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Molecular epidemiology and nitrofurantoin resistance determinants of nitrofurantoin-non-susceptible *Escherichia coli* isolated from urinary tract infections

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ABSTRACT

Objectives: The worldwide emergence of multidrug-resistant uropathogens has resulted in the revival of old antibiotics such as nitrofurantoin (NIT) for the treatment of uncomplicated urinary tract infections (UTIs). This study aimed to identify determinants of NIT resistance and to investigate the genetic diversity of NIT-resistant (NIT-R) *Escherichia coli* isolates.

Methods: Six NIT-R and three NIT-susceptible clinical *E. coli* isolates from patients with UTI were studied. The susceptibility of the isolates to various classes of antibiotics was evaluated by disk diffusion. The presence of plasmid-encoded efflux pump genes (*oqxA* and *oqxB*) was investigated by PCR. Nucleotide sequences of the *nfsA*, *nfsB* and *ribE* genes were determined. The genetic relatedness of the NIT-R isolates was evaluated by multilocus sequence typing (MLST).

Results: All six NIT-R isolates were characterised with high-level NIT resistance (MIC $\geq$ 512 mg/L) and they belonged to five distinct STs including ST131 (n = 2), ST73, ST405, ST10 and ST354 (n = 1 each). Amikacin, carbapenems, minocycline, tigecycline and fosfomycin were the most active agents against the studied uropathogens. The *oqxA* and *oqxB* genes were not detected in any isolate. All NIT-R isolates harboured inactivating genetic alterations in *nfsA* and *nfsB* [NfsA H11Y, S33N, S38Y, W212R substitutions, Δg638 (frameshift), Δa64-g73 (frameshift) and NfsB F84S, P45S, W94Stop, E197Stop substitutions, Δ*nfsB* locus]. The *ribE* gene of most isolates was unaffected, except for one isolate co-harbouring a deleterious RibE G85C substitution and NfsA/B alterations.

Conclusion: NIT resistance in the studied *E. coli* isolates was mainly mediated by *nfsA* and *nfsB* alterations.

1. Introduction

Escherichia coli is the most common uropathogenic bacterium causing community-acquired urinary tract infections (UTIs) [1,2]. The recent emergence and spread of multidrug-resistant *E. coli* isolates has led to renewed interests in older 'forgotten' antibiotics such as nitrofurantoin (NIT) for the management of uncomplicated UTIs.NIT is a synthetic bactericidal agent that is rapidly absorbed and is excreted in the urine to generate high therapeutic concentrations following oral administration [3]. It has a broad antibacterial spectrum and is particularly effective against the principal Gram-negative and Gram-positive uropathogens [3]. It became a preferred drug in the international consensus guidelines for UTI in 2010 and has been successfully used for a long time for the management of lower UTIs in adults, children and pregnant women [4]. Despite being in clinical use for decades, the exact mode of action of NIT is not completely understood, although it appears to involve the formation of reduced reactive intermediates that disrupt or alter bacterial ribosomal proteins and other macromolecules, halting vital bacterial biochemical processes [5]. The formation of these toxic intermediates is mediated by bacterial nitroreductases encoded by *nfsB* and *nfsA* genes [6].

Overall, clinical resistance to NIT remains low among *E. coli* isolates (<10%) and many multidrug-resistant organisms retain susceptibility to this agent [7,8]. To date, limited studies are available unravelling the mechanisms of NIT resistance among uropathogens. NIT resistance in *E. coli* is found to be mainly attributed to genetic

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alterations in *nfsA* and *nfsB* [9] and to lesser extent in the *ribE* gene encoding lumazine synthase, an essential enzyme involved in biosynthesis of the flavin mononucleotide, which is an important cofactor for NfsA and NfsB [10]. Furthermore, the plasmid-encoded efflux pump OqxAB has been found to mediate NIT resistance in *E. coli* isolates from human and animal sources [11].

Whilst the incidence of NIT-resistant (NIT-R) uropathogenic *E. coli* isolates is negligible among Iranian patients, little is known about the mechanisms mediating NIT resistance among *E. coli* from this geographic region. Therefore, the current study aimed to identify the determinants of NIT resistance and to investigate the genetic diversity of NIT-R *E. coli* isolates.

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

Six NIT-R and three NIT-susceptible (NIT-S) clinical *E. coli* isolates from patients with UTI were studied. Identification of the isolates to species level was performed by conventional biochemical tests [12]. The susceptibility of all of the isolates to various classes of antibiotics was determined by the Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13] using the following antibiotics: nalidixic acid (30 μg); ciprofloxacin (5 μg); levofloxacin (5 μg); gatifloxacin (5 μg); moxifloxacin (5 μg); gentamicin (10 μg); amikacin (30 μg); chloramphenicol (30 μg); ampicillin (10 μg); ceftriaxone (30 μg); cefepime (30 μg); ceftazidime (30 μg); imipenem (10 μg); meropenem (10 μg); doxycycline (30 μg); minocycline (30 μg); trimethoprim/sulfamethoxazole (SXT) (23.75/1.25 μg); fosfomycin (200 μg) (BBL™ Sensi-Disc™; Becton Dickinson, Sparks, MD, USA); and tigecycline (15 μg) (Mast Co., Bootle, UK). US Food and Drug Administration (FDA) interpretative criteria were used to determine tigecycline susceptibility by the disk diffusion method (susceptible, ≥19 mm; intermediate, 15–18 mm; resistant, ≤14 mm). All isolates were tested for extended-spectrum β-lactamase (ESBL) production using a phenotypic confirmatory test (the combined disk method) according to CLSI guidelines. Minimum inhibitory concentrations (MICs) of NIT were determined using Liofilchem® MIC Test Strips (Liofilchem, Roseto degli Abruzzi, Italy) containing a concentration gradient range of NIT (0.032–512 mg/L) and were interpreted according to CLSI guidelines as follows: susceptible, ≤32 mg/L; intermediate, 64 mg/L; and resistant, ≥128 mg/L [13]. NIT-S *E. coli* ATCC 25922 reference strain was used as quality control strain for antimicrobial susceptibility testing.

2.2. Identification of molecular determinants of nitrofurantoin resistance

To explore the molecular determinants mediating NIT resistance in the studied isolates, chromosomal DNA was extracted from all of the NIT-R and NIT-S isolates by the boiling method (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/278_mcr-multiplex-pcr-protocol-v2-oct16.pdf). PCR amplification of genes encoding the oxygen-insensitive NAD(P)H nitroreductases NfsA and NfsB as well as lumazine synthase RibE was performed using primers targeting amplification of coding sequences as well as some flanking regions (external primers). For isolates for which no amplification product was obtained using the external primers, internal primers targeting amplification of an internal region of the studied genes were used (Table 1). In addition, presence of the plasmid-encoded efflux pump OqxAB was examined by PCR method using the primers listed in Table 1. PCR products were sequenced and the nucleotide and deduced protein sequences were analysed at the National Center for Biotechnology Information (NCBI) website. The impact of identified amino acid substitutions on the biological function of the protein (i.e. neutral or deleterious) was further predicted using the Protein Variation Effect Analyzer (PROVEAN) software tool (http://provean.jcvi.org/index.php) [15].

2.3. Genotyping by multilocus sequence typing (MLST)

All NIT-R isolates were subjected to MLST as described previously [16]. PCR amplification and sequencing of internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were performed and alleles and sequence types (STs) were assigned in accordance with the *E. coli* MLST database website (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). All of the primer sequences of the seven genes are available at https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html.

3. Results

3.1. Bacterial genotyping and antimicrobial susceptibility testing

All six NIT-R isolates were characterised by high-level NIT resistance (MIC ≥ 512 mg/L) and they belonged to five distinct STs, including ST131 (n = 2 isolates), ST73, ST405, ST10 and ST354 (n = 1 isolate each) as determined by MLST (Table2). Antimicrobial

Table 1
Nucleotide sequences of primers used in this study.

Primer name	Sequence (5'→3')	T_a (°C)	Product size (bp)	Reference
nfsA-F1	ATTTTCTCGGCCAGAAGTGC	56	1036	
nfsA-R1	AGAATTTCAACCAGGTGACC			
nfsA-F2	TCTTGCCCCACAGCTGATG	58	893	
nfsA-R2	CTTACACGAATAGAGCGTTCC			
nfsB-F1	CAACAGCAGCCTATGATGAC	56	923	
nfsB-R1	CTTCGCGATCTGATCAACG			
nfsB-F2	CCCGCTAAATCTTCAACCTG	57	913	
nfsB-R2	AAAAGAGTGCGTCCAGGCTA			
nfsB-F3	TGCAAATCAGGAGAATCTGAG	55	846	[20]
nfsB-R3	TGGTCTGGCTAAACGCGATC			
nfsB-Int-F	CTTCAACGCTGTGATGACCTAC	57	566	This study
nfsB-Int-R	GCGTCATTCCACTAAGGCAT			
ribE-F	GCATTTAGTGGGTGCATGATC	58	700	[11]
ribE-R	GGAACTGGTATTCAACATCAGCG			
oqxA-F	GCGTCTCGGGATACATTGAT	55	482	
oqxA-R	GGCGAGGTTTTGATAGTGGA			
oqxB-F	CTGGGCTTCTCGCTGAATAC	57	498	[14]
oqxB-R	CAGGTACACCGCAAACACTG			

T_a, annealing temperature.

susceptibility testing showed that all of the NIT-R and NIT-S urinary *E. coli* isolates were susceptible to tigecycline, carbapenems (imipenem, meropenem) and amikacin and 8 (88.9%) were susceptible to fosfomycin. Whilst 83.3% (5/6) of the NIT-R isolates were also resistant to all quinolones (nalidixic acid, ciprofloxacin, levofloxacin, gatifloxacin, moxifloxacin), only 33.3% (1/3) of NIT-S isolates showed resistance to this family of antibiotics.

Rates of resistance to other antibiotics among the NIT-R and NIT-S isolates, respectively, were: gentamicin, 33.3% and 0%; SXT, 83.3% and 33.3%; chloramphenicol, 16.7% and 0%; ampicillin, 100% and 66.7%; ceftriaxone, 83.3% and 66.7%; cefepime, 33.3% and 66.7%; ceftazidime, 50.0% and 33.3%; minocycline, 0% and 0%; and doxycycline, 33.3% and 33.3% (Table 2). Also, 66.7% of NIT-R isolates (4/6) and NIT-S (2/3) were found to produce an ESBL (strains EC11, EC15, EC109, EC138, EC167 and EC168).

3.2. Mechanisms of nitrofurantoin resistance

To determine the molecular mechanisms conferring NIT resistance in the studied isolates, the presence of plasmid-encoded *oqxA* and *oqxB* genes was investigated by PCR and the nucleotide sequences of chromosomally encoded genes including *nfsA*, *nfsB* and *ribE* were determined. The plasmid-encoded genes *oqxA* and *oqxB* were not detected in any isolate, indicating that mechanisms other than antibiotic extrusion by this pump are mediating resistance in the studied isolates. Analysis of *nfsA* and *nfsB* gene sequences in the NIT-R isolates revealed several deleterious genetic alterations (Table 2). Whilst all NIT-R isolates harboured deleterious genetic alterations both in *nfsA* and *nfsB*, only one isolate harboured *ribE* alterations.

Analysis of the *nfsB* gene in two NIT-R isolates assigning to ST73 and ST405 revealed no amplification product using all three pairs of external primers targeting amplification of the *nfsB* coding sequence as well as some flanking regions, suggesting deletion of the *nfsB* locus. Absence of the *nfsB* locus in the genome of these isolates was confirmed by PCR using the *nfsB* internal primers. The NfsB protein was inactivated by premature termination due to nonsense mutations at codons 94 (W94stop, TGG < TGA) and 197 (E197Stop, GAA < TAA) in two NIT-R isolates assigned to ST10 and ST354. These alterations resulted in the production of a truncated and most likely non-functional 93- and 196-amino acid long protein, respectively, instead of a wild-type protein of 217 amino

Table 2
Characteristics of nitrofurantoin-resistant (NIT-R) and nitrofurantoin-susceptible (NIT-S) *Escherichia coli* isolated from urinary tract infections.

Isolate	NIT MIC (mg/L)	NfsA	NfsB	RibE	MLST	Susceptibility		
						S	I	R
EC11	≥512	ΔA64–G73 (frameshift)	G66D F84S V93A A174E	WT	ST131	GEN, AMK, CHL, IPM, MEM, MNO, TGC, SXT, FOS	DOX	CRO, FEP, AMP, CAZ, NAL, CIP, LVX, GAT, MFX
EC15	≥512	S38Y I117T K141E G187D A188V	Δ*nfsB* locus[a]	WT	ST73	GEN, AMK, CHL, IPM, MEM, DOX, MNO, TGC, NAL, CIP, LVX, GAT, MFX, CAZ, FOS	FEP	CRO, AMP, SXT
EC138	≥512	E58D Q72K I117T K141E G187D A188E W212R	Δ*nfsB* locus[a]	WT	ST405	GEN, AMK, IPM, MEM, FOS, TGC	MNO, CHL	CRO, FEP, AMP, CAZ, NAL, CIP, LVX, GAT, MFX, DOX, SXT
EC166	≥512	Δg638 (frameshift)	E197stop	WT	ST10	AMK, IPM, MEM, MNO, TGC, FEP, CRO, CAZ, FOS	None	NAL, CIP, LVX, GAT, MFX, CHL, AMP, SXT, DOX, GEN
EC168	≥512	S33N I117T K141E G187D	P45S G66D V93A A174E	G85C	ST131	AMK, MEM, IPM, DOX, MNO, TGC, FEP, CHL	None	CRO, AMP, CAZ, NAL, CIP, LVX, GAT, MFX, GEN, FOS, SXT
EC169	≥512	H11Y E58D I117T K141E Q147R G187D	V93A W94stop	WT	ST354	GEN, AMK, MEM, IPM, MNO, TGC, CAZ, FEP, CHL, FOS	DOX	CRO, AMP, NAL, CIP, LVX, GAT, MFX, SXT
EC48	32	E58D I117T K141E A172S G187D	V93A K130R I171F Q210H	WT	ND	GEN, AMK, IPM, MEM, DOX, MNO, TGC, NAL, CIP, LVX, GAT, MFX, CRO, FEP, CHL, FOS	CAZ, SXT	AMP
EC109	24	I117T K141E G187D	G66D V93A A174E	WT	ND	GEN, AMK, MEM, IPM, DOX, MNO, TGC, FOS	CHL, AMP	CRO, FEP, CAZ, NAL, CIP, LVX, GAT, MFX, SXT
EC167	16	E58D I117T K141E A172S G187D	V93A	WT	ND	GEN, AMK, IPM, MEM, CIP, LVX, GAT, MFX, FOS, SXT, CAZ, TGC, CHL	MNO	CRO, FEP, AMP, DOX, NAL

NIT, nitrofurantoin; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; S, susceptible; I, intermediate; R, resistant; WT, wild-type; ND, not determined; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; IPM, imipenem; MEM, meropenem; MNO, minocycline; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole; FOS, fosfomycin; DOX, doxycycline; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; CAZ, ceftazidime; NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; GAT, gatifloxacin; MFX, moxifloxacin.

[a] *nfsB* locus not amplifiable with all primers used in this study.

acids. In another two NIT-R isolates assigned to ST131 and ST10, *nfsA* deletions including guanine deletion at position +638 and a 10-nucleotide deletion (+64 to +73) were observed, which resulted in frameshifting in both cases and the introduction of several stop codons in the coding region of protein in the latter case. PROVEAN prediction of the amino acid changes showed that NfsA S38Y (TCC < TAC), W212R (TGG < AGG), S33 N (AGC < AAC) and H11Y (CAT < TAT) and NfsB F84S (TTC < TCC) and P45S (CCG < TCG) as well as RibE G85C (GGT < TGT) substitutions could have an inactivating impact on the function of the corresponding proteins (Table 3). However, other amino acid substitutions observed in NIT-R isolates as well as all substitutions identified in NIT-S isolates were categorised as neutral changes that are not likely to be involved in NIT resistance development in the studied isolates.

3.3. Nucleotide sequence accession nos

The nucleotide sequences of the mutated *nfsA*, *nfsB* and *ribE* genes have been deposited in the GenBank nucleotide sequence database under accession nos. MN256119–MN256126, MN215887, MN381176, and MN273513–MN273519.

4. Discussion and conclusions

Despite being in clinical use for decades, NIT has retained its activity against multidrug-resistant urinary *E. coli* isolates and the incidence of resistance to this old antibiotic has remained relatively low [17]. In the current study, MLST results revealed that the six NIT-R *E. coli* isolates were clonally diverse and were distributed into five distinct STs, with ST131 being the most frequent genotype (33.3%; n = 2 isolates) and the other STs being found in single isolates each (16.7%; n = 1). *Escherichia coli* ST131 has been found to contribute significantly to the spread of multidrug resistance in the community and often encodes multiple virulence products associated with mobile genetic elements and is responsible for a high proportion of urinary tract and bloodstream infections [18,19]. According to antimicrobial susceptibility testing results, amikacin, minocycline, tigecycline, carbapenems and fosfomycin were the most active agents against the studied uropathogens, which showed either a minor or no resistance rate. Analysis of NfsA, NfsB and RibE of NIT-R and NIT-S clinical *E. coli* strains demonstrated several amino acid substitutions. However, none of the substitutions identified in NIT-S isolates were found to have major implications for the functionality of the studied enzymes as predicted by PROVEAN tool and were categorised as neutral changes. Among the identified NfsA substitutions S38Y, W212R, S33N, H11Y, A188V, A188E, Q72K, Q147R, A172S, I117T, G187D, K141E and E58D, only the first four mutations, and among the NfsB substitutions P45S, F84S, K130R, I171F, G66D, V93A, A174E and Q210H only the first two mutations were predicted by the PROVEAN tool to have a deleterious impact on protein structure and were only harboured by the NIT-R isolates. We suggest that NfsA I117T, G187D, K141E and E58D and NfsB G66D, V93A and A174E substitutions cannot contribute to NIT resistance alone since these mutations also were found in NIT-S isolates and surprisingly the PROVEAN tool predicted these alterations as neutral changes. The NfsB nonsense mutations (W94stop, E197stop) in two NIT-R isolates resulted in the production of a truncated and most likely non-functional NfsB protein compared with the 217-amino acid wild-type protein and might explain the origin of NIT resistance in these isolates. The W94stop mutation has been also reported by Sandegren et al. among NIT-R isolates [20], reinforcing the hypothesis that position +282 in *nfsB* (codon 94) is a critical region that is prone to mutate upon resistance emergence. Production of truncated/inactivated NfsB or its absence in some NIT-R isolates might have resulted in the inability/reduced ability of NIT-R isolates to cause nitroreduction of NIT and the production of active intermediates from the compound. The lumazine synthase RibE of most of the studied isolates remained unaffected, except for one NIT-R isolate showing a G85C substitution that was predicted to have negative impact on protein structure. However, since this isolate also co-harboured a mutated NfsA and NfsB, the exact role of RibE mutation in resistance emergence in this isolate remains unknown. Studies demonstrating the role of RibE substitutions in the development of NIT resistance are scarce. It was initially described by Vervoort et al. who reported a laboratory-induced NIT-R *E. coli* isolate lacking NfsA/NfsB alterations but harbouring a 12-nucleotide deletion in the *ribE* gene [10]. However, they reported six NIT-R clinical *E. coli* isolates that lacked any RibE alterations [10]. Moreover, Ho et al. could not find any significant *ribE* mutations in NIT-R *E. coli* isolates originating from patients with UTI and from food-producing animals [11]. The *oqxAB* genes were not detected in any isolate in the current study, indicating that this efflux pump is not mediating NIT resistance in the studied isolates. Ho et al. studied the contribution of OqxAB to NIT resistance in *E. coli* from human and animal sources and reported a high prevalence of *oqxAB* among NIT-non-susceptible isolates (NIT-intermediate 11.5–45.5% and NIT-resistant 39.2–65.5%) [11]. In a study from China performed on 18 NIT-R clinical *E. coli* isolates, I117 T and G187D in NfsA as well as V93A, M57I and K122R in NfsB were the most common substitutions identified in the studied proteins and were predicted to have a neutral impact on protein structure [21]. In approximately 44% of resistant and 11% of intermediate isolates, but not in NIT-S isolates, the *oqxA* and *oqxB* genes were detected, which were found to contribute to elevated NIT MICs in isolates harbouring mutated *nfsA* and *nfsB* [21]. It has been demonstrated that *nfsA* alterations cause only low-level resistance and that the development of high-level resistance (MIC of 128 mg/L) occurs when inactivation of *nfsB* combines with *nfsA* inactivation [6,20]. These data showed that all NIT-R isolates were characterised by high-level NIT resistance (MIC $\geq$ 512 mg/L) and that NfsA and NfsB activities were probably knocked-out in all isolates.

In conclusion, NIT resistance in the studied isolates was mainly mediated by *nfsA* and *nfsB* alterations that were found in clonally

Table 3
Predicting the impact of amino acid substitutions on the biological function of NfsA, NfsB and RibE proteins using PROVEAN tool.

Protein	Variant	PROVEAN score	Prediction[a]	Isolates[b]
NfsA	**H11Y**	−5.746	Deleterious	NIT-R
	S33N	−2.711	Deleterious	NIT-R
	S38Y	−5.617	Deleterious	NIT-R
	E58D	−1.866	Neutral	NIT-R, NIT-S
	Q72K	−0.940	Neutral	NIT-R
	I117T	−0.634	Neutral	NIT-R, NIT-S
	K141E	1.207	Neutral	NIT-R, NIT-S
	Q147R	−1.170	Neutral	NIT-R
	A172S	−0.252	Neutral	NIT-S
	G187D	1.554	Neutral	NIT-R, NIT-S
	A188V	−0.338	Neutral	NIT-R
	A188E	0.694	Neutral	NIT-R
	W212R	−12.644	Deleterious	NIT-R
NfsB	**P45S**	−7.680	Deleterious	NIT-R
	G66D	−1.775	Neutral	NIT-R, NIT-S
	F84S	−5.862	Deleterious	NIT-R
	V93A	2.155	Neutral	NIT-R, NIT-S
	K130R	−0.516	Neutral	NIT-S
	I171F	−2.306	Neutral	NIT-S
	A174E	1.621	Neutral	NIT-R, NIT-S
	Q210H	−1.878	Neutral	NIT-S
RibE	**G85C**	−8.850	Deleterious	NIT-R

NIT-R, nitrofurantoin-resistant; NIT-S, nitrofurantoin-susceptible.
[a] PROVEAN score cut-off = −2.5.
[b] Isolates in which the specific amino acid substitution has been identified.

diverse uropathogens. The high rate of resistance to quinolones observed in this study is an alarming issue for infection control programmes, necessitating restriction of empirical prescription of critically important antibiotics such as fluoroquinolones to the most severe infections as well as their application according to antimicrobial susceptibility testing results.

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Competing interests

None declared.

Ethical approval

Not required.

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