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Research Paper

Quinolone resistance and ornithine decarboxylation activity in lactose-negative *Escherichia coli*

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Abstract

Quinolones and fluoroquinolones are widely used to treat uropathogenic *Escherichia coli* infections. Bacterial resistance to these antimicrobials primarily involves mutations in *gyrA* and *parC* genes. To date, no studies have examined the potential relationship between biochemical characteristics and quinolone resistance in uropathogenic *E. coli* strains. The present work analyzed the quinolone sensitivity and biochemical activities of fifty-eight lactose-negative uropathogenic *E. coli* strains. A high percentage of the isolates (48.3%) was found to be resistant to at least one of the tested quinolones, and DNA sequencing revealed quinolone resistant determining region *gyrA* and *parC* mutations in the multi-resistant isolates. Statistical analyses suggested that the lack of ornithine decarboxylase (ODC) activity is correlated with quinolone resistance. Despite the low number of isolates examined, this is the first study correlating these characteristics in lactose-negative *E. coli* isolates.

Key words: ODC, *gyrA*, *parC*, uropathogenic.

Introduction

Urinary tract infections (UTIs) are the second cause of antimicrobial prescriptions in South Brazil and are one of the major causes of office visits and hospitalization in the United States; these infections primarily affect women, pregnants and elderly people (Foxman, 2002; Tavares *et al.*, 2008). *Escherichia coli* is the main agent of UTIs, especially in community-acquired UTIs, and quinolones and fluoroquinolones have been used extensively to treat these infections (Ronald, 2003; Van Bambeke *et al.*, 2005). Since these antimicrobials agents were introduced, resistant strains have emerged and spread around the world (Schito *et al.*, 2009). Many studies have sought to understand the mechanisms of resistance, to develop more efficient antibiotics, and more recently, to relate resistance to biochemical or genetic characteristics (Lemos *et al.*, 2011; Rodriguez-Martínez *et al.*, 2011). The main mechanism of quinolone resistance involves mutations in the quinolone resistance determining region (QRDR) of *gyrA* and *parC* genes. The most common mutations are within the Ser83 and Asp87 codons in *gyrA* and within the Gly78, Ser80 and Glu84 codons in *parC*. The *gyrA* and *parC* genes encode subunits of DNA gyrase and topoisomerase IV, two enzymes involved in DNA supercoiling and DNA decatenation, respectively (Hooper, 2000). In *E. coli*, DNA gyrase is more susceptible to inhibition by quinolones than topoisomerase

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IV. In gram-negative bacteria, a single mutation in *gyrA* can reduce the susceptibility of DNA gyrase, furthermore additional mutations in *gyrA* or in *gyrB* and *parC* can increase resistance to the antibiotic (Jacoby, 2005; Minarini *et al.*, 2012).

The frequency of lactose-negative (*lac*$^-$) *E. coli* phenotype has shown to be very low, ranging from 5 to 10% (Winn *et al.*, 2006; Oliveira *et al.*, 2006). To characterize this group, the present work selected and evaluated uropathogenic *E. coli* isolates from UTIs regarding their quinolone resistance and biochemical activity profiles.

Materials and Methods

Bacterial sample

Fifty-eight *lac*$^-$ *E. coli* isolates from the urine of UTI patients from the Ponta Grossa, Brazil region were analyzed. These isolates were selected from inpatients and outpatients from 2008 to 2010. The urinary bacterial concentration used to diagnose a urine infection was > 10^5 colony-forming units per milliliter. The bacteria were stored until their use at -20 °C in BHI medium (Himedia, Mumbai, India) containing 15% glycerol. The *lac*$^-$ phenotype was confirmed in MacConkey agar (BD, Sparks, MD, EUA). *E. coli* ATCC 25922 was used as a control in the antibiotic susceptibility and biochemical tests.

Antibiotic susceptibility tests

The susceptibility of the *E. coli* isolates to the quinolones nalidixic acid (Nal), ciprofloxacin (Cip), norfloxacin (Nor) and ofloxacin (Ofx), and the beta-lactams cefotaxime (Ctx), ceftazidime (Ctz), aztreonam (Atm) and amoxicillin clavulanate (Amc) was determined using the disk diffusion method following the recommendations of the Clinical and Laboratory Standards Institute (2010). Disks (Laborclin, Pinhais, PR, Brazil) were stored at -20 °C until their use.

Biochemical characterization

The *E. coli* biotype was determined using the *Enterobacteriaceae* identification kit (Newprov, Pinhais, PR, Brazil) according to the manufacturer's instructions. This kit, approved by the Agência Nacional de Vigilância Sanitária (ANVISA), provides the following ten differential biochemical tests: L-tryptophan deamination; sulfidric acid, indole and gas production; glucose and rhamnose fermentation; lysine and citrate utilization; ornithine decarboxylation and motility.

Detection of *gyrA* and *parC* mutations

Multiplex PCR was used to amplify the *gyrA* and *parC* regions. Single *E. coli* colonies grown on MacConkey agar (BD, Sparks, MD, USA) were suspended in 15 μL of sterile deionized water and disrupted after 15 min of incubation at 95 °C. Then, the following reagents were added to a final volume of 30 μL: Taq DNA polymerase Invitrogen buffer (1), magnesium chloride (2.5 mM), deoxyribonucleotide triphosphates (dNTPs) (Invitrogen, Carlsbad, California, USA) (0.2 mM), primers (IDT, Coralville, Iowa, USA) (0.2 μM of GyrA primer and 0.4 μM of ParC primer) and Taq DNA polymerase (0.75 U). The primer sequences are listed in Table 1.

The genes were amplified using the following the thermal cycling profile: 2 min at 95 °C and 35 cycles of 30 s at 95 °C, 60 s at 55.4 °C and 60 s at 72 °C. The PCR products were separated on a 1 x TAE, 2% agarose gel and quantified using UVP Labwork Software (UVP Inc.).

The amplification products (20 μL) were treated with 10 U of exonuclease I (Biolabs, Ipswich, New England) and 1.0 U of alkaline phosphatase (USB, Cleveland, Ohio, USA) at 37 °C for 90 min. Then, the enzymes were inactivated at 80 °C for 30 min (Werle *et al.*, 1994).

The treated PCR products (5 μL) were sequenced using 0.5 μL of primer (10 μM), 1 μL of Big Dye Terminator mix (Applied Biosystems, Carlsbad, California, USA), 3 μL of Big Dye Buffer (1X) and ultrapure H_2O to a final volume of 10 μL using the following program: 2 min at 96 °C and 35 cycles of 45 s at 96 °C, 30 s at 55.4 °C and 4 min at 60 °C. The sequencing PCR products (10 μL) were precipitated using 2 μL of ammonium acetate (7.5 M), 60 μL of absolute ethanol and 10 μL of ultrapure water followed by 45 min of centrifugation. The supernatant was discharged and the precipitate was washed with 70% ethanol, dried and dissolved in deionized formamide. Sequencing was performed using a 24-capillary 3500xL System (Applied Biosystems, Carlsbad, California, USA). Reads were trimmed for the removal of low quality bases using the Phred program (Ewing *et al.*, 1998). To detect nucleotide mutations, the DNA sequences were aligned using Clustal W (Thompson *et al.*, 1994) against the wild-type

Table 1 - Primer sequences

Primer	Sequence	Reference
GyrA F	5'AAATCTGCCCGTGTCGTTGGT 3'	Rodriguez-Martínez *et al*., 2006
GyrA R	5'GCCATACCTACGGCGATACC 3'	
ParC F	5'GTATGCGATGTCTGAACT 3'	Cattoir *et al*., 2006
ParC R	5'TTCGGTGTAACGCATTGC 3'	

F = forward; R = reverse.

gyrA or *parC* gene nucleotide sequences from *E. coli* K12 substr. MG1655 (accession numbers 946614 and 947499, respectively).

Statistical analysis

Statistical analyses to correlate antibiotic resistance and biochemical characteristics were performed using Yates' chi-squared test because it is recommended for small sample numbers (Graphpad Prism 6.0). P value < 0.01 were considered statistically significant.

Results and Discussion

Forty-eight percent of the *lac⁻* *E. coli* isolates (28/58) were able to grow in the presence of at least one quinolone. Among the resistant isolates, seventy-nine percent (22/28) were resistant to all quinolones tested, and 21% (6/28) were resistant only to nalidixic acid. In contrast, only one isolate displayed intermediate resistance to the beta-lactams cefotaxime and ceftazidime. Our data showed high quinolone resistance rates among the isolates. Other studies that analyzed uropathogenic *E. coli* isolates in the same area revealed a norfloxacin resistance rate of 11.6% in Ponta Grossa (Bail *et al.*, 2006) and 13.8% in Curitiba (Ito *et al.*, 2008). In Fortaleza in the northeast of Brazil, a lower resistance rate of 7.5% was described for norfloxacin (Araújo *et al.*, 2011). In Europe, a multicentric study showed a ciprofloxacin resistance rate from 1.4% to 12.9% (Schito *et al.*, 2009), whereas a higher rate (31%) was registered in the hospitalized population in Ribeirão Preto, Brazil (Santo *et al.*, 2006). Because our isolates were primarily of community origin, the elevated resistance was unexpected. The available literature analyzed all uropathogenic *E. coli* isolates without determining the lactose phenotype of the isolates; our work suggests that the quinolone resistance frequency is increased in *lac⁻* *E. coli* strains.

Biochemical tests identified nine *E. coli* biotypes. They presented a common behavior in the majority of the tests except Ornithine Decarboxylase (ODC), Motility (Mot), Gas production (Gas) and Rhamnose fermentation (Rha) (Figure 1). The most common biotypes (72%), 981, 991 and 971, were able to ferment glucose and rhamnose, to produce gas and indole and to metabolize lysine and citrate. On the other hand, they presented a distinguished profile in the ornithine decarboxylase and motility tests. Most of the ornithine decarboxylase positive (ODC^+) *E. coli* isolates (26/36) showed to be sensitive to quinolone while most of the ODC^- *E. coli* isolates (18/22) showed to be resistant to it. Statistical analyses revealed a relationship between ODC and quinolone resistance ($p < 0.01$); no relationship was identified between motility and the same resistance parameter ($p > 0.05$). Several studies relating biological and genetic characteristics with antibiotic resistance have been performed over the last years (Bashir *et al.*, 2011). Ferjani *et al.* (2011) showed a direct relationship among virulence determinants, phylogenetic groups and susceptibility to fluoroquinolones. A Brazilian study related *E. coli* carbohydrate fermentation to virulence factors. Dulcitol-positive and raffinose-negative isolates were found to be more virulent than other isolates (Lemos *et al.*, 2011). Vila *et al.* (2002) related quinolone resistance and virulence factors, suggesting that quinolone-resistant strains are less virulent. The present study is the first to relate biochemical characteristics to quinolone resistance in lactose-negative *E. coli* isolates.

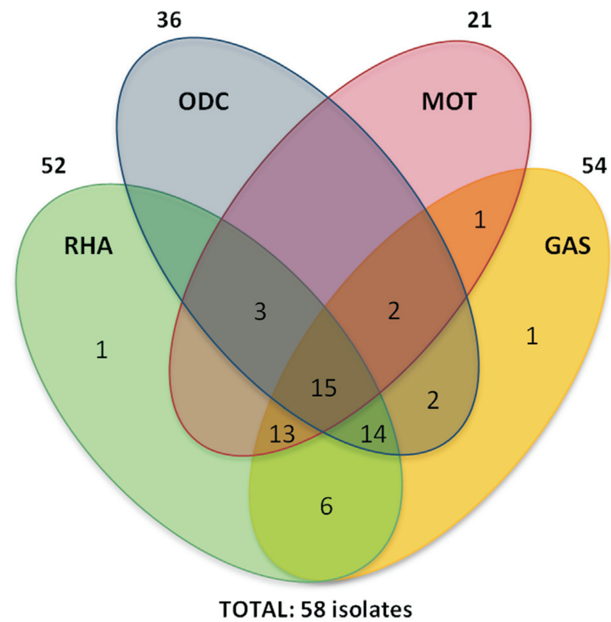

Figure 1 - Biochemical activities of the studied uropathogenic *E. coli* isolates. The numbers indicate the number of isolates that presented the indicated activity or group of activities. ODC = ornithine decarboxylase; MOT = motility; RHA = rhamnose fermentation; GAS = gas production.

Because the primary mechanism of quinolone resistance involves mutations in the QRDR of the *gyrA* and *parC* genes (Jacoby, 2005), these regions of the isolates' DNA were sequenced. One quinolone-sensitive *lac⁻* isolate was also included as a negative control. Of the twenty-eight quinolone resistant isolates analyzed, six could not be molecularly analyzed due to the low quality of their sequences. Their nucleotide substitutions are shown in Table 2.

The quinolone-sensitive isolate presented the same QRDR *gyrA* and *parC* sequence as the wild-type strain. The five exclusive nalidixic acid-resistant (Nal^R) isolates presented either the codon 83 mutation Ser → Leu (2/5), the codon 87 mutation Asp → Tyr (1/5) or no mutation in the sequenced area (2/5). They did not contain any QRDR *parC* mutations. It was expected that these exclusive Nal^R bacteria would not have many mutations as it is known that single mutations in the *gyrA* gene of gram-negative bacteria are able to generate this phenotype (Komp-Lindgren *et al.*, 2003) and that *parC* acts as a second mutation target that amplifies this resistance (Everett *et al.*, 1996). Because no mutations were found in two of the Nal^R isolates, their resistance could be explained either by mutations outside of

Table 2 - Quinolone resistance profile and molecular characterization of the studied *E. coli* isolates.

Antibiotic resistance profile	Number of isolates#	GyrA amino acids							ParC amino acids						
		82	83	84	85	86	87	88	79	80	81	82	83	84	85
E. coli wild-type sequence*		Asp	Ser	Ala	Val	Tyr	Asp	Thr	Asp	Ser	Ala	Cys	Tyr	Glu	Ala
Nal^S, Cip^S, Nor^S, Ofx^S	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nal^R	2	-	Leu	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	Tyr	-	-	-	-	-	-	-	-
Nal^R, Cip^R, Nor^R, Ofx^R	15	-	Leu	-	-	-	Asn	-	-	Ile	-	-	-	-	-
	1	-	Leu	-	-	-	Asn	-	-	Ile	-	-	-	Val	-
	1	-	Leu	-	-	-	Asn	-	-	-	-	-	-	Lys	-

**E. coli* QRDR *gyrA* and *parC* wild-type sequence from GenBank. # Six quinolone-resistant isolates could not be molecularly analyzed due to the low quality of their sequences. Nal = Nalidixic acid; Cip = Ciprofloxacin; Nor = Norfloxacin; Ofx = Ofloxacin; ^{S} = sensitive; ^{R} = resistant.

QRDR regions or other mechanisms that were not explored here, such as changes in permeability or efflux pump activity (Friedman *et al.*, 2001).

The seventeen sequenced *E. coli* isolates that showed resistance to all of the tested quinolones presented GyrA substitution at codons 83 (Ser $\rightarrow$ Leu) and 87 (Asp $\rightarrow$ Asn) and ParC substitution at codon 80 (Ser $\rightarrow$ Ile) and 84 (Glu $\rightarrow$ Val or Glu $\rightarrow$ Lys). Other studies have detected these QRDR GyrA and ParC amino acid mutations (Chen *et al.*, 2001; Mavroidi *et al.*, 2012). These changes were shown to reduce the affinity of the drug for their targets, thus resulting in bacterial growth even in the presence of quinolone (Bernard *et al.*, 2001).

Silva and Mendonça (2012) suggested that the GyrA codon 83 mutation generates supercoiling DNA alterations that could modify the expression of virulence factors. In addition, Weber *et al.* (2013) demonstrated that alterations in supercoiling affect fundamental cellular processes, including transcription. Based on these observations, it is possible that the GyrA codon 83 mutation that was detected in all of the analyzed multi-resistant isolates could be preventing the transcription of the *lac* operon genes and the *speC* gene, thus generating *lac*$^-$ and ODC$^-$ phenotypes, respectively. Because we cannot rule out the possibility that these genes are absent from the genome of the isolates, further studies are necessary.

A recent work showed that sublethal concentrations of fluoroquinolones were able to produce oxidative stress. To prevent DNA damage caused by reactive oxygen species (ROS) in the bacterial cell, ODC is upregulated, and the polyamine concentration increases (Umezawa *et al.*, 1997; Tkachenko *et al.*, 2011). Therefore, it might be interesting to measure ROS production in the studied isolates in future experiments.

In conclusion, the studied uropathogenic lactose-negative *E. coli* isolates showed a high quinolone resistance rate and indicated that there is a relationship between the absence of ornithine decarboxylase activity and quinolone resistance. The present work could serve as the basis for more comprehensive studies including a greater number of isolates from different localities to confirm our results.

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