Effect of 1-(1-naphthylmethyl)-piperazine on antimicrobial agent susceptibility in multidrug-resistant isogenic and veterinary *Escherichia coli* field strains

M. L. Marchetti,[1,2] J. Errecalde[1,3] and N. Mestorino[1,3]

Correspondence
N. Mestorino
noram@fcv.unlp.edu.ar

[1]Department of Pharmacology, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118 CC 296, 1900 La Plata, Buenos Aires, Argentina

[2]Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET, Buenos Aires, Argentina

[3]Department of Pharmacology, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Buenos Aires, Argentina

The objective of this study was to evaluate the interaction of the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP) when combined with different families of antimicrobial agents against isogenic strains and multidrug-resistant (MDR) *Escherichia coli* field strains isolated from animals. Laboratory isogenic strains of *E. coli* with different levels of expression of efflux pumps were used as quality controls. Ten MDR *E. coli* strains were collected from healthy animals in a cross-sectional study in four commercial dairy farms. The MICs of florfenicol, ciprofloxacin, tetracycline and ampicillin were determined by a serial microdilution method in Luria–Bertani broth in the presence or absence of NMP. NMP used with ampicillin exerted no effect on the isogenic or field strains. In most of the field MDR *E. coli* strains and in an *acrAB*-overexpressing (AG112) isogenic strain, the MICs of florfenicol, ciprofloxacin and tetracycline decreased at least fourfold when the antimicrobial was combined with the highest NMP concentrations. In the wild-type strain (AG100), there were no decreases of more than twice the MIC, whilst in strain AG100A, an efflux pump-deficient strain, the MIC did not change, regardless of the concentration of NMP used with these three antimicrobials. Thus, ampicillin was not affected by the efflux pump mechanism, whereas ciprofloxacin, tetracycline and florfenicol were shown to be substrates of efflux pumps, with a consequent significant reduction in MICs. Resistance could not be completely reversed in the *E. coli* field strains by NMP, probably because other resistance mechanisms were also present. However, in strain AG112, the MIC results demonstrated that NMP expressed an important synergistic activity with florfenicol. The reduction in florfenicol MIC value was sufficient to reverse antimicrobial resistance completely for AG112.

Received 28 October 2011
Accepted 10 February 2012

INTRODUCTION

Efflux pumps are membrane transporters that are widely distributed among micro-organisms. These systems can confer resistance to a given class of drug (specific drug resistance), but some of them, called multiple drug resistance (MDR) efflux pumps, can handle a wide variety of structurally unrelated compounds. Bacterial MDR has become a serious problem in human and veterinary medicine, not only in pathogenic but also in commensal bacteria (Delcour, 2009; Masuda *et al.*, 2000; Moreira *et al.*, 2004; Nikaido, 1996; Poole, 2005).

MDR in Gram-negative bacteria may be caused by over-expression of resistance–nodulation–cell division (RND)-type efflux pumps. These systems are protein exporters involved in the transport of lipophilic or amphiphilic molecules or toxic divalent cations with a broad spectrum of substrates (Renau *et al.*, 2002; Van Bambeke *et al.*, 2003). RND efflux pumps are organized as multicomponent systems, in which the efflux pump located in the inner membrane works in conjunction with a periplasmic fusion protein and an outer-membrane protein (Van Bambeke *et al.*, 2003, 2010). The AcrAB multidrug efflux system is the main efflux pump in *Escherichia coli* and belongs to the

Abbreviations: EPI, efflux pump inhibitor; MDR, multidrug resistant/resistance; MEC, minimum effective concentration; NMP, 1-(1-naphthylmethyl)-piperazine; PAβN, Phe-Arg-β-naphthylamide; RND, resistance–nodulation–cell division.

RND family. This multidrug efflux pump system is responsible for resistance to tetracycline, chloramphenicol, ampicillin, nalidixic acid and rifamicin (Moreira *et al.*, 2004; Nikaido, 1996; Webber & Piddock, 2003).

It has been demonstrated that efflux pump inhibition can increase intracellular substrate accumulation. For example, ethidium bromide accumulation assays have been used to investigate new alternatives to reverse MDR in bacteria (Kern *et al.*, 2006).

Efflux pump inhibitors (EPIs) are drugs able to modify resistance by blocking bacterial pumps. Several arylpiperidines and other compounds capable of reversing MDR in *E. coli* and other members of the *Enterobacteriaceae* have been studied extensively (Bean & Wareham, 2009; Bohnert & Kern, 2005; Coban *et al.*, 2009; Kern *et al.*, 2006).

Phe-Arg-β-naphthylamide (PAβN) is an EPI that has been studied by a number of authors (Bohnert & Kern, 2005; Pannek *et al.*, 2006; Sáenz *et al.*, 2004). It has been shown that PAβN is able to reverse antimicrobial resistance in some selected MDR Gram-negative bacteria. However, PAβN showed intrinsic antibacterial activity against *E. coli* strains without expression of AcrAB efflux pumps, and exerted its effect through additional mechanisms unrelated to pump inhibition (Bohnert & Kern, 2005; Pannek *et al.*, 2006; Sáenz *et al.*, 2004).

Some authors have identified 1-(1-naphthylmethyl)-piperazine (NMP) as moderately active in reversing MDR in *E. coli* overexpressing RND-type efflux pumps but not in pump-deficient mutants (Bohnert & Kern, 2005; Kern *et al.*, 2006; Schumacher *et al.*, 2006). They suggested that NMP is able to reduce the MICs of two or more antibiotics in efflux pump-overexpressing strains but has no effect on efflux pump-deficient strains. Although NMP has been shown to exhibit less intrinsic activity than PAβN, Bohnert & Kern (2005) considered that it is the most potent compound against *E. coli.*

To date, levofloxacin, tetracycline, chloramphenicol, oxacillin, clarithromycin, rifampicin and linezolid have been studied in combination with NMP against *E. coli* (Bean & Wareham, 2009; Coban *et al.*, 2009; Kern *et al.*, 2006; Schumacher *et al.*, 2006). However, there are few data about ampicillin, ciprofloxacin and florfenicol. These are antimicrobials widely used in veterinary treatments and are potential antimicrobial resistance selectors.

The objective of this study was to evaluate the interaction of the EPI NMP when combined with structurally unrelated antimicrobial agents (e.g. β-lactam antibiotics, quinolones and tetracyclines) against genetically known isogenic strains and MDR *E. coli* field strains isolated from animals.

METHODS

Bacterial strains. Laboratory strains included a wild-type strain (AG100) and two isogenic mutants: an RND-type pump-deficient strain (AG100A) and an *acrAB*-overexpressing strain (AG112) with the expression profile of an MDR efflux pump (Cohen *et al.*, 1993; George & Levy, 1983; Okusu *et al.*, 1996). These three genetically known isogenic *E. coli* strains were used as controls for validating the assay. They were kindly donated by Professor Hiroshi Nikaido (University of California, Berkeley, CA, USA) and Laura McMurry (Tufts University School of Medicine, Boston, MA, USA).

Ten *E. coli* field MDR isolates were obtained from the faeces of a variety of healthy animals (dairy cattle, calves and companion animals) using culturette swabs in a previous cross-sectional study carried out on four commercial farms in Buenos Aires province (Tandil, San Vicente, Trenque Lauquen and Luján) (Office International des Epizooties, 2008).

After biochemical typing, strains confirmed to be *E. coli* and resistant to three or more antimicrobials were selected as MDR *E. coli. E. coli* ATCC 25922 was used as a quality control.

Chemicals and media. NMP was obtained from Sigma-Aldrich. Luria–Bertani (LB) broth was prepared as 10 g tryptone l^{-1}, 5 g yeast extract l^{-1} and10 g NaCl l^{-1} in distilled water. Ampicillin (96 %, w/w) was from Fluka, tetracycline (97.03 %, w/w) and ciprofloxacin (99.8 %, w/w) were from Parafarm and florfenicol (99.3 %, w/w) was from Romikin.

Susceptibility testing. Susceptibility to four different families of antimicrobial substrates of efflux pump systems (florfenicol, tetracycline, ciprofloxacin and ampicillin) was studied in the presence or absence of NMP in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008, 2009).

MICs were determined in 96-well microtitre plates using a twofold standard broth microdilution method (CLSI, 2009) and all determinations were carried out in triplicate. LB broth was used instead of Mueller–Hinton broth due to lability of the isogenic reference strains.

The antimicrobial dilution series tested for the MDR strains was from 256 to 0.007 μg ml^{-1}. However, for the isogenic strains and *E. coli* ATCC 25922, the dilution series varied according to the susceptibility of the particular strain to the antimicrobial being evaluated.

Each antimicrobial was dispensed alone in the first row of a microtitre plate and was combined with NMP in the remaining rows. NMP was tested at five concentrations (6.25, 12.5, 25, 50 and 100 μg ml^{-1}) to determine its minimum effective concentration (MEC), the minimum concentration of EPI that produced the maximum reduction in substrate MIC (Tables 1 and 2).

To measure the antimicrobial activity of NMP, it was also dispensed alone in the last row for each strain over a dilution range of 1.56–800 μg ml^{-1}.

An inoculum of each bacterium was prepared by making a direct saline suspension of colonies selected from a 24 h agar plate. The suspension was adjusted to match a 0.5 McFarland standard (1×10^8–2×10^8 c.f.u. ml^{-1}) and then diluted in LB broth to obtain a concentration of 5×10^4 c.f.u. ml^{-1} after inoculation in each well (CLSI, 2008, 2009).

RESULTS

Effect of NMP on the isogenic reference strains

When NMP was combined with ampicillin, there was no change in MIC for any of the isogenic strains at any of the NMP concentrations tested, even in the *acrAB*-

Table 1. Effect of NMP on antimicrobial MICs for the isogenic strains AG100, AG100A and AG112

AMP, Ampicillin; CIP, ciprofloxacin; TET, tetracycline; FLF, florfenicol.

Strain	Genotype	AcrAB efflux pump expression	Antimicrobial	Antimicrobial MICs (μg ml^{-1}) against different concentrations of NMP (μg ml^{-1})					NMP MIC (μg ml^{-1})	Fold decrease in MIC	
				0	6.25	12.5	25	50	100		50 μg NMP ml^{-1}	100 μg NMP ml^{-1}
AG100	Wild-type	Normal	AMP	2	2	2	2	2	2	400	1	1
			CIP	0.015	0.015	0.015	0.007	0.007	0.007	400	2	2
			TET	1	1	1	0.5	0.5	0.5	400	2	2
			FLF	8	8	4	4	2	1	400	4	8
AG100A	AG100Δ*acrAB*	Deletion	AMP	1	1	1	1	1	1	400	1	1
			CIP	0.015	0.015	0.015	0.015	0.015	0.015	400	1	1
			TET	0.5	0.5	0.5	0.5	0.5	0.5	400	1	1
			FLF	1	1	1	1	1	1	400	1	1
AG112	AG100 *marR*	Overexpression	AMP	4	4	4	4	4	4	400	1	1
			CIP	0.062	0.062	0.062	0.031	0.031	0.015	400	2	4
			TET	8	8	8	4	2	0.5	400	4	16
			FLF	32	32	16	16	2	1	400	16	32

overexpressing strain, AG112 (Table 1). For the other antimicrobials studied, MICs were reduced at least twofold when they were combined with the two higher concentrations of NMP (50 and 100 μg ml^{-1}) in strains AG100 (wild-type) and AG112, but were unaffected in the deletion mutant strain, AG100A.

For florfenicol, a concentration of 50 μg NMP ml^{-1} reduced the MIC by at least 16-fold in AG112, whilst a concentration of 100 μg ml^{-1} reduced it by 32-fold. In the case of AG100 (wild-type strain), the florfenicol MIC was reduced four- and eightfold when combined with 50 and 100 μg NMP ml^{-1}, respectively.

For tetracycline, the addition of 50 μg NMP ml^{-1} decreased the MIC against AG112 fourfold, whilst 100 μg NMP ml^{-1} decreased the MIC 16-fold. For AG100, the tetracycline MIC decreased only twofold in the presence of the EPI, at the higher concentrations.

Finally, the ciprofloxacin MIC decreased two- and fourfold against AG112 when it was combined with 50 and 100 μg NMP ml^{-1}, respectively. For strain AG100, the MIC of ciprofloxacin decreased twofold with the higher NMP concentrations, similar to the results for tetracycline.

NMP had no synergistic activity with the antimicrobials studied against the pump-deficient strain AG100A: there were no changes in the MICs of the antimicrobials with or without NMP.

Finally, when NMP was evaluated without antimicrobials, its MIC was 400 μg ml^{-1} for all three isogenic strains.

According to the susceptibility breakpoints established by the Clinical and Laboratory Standards Institute for the antimicrobials studied, the MECs of NMP in strain AG112 were 50 μg ml^{-1} for florfenicol and tetracycline and 100 μg ml^{-1} for ciprofloxacin.

Changes in antimicrobial susceptibility of MDR *E. coli* field strains

There were no changes in the MIC of ampicillin in any of the MDR/ampicillin-resistant *E. coli* field strains when this antimicrobial was combined with NMP at any of the concentrations tested (Table 2), as with the isogenic control strains.

In most of the MDR/florfenicol-resistant *E. coli* field strains, the MIC of florfenicol decreased at least fourfold when this antimicrobial was combined with the highest NMP concentrations. With one MDR/florfenicol-resistant *E. coli* strain, the florfenicol MIC was reduced eight- and 16-fold with 50 and 100 μg NMP ml^{-1}, respectively (Table 2, sample dairy cattle 1).

For all MDR/tetracycline-resistant *E. coli* field strains, the tetracycline MIC was reduced at least fourfold with 100 μg NMP ml^{-1}. However, when the antimicrobial was combined with 50 μg NMP ml^{-1}, only six of the ten MDR/

Table 2. Effect of NMP on antimicrobial MICs for MDR *E. coli* field strain isolates

STX, Trimethoprim/sulfamethoxazole; see Table 1 for other abbreviations.

Location	Sample	Resistance phenotype	Antimicrobial	MIC (μg ml^{-1}) in the presence of NMP (μg ml^{-1}) at:						NMP MIC (μg ml^{-1})	Fold decrease in MIC	
				0	6.25	12.5	25	50	100		50 μg NMP ml^{-1}	100 μg NMP ml^{-1}
San Vicente	Dairy cattle 1	FLF CIP SXT AMP TET	FLF	16	16	16	8	2	1	⩾800	8	16
			AMP	256	256	256	256	256	256	⩾800	1	1
			CIP	32	32	32	16	8	8	⩾800	4	4
			TET	64	64	64	32	16	16	⩾800	4	4
	Dairy cattle 2	FLF SXT TET	FLF	256	256	128	128	64	64	⩾800	4	4
			TET	256	128	128	128	64	32	⩾800	4	8
Luján	Dog 1	FLF SXT AMP TET	FLF	256	256	256	256	128	128	⩾800	2	2
			AMP	256	256	256	256	256	256	⩾800	1	1
			TET	256	256	256	128	128	64	⩾800	2	4
	Dog 2	SXT AMP TET	AMP	256	256	256	256	256	256	⩾800	1	1
			TET	128	128	128	64	64	32	⩾800	2	4
Trenque Lauquen	Dairy cattle 1	FLF SXT TET	FLF	256	128	128	128	64	64	⩾800	4	4
			TET	64	64	64	32	16	16	⩾800	4	4
	Dairy cattle 2	FLF AMP TET	FLF	256	128	128	128	64	64	⩾800	4	4
			AMP	256	256	256	256	256	256	⩾800	1	1
			TET	256	256	128	128	64	64	⩾800	4	4
	Calf 1	CIP AMP TET	AMP	256	256	256	256	256	256	⩾800	1	1
			CIP	128	128	128	64	64	32	⩾800	2	4
			TET	256	256	256	256	64	64	⩾800	4	4
	Calf 2	CIP AMP TET	AMP	256	256	256	256	256	256	⩾800	1	1
			CIP	128	128	128	64	64	32	⩾800	2	4
			TET	256	256	256	128	128	64	⩾800	2	4
	Calf 3	CIP AMP TET	AMP	256	256	256	256	256	256	⩾800	1	1
			CIP	128	128	128	64	64	32	⩾800	2	4
			TET	256	256	256	128	128	64	⩾800	2	4
	Calf 4	CIP AMP TET	AMP	256	256	256	256	256	256	⩾800	1	1
			CIP	128	128	128	64	32	32	⩾800	4	4
			TET	256	256	128	128	64	64	⩾800	4	4

tetracycline-resistant *E. coli* strains achieved the same MIC reduction.

In the case of ciprofloxacin, a concentration of 100 μg ml^{-1} of the EPI was able to produce a fourfold reduction in the MIC of the quinolone in all of the MDR/ciprofloxacin-resistant *E. coli* field strains. However, the combination of ciprofloxacin with 50 μg NMP ml^{-1} produced a similar result only in two of the studied strains.

The MIC of NMP without antimicrobial was $\geqslant$800 μg ml^{-1} in all of the MDR *E. coli* field strains.

DISCUSSION

As shown by the MIC results, ampicillin was not affected by efflux pump overexpression in *E. coli*. Although several authors have reported that β-lactams are substrates of RND efflux pumps (Li *et al.*, 1998; Nakae *et al.*, 1999), in the present study, inhibition of the EPI did not affect the MIC results. The combination of efflux pump systems with β-lactamase enzymes allows bacteria to avoid enzymic saturation, collaborating in antimicrobial resistance (Bina *et al.*, 2009; Mazzariol *et al.*, 2000; Nakae *et al.*, 1999; Van Bambeke *et al.*, 2003, 2010).

Although strain AG112 is not an important β-lactamase producer, it can express an ampicillin-resistant phenotype. However, the efflux system by itself is unable to express high resistance levels against β-lactams (Bina *et al.*, 2009; Li *et al.*, 1998; Lomovskaya *et al.*, 2001; Nakae *et al.*, 1999).

In contrast, the MIC results demonstrated that florfenicol, tetracycline and ciprofloxacin are common substrates of efflux pump systems. In general, when combining antimicrobials with NMP at the highest concentrations (50 and 100 μg ml^{-1}), the MICs decreased at least fourfold, not only in the isogenic *E. coli* strains but also in the *E. coli* field isolates with an MDR phenotype. Similar results have been obtained by some authors for the MICs of fluoroquinolones with NMP and other EPIs against *Pseudomonas aeruginosa* (Kriengkauykiat *et al.*, 2005; Lomovskaya *et al.*, 2001; Renau *et al.*, 2002) and *E. coli* (Kern *et al.*, 2006; Sáenz *et al.*, 2004; Schumacher *et al.*, 2006).

As efflux pump overexpression is the only resistance mechanism present in strain AG112, the MIC results demonstrated that NMP expressed an important synergistic activity with florfenicol. The reduction in MIC value was sufficient to completely reverse the antimicrobial resistance of this strain.

In most cases, the MEC of NMP was 50 μg ml^{-1}, and it was 100 μg ml^{-1} in the rest of the strains (Table 2).

Several studies have used 100 μg NMP ml^{-1} in *E. coli* (Bohnert & Kern, 2005; Kern *et al.*, 2006; Schumacher *et al.*, 2006), *Acinetobacter baumannii* (Pannek *et al.*, 2006) and *Campylobacter* species (Hannula & Hänninen, 2008) to obtain better inhibition in RND-type overexpressing efflux pump systems.

The result of the MIC of NMP without an antimicrobial revealed that this EPI had no intrinsic antimicrobial activity, even at high concentrations, agreeing with the results of other authors (Kern *et al.*, 2006; Pannek *et al.*, 2006).

Despite the important decrease observed in the MIC values of florfenicol, tetracycline and ciprofloxacin in the MDR *E. coli* field strains, combinations of antimicrobial and NMP were unable to completely reverse the antimicrobial resistance.

Our demonstration of the inhibitory effect of NMP against MDR *E. coli* field strains requires genotypic confirmation. Further studies are planned to explore other mechanisms that may have contributed to MDR in our strains such as target mutations, β-lactamase production and loss of outer-membrane porins.

Conclusion

There is increasing evidence for a significant role of efflux pumps in antibiotic resistance in bacteria (Elkins & Nikaido, 2002; Everett *et al.*, 1996; Nikaido *et al.*, 2008; Van Bambeke *et al.*, 2003; Ziha-Zarifi *et al.*, 1999). In the present study, we demonstrated that the EPI NMP can partially reverse antimicrobial resistance in MDR *E. coli* field strains. This probably occurs because efflux pump overexpression by itself is unable to express a high-level resistance phenotype. In contrast, the association of overexpression of these genes with other antimicrobial resistance mechanisms may confer not only high-level but also broad-spectrum resistance (Van Bambeke *et al.*, 2003; Webber & Piddock, 2003).

In contrast, we demonstrated that inhibition of efflux pump overexpression had a significant role in florfenicol resistance. NMP could be a promising tool to reverse antimicrobial resistance completely when florfenicol is expressed in bacteria with an MDR phenotype.

The effect of efflux pumps needs to be considered in the design of future antibiotics and the role of inhibitors assessed in order to maximize the efficacy of current and future antimicrobials.

ACKNOWLEDGEMENTS

We are grateful to Professor Hiroshi Nikaido from the University of California, Berkeley, USA, and Laura McMurry from Tufts University School of Medicine, Boston, MA, for giving us the isogenic strains. The authors thank CONICET for its collaboration by granting a 2004–2008 doctoral scholarship. Research at the Pharmacology Department, Faculty of Veterinary Science, is partially supported by the Universidad Nacional de La Plata (Buenos Aires, Argentina) (V180). Bárbara Huber and Griselda Haag are also acknowledged for their laboratory work collaboration.

REFERENCES

Bean, D. C. & Wareham, D. W. (2009). Paradoxical effect of 1-(1-naphthylmethyl)-piperazine on resistance to tetracyclines in multidrug-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother* **63**, 349–352.

Bina, X. R., Philippart, J. A. & Bina, J. E. (2009). Effect of the efflux inhibitors 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-β-naphthylamide on antimicrobial susceptibility and virulence factor production in *Vibrio cholerae*. *J Antimicrob Chemother* **63**, 103–108.

Bohnert, J. A. & Kern, W. V. (2005). Selected arylpiperazines are capable of reversing multidrug resistance in *Escherichia coli* overexpressing RND efflux pumps. *Antimicrob Agents Chemother* **49**, 849–852.

CLSI (2008). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals*, 3rd edn; Approved Standard. M31-A3. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2009). *Performance Standards for Antimicrobial Susceptibility Testing*, 19th Informational Supplement. M100-S19. Wayne, PA: Clinical and Laboratory Standards Institute.

Coban, A. Y., Bayram, Z., Sezgin, F. M. & Durupinar, B. (2009). [Effect of efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine to MIC values of ciprofloxacin in ciprofloxacin resistant Gram-negative bacteria]. *Mikrobiyol Bul* **43**, 457–461 (in Turkish).

Cohen, S. P., Hächler, H. & Levy, S. B. (1993). Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *J Bacteriol* **175**, 1484–1492.

Delcour, A. (2009). Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* **1794**, 808–816.

Elkins, C. A. & Nikaido, H. (2002). Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. *J Bacteriol* **184**, 6490–6498.

Everett, M. J., Jin, Y. F., Ricci, V. & Piddock, L. J. (1996). Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother* **40**, 2380–2386.

George, A. M. & Levy, S. B. (1983). Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. *J Bacteriol* **155**, 531–540.

Hannula, M. & Hänninen, M. L. (2008). Effect of putative efflux pump inhibitors and inducers on the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli*. *J Med Microbiol* **57**, 851–855.

Kern, W. V., Steinke, P., Schumacher, A., Schuster, S., von Baum, H. & Bohnert, J. A. (2006). Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of *Escherichia coli*. *J Antimicrob Chemother* **57**, 339–343.

Kriengkauykiat, J., Porter, E., Lomovskaya, O. & Wong-Beringer, A. (2005). Use of an efflux pump inhibitor to determine the prevalence of efflux pump-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **49**, 565–570.

Li, X.-Z., Zhang, L., Srikumar, R. & Poole, K. (1998). β-Lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **42**, 399–403.

Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S. & other authors (2001). Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob Agents Chemother* **45**, 105–116.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H. & Nishino, T. (2000). Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **44**, 3322–3327.

Mazzariol, A., Cornaglia, G. & Nikaido, H. (2000). Contributions of the AmpC β-lactamase and the AcrAB multidrug efflux system in intrinsic resistance of *Escherichia coli* K-12 to β-lactams. *Antimicrob Agents Chemother* **44**, 1387–1390.

Moreira, M., Souza, E. & Moraes, C. (2004). Multidrug efflux systems in Gram-negative bacteria. *Braz J Microbiol* **35**, 19–28.

Nakae, T., Nakajima, A., Ono, T., Saito, K. & Yoneyama, H. (1999). Resistance to β-lactam antibiotics in *Pseudomonas aeruginosa* due to interplay between the MexAB-OprM efflux pump and β-lactamase. *Antimicrob Agents Chemother* **43**, 1301–1303.

Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. *J Bacteriol* **178**, 5853–5859.

Nikaido, E., Yamaguchi, A. & Nishino, K. (2008). AcrAB multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by RamA in response to environmental signals. *J Biol Chem* **283**, 24245–24253.

Office International des Epizooties (2008). *Collection and Shipment of Diagnostic Specimens. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, 6th edn. Paris: Office International des Epizooties.

Okusu, H., Ma, D. & Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* **178**, 306–308.

Pannek, S., Higgins, P. G., Steinke, P., Jonas, D., Akova, M., Bohnert, J. A., Seifert, H. & Kern, W. V. (2006). Multidrug efflux inhibition *in Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-β-naphthylamide. *J Antimicrob Chemother* **57**, 970–974.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* **56**, 20–51.

Renau, T. E., Léger, R., Yen, R., She, M. W., Flamme, E. M., Sangalang, J., Gannon, C. L., Chamberland, S., Lomovskaya, O. & Lee, V. J. (2002). Peptidomimetics of efflux pump inhibitors potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. *Bioorg Med Chem Lett* **12**, 763–766.

Sáenz, Y., Ruiz, J., Zarazaga, M., Teixidó, M., Torres, C. & Vila, J. (2004). Effect of the efflux pump inhibitor Phe-Arg-β-naphthylamide on the MIC values of the quinolones, tetracycline and chloramphenicol, in *Escherichia coli* isolates of different origin. *J Antimicrob Chemother* **53**, 544–545.

Schumacher, A., Steinke, P., Bohnert, J. A., Akova, M., Jonas, D. & Kern, W. V. (2006). Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of Enterobacteriaceae other than *Escherichia coli*. *J Antimicrob Chemother* **57**, 344–348.

Van Bambeke, F., Glupczynski, Y., Plésiat, P., Pechère, J. C. & Tulkens, P. M. (2003). Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. *J Antimicrob Chemother* **51**, 1055–1065.

Van Bambeke, F., Pages, J.-M. & Lee, V. J. (2010). Inhibitors of bacterial efflux pumps as adjuvants in antibacterial therapy and

diagnostic tools for detection of resistance by efflux. *Front Anti-Infect Drug Discov* **1**, 138–175.

Webber, M. A. & Piddock, L. J. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* **51**, 9–11.

Ziha-Zarifi, I., Llanes, C., Köhler, T., Pechere, J. C. & Plesiat, P. (1999). In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. *Antimicrob Agents Chemother* **43**, 287–291.