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RESEARCH ARTICLE

Transferable mechanisms of quinolone resistance are more frequent among enterotoxigenic *Escherichia coli* isolates displaying low-level quinolone resistance

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ABSTRACT

This study analysed the mechanisms of quinolone resistance among enterotoxigenic *Escherichia coli* (ETEC) in a periurban area of Lima, Peru. The susceptibility to nalidixic acid and ciprofloxacin, the role of Phe-Arg-β-Naphtylamyde inhibitable-(PAβN) efflux pumps, the presence of mutations in *gyrA* and *parC* as well as the presence of *aac(6')Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrVC* and *oqxAB* were determined in 31 ETEC from previous case/control studies of children's diarrhoea. Discordances between disk diffusion, with all isolates showing intermediate or fully resistance to nalidixic acid, and minimal inhibitory concentration (MIC), with 7 isolates being below considered resistance breakpoint, were observed. Twenty-one isolates possessed *gyrA* mutations (19 S_{83}L, 2 S_{83}A). AAC(6') Ib-cr, QnrS, QnrB and QepA were found in 7, 6, 2 and 1 isolates respectively, with 3 isolates presenting 2 transferable mechanisms of quinolone resistance (TMQR) concomitantly. TMQR were more frequent among isolates with MIC to nalidixic acid ranging from 2 to 16 mg/L (p=0.03), while *gyrA* mutations were more frequent among isolates with nalidixic acid MIC ≥ 128 mg/L (p=0.0002). In summary, the mechanisms of quinolone resistance present in ETEC isolates in Peru have been described. Differences in the prevalence of underlying mechanisms associated with final MIC levels were observed. The results suggest two different evolutive strategies to survive in the presence of quinolones related to specific bacterial genetic background.

Keywords: Epidemiology; public health; GyrA; Qnr; diarrheogenic *Escherichia coli*.

INTRODUCTION

Quinolones are a family of synthetic antibacterial agents which were introduced into clinical practice in the 1960's (Ruiz, 2019). The first reports of resistance to quinolones date from the same decade, but until the late 1980's and early 1990's there were few reports on resistance to these agents (Ruiz, 2019). Thereafter, levels of quinolone resistance increased rapidly and uncontrollably up to the current alarming levels (Ruiz, 2019).

The most common and largely studied mechanism of resistance to quinolones is the development of quinolone target mutations, with those present in *gyrA* and *parC* being the most widely described in *Enterobacteriaceae* (Ruiz, 2003). After 1998, another actor appeared on the scene: the first transferable mechanism of quinolone resistance (TMQR) (Ruiz, 2019). Since then, more than 10 different TMQR families accounting for 3 different mechanisms of action (target protection, antibiotic efflux, and antibiotic modification) have been described (Ruiz, 2019). Of note, the presence of amino acid substitutions in key points of quinolone targets usually results in higher levels of resistance to quinolones than the presence of TMQR, with an additive effect of different mechanisms of resistance in final resistance levels (Ruiz, 2003, 2019).

Overall, the levels of antibiotic resistance in Peru are extremely high irrespective of sample origin, with quinolones not being an exception (Palma *et al*., 2017; Ymaña *et al*., 2022). In addition to the presence of target mutations, previous reports in the country have shown the dissemination of TMQR, mainly *aac(6')Ib-cr* and *qnrB* determinants, with *qnrS* being reported less frequently (Pallecchi *et al*., 2009; Pons *et al*., 2014; Palma *et al*., 2017).

While quinolones remain outside the usual antibacterial armamentarium to treat infections in children, the presence of a relevant number of quinolone-resistant *E. coli* isolates have been

observed in both commensal and diarrhoeagenic *E. coli* from faeces of children (Pons *et al*., 2014; Medina *et al*., 2015). Nevertheless, despite its relevance as a cause of diarrhoea in Peru (Medina *et al*., 2015), the number of enterotoxigenic *Escherichia coli* (ETEC) analysed remains low. To the best of our knowledge, molecular data about the mechanisms of quinolone resistance are only available for 3 Peruvian ETEC isolates (Pons *et al*., 2014). Therefore, data on the mechanisms of resistance, especially regarding TMQR present in ETEC isolates in Peru remain obscure. In this scenario, the aim of this study was to determine the mechanisms of quinolone resistance in ETEC strains isolated from Peruvian children.

MATERIAL AND METHODS

Microorganisms

Thirty-one nalidixic acid (Nal) non-susceptible ETEC isolated during previous studies were recovered from frozen stocks and included in the analysis (Medina *et al*., 2015). Prior to further analysis, the susceptibility to Nal and ciprofloxacin (Cip) was confirmed by disk diffusion (Oxoid, Basingstoke, United Kingdom) in the isolates growing from frozen stocks in accordance with Clinical Laboratory Standard Institute guidelines (CLSI, 2021).

Presence of *gyrA* and *parC* mutations

Fragments of 343bp of *gyrA* and 395 of *parC* containing the so-called "quinolone-resistance determining regions" were amplified by PCR using primers (TIB Molbiol, Berlin, Germany) and procedures previously established (Table 1) (Palma *et al*., 2017). Amplified *gyrA* and *parC* products were gel-recovered (Omega Bio Tek, Norcross, GA) and sequenced (Macrogen, Seoul, South Korea) to establish the presence of point mutations.

Presence of transferable mechanisms of quinolone resistance

The presence of *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *oqxAB* was established by polymerase chain reaction (PCR) using previously described primers (TIB Molbiol) and conditions (Kim *et al*., 2009; Palma *et al*., 2017), while specific primers were designed to amplify a fragment of 499 bp of *qnrVC* following the PCR scheme: 95°C - 5 min, 30 x (95°C - 1 min, 55°C - 1 min, 72°C - 1 min), 72°C - 5 min (Table 1). The presence of *aac(6')Ib-cr* was established by PCR followed by enzymatic digestion with *Bts*C1 (New England Biolabs, Beijing, China) as described previously (Pons *et al*., 2014).

Role of Phe-Arg-β-Naphtylamyde inhibitable efflux pumps

Additionally, the role of efflux pumps was evaluated by determining the minimal inhibitory concentration (MIC) of Nal by agar microdilution as for CLSI guidelines (CLSI, 2021) in the presence and absence of 20 mg/L of Phe-Arg-β-Naphtylamyde (PAβN) (Sigma, Saint Louis, USA) as previously described (Pons *et al*., 2014).

Statistical analysis

Statistical analysis was performed with the GraphPad package (Boston, USA) using the Chi square test, with a p value < 0.05 being considered statistically significant.

RESULTS

The disk-diffusion results showed that no isolate was resistant to Cip and only 6 (19.4%) were categorised as having intermediate resistance, with 10 (32.3%) and 21 (67.7%) isolates presenting resistance and intermediate resistance to Nal. All isolates showing resistance to Nal by disk diffusion were confirmed as resistant when the MIC was performed in the absence of PAβN, while those classified as having intermediate resistance showed MICs ranging from 2 to 64 mg/L (Table 2). Thus, 10 isolates were classified as having intermediate resistance to Nal by disk diffusion. Of these, 7 possessed a MIC of 2 - 16 mg/L, and the remaining 3 had a MIC of 32 - 64 mg/L. When PAβN was added to the MIC, all isolates were classified as susceptible to Nal with MIC values of 2 -16 mg/L (Table 2).

The most common mechanism of quinolone resistance was the presence of alterations at position 83 of GyrA, which were detected in 20 out of 31 isolates (S_{83}A in 2 cases, S_{83}L in 20 cases), in all cases but one in isolates presenting Nal MIC levels $\geq$ 32 mg/L.

The most common TMQR were AAC(6')Ib-cr and QnrS found in 7 and 6 isolates respectively (Figure 1). Additionally, QnrB, was detected in 2 cases and QepA in another (Table 2). Three isolates presented 2 TMQR concomitantly, in 2 cases QnrS plus AAC(6')Ib-cr, and in the another the combination QnrB plus AAC(6')Ib-cr.

The presence of the different mechanisms of resistance varies among isolates with different MIC levels. Thus, TMQR were more frequents among isolates with Nal MIC levels ranging from 2 to 16 mg/L, with 6 out of 7 isolates possessing at least one TMQR (p=0.03). Meanwhile, *gyrA* mutations were more frequents among isolates

Table 1. Primers used in the study

Gene	Primer sequence		Size (bp)	Ann (°C)	Reference
	Forward	Reverse			
gyrA	AAATCTGCCCGTGTCGTTGGT	GCCATACCTACGGCGATACC	343	55	
parC	AAACCTGTTCAGCGCCGCATT	GTGGTGCCGTTAAGCAAA	395	59	
qnrA	AGAGGATTTCTCACGCCAGG	TGCCAGGCACAGATCTTGAC	580	55	
qnrB	GGMATHGAAATTCGCCACTG	TTTGCYGYYCGCCAGTCGAA	264	57	Palma *et al*., 2017
qnrS	GCAAGTTCATTGAACAGGGT	TCTAAACCGTCGAGTTCGGCG	428	55	
qnrC	GGGTTGTACATTTATTGAATCG	CACCTACCCATTTATTTTCA	307	50	
qnrD	TTTTCGCTAACTAACTCGC	GAAAGGATAAACAGGCAAAT	1084	50	
qnrVC	CIAACCTCCGIGATACACA	TGCCACGAICAIATTTTTACACC	499	55	This Study
oqxA	CTCGGCGCGATGATGCT	CCACTCTTCACGGGAGACGA	392	57	Kim *et al*., 2009
oqxB	TTCTCCCCCGGCGGGAAGTAC	CTCGGCCATTTTGGCGCGTA	512	64	
qepA	CGTGTTGCTGGAGTTCTTC	CTGCAGGTACTGCGTCATG	403	60	Palma *et al*., 2017
aac(6')-Ib-cr[1]	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	482	55	

bp: base pair, Ann: Annealing temperature (°C).

[1] The primers detected the *aac(6')Ib-cr* gene; the *aac(6')Ib-cr* variant was determined after PCR digestion with the *Bts*C1 enzyme (Pons *et al*., 2014; Palma *et al*., 2017).

Table 2. Characteristics of the isolates analysed

N	MIC		Disk Diffusion		GyrA	QnrB	QnrS	AAC(6')Ib-cr	QepA
	Nal	Nal+P	Nal	Cip					
1	2	2	I	I	Wt	–	+	–	–
1	2	2	I	S	Wt	–	–	–	–
1	2	2	I	S	Wt	–	+	–	–
1	8	4	I	S	Wt	–	+	+	–
1	16	8	I	S	Wt	+	–	–	–
1	16	8	I	S	Wt	–	+	–	–
1	16	8	I	I	$S_{83}L$	–	+	+	–
1	32	8	I	S	Wt	–	–	+	–
1	32	8	I	S	$S_{83}A$	–	–	–	–
1	32	8	R	S	Wt	–	–	–	–
1	32	8	R	I	Wt	+	–	+	–
1	64	8	I	S	$S_{83}A$	–	–	–	–
1	128	8	R	S	$S_{83}L$	–	–	–	–
1	128	8	R	I	$S_{83}L$	–	–	–	–
1	256	16	R	S	Wt	–	+	–	–
1	256	8	R	S	Wt	–	–	–	–
7	256	8	R	S	$S_{83}L$	–	–	–	–
1	256	8	R	I	$S_{83}L$	–	–	–	–
1	256	8	R	S	$S_{83}L$	–	–	–	+
2	256	8	R	S	$S_{83}L$	–	–	+	–
1	>256	8	R	S	$S_{83}L$	–	–	+	–
1	>256	8	R	I	$S_{83}L$	–	–	–	–
1	>256	16	R	S	$S_{83}L$	–	–	+	–
1	>256	16	R	S	$S_{83}L$	–	–	–	–

N: number; Nal: nalidixic acid; Nal+P: nalidixic acid + Phe-Arg-β-Naphtylamyde; Cip: ciprofloxacin; I: intermediate; S: susceptible; R: resistant; WT: wild type. No *qnrA*, *qnrC*, *qnrD*, *qnrVC* or *oqxAB* genes were detected.

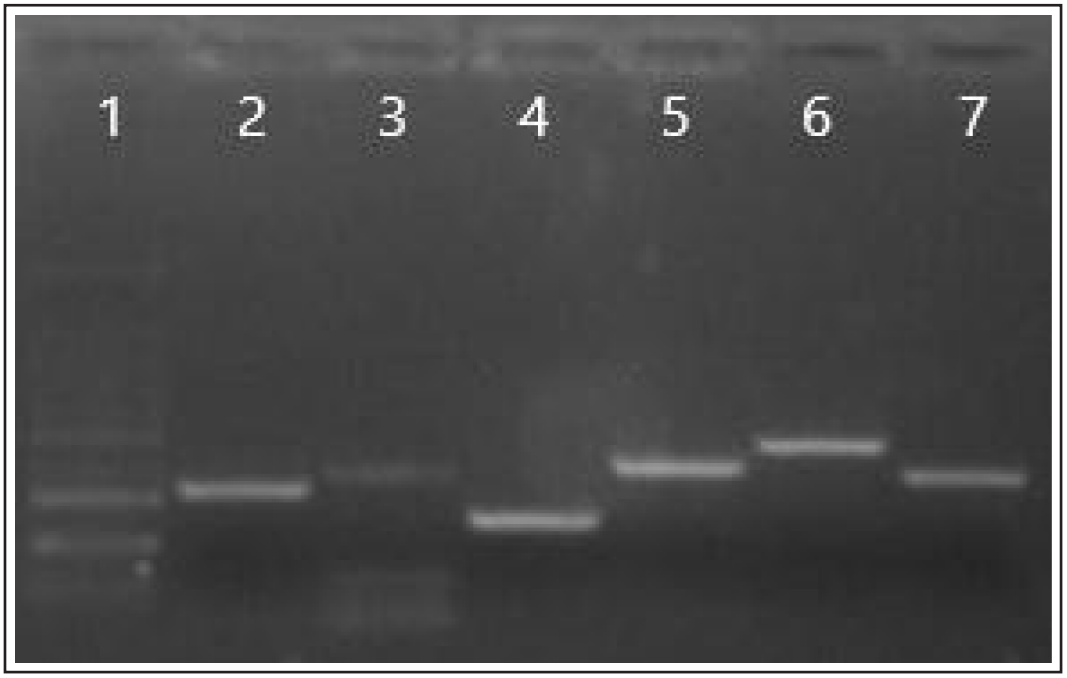

Figure 1. Amplification of quinolone resistance determinants.
Lane 1: 100-1500 pb DNA ladder (Canvax Reagents S.L, Valladolid, Spain); Lane 2: *gyrA* (343bp); Lane 3; *parC* (395bp); Lane 4; *qnrB* (264bp); Lane 5: *qnrS* (428bp); Lane 6: *aac6'(Ib)-cr* (482bp); Lane 7: *qepA* (403bp).

Table 3. Association of MIC levels with the presence of chromosomal or transferanle mechanisms of quinolone resistance

Nal	N	GyrA	TMQR
2-16	7	1 (14.2%)	6 (85.7%)
32-64	5	2 (40.0%)	3 (60.0%)
≥ 128	19	18 (94.7%)	5 (26.3%)
p		<0.001	0.03

Nal: MIC to Nal expressed in mg/L; N: Number; GyrA: isolates with an amino acid codon substitution in GyrA; TMQR: Transferable mechanism of quinolone resistance, when a isolate posses more than one TMQR only is considered once.

with Nal MIC levels ≥ 128 mg/L, with 18 out of 19 isolates (p<0.001) possessing a mutation at amino acid codon 83 of *gyrA* (Table 3).

DISCUSSION

Quinolones have been widely used worldwide, with this resulting in a high selective pressure over microorganisms and leading to the selection of quinolone-resistant microorganisms (Ruiz, 2019). Currently quinolone resistance levels are worrying, with descriptions of quinolone-resistant microorganisms in clinical settings, food samples, livestock, wild animals or environments (Pons *et al*., 2014; Palma *et al*., 2017; Castillo *et al*., 2022; Ymaña *et al*., 2022).

In the present study, and in agreement with previous reports (Saénz *et al*., 2004; Pons *et al*; 2014; Zurfluh *et al*., 2014), the presence of a single mutation in *gyrA* was unable to reach resistance levels to Nal when PAβN was added. This finding highlights the impact and hidden relevance of efflux pumps at basal levels of Nal resistance. At amino acid codon 83 was observed the presence of mutations leading to the presence of S_{83}L and S_{83}A. These amino acid substitutions have been detected in previous studies, with the substitution S_{83}A usually resulting in lesser effects in the final MIC levels than the substitution S_{83}L, as in present study (Ruiz, 2003; Pons *et al*., 2014; Palma *et al*., 2017).

In contrast to QnrS, which was not found among other diarrhoeagenic *E. coli* from Peru, AAC(6')Ib-cr has been observed as the most frequent TMQR in other diarrhoeagenic *E. coli* from the area (Pons *et al*., 2014). Notwithstanding, QnrS has been described in Peru among *E. coli* isolates from both healthy and ill individuals (Pallecchi *et al*., 2009; Palma *et al*., 2017). Meanwhile QnrB has largely been described in Lima and other Peruvian regions (Pallecchi *et al*., 2009; Pons *et al*., 2014; Palma *et al*., 2017), and is probably the most common of the Qnr determinants worldwide (Ruiz, 2019), QepA is a rarely described TMQR in Peru, being first described in the country in 2015 (Rincon *et al*., 2015). To the best of our knowledge, this is the second report of *qepA* in Peru.

All selected isolates were no-susceptible (intermediate or resistant) to Nal, but 7 of them were classified as susceptible when MIC to Nal was established. Discordances between the categorisation of Nal susceptibility using disk diffusion (intermediate) and MIC values (susceptible / resistant) has previously been described in *E. coli* and other *Enterobacteriaceae* (Mensa *et al*., 2008; Pons *et al*., 2014), warning about microorganisms with decreased susceptibility levels to quinolones. The presence of TMQR might explain this phenomenon. Thus, 5 out of 7 isolates presenting this discordant result possessed at least one TMQR, one isolate also presented an additional substitution in amino acid 83 (S_{83}L), and only one isolate remains without an identified mechanism of quinolone resistance.

Of note, significant differences in the prevalence of chromosomal and transferable mechanisms of resistance were observed. While seems reasonable that presence of *gyrA* mutations will be higher in isolates with high levels of resistance to quinolones, no apparent reason seems underly the accumulation of TMQR among isolates with MIC levels to Nal ranging between 2 and 16 mg/L. A plausible hypothesis to explain this phenomenon is a lesser effect on final bacterial fitness related to the presence of TMQR than to the presence of *gyrA* mutations. In this sense, the impact of *gyrA* mutations in bacterial fitness has been observed in other microorganisms (Fabrega *et al*., 2014), and differences in the prevalent mechanisms of quinolone resistance within isolates becoming to the same species have been related to specific genetic background, alerting about possible fitness compensatory mechanisms (Horna *et al*., 2019). While the presence of TMQR is considered a risk factor for the development of additional mechanisms of resistance, *E. coli* isolates lacking TMQR have been shown to have a greater facility for acquiring mutations in genes encoding quinolone targets than *E. coli* isolates possessing TMQR (Cesaro *et al*., 2008; Goto *et al*., 2015; Vinué *et al*., 2016).

Thus, microorganisms have two different strategies to survive in the presence of the concentrations of quinolones to which they are exposed. In this sense, it has been proposed that isolates with TMQRs, by already possessing a certain level of resistance to quinolones, would have a greater facility to develop mutations in genes other than those encoding for quinolone targets, being able to survive in the presence of concentrations of quinolones at would usually be exposed (Cesaro *et al*., 2008). The advantage of this strategy would be to avoid deleterious effects on fitness related to the presence of mutations in the targets of quinolones. This strategy would be especially beneficial in the presence of relatively low concentrations of quinolones. On the other side, isolates without TMQRs, would not reach sufficient levels of resistance without developing mutations in the quinolone targets. In addition, these isolates will be impacted by both the fitness cost related to target mutations, as well as the need to invest energy in plasmid replication. Thereby, the presence of TMQR will also be negatively selected in isolates carrying chromosomal mutations in quinolone's targets.

Regarding present isolates, quinolones are not used in the treatment of children infections, thereby ETEC have a limited exposure to quinolones, and the presence of mechanisms leading to low-levels of quinolone resistance with a low impact on bacterial fitness might be a good evolutive strategy. Furthermore, the use of other antimicrobial agents might favour the co-selection of genetic structures (Vien *et al*., 2012; Murase *et al*., 2022).

The main limitation of the study is the sample size. Nevertheless, the presence of different circulating quinolone resistance mechanisms is highlighted, with differences in the prevalence of chromosomal and transferable quinolone resistance mechanisms being observed.

In summary, the mechanisms of quinolone resistance present in ETEC isolates in Peru have been described, with differences in the underlying mechanisms associated with final MIC levels being established. Continuous surveillance of levels and related mechanisms of antimicrobial resistance is essential to adequately fight the increasing levels of antimicrobial resistance.

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Conflict of interes statement

The author declares that they have no conflict of interests.

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