Development and analysis of furazolidone-resistant *Escherichia coli* mutants

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Martínez-Puchol S, Gomes C, Pons MJ, Ruiz-Roldán L, Torrents de la Peña A, Ochoa TJ, Ruiz J. Development and analysis of furazolidone-resistant *Escherichia coli* mutants. APMIS 2015.

Furazolidone-resistant mutants were obtained from four clinical isolates of diarrhoeagenic *Escherichia coli*. The stability of the resistance and the frequency of mutation were established. The minimal inhibitory concentration of furazolidone, nitrofurantoin, nalidixic acid, ampicillin, chloramphenicol and tetracycline was established both in the presence and absence of the efflux pump inhibitor Phe-Arg-β-Naphtylamide. The presence of mutations in the nitroreductase genes *nfsA* and *nfsB* was analysed by PCR; sequencing and their enzymatic activity was assessed by a spectrophotometric assay. Alterations in outer membrane proteins were studied by SDS-PAGE. The frequency of mutation ranged from $<9.6 \times 10^{-10}$ to 9.59×10^{-7} . Neither an effect on efflux pumps inhibited by Phe-Arg-β-Naphtylamide nor cross-resistance with the antibiotics studied was observed. Nineteen mutants (52.94%) presented mutations in the nitroreductase-encoding genes: 17 in the *nfsA* gene (15 mutants with an internal stop codon, 2 with amino acid changes), 2 in the *nfsB* (all amino acid changes). Alterations in the outer membrane proteins OmpA and OmpW were also observed. Although more studies are necessary to find other resistance mechanisms, present data showed the low potential of selecting furazolidone-resistant mutants, together with the lack of cross-resistance with unrelated antimicrobial agents.

Key words: Nitrofurantoin; *Enterobacteriaceae*; resistance mechanisms.

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Furazolidone is a nitrofurantoin derivative of nitrofurantoin that possesses antibacterial activity against both Gram-positive and Gram-negative bacteria. Due to the fact that some metabolites of furazolidone may have carcinogenic effects in animal models, its use has been forbidden in different countries, including the United States, (1). Nonetheless, furazolidone has been extensively used in the treatment of different human infections. Thus, furazolidone is currently used for the treatment of parasitic infections by *Giardia lamblia* or *Entamoeba histolytica* (2, 3) or in the treatment of *Helicobacter pylori*

infections (4). Meanwhile, in South America it is considered an alternative treatment to the commonly prescribed antibiotics for children diarrhoea, because of the increasing levels of resistance to commonly used antibacterial agents (5–8), and due to its good activity against most *Enterobacteriaceae* and its low cost. Thus, the use of furazolidone is now being implemented for the treatment of these pathologies in different Latin American countries (9–11).

Although the specific mode of action of nitrofurans is unclear, it has been shown that *E. coli* strains resistant and susceptible to nitrofurans differ in their ability to reduce these compounds (12),

suggesting that they need to be activated for this nitroreductase activity to show its antibiotic effect. Few data on the mechanisms involved in the development of nitrofurantoin resistance are available to date, demonstrating that the development of furazolidone resistance is mediated by the inactivation of the nitroreductases present in *E. coli*. Thus, Whiteway *et al.* (13) showed that the increased resistance of these bacteria is caused by successive mutations, insertions or deletions in the nitroreductase-encoding genes *nfsA* and *nfsB*, resulting in a sequential decrease in the nitrofurantoin reducing capacity.

Thus, the aim of this study was to determine the ability of furazolidone to select resistant mutants, analysing their stability and underlying mechanisms of resistance.

MATERIALS AND METHODS

Bacterial strains

Four clinical isolates of *E. coli* (2 enteroaggregative *E. coli* - EAEC; and 2 enterotoxigenic *E. coli* - ETEC) isolated from faeces collected from children under 2-year old with diarrhoea from periurban communities of Lima, Peru (5). These isolates were selected because are representative of the main diarrhoeagenic *E. coli* pathotypes. The exact isolates were chosen at random from the collections of the Universidad Peruana Cayetano Heredia.

Antimicrobial susceptibility test

The susceptibility to furazolidone and nitrofurantoin was established using the disk diffusion method in accordance with the CLSI guidelines (14).

Minimal inhibitory concentration (MIC)

The MIC to furazolidone and nitrofurantoin and also to nalidixic acid, ampicillin, chloramphenicol and tetracycline was determined by the agar dilution method according to the CLSI (14). The MICs were also determined by adding 20 mg/L of Phenyl-Arginine- β -naphthylamide (PABN) to evaluate the role of the PABN inhibitable efflux pumps in the resistance to furazolidone. A control with this compound and with the antibiotic solvent (N-N-dimethylformamide) was also made to ensure that it did not interfere in the bacterial growth.

Development of furazolidone-resistant mutants

The isolates were grown in Luria-Bertani broth overnight and 100 μ L was inoculated onto Mueller-Hinton plates containing serial dilutions of furazolidone from 1 $\times$ MIC to 128 mg/L. The plates were incubated at 37 °C and read at 24 and 48 h. To determine the original inoculum serial dilutions were performed. The experiments were performed in duplicate. The frequency of mutation was established as previously described (15). The absence of contaminations was assessed by the study of clonal

relations between parental and derived mutant strains by REP-PCR as described elsewhere (16).

Determination of the stability of the furazolidone-resistant mutants selected

The stability of the selected mutants was established by making 20 consecutive passes without the antibiotic on MacConkey agar (17). Thereafter, the MIC to furazolidone was repeated to observe possible differences between the first and the twentieth pass.

Nitroreductase gene amplification and sequencing

The presence of mutations in the nitroreductase genes *nfsA* and *nfsB* was determined by PCR as described previously (13). The products were purified with Wizard SV Gel and the PCR Clean Up System (Promega, Madison, WI, USA) and sequenced (Macrogen, Seoul, Korea).

Nitroreductase activity assay

Protein extraction and the consequent enzymatic assay, performed in 96-well plates and in three independent assays, were made as described elsewhere (18).

Study of the outer membrane proteins

Alterations in outer membrane proteins were studied by analysing differences between the patterns of the parental strains and mutants showed in electrophoresis in 12% acrylamide gel, at constant 40 mA and 150 V. Proteins were extracted as described by Tavio *et al.* (19) and visualized with a Coomassie staining. When differences were observed in the intensity of the bands in the gel, the products were recovered and sent to sequencing (University of Barcelona, Barcelona, Spain) for the identification of the protein.

RESULTS

Antimicrobial susceptibility

The MIC in the parental strains ranged from 0.25 to 2 mg/L. After mutant selection, 34 different colonies, 10 *E. coli* EAEC and 24 *E. coli* ETEC, were randomly selected and confirmed as mutants by REP-PCR. The mutants showed increases up to 3-fold in their MIC with respect to parental strain 1 (from 0.5 to 4 mg/L for furazolidone and 4 to 32 mg/L for nitrofurantoin), up to 2-fold with respect to parental strains 2 and 4 (ranging from 2 to 8 mg/L and 1 to 8 mg/L for furazolidone and 2 to 16 mg/L and 4 to 32 mg/L for nitrofurantoin respectively), and up to 5-fold with respect to parental strain 3 (from 0.25 to 8 mg/L). All MIC levels were proportional with zone diameters obtained in the disk diffusion test (Table 1). Neither changes in the MIC levels with the addition

Table 1. Relationship among minimum inhibitory concentration, disk diffusion, nitroreductase gene mutations and porin alterations

n	MIC Fur	Fur (mm) ¹	NfsA	NfsB	Porines
1	2	20 (S)	Ser12Thr, Glu223Lys	wt	wt
1	2	20 (S)	wt	wt	↑ OmpA
1	4	16 (I)	STOP 76	wt	wt
14	4	16 (I)	STOP 76	wt	↓ OmpA ↓ OmpW ↓ OmpA
7	4	16 (I)	wt	wt	↓ OmpA
4	4	16 (I)	wt	wt	wt
1	8	16 (I)	Ile220Leu, Pro223Gln, Tyr230Ile	Gly191Ala	↓ OmpA
1	8	16 (I)	wt	Val190Gly	↓ OmpA
1	8	16 (I)	wt	Asp197Tyr	↓ OmpA
3	8	16 (I)	wt	wt	↓ OmpA

n, number of isolates; MIC, minimum inhibitory concentration (mg/L); Fur (mm), furazolidone disk diffusion; S, susceptible; I, intermediate; nfsA, mutations found in *nfsA* gene; nfsB, mutations found in *nfsB* gene.

¹Breakpoint for nitrofurantoin was used.

of PABN nor cross-resistance was observed with any of the antibiotics tested. Additionally, no differences in the MIC levels were obtained when the MIC was read at 24 or 48 h. The PABN and the solvent had no inhibitory effect on the growth. No effect of N-N-dimethylformamide or PABN in the bacterial growth was observed.

Stability and frequency of mutation of the furazolidone-resistant mutants selected

The frequency of mutation obtained ranged from $<9.6 \times 10^{-10}$ to 9.59×10^{-7} (Table 2). After making 20 consecutive passes without antibiotic the MIC obtained in the mutants was not different from the initial.

Study of the nitroreductase genes

Seventeen mutant strains (50%) presented mutations in the nitroreductase A encoding gene (*nfsA*), producing the generation of a STOP codon in 15 (88.2%) of these isolates. Punctual amino acid

changes in this gene were observed in the other two isolates. In the nitroreductase B gene (*nfsB*) a lower number of amino acid changes was generated in 3 isolates (8.8%), none with the generation of a STOP codon. One isolate (2.9%) presented amino acid changes in both nitroreductase genes, while 15 isolates (44%) remained without mutations. An overview of all these changes is specified in Table 1.

Nitroreductase activity

Of the two isolates with mutations from parental strain 1, one showed no differences in nitroreductase activity and the other (with a STOP codon in the *nfsA* gene) showed a 20% decrease. The mutant from parental strain 2 and one mutant from parental strain 3 whose NfsB had an amino acid change, showed a decrease in nitroreductase activity of 25–40%. On the other hand, the mutant from parental strain 3 with a double mutation in the *nfsA* and *nfsB* genes showed a decrease of almost 50% in activity. Finally, in the mutants from parental strain 4, all with a codon STOP in NfsA nitroreductase, a decrease in the activity of 5–65% was observed. Results are shown in Fig. 1 and expressed in relation to parental strains.

Outer membrane proteins

Sequencing showed that the two porins with a different profile expression in a polyacrylamide gel were OmpA and OmpW. The first showed both overexpression in one mutant and lower expression in 27 mutants (82.3%), while 14 mutants (41.2%) showed a decrease in the expression of OmpW, all compared with the parental strains (Table 1, Fig. 2).

DISCUSSION

The standard treatment used in cases of diarrhoea is limited to only fluid replacement. However, due to the specific virulence of the pathogen, the disease duration or the general status of the patient, which may also be influenced by nutritional status, the use of antibiotics is sometimes necessary (20). The

Table 2. Frequencies of mutation obtained for *Escherichia coli* mutants

Isolate	MIC	Inoculum	Concentration of furazolidone (mg/L) in selection plates								
			0.5	1	2	4	8	16	32	64	128
<i>E. coli</i> 1	0.5	3.95×10^8	c	c	c	3.8×10^{-9}	$<1.3 \times 10^{-9}$	$<1.3 \times 10^{-9}$	$<1.3 \times 10^{-9}$	$<1.3 \times 10^{-9}$	$<1.3 \times 10^{-9}$
<i>E. coli</i> 2	2	4.7×10^8	–	–	c	c	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$
<i>E. coli</i> 3	0.25	2.76×10^8	c	c	c	3.98×10^{-8}	$<1.8 \times 10^{-9}$	$<1.8 \times 10^{-9}$	$<1.8 \times 10^{-9}$	$<1.8 \times 10^{-9}$	$<1.8 \times 10^{-9}$
<i>E. coli</i> 4	1	5.21×10^8	–	c	c	9.59×10^{-7}	$<9.6 \times 10^{-10}$	$<9.6 \times 10^{-10}$	$<9.6 \times 10^{-10}$	$<9.6 \times 10^{-10}$	$<9.6 \times 10^{-10}$

MIC, minimum inhibitory concentration; C, confluent grown.

Dash: not performed.

Fig. 1. Relative nitroreductase activity of the mutant strains expressed in relation to the parental strains activity. Parental strains (dotted bars); mutant strains with mutations in their nitroreductase genes (white bars).

Fig. 2. Outer membrane protein profile. 1st lane: Parental strain; 2nd lane: Mutant strain with an OmpA overexpression; 3rd lane: Parental strain; 4th lane: Mutant strain with a decreased expression in OmpA and OmpW.

most widely used group of antimicrobials in infections caused by *Enterobacteriaceae* are currently β -lactams or cotrimoxazole, however, resistance levels to these antibiotics have been increasing over the years (21).

Although safe (22), quinolones, such as ciprofloxacin, which are extensively used in the treatment of adult diarrhoea, are sparingly used in the treatment of children diarrhoea. Moreover, since the 1990s, an increase in the levels of quinolone resistance has been described worldwide, affecting both commensal and pathogenic microorganisms (5, 6, 8). At present, the World Health Organization considers the problem of antimicrobial resistance a priority (23).

In this context, furazolidone is increasingly used in different low and middle income settings (9–11), as treatment for different diarrheic pathogens.

Present data showed that furazolidone generally presents low mutation frequencies, ranging from $<9.6 \times 10^{-10}$ to 9.59×10^{-7} , demonstrating the low potential of this antimicrobial agent for selecting resistant mutants. These frequencies of mutation were lower than those described for other antimicrobial agents such as rifaximin or rifampicin, and similar to that of azithromycin or ciprofloxacin (15, 17). The MIC of furazolidone obtained in the mutants reached levels of between 2 and 8 mg/L, being considered as intermediate in 35 of 37 mutants in which the MIC levels were equal or higher than 4 mg/L. Present data are in accordance with the fact that more than one mutation step is needed to achieve high levels of furazolidone resistance (13). Moreover, no differences in the MIC values were observed between the initial values and after 20 growth passes without antibiotic pressure, thus, the mutations acquired are time-stable. The additive effect of sequential target mutation has been previously described in the selection of resistance to other antimicrobial agents such as the quinolones (8). Due to this additive effect, the stability of selected mutants and the fact that intermediate MIC levels may be reached in a single step, and then a potential full-resistance may be selected in a subsequent mutation step; when used, the levels of furazolidone resistant need to be monitored in continuous manner.

In the presence of the efflux pump inhibitor PA β N, the expected decreases in the MIC values were not found, thus efflux pumps inhibited by PA β N do not play an important role in the acquisition of resistance to furazolidone, unlike other antibiotics previously described such as nalidixic acid, azithromycin or rifaximin (7, 15, 24). The MIC was determined both in the presence and the absence of inhibitor, reading the results at 24 and 48 h and no

differences were found, demonstrating that no inducible furazolidone-resistance mechanism was selected. Cross-resistance was not found showing that the use of furazolidone probably will not affect the levels of susceptibility to nalidixic acid, ampicillin, chloramphenicol and tetracycline.

The *nfsA* and *nfsB* genes were sequenced to analyse the mechanisms of resistance selected in our strains. Different to that observed previously (13), in which all first-step mutants presents alterations in the *nfsA* gene, in this study were found mutations in the *nfsA* or *nfsB* genes, or in both genes concomitantly. Thus, mutations in *nfsA* and/or *nfsB* were found in the 55.8% (19 of 34) of the mutants. However, although the presence of these alterations explains the rise in the MIC levels, these levels remained remarkably low. When a STOP codon was selected at amino acid codon 76 of NfsA, a truncated nitroreductase was produced, thereby not allowing the antibiotic to metabolize and consequently, remaining in the bacteria for a longer time without affecting it. This is explained by the inactivation of nitroreductases which causes the acquisition of resistance to nitrofurans as shown in Fig. 1 (12, 13). The generation of a terminal codon was not observed in the 3 isolates (8.8%) with alterations in the nitroreductase gene *nfsB*.

On assessing the activity of these enzymes, all the mutant strains that generated STOP codons in their *nfsA* gene and the double mutants showed an important decrease in their activity. It is important to also consider that some mutants without mutations in the nitroreductase genes had less activity, demonstrating the presence of other furazolidone-resistant mechanisms. Similar fact was observed in previous studies, in which both an increase in furazolidone-MIC levels and a decrease in the activity of nitroreductase activity were confirmed in absence of acquisition of target mutations (13). In this case, the authors adduce as explanations the possible presence of other enzymes possessing nitroreductase activity, presence of essential trans-acting factor or alterations affecting furazolidone uptake.

The study of the outer membrane proteins showed differences in the expression of either over or under OmpA expression. This may be due, not only to an adaptation to the antibiotic but also to the mutations in the resistant strains caused by the presence of furazolidone that could lead to an internal alteration in the cellular machinery due to the production of oxidative stress (25). However, in *E. coli* or *Citrobacter freundii* has been observed that the increase in the expression of OmpA may be associated with decreased susceptibility to tetracycline and carbapenems (26, 27), Smani et al. (28)

showed that the disruption of the *ompA* gene in *Acinetobacter baumannii* led to decreased MICs of chloramphenicol, aztreonam, and nalidixic acid. These authors propose that the loss of OmpA may disturb membrane processes, including the transport of antimicrobial agents. Moreover, the changes found in OmpW profile expression, which decreased in all mutant strains affected, could be the cause of the increment in MIC levels. Other studies have previously demonstrated that the OmpW porin could be overexpressed when resistance to some antimicrobial agents is selected (29), suggesting that this porin acts by expelling the antibiotic outside the cell leading to a higher antimicrobial tolerance (30). Nevertheless, in accordance with our results, other studies have suggested a different role for this porin, finding a decrease in its expression in cases of antimicrobial resistance (31), suggesting that this membrane protein could also be working as an entrance of the antibiotics into the bacteria.

Although more studies are necessary to determine non-identified resistance mechanisms, the low frequency of mutation together the low levels of resistance achieved in a single step highlight the low potential of selecting furazolidone-resistant mutants.

This work was supported by the Agencia Española de Cooperación Internacional al Desarrollo (AECID) [grants nos. D/024648/09 and D/030509/10] and by Generalitat de Catalunya, Departament d'Universitats, Recerca i Societat de la Informació (2014 SGR 26). JR has a fellowship from the programme I3 of the Instituto de Salud Carlos III (ISCIII, Spain) [grant no. CES11/012].

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