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

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Submitted: October 21, 2014; Approved: March 30, 2015.

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 MIC₅₀ of 4 μg/mL, 0.03125 μg/mL, and 0.03125 μg/mL, respectively, while the MIC₉₀ 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...
belong to β-lactams or fluoroquinolones groups (Aypak et al., 2009). Most widely used β-lactams include amino-
penicillins (ampicillin) and first-generation cephalosporins (cephalothin and cephalaxin), while new generation cepha-
losporins may be considered as reserve antibiotics. The production of β-lactamases is the key mechanism of resist-
tance to β-lactam antibiotics in gram-negative bacilli (Gut-
kind 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). Fur-
thermore, plasmid-mediated quinolone resistance (PMQR)
genesis determinants of the components of a system for
horizontal gene transfer (HGT). Resistance
integrons (or mobile integrons) are elements that contain
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-
geneneration cephalosporins and quinolones resistance deter-
minants 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 Enterobac-
teriaceae isolates, which have been included in this study.

The isolates were identified using conventional bio-
chemical and physiological tests. The antibiotic suscep-
tibility 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), ampicil-
lin/sublactam (AMS), cephalothin (CTN), third-generation
cephalosporins (3GC) as cefotaxime (CTX), cefazidime
(CAZ), and other antibiotics such as gentamicin (GEN),
ciprofloxacin (CIP), nitrofurantoin (NIT), and trimetho-
prim/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 us-
ing 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 (blaDHA and
blaCMY for AmpC genes) were studied by PCR using spe-
cific primers (Table 1). The confirmation of aac(6’)-Ib-cr
variant was performed by RFLP-PCR using BseGI 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 fluoro-
quinolone-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 carry-
ing 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, 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 resis-
tant and showed synergistic effect between 3GC and
phenylboronic acid suggesting the presence of AmpC β-lacta-
mase. This isolate belonged to the phylogenetic group B1.
PCR and subsequent sequencing revealed that this isolate
carried the bla\textsubscript{CMY-2} gene (a plasmid AmpC enzyme, AmpCp).

The search for PMQR determinants ruled out the presence of \textit{qnr} genes, \textit{qepA} efflux pump, and allelic variant \textit{aac(6')-Ib-cr} over all of the isolates analyzed. Only acetylation variant, \textit{aac(6')-Ib} with activity towards aminoglycosides was found in 5 of 71 isolates (3 \textit{K. pneumoniae} and 2 \textit{E. coli}). MIC\textsubscript{50} values to NAL, LEV, and CIP, determined for the 63 uropathogenic \textit{E. coli} isolates, were 4 µg/mL, 0.03125 µg/mL, and 0.03125 µg/mL, respectively; while the MIC\textsubscript{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 \textit{E. coli} (\(n = 13\)) have been found to contain two mutations in the \textit{gyrA} sequence (Ser83Leu and Asp87Asn) and at least one in \textit{parC} (Ser80Ile).

| Target       | Primer name     | Primers (5' → 3')                      | Amplicon size (bp) | Reference                                      |
|--------------|-----------------|----------------------------------------|--------------------|------------------------------------------------|
| \textit{intI1} | I5 (IntI1 F)    | ACCGCCAAGCTTTCACGACCAT                 | 930                | Di Conza et al., 2002                         |
|              | I3 (IntI1 B)    | GCGTTCCGTCAAGGTTCCTGG                 |                    |                                                 |
| \textit{intI2} | int2 F          | TTATTGCTGGAGATTAGGC                   | 223                | Goldstein et al., 2001                        |
|              | int2 R          | ACCTGTACGCTTGTTATC                    |                    |                                                 |
| \textit{intI3} | int3 F          | TGTCTTTGTAAGGCGAGGGTT                 | 600                | Goldstein et al., 2001                        |
|              | int3 R          | AGTTGGTGACGAGATGAGG                   |                    |                                                 |
| \textit{orf513} | 341A            | CGCCCACTTAAAACAAACG                   | 468                | Sabaté et al., 2002                           |
|              | 341B            | GAGGCTTTGTGGTGCAA                    |                    |                                                 |
| \textit{qnrA} | QnrAm-F         | AGAGGATTTTCTACGCCAGG                  | 580                | Cattoir et al., 2007                          |
|              | QnrAm-R         | TGCCAGCCCACAGATCTTGAC                 |                    |                                                 |
| \textit{qnrB} | QnrBm-F         | GMATHTGAATGGCGCAGCCTGTG               | 264                | Cattoir et al., 2007                          |
|              | QnrBm-R         | TTTGCYGGYCCGGACGTGCGG                 |                    |                                                 |
| \textit{qnrC} | qnrC-F          | GGGTTGTAACATTATGGAATC                 | 307                | Wang et al., 2009                             |
|              | qnrC-R          | TCCACTTTACGGAGTTCT                   |                    |                                                 |
| \textit{qnrD} | qnrD-F          | CGAGATCATTACGGGAGGTA                  | 581                | Covaco et al., 2009                           |
|              | qnrD-R          | AACAAAGGGAAGCCTGCGG                   |                    |                                                 |
| \textit{qnrS} | QnrSm-F         | GCAAATTCATTGAAACAGGGT                 | 428                | Cattoir et al., 2007                          |
|              | QnrSm-R         | TCTAACCAGGAGGCTGGG                    |                    |                                                 |
| \textit{qepA} | QepA-GF         | ACATCTACGGCTTCCTGCGGT                 | 502                | Rincón et al., 2013                           |
|              | QepA-GR         | AACTCTCTGACGGCCTGATC                  |                    |                                                 |
| \textit{aac(6')-Ib-cr} | AAC(6')-F | CGATTCCTATATCGTCGAGTG | 477 | Rincón et al., 2013 |
|              | AAC(6')-R        | TTAACCGGACTACGGCTGGTC                 |                    |                                                 |
| \textit{bla\textsubscript{CMY}} | CITM F | TGCCACGAACTGACGCAAGCAA | 462 | Pérez-Pérez and Hanson, 2002 |
|              | CITM R          | TTTTTCTGACGGCGCGCTGGCG                 |                    |                                                 |
| \textit{bla\textsubscript{DHA}} | DHAM F | AACCTTCACAGGTGTGCTGGGT | 405 | Pérez-Pérez and Hanson, 2002 |
|              | DHAM R          | CCGTACGACATTGGTTTGCC                  |                    |                                                 |

Table 2 - Antibiotic susceptibility profile of 71 studied isolates.

| Species                | AMP | AMS | CTN | CTX | CAZ | GEN | CIP | NIT | TMS |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| \textit{E. coli} (\(n = 63\)) | 42  | 15  | 13  | 1   | 1   | 7   | 13  | 2   | 20  |
| \textit{K. pneumoniae} (\(n = 6\)) | 6   | 3   | 3   | 0   | 0   | 3   | 4   | 4   | 3   |
| \textit{P. mirabilis} (\(n = 2\)) | 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 fluoroquinolone-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-resistant 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 sull gene is commonly located, which confers resistance to sulfonamides (Di Conza and Gutkind, 2010).

A unique 3GC resistant isolate harboring bla\textsubscript{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.

Acknowledgments

This work was supported by CAI+D - UNL to JDC. Positive controls were kindly ceded by Dr Nordman (qnrA, B and S), Dr Wang (qnrC) and Dr Kunikazu Yamane [qepA y aac(6')-Ib-cr].

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Associate Editor: Ana Lúcia da Costa Darini

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