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

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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, 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.

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 rifampicin (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; Saéz 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; Saéz 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 were 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 faces 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 and 10 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 × 108–2 × 108 c.f.u. ml–1) and then diluted in LB broth to obtain a concentration of 5 × 104 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-
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 \( \mu \text{g ml}^{-1} \)) in strains AG100 (wild-type) and AG112, but were unaffected in the deletion mutant strain, AG100A.

For florfenicol, a concentration of 50 \( \mu \text{g NMP ml}^{-1} \) reduced the MIC by at least 16-fold in AG112, whilst a concentration of 100 \( \mu \text{g NMP ml}^{-1} \) 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 \( \mu \text{g NMP ml}^{-1} \), respectively.

For tetracycline, the addition of 50 \( \mu \text{g NMP ml}^{-1} \) decreased the MIC against AG112 fourfold, whilst 100 \( \mu \text{g NMP ml}^{-1} \) 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 \( \mu \text{g NMP ml}^{-1} \), 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 \( \mu \text{g ml}^{-1} \) 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 \( \mu \text{g ml}^{-1} \) for florfenicol and tetracycline and 100 \( \mu \text{g ml}^{-1} \) for ciprofloxacin.

### Changes in antimicrobial susceptibility of MDR \textit{E. coli} field strains

There were no changes in the MIC of ampicillin in any of the MDR/ampicillin-resistant \textit{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 \textit{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 \textit{E. coli} strain, the florfenicol MIC was reduced eight- and 16-fold with 50 and 100 \( \mu \text{g NMP ml}^{-1} \), respectively (Table 2, sample dairy cattle 1).

For all MDR/tetracycline-resistant \textit{E. coli} field strains, the tetracycline MIC was reduced at least fourfold with 100 \( \mu \text{g NMP ml}^{-1} \). However, when the antimicrobial was combined with 50 \( \mu \text{g NMP ml}^{-1} \), 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.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample</th>
<th>Resistance phenotype</th>
<th>Antimicrobial</th>
<th>MIC (µg ml⁻¹) in the presence of NMP (µg ml⁻¹) at:</th>
<th>NMP MIC (µg ml⁻¹)</th>
<th>Fold decrease in MIC (%)</th>
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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 \(\geq 800\) 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 \(\beta\)-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 \(\beta\)-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 \(\beta\)-lactamase producer, it can express an ampicillin-resistant phenotype. However, the efflux system by itself is unable to express high resistance levels against \(\beta\)-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* (Kriengkauykit et al., 2005; Lomovskaya et al., 2001; Renau et al., 2002) and *E. coli* (Kern et al., 2006; Säenz 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, \(\beta\)-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.

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