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Short Communication

In vitro and in vivo persistence of IncN plasmids carrying *qnr* genes in uropathogenic *Escherichia coli* isolates

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Objectives: This study aimed to evaluate the persistence of the plasmid-mediated quinolone resistance (PMQR) among uropathogenic *Escherichia coli* strains grown under or without exposure to subinhibitory concentrations of ciprofloxacin. Based on that, we evaluated the possible spontaneous loss or maintenance of PMQR and the possible appearance of compensatory mutations in *gyrA* and *parC* genes.
Methods: Three uropathogenic *E. coli* strains harbouring chromosomal mutations in the *gyrA* and/or *parC* genes coupled with *qnrS1* or *qnrB2* determinants carried by distinct plasmid sizes and incompatibility N groups (IncN/ST1, IncN/ST5) were evaluated using in vitro and in vivo assays.
Results: PMQRs remained stable in all strains throughout the generations evaluated, independently of exposure to ciprofloxacin in both in vivo and in vitro assays. Analysis of *gyrA* and *parC* genes after in vivo and in vitro assays revealed that no changes occurred in quinolone-resistance determining regions (QRDR).
Conclusion: We demonstrated that IncN plasmids were persistent over 14 days in *E. coli* clinical strains independently of exposure to ciprofloxacin, as well as previous mutations in QRDR.
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1. Introduction

Resistance to fluoroquinolones, which are synthetic broad-spectrum antimicrobials used to treat a diversity of infectious diseases caused by Gram-negative bacteria, has increased drastically worldwide [1–3]. It is known that fluoroquinolone-resistant Gram-negative bacilli often carry plasmid-mediated quinolone resistance (PMQR) genes, such as the *qnr* determinants that encode a pentapeptide repeat protein that protects the quinolone targets, and/or mutations on DNA gyrase and topoisomerase IV encoding genes that impairs their action [4]. Its expression may be influenced by selective pressure caused by antimicrobial exposure. In this case, fluoroquinolone is considered a potent inducer of the SOS response, causing DNA damage or arresting replication forks by blocking DNA gyrase [5]. During the exposure, RecA activation induces cleavage of the LexA repressor [6]. However, the influence of this exposure on the maintenance of PMQR and the cumulative emergence of *gyrA* and *parC* mutations is not well known. In this context, this study aimed to evaluate the persistence of the PMQR among uropathogenic *Escherichia coli* strains grown under or without exposure to subinhibitory concentrations of ciprofloxacin. Based on that, we evaluated the possible spontaneous loss or maintenance of PMQR and the appearance of compensatory mutations in *gyrA* and *parC* genes using in vitro and in vivo assays.

2. Methods

2.1. Bacterial strains characterisation

Three uropathogenic *E. coli* strains harbouring chromosomal mutations in the *gyrA* and/or *parC* genes and *qnrS1* or *qnrB2* determinants carried by distinct plasmid sizes and the

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Table 1
Microbiological features of uropathogenic *Escherichia coli* strains harbouring distinct plasmid-mediated *qnr* genes included in this study.

Strain	Plasmid characteristics				QRDR mutations[a]		β-Lactamase content	MIC (μg/mL)
	PMQR	Size (kb)	Inc group	pMLST	*gyrA*	*parC*		CIP[b]
Ec79 (ST131)	*qnrS1*	~54	IncN	ST1	$Ser_{83}Leu$		bla_{TEM-1}	8 (4)
Ec234 (ST10)	*qnrB2*	~55	IncN	ST5	$Ser_{83}Phe$, $Asp_{87}Ala$	$Ser_{80}Ile$	–	16 (8)
Ec414 (ST10)	*qnrB2*	~76	IncN	ST1	$Gly_{91}Arg$		bla_{SHV-2}	0.5 (0.25)

CIP, ciprofloxacin; MIC, minimal inhibitory concentration; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone-resistance determining resistance; ST, sequence typing.
[a] Previous QRDR mutations observed in the *E. coli* strains.[b] ½ MIC used in the vitro assays for each *E. coli* strain.

incompatibility N group (IncN) were evaluated (Table 1). Two *E. coli* strains are part of a collection of 106 Enterobacteriales isolated from hospitalised patients [7], and one *E. coli* strain was isolated from a community-acquired urinary tract infection (UTI) [8]. All *E. coli* strains were recovered from distinct cities located in the state of Minas Gerais, Brazil. The bacterial identification was confirmed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS) using a Microflex LT mass spectrometer with Biotyper 3.3 software (Bruker Daltonics, Bremen, Germany), following the manufacturer's recommendations. The molecular typing was determined by multilocus sequence typing (MLST) as previously described at the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) [9]. The Inc groups of the PMQR carrying plasmids were determined using a molecular characterisation scheme as previously published [10], followed by Southern blot/hybridisation assays using specific probes and subtyped (ST) by multilocus sequence plasmid typing (pMLST). According to MLST analysis, the Ec79 strain belonged to ST131 and harboured an IncN/ST1 54-kb plasmid carrying *qnrS1*. In addition, Ec234 and Ec414 strains belonged to ST10 and harboured an IncN/ST5 55-kb and IncN/ST1 76-kb plasmids, respectively, both carrying *qnrB2* (Table 1).

2.2. In vitro assay in the presence and absence of subinhibitory concentrations of ciprofloxacin

First, the three *E. coli* strains (EC79, Ec234 and Ec414) were grown separately in MacConkey agar (BD Difco, Sparks, MD, USA) plates at 35 °C ± 2 °C overnight. Isolated colonies of each strain were then cultured into Tryptic Soy agar (TSA; BD Difco, Sparks, MD, USA) supplemented with 1/2 MIC of ciprofloxacin; i.e. a subinhibitory concentration [7,8], as shown in Table 1. Growth in ciprofloxacin-free agar was also evaluated in parallel as a control. For 14 consecutive days, isolated colonies were transferred in duplicate to new TSA agar plates to maintain a constant antimicrobial selective pressure and nutrient concentration. Colonies recovered at the 1st, 3rd, 7th and 14th day were stored in Brain Heart Infusion broth (BHI; BD Difco, Sparks, MD, USA) supplemented with 15% glycerol and stored at −80 °C. Plasmid DNAs of selected colonies were extracted using the Kieser protocol [11]. The plasmids were then analysed on 0.7% agarose gel after staining with ethidium bromide. *Escherichia coli* V517 and *E. coli* R861 reference strains were used as plasmid size markers.

2.3. In vivo assay using specific pathogen-free (SPF) chick infection model

For the SPF chick infection model, the animals were distributed in four isolator cabinets for poultry (https://www.alescobrasil.com.br/isolators), each one with eight chicks and were kept for 14 days. All birds were fed with feed and water ad libitum. Animals were randomly assigned to different therapeutic groups as follows: (i) groups 1 and 2, animals were exposed to ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) and were inoculated with Ec79 or Ec234 strains; (ii) groups 3 and 4, animals were not exposed to ciprofloxacin and were inoculated with Ec79 or Ec234 strains. Each animal received a pre-standardised infective dose of fresh bacterial culture and suspended in sterile saline [approximately 1.5×10^8 colony forming units (CFUs)/mL] that was inoculated via gavage prepared with isolated colonies. Groups 1 and 2 were exposed to ciprofloxacin at a concentration of 4 μg/mL (½ MIC) through water offered daily, 1 day postinfection [12,13]. Cloacal swabs were collected on the 3rd, 5th, 10th and 14th day and cultivated on MacConkey agar plates. After 24 h of incubation, isolated colonies were stored in BHI supplemented with 15% glycerol at −80 °C for the extraction of plasmids [11]. At the end of the 14th day trial, the animals were euthanised, and gut, kidney, cecum and liver organs were properly obtained for further analysis. The isolates were recovered from each organ in MacConkey agar plates with 4 μg/mL added ciprofloxacin.

2.4. Pharmacokinetic analysis for ciprofloxacin determination in chick blood

In addition, blood samples of each animal were collected for the analysis of the serum level of ciprofloxacin by high performance liquid chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan) using a Hypersil™ ODS C-18 (150 mm × 4.6 nm i.d., 5 μm particle size) analytical columns (Thermo Fisher Scientific, St. Louis, MO, USA). A Shimadzu fluorescence detector (excitation/emission wavelength of 278/450 nm) (Shimadzu Corp., Kyoto, Japan) was used. Two hundred and fifty microlitres of serum were treated with 250 μL acetonitrile and vortexed for 2 min and incubated for 15 min at 0 °C (ice). Then, this mixture was again vortexed for 2 min and subjected to centrifugation at 10,000 x g for 20 min at 4 °C. The supernatant (250 μL) was collected, filtered and analysed (20 μL) by HPLC using a mobile phase (acetic acid 2%/acetonitrile; 84/16; v/v) and run at a flow rate of 1.0 mL/min at 40 °C for 15 min. The calibration curve was performed on a pool of serum chicken using the following concentrations (μg/mL): 0.10, 0.25, 0.50, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00.

2.5. Analysis of mutations in the topoisomerase encoding genes

To verify the maintenance or appearance of new mutations in topoisomerase encoding genes of isolated colonies of Ec79, Ec234 and Ec414 strains obtained on the 7th and 14th day of in vitro assays, and colonies of Ec79 and Ec414 strains obtained from in vivo assay (14th day) were investigated. DNA extraction and polymerase chain reaction (PCR) were performed using sets of primers designed for amplification and sequencing of quinolone-resistance determining regions (QRDR) as described elsewhere [14]. PCR products were purified for sequencing using the GFX™ PCR kit (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK)

and the forward and reverse strands were sequenced on an ABI 3500 Genetic Analyzer using a Big-Dye Cycle Sequencing Terminator Kit (Thermo Fisher Scientific, Foster City, CA, USA). Raw sequences were reviewed by visual inspection using the Chromas v.1.45 (32-bit) software (Technelysium Pty Ltd, South Brisbane, Qld, Australia). QRDR nucleotide sequences in *gyrA* and *parC* genes were compared with the respective reference sequences deposited in the GenBank database (accession numbers from AF052253 to AF052260).

3. Results

The present study showed that the PMQRs remained stable in all strains, independently of exposure to ciprofloxacin in both in vivo and in vitro assays. Then, we performed an animal model assay, in which chicks were infected with Ec79 and Ec234 strains separately, to verify if the stability of PMQRs was persistent. During 14 consecutive days, the four groups with eight animals each were kept under exposure to ciprofloxacin (groups 1 and 2) or not (groups 3 and 4). The mean daily consumption of water containing ciprofloxacin 4 μg/mL was 35.5 mL for each group (Fig. 1A). During the trial, a total of nine chicks belonging to the groups that were not exposed to ciprofloxacin died, with six and three deaths related to animals infected with Ec234 and Ec79 strains, respectively. The serum antimicrobial levels in the treated chicks checked by HPLC were maintained during the 14 days (Fig. 1B), eliminating an initial concern that the activity of ciprofloxacin could have been diminished in the presence of light and/or at room temperature. Plasmid persistence was investigated by plasmid extraction analyses of fresh colonies of *E. coli* recovered from the kidney of the animals at the 14th day of being assayed. Plasmids carrying *qnrB2* and *qnrS1* genes were persistent and stable throughout all the generations evaluated. In addition, the presence of PMQR was confirmed by PCR for *qnrS* or *qnrB* genes in the isolates recovered from the chick groups. Moreover, *E. coli* isolates recovered from the microbiota of the chicks included in all groups were also screened before the in vivo assay. Further analysis demonstrated the absence of *qnr* genes in such isolates. Although a diversity of plasmids could be observed in the wild-type *E. coli* strains (data not shown), none of them were similar with those verified in the uropathogenic *E. coli* strains.

To verify the occurrence of possible additional or compensatory changes in QRDR, isolates obtained by in vitro and in vivo assays of all groups had the *gyrA* and *parC* genes sequenced and analysed. The Ec79 and Ec414 strains originally exhibited mutations Ser_{83}Leu and Gly_{91}Arg in the *gyrA* gene, respectively, while the Ec234 strain

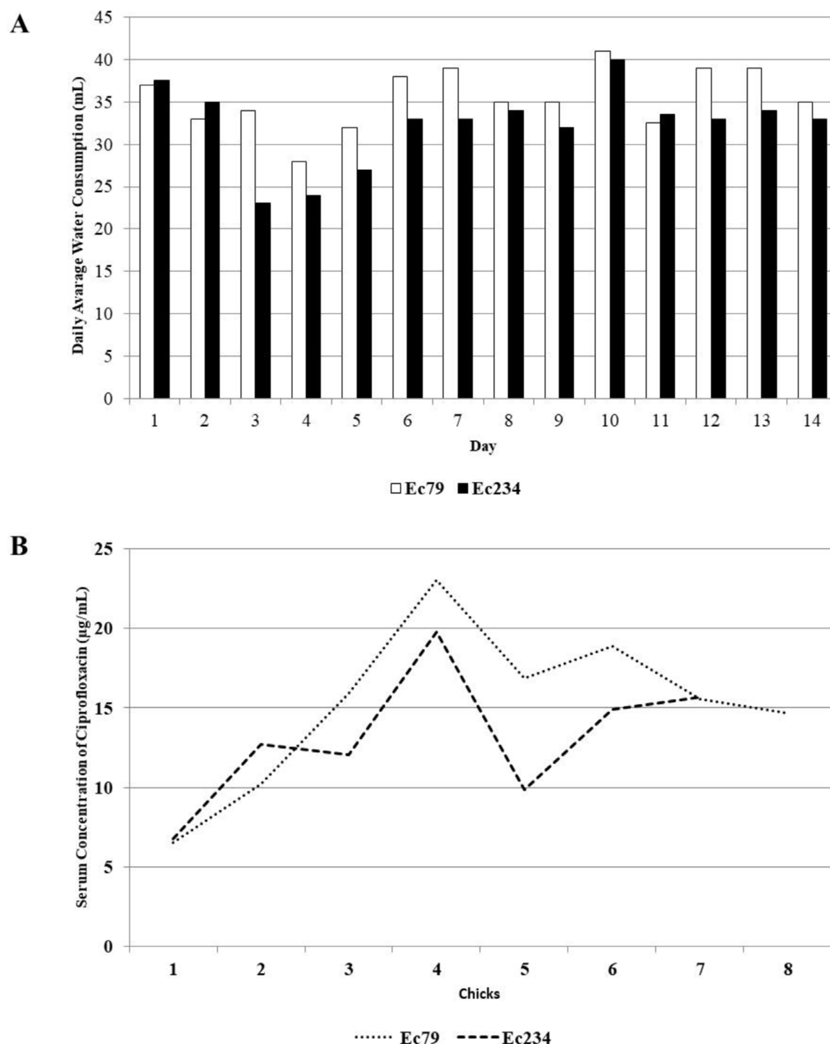

Fig. 1. Daily average water consumption (mL) supplemented with fixed concentration of ciprofloxacin (4 μg/mL) (A) and serum levels of ciprofloxacin after 14 days of exposure (B) in the in vivo assays. For the Ec234 group, one serum level of ciprofloxacin was not obtained.

presented two mutations in *gyrA* (Ser_{83}Phe and Asp_{87}Ala) and one mutation Ser_{80}Ile in *parC* (Table 1). However, analysis of the *gyrA* and *parC* genes for *E. coli* strains obtained during both in vivo and in vitro assays revealed no additional mutations in QRDR.

4. Discussion

The IncN plasmids can carry several antimicrobial resistance genes, including *qnr* determinants, and they present a broad distribution among Enterobacteriales isolates [15]. Moodley and Guardabassi demonstrated using in vitro and in vivo assays the ability of these plasmids to be easily conjugated, indicating a high stability of those elements [16].

In the present study, no plasmid loss was observed after 14 consecutive days of exposure to ciprofloxacin, or even in the absence of selective pressure, in both in vitro and in vivo assays. For the strains exposed to 4 μg/mL of ciprofloxacin, the persistence of the PMQRs would be expected due to the selective pressure exerted by ciprofloxacin. However, even though we know that the strains have significant mutations in QRDR that lead to fluoroquinolone resistance, it was assumed that isolates without PMQR could be preferentially selected because of better bacterial fitness, which was not observed. Interestingly, PMQRs were stable during the experiments, even in the absence of antimicrobial exposure. No isolate demonstrated the loss of the PMQR or additional compensatory mutations in QRDR.

In this study, no loss of PMQRs carrying different *qnr* determinants was observed. These results are similar to those of a previous study that reported the stability of a plasmid harbouring *qnr* [15]. Allou and colleagues evaluated the persistence of the PMQR carrying *qnrA1* and *qnrS1* genes present in *E. coli* after successive cultures on drug-free Mueller–Hinton agar and by in vivo infection models [17]. These authors reported an in vitro loss of only 0.8% and 0.3% of plasmids carrying *qnrA1* and *qnrS1* genes, respectively. However, plasmid loss values were slightly higher, varying from 4.5% to 4.2% for plasmids harbouring *qnrA1* and *qnrS1* in the rat UTI model after 10 days of infection [17]. Machuca and colleagues performed an in vivo and in vitro competition study that indicated no effect on the growth of *E. coli* strains harbouring *qnrA1*, *qnrB1* and *qnrC1* [18]. However, *qnrD1* and *qnrS1* genes alone were able to improve the bacterial fitness, an effect that became even more pronounced in the presence of specific mutations in *gyrA* and *parC*, whereas these mutations associated with the presence of *qnrB1* and *qnrC1* resulted in a decreased bacterial fitness [18]. Two other previous studies also reported the persistence of plasmids carrying distinct *qnrA* variants and *qnrS1* in the presence and absence of selective pressure [19,20]. Such results indicated that a low cost is necessary by the bacterial cell to maintain the PMQR and, consequently, a high persistence of these plasmids. This cost can vary according to the structural variant of Qnr and the presence of mutations in QRDR.

In our study, analysis of *gyrA* and *parC* after in vivo and in vitro assays revealed no changes in the QRDR. The three *E. coli* clinical lineages evaluated in the present study showed the same mutations in QRDR, demonstrating the stability of these mutations even in the absence of ciprofloxacin selective pressure. No additional mutations in QRDR were detected that could confer an enhancement in the fluoroquinolone resistance phenotype after ciprofloxacin exposure, or even the return to the wild-type sequence, i.e. susceptibility to quinolones, even when it could confer advantage on bacterial fitness in a medium free of selective pressure, in which a better bacterial fitness could be required.

In conclusion, we demonstrated that IncN plasmids were persistent in *E. coli* clinical strains independently of exposure to ciprofloxacin, as well as the previous mutations in QRDR. Although we cultured the isolates for only 14 days, our in vitro and in vivo findings are consistent and suggest that resistance to fluoroquinolones may not revert even after restricting the use of quinolones.

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Competing interests

A.C.G. recently received research funding and/or consultation fees from Bayer, Cristália, InfectoPharm, Eurofarma, Pfizer, MSD and Zambon. Other authors have nothing to declare. This study was not financially supported by any Diagnostic/Pharmaceutical company.

Ethical approval

This study was approved by the Committee on Ethics in the Use of Animals (CEUA) of São Paulo University College of Veterinary Medicine (FMVZ-USP) under process number 3247270614.

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