Evaluation of the Susceptibility Profiles, Genetic Similarity and Presence of *qnr* Gene in *Escherichia coli* Resistant to Ciprofloxacin Isolated in Brazilian Hospitals

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Increasing quinolone resistance has been reported worldwide, mainly among clinical isolates of *Escherichia coli*. The objectives of this study were to determine the susceptibility profile, the genetic relatedness, and the prevalence of the *qnr* gene among ciprofloxacin-resistant *Escherichia coli* isolated from distinct Brazilian hospitals. A total of 144 ciprofloxacin-resistant *Escherichia coli* were isolated from 17 Brazilian hospitals between January/2002 and June/2003. The antimicrobial susceptibility testing was performed by microdilution according to NCCLS. The presence of the *qnr* gene was initially screened by colony blotting, and then confirmed by PCR followed by DNA sequencing. Ninety-five urinary ciprofloxacin-resistant *Escherichia coli* were further selected for molecular typing by pulsed-field gel electrophoresis (PFGE). Imipenem and meropenem showed the highest susceptibility rates (100.0% for both compounds) followed by amikacin (91.0%) and piperacillin/tazobactan (84.8%). A single ciprofloxacin-resistant *Escherichia coli* isolate was positive for *qnr* among the 144 ciprofloxacin-resistant *Escherichia coli*. Forty-six PFGE patterns were observed among the 95 ciprofloxacin-resistant *Escherichia coli* type. This study shows that therapeutic options are limited for treatment of ciprofloxacin-resistant *Escherichia coli* due to the presence of additional mechanisms of antimicrobial resistance, such as ESBL production. The *qnr* gene was uncommon among ciprofloxacin-resistant *Escherichia coli* clinical isolates, but its identification might indicate the emergence of this mechanism of quinolone resistance in Brazil. The great genomic variability found among the ciprofloxacin-resistant *Escherichia coli* highlights the importance of the appropriate use of quinolone to restrict the selection of resistant isolates.

Key-Words: Quinolone resistance, *Escherichia coli*, PFGE, qnr.

Escherichia coli is a leading cause of nosocomial as well as community-acquired infections, especially urinary tract infections (UTI) [1,2]. Recent studies have shown a considerable increase in the prevalence of resistance to ciprofloxacin among clinical isolates of *Escherichia coli* [3,4]. Although quinolone resistance generally results from chromosomal mutation in *gyr*A and *par*C genes, recent studies showed that low-level resistance can be also plasmid mediated by the acquisition of the *qnr* gene mediated by plasmid pMG252 [5-7]. This plasmid was first report in 1998 in a *Klebsiella pneumoniae* clinical strain isolated in 1994 at the University of Alabama at Birmingham Medical Center [8], but has still not been detected among isolates from South America.

The objectives of this study were to determine the susceptibility profile, the genetic relatedness, and the frequency of the *qnr* gene among ciprofloxacin-resistant *Escherichia coli* (CR-EC) isolated from distinct Brazilian hospitals.

Materials and Methods

Participant Institutions

A total of seventeen medical centers participated in the study. The centers were located throughout eight Brazilians estates: Bahia (one center), Minas Gerais (two centers), Brasília (one center), Ceará (two centers), Maranhão (one center), Paraná (two centers), Rio Grande do Sul (one center), Santa Catarina (one center) and São Paulo (six centers) (Figure 1).

Received on 16 August 2006; revised 15 December 2006.
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The Brazilian Journal of Infectious Diseases 2007;11(1):40-43.

Bacterial Isolates

The Brazilian hospitals were requested to send CR-EC isolates to the coordinating laboratory. From January 2002 to June 2003, a total of 144 clinical isolates of CR-EC were isolated from the 17 hospital-based laboratories. Only one isolate per patient was included in the study. The isolates were identified at the participating institution by the routine methodology in use at each laboratory. Confirmation of species identification was performed by biochemical conventional methods if necessary.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing was performed using the reference broth microdilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS 2003). The minimal inhibitory concentration (MIC) was defined as the lowest antimicrobial concentration able to totally inhibit bacterial growth. Quality control was performed by testing *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 70603.

According to NCCLS criteria, MICs ≥ 2 μg/mL for cefotaxime and/or ceftriaxone and/or ceftazidime and/or aztreonam were considered as extended-spectrum β-lactamase (ESBL) producers [9]. The ESBL phenotype was confirmed by using the addition of clavulanic acid (Oxoid® Basingstoke, England) according to manufacture recommendation.

Screening for the *qnr* Gene in Clinical Strains EC-QR

All clinical strains of EC-QR were screened for the *qnr* gene by the colony blotting and Southern hybridization methods using the kit "Ready to go" (Amersham Pharmacia Biotech Inc., New Jersey, EUA), in accordance with the manufacture's recommendations. The *qnr* probe was made from plasmid pMG252 by PCR amplification with primers *sense* 5' GATAAAGTTTTTCAGCAAGAGG 3' and *anti-sense* 5' ATCCAGATCGGCAAAGGTTA 3'.

PCR Detection of *qnr* Gene

The gene was amplified by using primers QP1 (5' GATAAAGTTTTTCAGCAAGAGG 3'starting at the 12th *qnr* nucleotide) and QP2 (5' ATCCAGATCGGCAAA GGTTA 3') to produce a 593-bp product, as described by Jacoby et al. [6]. PCR conditions were 94°C for 1 min, 57°C for 30 s, and 72°C for 1 min for 30 cycles.

Quality Control

The QnrA-producing *Klebsiella pneumoniae* pMG252 and *Escherichia coli* ATCC 25922 were used as positive and negative quality controls for hybridization and PCR experiments, respectively.

Nucleotide Sequence of *qnr* Gene

Sequencing was performed using Big Dye terminator version II (BigDye™ Terminators v2.0 – Cycle Sequencing Kit – Applied biosystems-ABI PRISM®, California, EUA) and in equipment an ABI PRISM 377 DNA Analyzer (Perkin Elmer®, California, EUA) reading was performed. The nucleotide sequence has been submitted to the GenBank database and assigned accession number AM295981.

Evaluation of Genetic Similarity in CR-EC Isolates

CR-EC collected from urinary tract infections were selected for molecular typing by Pulsed-field gel electrophoresis (PFGE). From each medical center, a maximum of 10 CR-EC isolates were included for analysis. Genomic DNA was prepared for restriction fragments analysis as described by Pfaller [10] and digested with SpeI restriction enzyme (New England Biolab, Inc., Beverly, USA). PFGE was performed on the CHEF DR III (Bio-Rad, Richmond, CA), under the following conditions: 1% agarose, 13ºC, 200V for 23h. The obtained fragments were analyzed according to the Pfaller criteria (1992) [10].

Results

Among the 144 CR-EC, a total of 76.4% and 3.5% were isolated, respectively, from patients with urinary tract infections and bloodstream infections. The remaining isolates (n=29) were recovered from peritoneal fluids, bronchoalveolar wash, catheter tip and multiple abscesses. A total of 43% of all CR-EC from urinary tract infections were collected from outpatients, reflecting the increasing frequency of quinolone resistance among community-acquired infections in the Brazilian medical centers. Although data from the SENTRY program in Latin America has already detected an increasing frequency of CR-EC in this region, these data were collected from hospitalized patients [1]. This high frequency of CR-EC found in the present study contrasts with data from other countries, such as the United States, where the prevalence of quinolone resistance among UTI isolates ranges from 0.7% in 1996 to 2.5% in 2001 [2]. The current data may be related to the increased use of quinolones for the treatment of community-acquired infections, such as UTI and respiratory tract infections in Brazil.

The antimicrobial activity and susceptibility profile of all 144 CR-EC is summarized in Table 1. The best coverage was achieved by the tested carbapenems, imipenem and meropenem (100.0%), followed by amikacin (91%) and ceftazidime (91%), and piperacilin/tazobactan (84.8%). The decreased susceptibility rates to cefoxitin (47.6%) may reflect the presence of alternate resistance mechanisms, such as alterations in outer membrane permeability, AmpC hyperproduction [11,12] or the acquisition of plasmid-borne genes mediating the expression of other class C β-lactamases [13-15].

Based on the NCCLS/CLSI criteria, 22.9% (n=33) of the CR-EC isolates collected between 2002 and 2003 could be considered ESBL producers. The production of ESBL was variable according to sex, age and infection origin, as shown in Table 2. Although there was no difference in relation to sex and infection origin, isolates recovered from patients older than 60 years old presented an increased chance of harboring the ESBL phenotype (p=0,006). These data is in accordance to a previous study by Eom at al. (2002) [16], where advanced age was a risk factor associated with the acquisition of quinolone-resistant E. coli among patients with UTI.

In this study, CR-EC harboring the ESBL phenotype could be detected in most of participating medical centers in Brazil (76.4%), indicating these multi-drug-resistant isolates are a reality in many geographical regions within the country. This increased ESBL production rate among CR-EC is worrisome, since the treatment of these multi-drug-resistant isolates is limited to the available carbapenems.

A total 144 CR-EC isolates were screened by colony blotting and Southern hybridization method. Only one clinical isolate yielded a hybridization signal with the *qnr* gene probe as showed in the Figure 2. This isolate was sent by a hospital in Porto Alegre, from an 80 year- old patient with urinary tract infection. Among the tested antimicrobial agents, this sample was resistant to ciprofloxacin, nalidixic acid, piperacillin/tazobactam, and cefoxitin. The ESBL screening and confirmatory tests were both negative for this specific sample. The *qnr* gene was sought by PCR by using specific primers in this strain, which was positive for screening test (Figure 3). After performing the nucleotide sequencing, a sequence of 493 bp fragment was obtained, identical to those of the original plasmid pMG252, as previously described by Jacoby et al. in 2003 [6].

Table 1. Antimicrobial potency and spectrum for selected antimicrobial agents tested against CR-EC isolates in 17 Brazilian laboratory-based hospitals

Antimicrobial agents	μg/mL		% Susceptibility
	MIC_{50}	MIC_{90}	
β-lactams			
Aztreonam	≤1	>32	77.9
Ceftriaxone	≤1	>64	75.9
Ceftazidime	≤1	8	91.0
Cefoxitin	4	32	52.4
Cefepime	≤1	>32	80.0
Ampicillin/Sulbactam	32	>64	27.6
Piperacilin/Tazobactam	≤8	128	84.8
Carbapenems			
Imipenem	≤0,25	≤0,25	100.0
Meropenem	≤0,25	≤0,25	100.0
Quinolones			
Nalidix acid	≥32	>32	0.0
Gatifloxacin	≥8	≥8	3.4
Aminoglycosides			
Gentamicin	≤2	>16	65.7
Amikacin	≤8	16	91.0

Figure 1. The participating medical centers are marked on the map with a circle signal.

Table 2. Demographic characteristics of the 33 ciprofloxacin-resistant ESBL-producing *E. coli* isolates

	ESBL producing (%)
Sex	
Male	16 (48.5)
Female	17 (51.5)
Age [a]	
≤ 1	2 (6.1)
2-12	1 (3.0)
13-60	8 (24.2)
>60	16 (48.5)
Infection origin	
Community	5 (15.2)
Nosocomial	7 (21.2)
Indeterminated	21 (63.6)

[a] Age information was missing from 6 isolates.

Figure 2. Screening for the *qnr* gene by colony blotting technique.

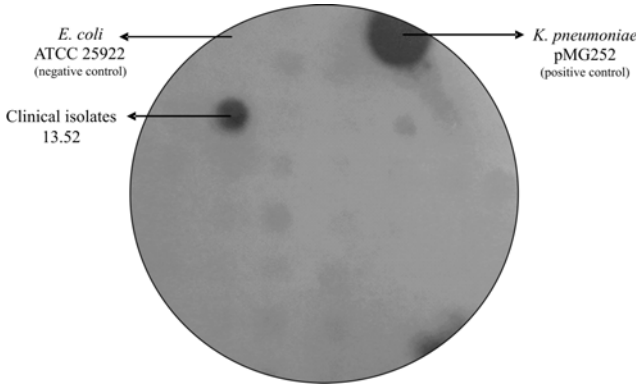
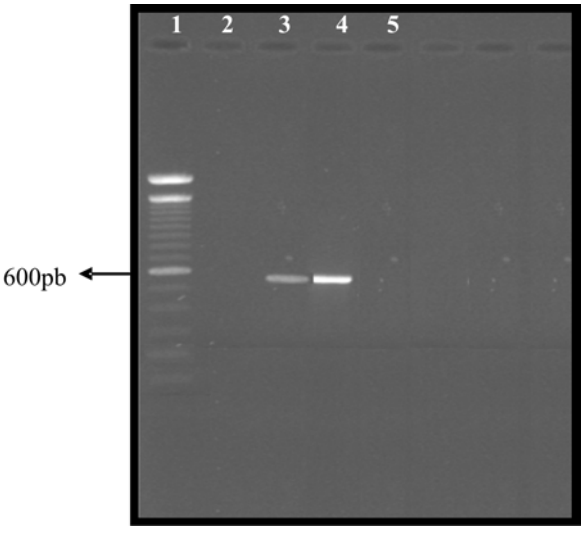


Figure 3. Detection of *qnr* gene by PCR. Line 1, Molecular weight marker; Line 2, negative quality control strain (ATCC 25922 *E. coli*); Line 3, clinical isolate (13.52); Line 4, positive quality control strain (pMG252 *K. pneumoniae*) and Line 5, master-mix solution.



Discussion

The current study identified, for the first time, the presence of the *qnr* gene in a clinical isolate of *E. coli* from a city in Brazil. This finding may reflect the ability and the possibility of acquisition of this unusual genotype by Brazilian isolates of *E. coli*. The low frequency of *qnr* gene isolation was also described by other authors. After evaluating a total of 91 *E. coli* isolates from the United States, Jacoby et al. (2003) [6] could identify only one isolate harboring the *qnr* gene. In China, a study by Wang et al. [7] also found a low prevalence (8%) of this genotype among the high-level quinolone resistant *E. coli*.

Similar PFGE profiles were detected among patients from the same medical center, possibly indicating a patient-to-patient transmission of resistant isolates. On the other hand, a total of 46 distinct PFGE patterns were detected among the 95 CR-EC isolated from urinary tract infections, indicating an elevated genetic diversity. Therefore, this increased frequency of CR-EC may be associated with the selection of resistant *E. coli* isolates from the patients' own flora. These isolates may be associated to the previous use of fluoroquinolones by the patient, or by the ingestion of food products contaminated with resistant pathogens. This last hypothesis was confirmed by a study in Spain, which found an elevated number of quinolone-resistant *E. coli* colonizing poultry and pork [3]. Additional PFGE studies are necessary to establish the temporal and geographical distribution of these resistant isolates, in order to determine the mode of dissemination.

Fluoroquinolone usage and prescription should be restricted among community and hospitalized patients, mainly among populations with an increased risk of developing this resistance phenotype. Additional surveillance studies are necessary to understand the dissemination of quinolone resistance in Brazil, to help implementing appropriate control measures, as well as to guide the adequate use of antimicrobial agents in this geographic region.

Acknowledgements

We would like thank Dr. Afonso Barth and Lalitagauri Deshpande for providing the clinical isolates and positive quality control strain, respectively. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq.

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