Interplay between pathogenicity island carriage, resistance profile and plasmid acquisition in uropathogenic Escherichia coli

Vera Calhau,1,2 Sara Domingues,1 Graça Ribeiro,2 Nuno Mendonça1 and Gabriela Jorge Da Silva1

This study aimed to characterize the relationship between pathogenicity islands (PAIs), single virulence genes and resistance among uropathogenic Escherichia coli, evaluating the resistance plasmid carriage fitness cost related to PAIs. For 65 urinary E. coli, antimicrobial susceptibility and extended-spectrum beta-lactamase production were determined with the Vitek 2 Advanced Expert system. Phylogroup determination, detection of PAIs and virulence genes papAH, papaC, sfa/foc, afa/dra, iutA, kpsMIII, cnf1, eaeA, hlyA, stx1 and stx2, plasmid replicon typing and screening for plasmidic resistance determinants qnr, aac(6’)-Ib-cr, qepA and blaCTX-M were carried out by PCR. Conjugation was performed between a donor carrying IncF, IncK and blaCTX-M-15, and receptors carrying one to six PAIs. The relative fitness of transconjugants was estimated by pairwise competition experiments. PAI IV536 (68%), gene iutA (57%) and resistance to ampicillin were the most prevalent traits. PAI I536, PAI II536, PAI III536 and PAI IIJ96 were exclusively associated with susceptibility to amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole, and were more prevalent in strains susceptible to ampicillin and cefalotin. PAI IV536, PAI II CFT073 and PAI ICFT073 were more prevalent among isolates showing resistance to amoxicillin/clavulanic acid, cefotaxime, ceftazidime and gentamicin. An inverse relationship was observed between the number of plasmids and the number of PAIs carried. Transconjugants were obtained for receptors carrying three or fewer PAIs. The mean relative fitness rates of these transconjugants were 0.87 (two PAIs), 1.00 (one PAI) and 1.09 (three PAI). The interplay between resistance, PAI carriage and fitness cost of plasmid acquisition could be considered PAI specific, and not necessarily associated with the number of PAIs.

INTRODUCTION

Uropathogenic Escherichia coli (UPEC) strains are the most frequent cause of community- and hospital-acquired urinary tract infections (UTIs). These strains express several virulence factors that contribute to the efficient colonization and infection of the host urinary tract, such as adhesins, siderophores and toxins (Wiles et al., 2008). Some of these virulence determinants are usually encoded in chromosomal DNA segments known as pathogenicity islands (PAIs), which are mobile genetic elements that can be transferred horizontally, thus, dramatically changing the phenotype of the receptor (Hacker et al., 1997; Gal-Mor & Finlay, 2006).

The antimicrobial resistance reported in UPEC during the last few decades has become a major concern. The acquisition of resistance genes through mobile genetic elements, such as plasmids, is the major force of the antimicrobial resistance dissemination, enhancing the survival capacity of bacteria under antibiotic pressure (Bennett, 2008). In clinical management, it is usually accepted that resistance, especially multidrug resistance, equates to the virulence of the strain. However, molecular studies have indicated an inverse relationship between the distribution of virulence factors and antimicrobial resistance, particularly to quinolones (Vila et al., 2002; Johnson et al., 2003; Moreno et al., 2006; Piatti et al., 2008).

Phylogenetic analysis of extra-intestinal pathogenic E. coli strains showed that they cluster into four main groups. Virulent strains belong mainly to phylogroup B2 and, to a
lesser extent, to group D, and the majority of the commensal strains belong to groups A and B1 (Clermont et al., 2000). Eventually, the less virulent strains may be more prone to acquire resistance determinants. This has been demonstrated for resistance to quinolones (Vila et al., 2002). However, this interplay between virulence and resistance has been reported for single genes and not for genes located in higher segments of DNA. To the best of our knowledge, the relation between the main PAIs detected in UPEC strains and antimicrobial resistance has not been addressed. We hypothesize that maintenance of PAIs and plasmids carrying resistance genes will impose a biological cost on the host cell. In fact, most studies show a fitness burden associated with the presence of resistance plasmids (Björkman & Andersson, 2000). Therefore, the main objectives of this study were to characterize the relationship between the presence of virulence determinants, including PAIs, the antimicrobial-resistance profile and the phylogenetic background in a collection of clinical E. coli isolated from urine samples in a university hospital. Additionally, we intended to evaluate the fitness cost conferred by the acquisition of a resistance plasmid in strains harbouring different number of diverse PAIs.

**METHODS**

**Bacterial isolates and susceptibility testing.** A total of 65 non-duplicate E. coli isolates were collected from urine samples during November and December 2007 from different wards of the Coimbra Hospital and University Centre (CHUC), Coimbra, Portugal. The antimicrobial-susceptibility profiles and the preliminary identification of extended spectrum β-lactamase-producing isolates were determined by the Clinical Pathology Service (University Hospital of Coimbra) using the Vitek 2 Advanced Expert system (bioMérieux). The results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010). E. coli ATCC 25922 was used as a quality control strain.

**Phylogenetic analysis.** Determination of the major E. coli phylogenetic groups (A, B1, B2 and D) was performed by using a simple PCR-multiplex-based technique, which screens for the genes chuA and yjaA, and the DNA fragment tspE4.C2 (Clermont et al., 2000; Mendonça et al., 2011).

**Detection of PAI markers and virulence genes.** PAI markers were detected using the method of Bronowski et al. (2008), based on the technique described by Sabaté et al. (2006). The method consists of three multiplex-PCRs that allow the detection of eight PAIs, encoding different virulence genes: PAI I536 z-haemolysin, CS12 fimbriae and F17-like fimbrial adhesin; PAI II536, z-haemolysin and P-related fimbriae; PAI IICFT073, S-fimbriae and an iron siderophore system; PAI IV536, versiniabactin siderophore system; PAI I536, z-haemolysin and P-fimbriae; PAI II536, z-haemolysin, Prs-fimbriae and cytotoxic necrotizing factor 1; PAI IIICFT073, z-haemolysin, P-fimbriae and aerobactin; and PAI IIIICFT073, P-fimbriae and iron-regulated genes.

Other virulence genes that may be present in extra-intestinal pathogenic E. coli, such as papA4, papC (P-fimbriae structural subunit and assembly), sfa/foc (S and F1C fimbriae), sfa/dra (Dr-binding adhesins), intA (aerobactin receptor), kpsMII (group 2 capsules) and cnf1 (cytotoxic necrotizing factor 1), were also screened by PCR (Johnson & Stell, 2000), as well as specific genes of intestinal pathogenic E. coli, including the enterohaemorrhagic E. coli-associated virulence genes eaeA ( intimin), hlyA (pore-forming cytolyisin) and stx1 and stx2 (Shiga-like toxins) (Ram et al., 2008). The PCR products were purified using the QiAquick PCR purification kit (Qiagen), according to the manufacturer’s instructions, and amplicons were further sequenced at Macrogen.

**Antimicrobial-resistance determinant detection.** The blaCTX-M, blaTEM and blaSHV genes encoding β-lactamases, and the plasmid-mediated quinolone-resistance determinants qacE·d·II-cr, qepA, B and S, and qepA, were screened with specific primers by PCR (Park et al., 2006; Cattoir et al., 2007; Mendonça et al., 2007; Ma et al., 2009). All the amplicons were purified with ExoSAP-IT (USB) and further sequenced at Macrogen.

**Plasmid replicon typing.** Plasmid replicons were identified by the PCR-based replicon typing scheme described by Carattoli et al. (2005), which consists of five different multiplex and three simplex PCRs, and allows the detection of the major replicon families in Enterobacteriaceae: HI2, H11, 11-γ, X, L/M, N, FIA, FIB, FIC,W, Y, P, A/C, T, K and B/O.

**Biological fitness assessment.** Conjugation assays were used to transfer a plasmid carrying a resistance determinant gene into recipients with diverse number of PAIs. Strain Ec161B (Calhau et al., 2013) was used as the plasmid donor. E. coli strains with one to six PAIs were used as receptors: Ec396 (PAI IV536), Ec93 (PAI IV536, PAI IIJ96), Ec107 (PAI IV536, PAI I536 and PAI II536), Ec112 (PAI I536, PAI II536, PAI IV536 and PAI II536), Ec270 (PAI I536, PAI II536, PAI IV536, PAI IIJ96, PAI ICFT073, and PAI IIIICFT073). Brieﬂy, donor and recipient strains were grown in Luria–Bertani (LB) broth overnight and mating assays were performed on LB agar using 100 µl receptor and 50 µl donor, and incubated at 37 °C overnight. A suspension of the grown culture was made in 500 µl of physiological serum and 25 µl was dispersed into selective plates. The transconjugants were selected on LB agar plates containing 10 µg tetracycline ml⁻¹ plus 1 µg cefotaxime ml⁻¹ or 50 µg nalidixic acid ml⁻¹ plus 1 µg cefotaxime ml⁻¹, according to the receptor resistance proﬁle. The acquisition of the plasmid was conﬁrmed by PCR.

The relative fitness (W) of plasmid-carrying transconjugants was estimated by pairwise competition experiments between the transconjugants and the receptor strain in S2 minimal medium for 24 h, as described elsewhere (Ray et al., 2009), with the exception that 25 mg glucose ml⁻¹ was added to the medium (Enne et al., 2005). Brieﬂy, a single c.f.u. was inoculated in the medium and incubated at 37 °C overnight with good aeration (225 r.p.m.) (day −1). On the next day (day 0), cultures were diluted 1 : 10 in 0.9% NaCl and an equal amount of each competitor was transferred into the medium. The initial cell density [A(0) and B(0)] was determined by plating different dilutions of the wild-type strain and the transconjugants on antibiotic-free LB agar and on LB agar supplemented with 1 µg cefotaxime ml⁻¹, respectively. The competition culture was incubated at 37 °C with agitation at 225 r.p.m. After 24 h, the competition assay was stopped (day 1). The culture was diluted and different dilutions were plated on LB agar with and without antibiotic selection, to calculate the final density [A(1) and B(1)] of each competitor. Twelve to fifteen competition replicates were done for each transconjugant.

The relative fitness (W) was calculated as the ratio of the Malthusian parameter of each competitor: \( W_A = \frac{N_A}{N_B} \), where \( N_A = \ln(A(1)) - \ln(A(0)) \), \( M_B = \ln(B(1)) - \ln(B(0)) \), \( A(0) \) or \( B(0) \) was estimated density of A or B at day 0 (cells ml⁻¹), and \( A(1) \) or \( B(1) \) was estimated density of A or B at day 1 (cells ml⁻¹).

**Statistical analysis.** The chi-squared test and t-test were applied using IBM SPSS statistics version 22.0. A P ≤ 0.05 was considered
RESULTS

Strain origin and phylogeny

*E. coli* strains were obtained from patients suffering from UTIs, presenting ages ranging from 14 to 98 years. The majority of the strains were isolated from the female gender (72%). Fifty-seven per cent of the isolates were of nosocomial origin, while the remaining 43% were from community UTIs. The most prevalent phylogroup was B2 (46%), followed by group A (28%), B1 (15%) and phylogenetic group D (11%) (Table 1).

Virulence characterization

The PAIs most frequently detected was PAI IV536 (68%), followed by PAI IICFT073 (42%) and PAI ICFT073 (42%) (Table 1). PAI I536 and PAI I96 were detected in 9% of the isolates; PAI II536 in 6% and PAI III536 in 3% (n=2) of the isolates. PAI I96 was not detected. PAI I536, PAI II536, PAI III536 and PAI I96 were exclusively detected in isolates from phylogroup B2, and PAI IV536 was detected among all the phylogroups, though it was more prevalent among phylogroups B2 (100%) and D (86%). PAI IICFT073 and PAI ICFT073 were only detected among isolates belonging to phylogroups B2 and D (80% of B2 and 43% of D; and 90% of B2 and 14% of D, respectively).

All the screened virulence genes were detected in the isolates with the exception of *hlyA* and stx2 (Table 1). The virulence gene most frequently detected was *iutA* (57%), followed by *kpsMTII* (52%), *papC* (29%), *cnfI* (26%) and *papAH* (22%). The least frequent genes were *sfa/focDE* (14%), and *afa/dra*, *eae* and *stx1*, each detected in 3% of the isolates. The majority of the virulence genes were more frequently identified among isolates from the B2 phylogroup. Exceptions were *papAH* (43%), *papC* (57%) and *afa/dra* (14%) genes, more prevalent among isolates from phylogenetic group D, and *stx1* gene, detected in one isolate from phylogroup A and in another from the B2 group.

Phenotypic and genotypic resistance profiles

Resistance to ampicillin was the most prevalent (74%), followed by resistance to ciprofloxacin (69%), cefalotin (57%), trimethoprim/sulfamethoxazole (48%), amoxicillin/clavulanic acid (42%) and gentamicin (29%). Resistance to cefotaxime and ceftazidime were the least detected among the isolates (19% each).

The plasmidic resistance determinants *aac(6')-Ib-cr* (49%) and *blaCTX-M* (19%) were the most prevalent, while *qnrA* and *qnrS* genes were only detected in 3% of the strains. The *qnrB* and *qepA* genes were not identified.

The *aac(6')-Ib-cr* gene was detected in 44% of the strains resistant to ciprofloxacin.

Relation between the phylogenetic background and virulence genes and the phenotypic resistance

The distribution of the phylogroups and virulence traits among strains susceptible or resistant to different antibiotics is shown in Table 2. Phylogroups A, B1 and D were more prevalent among isolates susceptible to amoxicillin/clavulanic acid, cefotaxime, ceftazidime and gentamicin, while group B2 was more prevalent in isolates resistant to these antibiotics. The reverse was observed for...
### Table 2. Distribution of phylogroups, PAIs and virulence genes among susceptible and resistant strains

<table>
<thead>
<tr>
<th>Phylogenetic group/PAI/virulence gene</th>
<th>AMP</th>
<th>KF</th>
<th>CTX</th>
<th>CAZ</th>
<th>AMC</th>
<th>CIP</th>
<th>GEN</th>
<th>SXT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (n=17)</td>
<td>R (n=48)</td>
<td>S (n=28)</td>
<td>R (n=37)</td>
<td>S (n=53)</td>
<td>R (n=12)</td>
<td>S (n=38)</td>
<td>R (n=27)</td>
</tr>
<tr>
<td>Phylogenetic group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>18 (3)</td>
<td>31 (15)</td>
<td>43 (12)</td>
<td>16 (6)</td>
<td>32 (17)</td>
<td>8 (1)</td>
<td>32 (17)</td>
<td>8 (1)</td>
</tr>
<tr>
<td>B1</td>
<td>12 (2)</td>
<td>17 (8)</td>
<td>11 (3)</td>
<td>19 (7)</td>
<td>19 (10)</td>
<td>0 (0)</td>
<td>19 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B2</td>
<td>59 (10)</td>
<td>42 (20)</td>
<td>36 (10)</td>
<td>54 (20)</td>
<td>36 (19)</td>
<td>92 (11)</td>
<td>36 (19)</td>
<td>92 (11)</td>
</tr>
<tr>
<td>D</td>
<td>12 (2)</td>
<td>10 (5)</td>
<td>11 (3)</td>
<td>11 (4)</td>
<td>13 (7)</td>
<td>0 (0)</td>
<td>13 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAIs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI I336</td>
<td>24 (4)</td>
<td>4 (2)</td>
<td>14 (4)</td>
<td>5 (2)</td>
<td>11 (6)</td>
<td>0 (0)</td>
<td>11 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI II336</td>
<td>18 (3)</td>
<td>2 (1)</td>
<td>11 (3)</td>
<td>3 (1)</td>
<td>8 (4)</td>
<td>0 (0)</td>
<td>8 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI III336</td>
<td>6 (1)</td>
<td>2 (1)</td>
<td>4 (1)</td>
<td>3 (1)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI IV336</td>
<td>82 (14)</td>
<td>63 (30)</td>
<td>57 (16)</td>
<td>76 (28)</td>
<td>62 (33)</td>
<td>92 (11)</td>
<td>62 (33)</td>
<td>92 (11)</td>
</tr>
<tr>
<td>PAI I1CF073</td>
<td>52 (9)</td>
<td>38 (18)</td>
<td>27 (8)</td>
<td>51 (19)</td>
<td>34 (18)</td>
<td>75 (9)</td>
<td>34 (18)</td>
<td>75 (9)</td>
</tr>
<tr>
<td>PAI II1CF073</td>
<td>41 (7)</td>
<td>44 (21)</td>
<td>25 (7)</td>
<td>57 (21)</td>
<td>32 (17)</td>
<td>92 (11)</td>
<td>32 (17)</td>
<td>92 (11)</td>
</tr>
<tr>
<td>PAI I986</td>
<td>24 (4)</td>
<td>4 (2)</td>
<td>14 (4)</td>
<td>5 (2)</td>
<td>11 (6)</td>
<td>0 (0)</td>
<td>11 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Virulence genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>papAH</td>
<td>47 (8)</td>
<td>13 (6)</td>
<td>29 (8)</td>
<td>16 (6)</td>
<td>26 (14)</td>
<td>0 (0)</td>
<td>26 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>papC</td>
<td>53 (9)</td>
<td>21 (10)</td>
<td>32 (9)</td>
<td>27 (10)</td>
<td>32 (17)</td>
<td>17 (2)</td>
<td>32 (17)</td>
<td>17 (2)</td>
</tr>
<tr>
<td>afa/draBC</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>4 (1)</td>
<td>3 (1)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>sfA/focDE</td>
<td>29 (5)</td>
<td>8 (4)</td>
<td>21 (6)</td>
<td>8 (3)</td>
<td>15 (8)</td>
<td>8 (1)</td>
<td>15 (8)</td>
<td>8 (1)</td>
</tr>
<tr>
<td>kpsMTII</td>
<td>47 (8)</td>
<td>54 (26)</td>
<td>32 (9)</td>
<td>68 (25)</td>
<td>45 (24)</td>
<td>83 (10)</td>
<td>45 (24)</td>
<td>83 (10)</td>
</tr>
<tr>
<td>intA</td>
<td>53 (9)</td>
<td>58 (28)</td>
<td>39 (11)</td>
<td>70 (26)</td>
<td>47 (25)</td>
<td>100 (12)</td>
<td>47 (25)</td>
<td>100 (12)</td>
</tr>
<tr>
<td>cnf1</td>
<td>47 (8)</td>
<td>19 (9)</td>
<td>32 (9)</td>
<td>22 (8)</td>
<td>28 (15)</td>
<td>17 (2)</td>
<td>28 (15)</td>
<td>17 (2)</td>
</tr>
<tr>
<td>eae</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>5 (2)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>stxl</td>
<td>6 (1)</td>
<td>2 (1)</td>
<td>7 (2)</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
<td>4 (2)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

AMC, Amoxicillin/clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; GEN, gentamicin; KF, ceftolizin; SXT, trimethoprim/sulfamethoxazole.
trimethoprim/sulfamethoxazole, as phylogroups A, B1, and D were more prevalent in the resistant isolates (42 vs 15 %, 23 vs 9 % and 16 vs 6 %, respectively), while phylogroup B2 was more prevalent among the susceptible isolates (71 vs 19 %). Considering resistance to ampicillin and ciprofloxacin, phylogenetic group A was more prevalent among susceptible strains compared to the resistant isolates (31 vs 18 % for ampicillin and 33 vs 15 % for ciprofloxacin). Phylogroup B1 was also more prevalent among isolates susceptible to these antibiotics (17 vs 12 % and 20 vs 5 %, respectively). Contrarily, phylogroups B2 (59 vs 42 % for ampicillin and 55 vs 42 % for ciprofloxacin) and D (12 vs 10 % and 25 vs 4 %, respectively) were more prevalent among resistant isolates.

The majority of virulence genes were more prevalent among susceptible isolates (Table 2). Exceptions were observed for iutA, kpsMTII, afa/dra and eae genes: the iutA gene was more common in resistant isolates except for trimethoprim/sulfamethoxazole (71 % of susceptible vs 42 % of resistant); kpsMTII was more frequent among resistant isolates except for ciprofloxacin (60 % in susceptible vs 49 % in resistant); and afa/dra was more prevalent among ampicillin-resistant strains (4 vs 0 %); and eae was most frequently detected among strains resistant to ampicillin (4 vs 0 %), amoxicillin/clavulanic acid (4 vs 0 %), gentamicin (5 vs 0 %), cefalotin (5 vs 0 %) and ciprofloxacin (4 vs 0 %) and gentamicin (74 vs 30 %, P=0.005), and in isolates susceptible to trimethoprim/sulfamethoxazole (65 vs 19 %, P<0.001). Finally PAI IV536 was more prevalent among isolates susceptible to ampicillin (82 vs 63 %), ciprofloxacin (90 vs 58 %, P=0.010) and trimethoprim/sulfamethoxazole (85 vs 48 %, P=0.001), and resistant to amoxicillin/clavulanic acid (70 vs 66 %), cefotaxime (92 vs 62 %, P=0.049), ceftazidime (92 vs 62 %), cefalotin (76 vs 57 %) and gentamicin (79 vs 63 %) (Table 2).

All isolates carried up to six PAIs. Isolates resistant to amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole did not present more than three PAIs (Table 2).

### Distribution of PAIs according to the resistance profile

PAI I536, PAI II536, PAI III536 and PAI IIJ96 were exclusively found among strains susceptible to amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole, and were more prevalent in strains susceptible to ampicillin and cefalotin (Table 2). Nonetheless, only the absence of PAI I536, PAI II536 and PAI IIJ96 in isolates resistant to ciprofloxacin (P<0.001 each), and of PAI IV536 and PAI IIJ96 in isolates resistant to trimethoprim/sulfamethoxazole (P=0.014 each), were considered statistically significant. The remaining PAIs detected in this study were found in both resistant and susceptible isolates, for all the considered antibiotics. PAI ICFT073 was more prevalent in isolates susceptible to ampicillin (32 vs 38 %), ciprofloxacin (50 vs 38 %) and trimethoprim/sulfamethoxazole (39 vs 23 %, P=0.003), and in isolates resistant to amoxicillin/clavulanic acid (44 vs 40 %), cefotaxime (75 vs 34 %, P=0.009) ceftazidime (75 vs 34 %, P=0.029), cefalotin (51 vs 27 %) and gentamicin (63 vs 33 %). PAI IIICFT073 was more prevalent among isolates resistant to ampicillin (44 vs 41 %), amoxicillin/clavulanic acid (56 vs 34 %), cefotaxime (92 vs 32 %, P<0.001), ceftazidime (92 vs 32 %, P<0.001), cefalotin (57 vs 35 %, P=0.002), ciprofloxacin (44 vs 40 %) and gentamicin (74 vs 30 %, P=0.005), and in isolates susceptible to trimethoprim/sulfamethoxazole (65 vs 19 %, P<0.001). Finally PAI IV536 was more prevalent among isolates susceptible to ampicillin (82 vs 63 %), ciprofloxacin (90 vs 58 %, P=0.010) and trimethoprim/sulfamethoxazole (85 vs 48 %, P=0.001), and resistant to amoxicillin/clavulanic acid (70 vs 66 %), cefotaxime (92 vs 62 %, P=0.049), ceftazidime (92 vs 62 %), cefalotin (76 vs 57 %) and gentamicin (79 vs 63 %) (Table 2).

### Plasmid carriage and virulence markers

The plasmid group most frequently detected was IncF (85 %), followed by IncK (59 %). IncX, IncL/M and IncT were not detected. IncHI was exclusively detected in strains from phylogroups B1, IncN in phylogenetic group B2 and IncA/C in isolates from phylogroup A (Table 1).

The relation between the number of PAIs and the number of plasmids carried by the isolates is shown in Table 3. Strains carrying more than three plasmids contained three or fewer PAIs, while strains with three or fewer identified plasmids harboured up to six PAIs (Table 3).

**Table 3. Distribution of isolates according to the number of PAIs and number of identified plasmids**

<table>
<thead>
<tr>
<th>No. of PAIs</th>
<th>Prevalence % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤1 (n=19)</td>
</tr>
<tr>
<td>0</td>
<td>10 (2)</td>
</tr>
<tr>
<td>1</td>
<td>16 (3)</td>
</tr>
<tr>
<td>2</td>
<td>16 (3)</td>
</tr>
<tr>
<td>3</td>
<td>53 (10)</td>
</tr>
<tr>
<td>4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>6</td>
<td>5 (1)</td>
</tr>
</tbody>
</table>

**Evaluation of the biological cost conferred by plasmid acquisition in transconjugants**

Considering the results obtained, which seemed to indicate an inverse relationship between the amount of plasmids and the number of PAIs carried simultaneously in the same isolate, fitness studies were performed. Transconjugants carrying a plasmid were only obtained in three receptors harbouring three or fewer PAIs. PCRs confirmed the presence of IncK and blaCTX-M-15 in the transconjugants. The fitness cost of the same plasmid in the transconjugants was evaluated and the mean relative fitness ranged from...
Among the isolates. This observation emphasizes the potential role of food reservoirs and foodborne transmission of *E. coli* and the development of UTIs (Vincent et al., 2010).

The *intA* gene, encoding the aerobactin receptor, which is part of a siderophore system, was the most detected virulence gene. The production of aerobactin has been shown to facilitate growth in urine (Johnson et al., 1988), a medium with limited iron conditions, in which siderophores may be extremely useful and constitute an important virulence factor in UTI development.

The isolates presented high levels of resistance to amoxicillin, third-generation cephalosporins, aminoglycosides and fluoroquinolones compared to the mean rates of resistance reported in Portugal in 2007 (EARSS, 2007). The resistance to third-generation cephalosporins was associated with the production of the CTX-M-15 β-lactamase and with phylogenetic group B2, which is in concordance with other reports (Coque et al., 2008; Karisik et al., 2008).

Prevalence of phylogroup B2 was observed among the isolates susceptible to amoxicillin, ciprofloxacin and trimethoprim/sulfamethoxazole, supporting the old paradigm of a trade-off between resistance and virulence (Johnson et al., 2003, 2005; Houdouin et al., 2006; Moreno et al., 2006; Piatti et al., 2008). Nonetheless, the majority of the isolates resistant to amoxicillin and ciprofloxacin also belong to phylogroup B2. A shift towards non-B2 phylogroups was only observed for trimethoprim/sulfamethoxazole.

A positive relation was observed for phylogroup B2 and the most frequent PAIs, PAI IV536, PAI II536, PAI III536 and PAI II96 were detected. The remaining PAIs were also more prevalent among strains from this group. Sabaté et al. (2006) similarly reported the occurrence of PAI II536 and III536 only among B2 strains, and PAI I536 and II96 among B2 and B1 strains. PAI I536 and PAI III536 have been described as the most unstable islands and, thus, more prone to be excised (Middendorf et al., 2004). This may explain the low prevalence of these islands observed in this study. The fact that these PAIs have only been detected among B2 strains may lead us to speculate that this phylogroup possibly provides a genetic background that may somehow contribute to enhance the stability of these islands in the bacterial genome. In addition, the virulence genes detected were also more frequent in the B2 phylogroup, with the exception of *papAH* and *papC*, more common in phylogroup D, considered the second most virulent. Nonetheless, the reduced number of strains belonging to this phylogenetic group compared to the remaining phylogroups might influence the results. The higher concentration of virulence factors within isolates from phylogroup B2 is mainly due to a genetic background that allows numerous horizontal gene transfer events (Escobar-Páramo et al., 2004), which may explain the higher prevalence of detected virulence factors and PAIs among this group.

Although rare, the *eae* and *stxl* genes were also detected among the isolates. This observation emphasizes the possible role of food reservoirs and foodborne transmission of *E. coli* and the development of UTIs (Vincent et al., 2010).
The presence of virulence genes was generally more frequent among strains susceptible to the tested antibiotics, which was in agreement with other studies (Johnson & Stell, 2000; Vila et al., 2002; Moreno et al., 2006; Piatti et al., 2008). Nonetheless, this tendency was not observed for the iutA, afa/dra and eae genes, as they were generally more prevalent among resistant isolates. This could be associated with the fact that these genes may be carried in plasmids, which also contain resistance determinants.

The results seemed to indicate an inverse tendency between the number of plasmids and the number of PAs. This led us to hypothesize that the presence of more PAs may be a factor that could affect the acquisition of plasmids. The fact that we could not obtain transconjugants using receptors with more than three PAs seemed to confirm this hypothesis. However, the in vitro fitness studies performed indicated that, at least for receptors harbouring three or fewer PAs, the carriage of a higher number of islands was not directly associated with a higher fitness cost conferred by the acquisition of the plasmid. The absence of a clear fitness burden imposed by the initial acquisition of a resistance-encoding plasmid differs from most of the fitness studies, where it is associated with a biological cost (Dionisio et al., 2005; Enne et al., 2005). Thus, other factors, which may be possibly related to the genetic background of the bacteria, may be responsible for this tendency. Nonetheless, the transconjugants carried only the more prevalent PAs. The results showed that these were associated with plasmidic resistance determinants, namely genes encoding resistance to third-generation cephalosporins. In fact, the combination of three PAs carried by the transconjugant with a fitness advantage has already been reported as being a prevalent combination of PAs for clone ST131 (Calhau et al., 2013). Therefore, the trade-off between the plasmid number and the number of PAs may be related to the presence or absence of specific PAs rather than their number. This study confirms previous reports of the trade-off between resistance and virulence, and provides new insights into the interplay between resistance and virulence encoded by PAs.

ACKNOWLEDGEMENTS

All authors contributed equally to this work. N. M. was supported by a grant from the Fundação para a Ciência e a Tecnologia, Lisbon, Portugal (SFRH/BPD/45815/2008). This work was supported financially by a European Society of Clinical Microbiology and Infectious Diseases 2010 research grant, the Fundação para a Ciência e Tecnologia (grant no. PEst-CE/SAU/UI0177/2014), and by the Center for Pharmaceutical Studies, University of Coimbra, Portugal. The authors would like to thank all the members of the Bacteriology Laboratory of the Clinical Pathology Service of the University Hospital of Coimbra for the collaboration over the isolation and identification of bacteria. Positive controls used in plasmid replication typing were kindly provided by Alessandra Carattoli, Instituto Superior di Sanità, Rome, Italy.

REFERENCES


