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Research Paper

Susceptibility to β-lactams and quinolones of Enterobacteriaceae isolated from urinary tract infections in outpatients

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Abstract

The antibiotic susceptibility profile was evaluated in 71 Enterobacteriaceae isolates obtained from outpatient urine cultures in July 2010 from two health institutions in Santa Fe, Argentina. The highest rates of antibiotic resistance were observed for ampicillin (AMP) (69%), trimethoprim/sulfamethoxazole (TMS) (33%), and ciprofloxacin (CIP) (25%). Meanwhile, 21% of the isolates were resistant to three or more tested antibiotics families. Thirty integron-containing bacteria (42.3%) were detected, and a strong association with TMS resistance was found. Third generation cephalosporin resistance was detected in only one *Escherichia coli* isolate, and it was characterized as a bla_{CMY-2} carrier. No plasmid-mediated quinolone resistance (PMQR) was found. Resistance to fluoroquinolone in the isolates was due to alterations in QRDR regions. Two mutations in GyrA (S83L, D87N) and one in ParC (S80I) were observed in all CIP-resistant *E. coli*. It was determined to be the main phylogenetic groups in *E. coli* isolates. Minimum Inhibitory Concentration (MIC) values against nalidixic acid (NAL), levofloxacin (LEV), and CIP were determined for 63 uropathogenic *E. coli* isolates as MIC_{50} of 4 μg/mL, 0.03125 μg/mL, and 0.03125 μg/mL, respectively, while the MIC_{90} values of the antibiotics were determined as 1024 μg/mL, 64 μg/mL, and 16 μg/mL, respectively. An association between the phylogenetic groups, A and B1 with fluoroquinolone resistance was observed. These results point to the importance of awareness of the potential risk associated with empirical treatment with both the families of antibiotics.

Key words: urine tract infection, outpatient, β-lactam resistance, fluoroquinolone resistance, integrons.

Introduction

Urinary tract infections (UTIs) are the second most common cause of human infections, next to respiratory tract infections (Foxman, 2003). In Argentina, UTIs are the most frequent reasons behind an outpatient medical consultation. Furthermore, 95% of UTIs are caused by a single microbial species, *Escherichia coli*, which is a main etiologic agent; while other species such as *Klebsiella* spp. and *Proteus* spp. have also been reported occasionally (Auer *et al.*, 2010). Among *E. coli*, four major phylogenetic groups (A, B1, B2, and D) have been identified as causal agents of extra-intestinal infections. Usually, commensal strains belong to A and B1 groups and contain low number of virulence determinants, while extra-intestinal pathogenic strains belong mainly to B2 group and to a lesser extent to D group and contain genes encoding virulence factors responsible for promoting colonization, adhesion, invasion, and evasion of the defense mechanisms of the human host (Clermont *et al.*, 2000).

Currently, most antibiotic treatments for UTIs are empirical, particularly for those acquired in the community. In general, most of the prescribed antimicrobial agents

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belong to β-lactams or fluoroquinolones groups (Aypak *et al.*, 2009). Most widely used β-lactams include aminopenicillins (ampicillin) and first-generation cephalosporins (cephalothin and cephalexin), while new generation cephalosporins may be considered as reserve antibiotics. The production of β-lactamases is the key mechanism of resistance to β-lactam antibiotics in gram-negative bacilli (Gutkind *et al.*, 2013).

On the other hand, ciprofloxacin and norfloxacin are the fluoroquinolones commonly prescribed for treatment of UTIs. Different chromosomally encoded mechanisms of quinolone resistance have been established, viz. mutations in quinolone resistance determining regions (QRDR) of *gyrA* and *parC* genes and decreased accumulation of the drug due to impermeability of the outer membrane and/or over-expression of efflux pump systems (Ruiz, 2003). Furthermore, plasmid-mediated quinolone resistance (PMQR) genes have recently been described in Enterobacteriaceae species, including *qnr* genes (*qnrA*, *qnrB*, *qnrS*, *qnrC,* and *qnrD*), the modified acetyltransferase *aac(6')-Ib-cr,* and the efflux pumps *qepA* and *oqxAB* (Andres *et al.*, 2013). Most of these determinants could be associated with resistance integrons which may be embedded in elements related to horizontal gene transfer (HGT). Resistance integrons (or mobile integrons) are elements that contain genetic determinants of the components of a system for site-specific recombination that recognizes and captures resistance genes in mobile cassettes (Di Conza and Gutkind 2010). Class 1, 2, and 3 integrons are widely associated with resistance determinants in human clinical isolates (Boucher *et al.*, 2007).

The aim of this study was to determine the antibiotic susceptibility profile in Enterobacteriaceae isolated from outpatient urine cultures and evaluate their association with the presence of resistance integrons. In addition, third-generation cephalosporins and quinolones resistance determinants were characterized, and phylogenetic group of *E. coli* isolates was determined.

Materials and Methods

The study was carried out in Santa Fe city in July 2010. A total of 260 urine cultures from outpatients with symptoms of UTIs were included in this report. Etiologic agents were found in 85 out of 260 (33%) samples, and 71 out of 78 (91%) gram-negative bacilli were Enterobacteriaceae isolates, which have been included in this study.

The isolates were identified using conventional biochemical and physiological tests. The antibiotic susceptibility profile was determined by disk diffusion according to CLSI guidelines (CLSI 2010) and *Sociedad Argentina de Bacteriología, Micología y Parasitología Clínica* (SADEBAC) recommendations (Famiglietti *et al.*, 2005). The antibiotics tested were ampicillin (AMP), ampicillin/sulbactam (AMS), cephalothin (CTN), third-generation cephalosporins (3GC) as cefotaxime (CTX), ceftazidime (CAZ), and other antibiotics such as gentamicin (GEN), ciprofloxacin (CIP), nitrofurantoin (NIT), and trimethoprim/sulfamethoxazole (TMS). The minimum inhibitory concentration (MIC) of nalidixic acid (NAL), levofloxacin (LEV), and CIP was determined by agar dilution method as recommended by CLSI guideline (CLSI 2010).

Phenotypic identification of extended spectrum (ESBL) and AmpC β-lactamases were performed in those isolates that showed resistance to 3GC by synergy tests using CTX and CAZ and compared with CTX/clavulanic acid and CAZ/clavulanic acid-containing disks (CLSI 2010) or with phenylboronic acid disks (Britania Lab, Argentina) (Yagi *et al.*, 2005), respectively.

The presence of class 1, 2, and 3 integrons, unusual class 1 integrons, PMQR (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD, qepA,* and *aac(6')-Ib-cr*), and β-lactamases (bla_{DHA} and bla_{CMY} for AmpC) genes were studied by PCR using specific primers (Table 1). The confirmation of *aac(6')-Ib-cr* variant was performed by RFLP-PCR using *BseG I* enzyme (Fermentas, Thermo Fisher Scientific Inc., Massachusetts, USA) and sequencing (Rincón *et al.*, 2013). The presence of mutations in the QRDR regions was studied in fluoroquinolone-resistant *E. coli* by amplification and sequencing of *gyrA* and *parC* genes (Rodríguez-Martínez *et al.*, 2006).

Finally, the phylogenetic group of all *E. coli* isolates was determined by PCR according to the method described by Clermont *et.al.* 2000.

Results

Out of all Enterobacteriaceae recovered (n = 71), 63 were identified as *E. coli* (88%), 6 as *K. pneumoniae* (9%) and 2 as *P. mirabilis* (3%).

The antibiotic susceptibility profile of 71 isolates studied is summarized in Table 2. It should be emphasized that 15 (21%) isolates were resistant to three or more tested antibiotics groups.

This study showed that 30 (42%) isolates were carrying integrons. Of these 30 isolates, 23 had class 1 integrons (77%), one had class 1 unusual integron (positive *orf513*), and 9 (30%) had class 2 integrons, highlighting the fact that two of *E. coli* isolates (6.7%) shared both classes of integrons. None of the isolates were found to contain class 3 integrons. Fisher's exact test failed to find any association between the presence of integrons and resistance to AMP, AMS, CTN, CTX, CAZ, GEN, CIP, or NIT ($p > 0.05$). However, a strong association between resistance to TMS and the presence of integrons ($p = 0.0003$) was observed.

Only one *E. coli* isolate was both CTX and CAZ resistant and showed synergistic effect between 3GC and phenylboronic acid suggesting the presence of AmpC β-lactamase. This isolate belonged to the phylogenetic group B1. PCR and subsequent sequencing revealed that this isolate

Table 1 - PCR primers used to detect integrons or resistance genes and the expected sizes of amplicon.

Target	Primer name	Primers (5' → 3')	Amplicon size (bp)	Reference
intI1	I5 (IntI1 F)	ACCGCCAACTTTCAGCACAT	930	Di Conza *et al*., 2002
	I3 (IntI1 B)	GCGTTCGGTCAAGGTTCTGG		
intI2	intI2 F	TTATTGCTGGGATTAGGC	223	Goldstein *et al*., 2001
	intI2 R	ACGGCTACCCTCTGTTATC		
intI3	intI3 F	TGTTCTTGTATCGGCAGGTG	600	Goldstein *et al*., 2001
	intI3 R	AGTGGGTGGCGAATGAGTG		
orf513	341A	CGCCCACTCAAACAAACG	468	Sabaté *et al*., 2002
	341B	GAGGCTTTGGTGTAACCG		
qnrA	QnrAm-F	AGAGGATTTCTCACGCCAGG	580	Cattoir *et al*., 2007
	QnrAm-R	TGCCAGGCACAGATCTTGAC		
qnrB	QnrBm-F	GGMATHGAAATTCGCCACTG	264	Cattoir *et al*., 2007
	QnrBm-R	TTTGCYGYYCGCCAGTCGAA		
qnrC	qnrC-F	GGGTTGTACATTTATTGAATC	307	Wang *et al*., 2009
	qnrC-R	TCCACTTTACGAGGTTCT		
qnrD	qnrD-F	CGAGATCAATTTACGGGGAATA	581	Covaco *et al*., 2009
	qnrD-R	AACAAGCTGAAGCGCCTG		
qnrS	QnrSm-F	GCAAGTTCATTGAACAGGGT	428	Cattoir *et al*., 2007
	QnrSm-R	TCTAAACCGTCGAGTTCGGCG		
qepA	QepA-GF	ACATCTACGGCTTCTTCGTCG	502	Rincón *et al*., 2013
	QepA-GR	AACTGCTTGAGCCCGTAGATC		
aac(6')-Ib-cr	AAC(6')-F	CGATCTCATATCGTCGAGTG	477	Rincón *et al*., 2013
	AAC(6')-R	TTAGGCATCACTGCGTGTTC		
bla_{CMY}	CITM F	TGGCCAGAACTGACAGGCAAA	462	Pérez-Pérez and Hanson, 2002
	CITM R	TTTCTCCTGAACGTGGCTGGC		
bla_{DHA}	DHAM F	AACTTTCACAGGTGTGCTGGGT	405	Pérez-Pérez and Hanson, 2002
	DHAM R	CCGTACGCATACTGGCTTTGC		

carried the bla_{CMY-2} gene (a plasmid AmpC enzyme, AmpCp).

The search for PMQR determinants ruled out the presence of *qnr* genes, *qepA* efflux pump, and allelic variant *aac(6')-Ib-cr* over all of the isolates analyzed. Only acetylating variant, *aac(6')-Ib* with activity towards aminoglycosides was found in 5 of 71 isolates (3 *K. pneumoniae* and 2 *E. coli*). MIC_{50} values to NAL, LEV, and CIP, determined for the 63 uropathogenic *E. coli* isolates, were 4 μg/mL, 0.03125 μg/mL, and 0.03125 μg/mL, respectively; while the MIC_{90} values for the same antibiotics were 1024 μg/mL, 64 μg/mL, and 16 μg/mL, respectively.

The absence of PMQR in these isolates makes one to suspect that fluoroquinolone resistance in these isolates was due to mutations in the QRDR regions. As expected, all fluoroquinolone-resistant *E. coli* (n = 13) have been found to contain two mutations in the *gyrA* sequence (Ser83Leu and Asp87Asn) and at least one in *parC* (Ser80Ile). A sin-

Table 2 - Antibiotic susceptibility profile of 71 studied isolates.

Species	Number of resistant isolates (%)								
	AMP	AMS	CTN	CTX	CAZ	GEN	CIP	NIT	TMS
E. coli (n = 63)	42	15	13	1	1	7	13	2	20
K. pneumoniae (n = 6)	6	3	3	0	0	3	4	4	3
*P. mirabili*s (n = 2)	1	0	0	0	0	0	1	2	1
Total (n = 71)	49 (69%)	18 (25%)	16 (22%)	1 (1.4%)	1 (1.4%)	10 (14%)	18 (25%)	8 (11%)	24 (33%)

AMP: ampicillin, AMS: ampicillin/sulbactam, CTN: cephalothin, CTX: cefotaxime, CAZ: ceftazidime, GEN: gentamicin, CIP: ciprofloxacin, NIT: nitrofurantoin, TMS: trimethoprim/sulfamethoxazole.

gle isolate showed a second substitution in *parC* (Glu84Gly).

The distribution of the phylogenetic groups of the 63 *E. coli* isolates was 16 A, 11 B1 , 11 B2, and 25 D, showing a higher percentage of isolates belonging to B2 and D groups (57%) with respect to those linked to commensal strains (A and B1 groups: 43%).

When assessing the association between fluoroquinolone susceptibility profile and its distribution into the four phylogenetic groups, a significant difference was observed ($p = 0.0111$). Further analysis showed that 10 of 13 fluoroquinolones resistant isolates (76.9%) belonged to the phylogenetic groups, A and B1, while 33 of 50 non-resistant fluoroquinolone isolates (66.0%) belonged to the groups, B2 and D ($p = 0.0100$). These results suggest that fluoroquinolone-susceptible *E. coli* strains would have more virulence determinants since they belong to the phylogenetic groups, B2 and D. In contrast, there was a strong association between fluoroquinolone-resistance strains and A and B1 phylogenetic groups, suggesting that the presence of these resistance mechanisms would favor *E. coli* clones to become successful commensals.

Discussion

As expected, species distribution of Enterobacteriaceae showed that *E. coli* is the predominant bacteria in cases of UTI (Auer *et al.*, 2010).

The high prevalence (42%) of integrons found in studied isolates should be considered as a wake-up call, because of the latent ability of these genetic platforms to recruit novel resistance mechanisms and promote the emergence of multidrug resistant isolates. On the other hand, the presence of integrons was found to be associated with TMS resistance, a fact which can be determined by analyzing 3-terminal conserved region of class 1 integrons where the *sul1* gene is commonly located, which confers resistance to sulfonamides (Di Conza and Gutkind, 2010).

A unique 3GC resistant isolate harboring $bla_{\text{CMY-2}}$ gene was detected among the isolates derived from these patients. Within AmpCp, this β-lactamase is the most widely distributed in the world and has previously been described in UTIs caused by *E. coli* from outpatients in Argentina (Cejas *et al.*, 2012).

This study has demonstrated the absence of PMQR determinants in Enterobacteriaceae causing outpatient UTIs, regardless of whether these isolates are susceptible or resistant to fluoroquinolones. Although there are many reports describing the presence of these PMQR determinants in Argentina (Andres *et al.*, 2013; Rincón *et al.*, 2013; Rincón *et al.*, 2014), comparisons with our work should be carefully made due to the difference in criteria of selection of the bacteria used in these studies. The lack of statistical association between the presence of integrons and CIP resistance is consistent with the absence of PMQR determinants, particularly of allelic variant *aac(6')-Ib-cr*, which has been described as cassettes in the variable region of class 1 integrons (Di Conza and Gutkind, 2010).

Interestingly, in this work, a strong association between fluoroquinolone-resistant *E. coli* and A and B1 phylogenetic groups (considered commensal) was observed. Other studies have shown that acquisition of resistance determinants and the expression of a multidrug resistance phenotype is associated with a decrease in virulence of *E. coli* isolates (Molina-López, 2011). Furthermore, some evidences suggest that quinolones resistance in *E. coli* may be associated with the loss of certain virulence factors such as expression of β-hemolysis and P fimbriae, a condition that can be attributed to a decrease in the activities of gyrase and topoisomerase due to mutations in the QRDR region responsible for resistance to these antibiotics (Drews *et al.*, 2005).

In conclusion, this study reports a detailed characterization of uropathogenic Enterobacteriaceae isolates derived from outpatients in Santa Fe city, Argentina. The highest degrees of resistance were observed for AMP, TMS and CIP. A high percentage of integrons (42%) was also detected. The ability of these genetic platforms to recruit antibiotic resistance cassettes efficiently is a potential threat to the emergence of multidrug-resistant isolates. In particular, all uropathogenic *E. coli* isolated did not show PMQR determinants, and mutations in QRDR regions were observed in those fluoroquinolone-resistant isolates.

Moreover, marked differences between fluoroquinolone-susceptible profile and phylogenetic groups in *E. coli* strains were observed. A subsequent analysis showed a correlation between fluoroquinolone resistant isolates and phylogenetic groups considered potentially less virulent (A and B1), and vice versa. Finally, periodic surveillance studies are recommended to review the use of β-lactams, fluoroquinolones, and TMS while choosing empirical treatment for UTIs.

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