, glucose; CPZ, chlorpromazine; PAβN, phenylalanine-arginine β-naphthylamide; NMP, 1-(1-naphthylmethyl)-piperazine; CCCP, cyanide-*m*-chlorophenylhydrazone. Values are representative of the rate of accumulation of Hoechst dye in the presence of the chemosensitisers and in the presence or absence of glucose. The grey shading indicates a significant effect (ratio without/with glucose >4) of energy (glucose) on the accumulation rate.

phenothiazines on biological membranes can boost the observed accumulation in association with the chemical properties of Hoechst dye [40]. Regarding CPZ, an effect is observed with strain EA122554. Taken together, these data illustrate the impact that chemosensitisers can have on the accumulation of dyes, which can be different from the effect on antibiotic activity.

4. Conclusion

Overall, the potential utility of a series of chemosensitisers in combination with antimicrobials to revert antimicrobial resistance in clinical isolates of *E. aerogenes* was explored. The isolates studied were also characterised, with target gene mutations identified. These genotypes most likely underpin the 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 Hoechst dye, CCCP-sensitive and activated by glucose, is demonstrated in strains EACRO, EA149399, EAPOL13200, EAART15737, EA118259 and EA137454. In strains EAPOL13200 and EA137454, PAβN can impair this efflux, whilst NMP is able to alter its activity in strains EAPOL13200 and EA149399. Regarding these effects, we may hypothesise that PAβN and NMP can have same activity behaviour in strain EA149399 and exhibit different action in strains EAPOL13200 and EA137454. These differences in the inhibitor activity may be due to different affinities for Hoechst dye, PAβN and NMP for the binding sites located inside the efflux pump, or by steric hindrances caused by fixation in the AcrB pockets [41,42].

The more important point is the correlation between accumulation studies and restoration of antibiotic activity. For fluoroquinolones it is clear that the presence of target mutations in the various isolates drastically impairs the recovery of ciprofloxacin susceptibility whatever the chemosensitiser used. Regarding CHL, resistance is efficiently reversed by the three chemosensitisers in three strains for which an effect of CCCP on Hoechst accumulation has been evidenced. This indicates that CPZ, PAβN and NMP are able to block CHL efflux in these strains and restore a partial or completely susceptible phenotype. Interestingly, no restoration of CHL susceptibility was obtained for strain EA149399 for which an efflux CCCP, PAβN and NMP sensitive is observed with Hoechst dye. The divergence observed between the restoration of susceptibility and dye accumulation is probably due to the difference in affinity/binding constant for the AcrB pump site. Alternatively, in the case of MIC determination, the effect of the antibiotic on its target initiating bacterial death is different to the Hoechst assay where the release of dye from DNA or membrane association is measured.

This divergence between dye and antibiotic must be taken into consideration when assessing the contribution of efflux in clinical isolates and the evaluation of new adjuvants or chemosensitisers. In this way, use of the recent concept 'structure intracellular concentration activity relationship', or SICAR will be more efficient to study the accumulation–activity correlation of clinically used or new antibiotics and the potential of new adjuvants/chemosensitisers [43].

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Competing interests

None declared.

Ethical approval

Not required.

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