Molecular typing of fluoroquinolone-resistant and fluoroquinolone-susceptible *Escherichia coli* isolated from blood of neutropenic cancer patients in a single center

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Objective: To investigate the molecular epidemiology of fluoroquinolone-resistant (FQ-R) and fluoroquinolone-susceptible (FQ-S) bacteremic *Escherichia coli* isolates from neutropenic patients by pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis.

Methods: Nineteen FQ-R and 27 FQ-S isolates of *E. coli*, obtained from patients on a hematologic ward over a 7-year period, were genotyped by PFGE and RAPD using two different random primers (1247 and 1283).

Results: PFGE analysis was able to type all FQ-S isolates and most (17/19, 89%) FQ-R isolates of *E. coli*. All isolates were genotypically unrelated, with the exception of two indistinguishable FQ-R isolates from different patients in the same period. RAPD analysis typed all isolates, including those FQ-R isolates untypable by PFGE, but was unable to distinguish between some isolates that were different by PFGE. Using primer 1247, RAPD analysis identified six pairs and one triad, while primer 1283 identified seven pairs and one triad of indistinguishable isolates.

Conclusions: No spread of epidemic FQ-R or FQ-S *E. coli* isolates was documented among neutropenic patients. RAPD analysis is a powerful genotyping method, but appeared to be less reproducible and discriminatory than PFGE for investigating *E. coli* isolates.

Key words: *Escherichia coli*, fluoroquinolone resistance, neutropenic patients, PFGE, RAPD

INTRODUCTION

Fluoroquinolones are potent antimicrobial agents used in the treatment and prophylaxis of community-acquired and nosocomial infections [1]. When the new fluorinated quinolones were introduced more than 10 years ago, optimism concerning their usefulness in the prophylaxis of febrile neutropenia was justified by their broad antibacterial spectrum, high intraluminal concentration, systemic bactericidal activity, good tolerability, and apparent lack of potential for development of resistance [1]. All these characteristics contribute to their widespread use for antibacterial chemoprophylaxis in neutropenic patients [2,3]. Although the risk of development of resistance was considered to be extremely low [4], after their introduction to clinical practice an increasing emergence of quinolone-resistant bacteria was documented [5–7], particularly in *Staphylococcus aureus* and *Pseudomonas aeruginosa* [8,9]. The emergence of high-level fluoroquinolone-resistant (FQ-R) strains (MIC >128 mg/L) has also been reported for various members of the Enterobacteriaceae family, including *Escherichia coli* [10–12]. A clear association was observed between increased use of quinolones and emergence of fluoroquinolone resistance. Neutropenic patients with cancer who receive prophylaxis with fluoroquinolones may be at risk of

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Table 1 Distribution from 1990 to 1996 of *E. coli* bacteremic isolates from a hematologic ward in Rome

Year	No. of *E. coli* bacteremias	No. (%) of *E. coli* FQ-R[a] isolates	No. of *E. coli* FQ-R[a] isolates studied	No. (%) of *E. coli* FQ-S[b] isolates	No. of *E. coli* FQ-S[b] isolates studied
1990	21	2 (10)	2	19 (90)	16
1991	10	2 (20)	1	8 (80)	5
1992	16	6 (37.5)	2	10 (62.5)	1
1993	33	16 (48)	-	17 (52)	-
1994	43	33 (77)	3	10 (23)	1
1995	27	21 (78)	5	6 (22)	3
1996	9	7 (78)	6	2 (22)	1

[a]FQ-R: fluoroquinolone-resistant strains; MIC≥4 mg/L.
[b]FQ-S: fluoroquinolone-susceptible strains; MIC≤1 mg/L.

developing *E. coli* bacteremia due to FQ-R strains [13], and resistant *E. coli* isolates from the hematologic ward in Rome, Italy appeared after the introduction of fluoroquinolone prophylaxis for neutropenia, increasing from 5% to 78% between 1990 and 1996. The increasing rate of breakthrough bacteremias with resistant *E. coli* seems to be due to the independent emergence of several new clones [14]. However, nosocomial transmission may contribute to these resistant strains becoming a cause of concern [15].

Phenotypic characterization of *E. coli* isolates has proved to be inadequate for identifying strain diversity or similarity, which thus requires genotypic analysis. Various different typing methods have been used to study the genotypes of bacteria involved in nosocomial infections. Pulsed-field gel electrophoresis (PFGE) is the method of reference, although it is costly, time-consuming, and requires expensive instrumentation and trained personnel [16]. PCR typing methods, e.g. random amplified polymorphic DNA (RAPD) analysis [17–19], are rapid and less expensive, but are not standardized because there is no consensus regarding the conditions, instruments, enzymes and primers to be used [20,21].

The aim of this study was to investigate the molecular epidemiology of FQ-R and fluoroquinolone-susceptible (FQ-S) isolates of *E. coli* causing bacteremia in neutropenic patients with two different typing methods, i.e. PFGE versus RAPD analysis.

MATERIALS AND METHODS

E. coli bacteremic isolates

FQ-R and FQ-S isolates of *E. coli* responsible for clinically significant bacteremia in patients admitted to the hematologic ward of the University 'La Sapienza' in Rome, Italy from 1990 to 1996 and available as stock cultures (−70°C) were analyzed. Not all isolates from this period were available for analysis, but 19 FQ-R and 27 FQ-S *E. coli* isolates were included in the study (Table 1). Blood cultures were initially performed by the BacTec method (Becton Dickinson, Sparks, MD, USA) and the isolates were identified as *E. coli* by a standard in-house method [22], including biochemical reactions determined by the API 20E system (BioMérieux, Marcy L'Etoile, France).

The classification of *E. coli* blood isolates as FQ-S or FQ-R isolates was determined from the MIC of ciprofloxacin. A standard broth microdilution procedure with cation-adjusted Muller–Hinton broth (Oxoid, Basingstoke, UK) and a final inoculum of 5×10^8 CFU/L was used, with breakpoint concentrations for susceptible (≤1 mg/L) and resistant (≥4 mg/L) strains, according to the NCCLS performance and interpretive guidelines [23]. All reagents, except where specified, were purchased from Sigma (St Louis, MO, USA).

PFGE

E. coli isolates were grown overnight in 10 mL of brain–heart infusion broth (BHI, Oxoid). After incubation, bacteria were harvested, washed twice in PIV buffer (1 M NaCl, 10 mM Tris-HCl, pH 7.6) and resuspended in PIV buffer to achieve an extinction coefficient of 0.9 at a wavelength of 550 nm. A 0.5-mL aliquot of this bacterial suspension was quickly mixed with 0.5 mL of 1.5% (w/v) Incert agarose (FMC, Rockland, ME, USA). After agarose plug casting, samples were transferred to 5 mL of lysing solution (6 mM Tris-HCl (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μg/mL of RNase, 1 mg/mL lysozyme) and incubated overnight for 24 h. The plugs were transferred to ESP solution (0.5 M EDTA (pH 9), 1% sodium lauroyl sarcosine, 100 μg/mL of proteinase K) and incubated for 48 h at 50°C. The plugs were washed twice with phenylmethylsulfonyl fluoride (PMSF) 1 mM in TE buffer (10 mM Tris-HCl, 1 mM EDTA

(pH 7.5)) and three times with TE buffer. *Xba*I (New England BioLabs, Beverly, MA, USA) was used for cleavage. Plugs were equilibrated in 250 µL of restriction buffer plus spermidine (1 mM), and cleavage was performed overnight with 40 U of enzyme.

Electrophoresis was performed in 1% (w/v) FastLane agarose (FMC), at 14°C in TBE buffer (89 mM Tris-borate, 2 mM EDTA (pH 8.5)). PFGE was performed in a Chef Mapper apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The pulse time was ramped from 1 to 50 s over 24 h at 200 V. Lambda concatemers (Bio-Rad) were used as DNA size markers. Gels were then stained with ethidium bromide and acquired digitally (Gel Doc 1000, Bio-Rad). Restriction fragment patterns were analyzed with MA Fingerprinting software (Bio-Rad). Similarity coefficient (S_{AB}) values were calculated directly by the software. Since band position alone was used, S_{AB} values ranged from 0 to 1.0, where 0 indicates that the fingerprints had no bands in common and 1.0 indicates that the fingerprints were identical. The unwarping of gels requires subjective input from the investigator, so pairs of strains with S_{AB} values ≥ 0.9 were regarded as highly similar and not distinguishable by PFGE. The cut-off value of 0.9 was based on previous experience of using the software to compare profiles of the same run on different gels with various degrees of gel warping [24,25].

RAPD analysis

RAPD fingerprinting was performed as described by Berg et al [26]. Overnight broth cultures of *E. coli* were centrifuged, and the pellet was diluted 10-fold in distilled water, boiled, and used as DNA template. As a negative control, a strain of *Acinetobacter baumannii* (LF6), untypable with primer 1247, was used. For PCR, the 10-nucleotide primers 1247 (AAGAGCCCGT) and 1283 (GCGATCCCCA), used previously to type FQ-R *E. coli* isolates [14], were used. PCR was carried out in 50-µL volumes containing 2.5 U of *Taq* polymerase (Finnzyme, Riihitontuntie, Finland), PCR buffer (10 mM Tris-HCl, pH 7.5), 50 mM KCl, 3 mM $MgCl_2$, 1.25 mM (each) deoxynucleoside triphosphates (Promega, Madison, WI, USA), 10 mM primer, and 5 µL of *E. coli* DNA template. The PCR consisted of 40 cycles comprising 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. Amplicons were visualized, after electrophoresis in 1.5% (w/v) agarose gels, under UV light. Analysis was as described for PFGE.

Discriminatory power

To compare the efficacy of the two typing methods (PFGE versus RAPD analysis), the discriminatory index of Hunter and Gaston was employed [27].This index represents the probability that two unrelated strains will be characterized as being of different types by a given typing system. Therefore, a discriminatory index value close to 1 indicates the most powerful method for typing a given species of bacteria.

Table 2 Ciprofloxacin MICs for *E. coli* isolates included in the study

Isolate identification	Patient identification	Isolation date	Ciprofloxacin MIC (mg/L)
L59	1	12 January 1990	0.5
L61	2	6 February 1990	0.5
L62	3	23 April 1990	2000
L64	4	25 May 1990	0.25
L66	5	4 June 1990	0.5
L67	6	23 July 1990	0.125
L68	7	13 August 1990	0.125
L69	8	7 September 1990	0.0625
L70	9	3 October 1990	0.5
L71	10	10 October 1971	0.125
L72	11	11 October 1990	0.125
L74	12	25 October 1990	0.125
L75	13	28 October 1990	0.125
L76	14	21 November 1990	0.125
L78	15	23 December 1990	0.125
L79	16	29 December 1990	0.125
L80	17	31 December 1990	2000
L82	18	25 February 1991	0.125
L83	19	16 March 1991	0.125
L87	20	2 September 1991	0.125
L90	21	17 December 1991	1000
L132	22	27 July 1991	0.125
L177	23	17 July 1991	1000
L194	24	30 July 1992	2000
L198	25	9 September 1992	0.125
L200	26	13 September 1992	0.125
L278	27	31 January 1994	0.125
L281	28	11 February 1994	2000
L282	29	7 February 1994	1000
L346	30	19 June 1995	2000
L347	23	1 October 1994	1000
L394	31	11 June 1995	1000
L402	32	19 July 1995	1000
L412	33	17 September 1995	1000
L421	34	9 October 1995	0.125
L426	35	21 October 1995	0.125
L428	36	28 October 1995	1000
L433	37	6 November 1995	0.125
L444	38	7 January 1996	1000
L445	39	9 January 1996	2000
L449	40	15 January 1996	1000
L451	41	18 January 1996	0.125
L454	42	12 February 1996	2000
L456	43	20 February 1996	2000
L457	44	20 February 1996	1000
L467	45	24 May 1996	0.125

RESULTS

The ciprofloxacin MICs for the *E. coli* isolates included in the study are shown in Table 2. PFGE typed 17 of the 19 isolates of FQ-R *E. coli* examined. Two isolates (L444 and L62) were not typable, showing only a diffuse DNA band of between 150 and 350 kb in size. Fifteen of the 17 typable isolates were unrelated. Two isolates (L454 and L456), obtained from different patients in the same period (February 1996), were indistinguishable (S_{AB}=0.9). Two other isolates (L177 and L347), obtained from the same patient during two episodes of bacteremia occurring 1 year apart, were different (S_{AB}=0.25).

All 27 FQ-S isolates were typable by PFGE and were unrelated. No relationship was found between FQ-S and FQ-R isolates. All FQ-R *E. coli* isolates were typable by RAPD analysis using the primer 1247, and five pairs and one triad of indistinguishable isolates were identified (L449–L177, L412–L457, L444–L445, L281–L428, L454–L456, L62–L98–L194).

All FQ-R strains were also typable with primer 1283, which was able to identify four pairs of indistinguishable isolates (L454–L456, L449–L177, L445–L444, L281–L428). These four pairs were identical to four of the five pairs identified with primer 1247. One of these pairs (L454–L456) was also found to be indistinguishable by PFGE analysis. The two isolates that were not typable with PFGE (L444 and L62) were successfully typed by RAPD analysis and were unrelated.

RAPD analysis with primer 1247 identified only one indistinguishable pair of FQ-S isolates (L68–L61), while primer 1283 identified three pairs and one triad of indistinguishable isolates (L426–L433, L59–L61, L200–L198, L79–L76–L132). No pairs or triads of indistinguishable FQ-S isolates were common to both primers.

The discriminatory index was 0.999 for PFGE, 0.992 for RAPD with primer 1247, and 0.991 for RAPD with primer 1283.

DISCUSSION

According to the PFGE results, horizontal spread of a single clone of FQ-R *E. coli* in the hematologic ward in Rome appears to be a rare event and does not represent a cause of concern in terms of infection control. Furthermore, no genotype correlation was found among FQ-S isolates and between FQ-R and FQ-S isolates. Horizontal transmission has, however, been described previously for several multiresistant enteric bacteria [28,29]. It is noteworthy that horizontal spread of resistant clones of *E. coli* was reported in the Cancer Center of Ulm [15]. However, reports suggest that resistance to quinolones in *E. coli* is mainly of multiclonal origin [14]. Oethinger et al evaluated FQ-R isolates of *E. coli* from several centers in Europe and the Middle East and found no correlation between strains provided by different hospitals, supporting the lack of horizontal transmission [14]. In the present study, PFGE analysis was able to distinguish all FQ-S and most (17/19, 89%) FQ-R *E. coli* isolates. Genotyping by PFGE is a powerful tool for studying the genetic diversity of *E. coli* strains and, as in the Ulm experience, a similar percentage of FQ-R strains were typable (13/16, 81%) [14].

RAPD analysis typed two FQ-R isolates that were untypable by PFGE, and so was a more sensitive technique. This could be explained by the fact that, although degraded, DNA from these two isolates was still available as a template for the primers used in RAPD analysis. The results obtained with RAPD using different primers yielded different results (four pairs of isolates found to be indistinguishable, but seven pairs or triads identified as different with the two primers). Many factors can affect the reproducibility of RAPD analysis, including Mg^{2+} concentration [30], batch-to-batch variation in primer synthesis [31], ratio of DNA template concentration to primer concentration, model of thermocycler used [20], and the supplier and concentration of *Taq* DNA polymerase [32]. Furthermore, RAPD is a combination of artifactual variations due to the low annealing temperature and true polymorphism [20]. In our experience, RAPD analysis was less discriminatory than PFGE for typing both FQ-R and FQ-S *E. coli* isolates. RAPD analysis was unable to distinguish between isolates found to be different by PFGE. In a previous report, some FQ-R isolates with identical RAPD genotypes had different PFGE genotypes [14].

A possible explanation for the different discriminatory power of the two genotyping methods may be that PFGE examines about 80% of the genomic DNA, while RAPD is able to analyze only 0.1–0.2% of DNA, as calculated by the ratio between the sum of the sizes of all bands generated by *XbaI* and the size of the *E. coli* chromosome for PFGE, and the sum of sizes of bands generated by RAPD and the size of the *E. coli* chromosome (G. Cardinali, personal communication).

As observed in this study, the discriminatory index (*D*) of Hunter and Gaston [29] is unable to discriminate between genotyping methods that group few strains in many types, such as the new genotyping methods. This characteristic reduces the capability of this index to evaluate the best method for typing a given species. However, with the limited number of isolates examined in this study, RAPD analysis had a more effective

typability, but was less discriminative than PFGE for characterizing bacteremic *E. coli* isolates.

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