Virulence factors associated with antimicrobial resistance determinants among *Escherichia coli* and *Klebsiella* spp.

Tese de doutoramento em Ciências Farmacêuticas, na área de especialização em Microbiologia e Parasitologia, orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva e pelo Doutor Nuno Ricardo Furtado Dias Mendonça, e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Abstract

_Escherichia coli_ and _Klebsiella_ spp. are important pathogens, responsible for several infectious diseases. These members of _Enterobacteriaceae_ family are of particularly concerning due to a high increase in their resistance to antimicrobials. The detection of more resistant strains brings into question if this enhancement of resistance may be accompanied by an increase in virulence. If so, extremely pathogenic strains would start to emerge, and no antibiotic therapy would be available to fight them, leading to serious public health problems. Thus, the main objective of this research was to understand the relation between virulence and resistance among _E. coli_ collected from different origins and _Klebsiella_ spp.

The interplay between resistance and virulence was studied among _Uropathogenic E. coli_ (UPEC). _E. coli_ strains are classified into four main phylogenetic groups. More virulent strains belong mainly to phylogroup B2 and, to a lesser extent, to group D, and most of the commensal strains belong to groups A and B1. During the characterization of strains, a new genotype was discovered in the Clermont method, which is used to assess phylogenetic groups. This _yjaA/ tspE4.C2_ genotype was assigned to phylogroup B2.

Pathogenicity islands (PAIs) are mobile genetic elements that carry virulence genes and may increase the virulence of bacteria. PAI _I_536, PAI _II_536, PAI _III_536 and PAI _II_96 were found to be associated to phylogroup B2. A trade-off between virulence and resistance was observed for these islands, as they were generally found among susceptible strains. The same was observed for the majority of individual virulence genes including adhesins, toxins and siderophore systems. Contrarily, a correlation was observed for the PAI _IV_536, PAI _ICFT073_ and PAI _II_CFT073_ and resistance to amoxicillin-clavulanic acid, cephalothin, cefotaxime, ceftazidime and gentamicin. This combination of PAIs, along with IncF plasmid, _blaCTX-M-15_ and _aac(6')-lb-cr_ genes, was also found to be prevalent among the UPEC CTX-M-15 producers assigned to the sequence type ST131.

It was observed that strains with a higher number of identified plasmids carried less PAIs. It was hypothesised that the biological cost of the carriage of a resistance plasmid was higher to the cell, according to the number of PAIs carried. Conjugation assays and fitness studies were performed, and the latter indicated that the carriage
Abstract

of a higher number of PAIs did not implicate a direct rise in the fitness cost of the acquisition of the resistance plasmid, at least in strains harbouring until three PAIs.

Participation on an international study with *E. coli* isolates from Nigeria, allowed comparing the characteristics of these isolates with the Portuguese ones. Even though regional differences were observed, clone ST131, IncF plasmids and *bla<sub>CTX-M-15</sub>* were common in the two countries. This supports the fact that clone ST131 and the referred resistance determinant are widely and successfully disseminated worldwide.

The same *E. coli* ST131 clone, carrying resistance determinants *bla<sub>CTX-M-15</sub>*,* bla<sub>TEM-type</sub>*,* qnrS* and *aac(6')-lb-cr*, IncF and IncP plasmids, and virulence factors PAI IV<sub>536</sub>, PAI I<sub>CFT073</sub>, PAI II<sub>CFT073</sub>, *iutA*, *sfa/foc* and *papAH*, was also identified at the effluent of a hospital wastewater treatment plant (WWTP). This shows that WWTPs may be contributing to the dissemination of virulent and resistant bacteria into water ecosystems, constituting, thus, an environmental and public health risk.

The interplay between resistance and virulence was also assessed for *K. pneumoniae* and *K. oxytoca*. PAI IV<sub>536</sub> was statistically associated to resistance to gentamicin and sulfamethoxazole-trimethoprim among *K. pneumoniae*. The presence of PAI II<sub>CFT073</sub> was firstly identified among *K. oxytoca* in this study. It was significantly associated with resistance to cephalothin, cefotaxime, ceftazidime, ciprofloxacin, and gentamicin. Contrarily to the observed in *E. coli*, the trade-off between resistance and virulence did not seem to exist. In fact, among *K. pneumoniae*, higher prevalence of virulence factors was detected among isolates resistant to ciprofloxacin and gentamicin and at a lesser extent to cefotaxime and sulfamethoxazole-trimethoprim. Furthermore, higher prevalence of virulence genes was observed in *K. oxytoca* resistant to sulfamethoxazole-trimethoprim.

Multidrug resistant *K. pneumoniae* isolates from an outbreak in a renal transplant unit were studied and characterized for virulence factors, resistance determinants and genetic relatedness. Two main genetic lineages were detected excluding the hypothesis of an outbreak caused by a single internal clone. PAI III<sub>536</sub> and PAI II<sub>CFT073</sub> were detected for the first time in *K. pneumoniae* during this study.

Overall, the results presented in this dissertation indicated that, although it was detected a general trade-off between resistance and virulence among *E. coli*, the same was not usually verified for *Klebsiella* spp. isolates. Nonetheless, despite of the trade-off verified in *E. coli*, clone ST131, found among Portuguese and Nigerian
isolates in this study, is an example that in this species, virulence and resistance features may co-exist. The interplay between resistance and virulence seems to be, therefore, influenced by the genus and species, the virulence factor, and the resistance phenotype and genotype.
Resumo

*Escherichia coli* e *Klebsiella* spp. são importantes agentes patogênicos, responsáveis por diversas doenças infecciosas. Estas bactérias da família *Enterobacteriaceae* são particularmente preocupantes, devido ao crescente aumento da sua resistência aos antimicrobianos. A detecção de estirpes mais resistentes leva a ponderar sobre um eventual aumento simultâneo da virulência. Caso isto se verifique, a emergência de estirpes extremamente patogênicas, para as quais não haverá alternativas terapêuticas, levaria a sérios problemas de Saúde Pública. Desta forma, o principal objectivo desta investigação foi compreender a relação entre virulência e resistência em *E. coli* de diferentes origens e em isolados do género *Klebsiella*.

A relação entre resistência e virulência foi estudada em *E. coli* uropatogénicas. As estirpes de *E. coli* podem ser classificadas em quatro grupos filogenéticos principais. As estirpes mais virulentas pertencem maioritariamente ao grupo B2, seguido do D, enquanto que a maioria das comensais pertencem aos grupos A e B1. Durante a caracterização das estirpes, foi descoberto um novo genótipo usando o método de Clermont, utilizado para identificar os filogrupos. Este genótipo *yjaA/ tscpE4.C2* foi atribuído ao grupo filogenético B2.

As ilhas de patogenicidade (PAIs) são elementos genéticos móveis que transportam genes de virulência e, consequentemente, aumentam a virulência bacteriana. Neste trabalho, as ilhas PAI I_{536}, PAI II_{536}, PAI III_{536} e PAI II_{196} foram associadas ao filogrupo B2. Relativamente a estas ilhas, observou-se uma relação inversa entre resistência e virulência, uma vez que foram geralmente encontradas em estirpes susceptíveis. O mesmo foi observado para a maioria dos genes individuais de virulência, nomeadamente de adesinas, toxinas e sideróforos. Contrariamente, observou-se uma correlação entre a PAI IV_{536}, PAI I_{CFT073} e PAI II_{CFT073} e as resistências à amoxicilina-ácido clavulânico, cefalotina, cefotaxima, ceftazidima e gentamicina. Esta combinação de PAIs juntamente com um plasmídeo IncF, e os genes *bla_{CTX-M-15}* e *aac(6')-lb-cr*, foram também mais prevalentes nos isolados de *E. coli* uropatogénicas produtoras de CTX-M-15, identificadas como pertencendo ao clone ST131.

Observou-se que as estirpes com maior número de plasmídeos identificados tinham menor número de PAIs. Colocou-se então a hipótese de que a presença de
um plasmídeo de resistência e um maior número de PAIs levaria a um custo biológico para a célula. Assim, efectuaram-se ensaios de conjugação e estudos de fitness, tendo sido concluído que a presença de maior número de ilhas não implicava um aumento directo no custo biológico da aquisição do plasmídeo de resistência, pelo menos em estirpes com três ou menos PAIs.

A participação num estudo internacional com estirpes de *E. coli* isoladas na Nigéria permitiu comparar as características destes isolados com os isolados portugueses. Apesar de se terem observado diferenças regionais, o clone ST131, plasmídeo IncF e o gene *bla*CTX-M-15 foram comuns nestes dois países, suportando o facto que este clone, e os respectivos determinantes de resistência, se encontrarem disemnados mundialmente.

O mesmo clone ST131, contendo os determinantes de resistência *bla*CTX-M-15, *bla*TEM-type, *qnrS* e *aac(6’)-Ib-cr*, plasmídeos IncF e IncP, e os factores de virulência PAI IV536, PAI ICF073, PAI IIICF073, *iutA*, *sfa/foc* e *papAH* foi detectado no efluente de uma Estação de Tratamento de Águas Residuais (ETAR) hospitalar, demonstrando-se que as ETARs podem estar a contribuir para a disseminação de bactérias resistentes e virulentas para os ecossistemas naturais, constituindo assim, um risco para a saúde pública e ambiental.

A relação entre resistência e virulência foi também estudada para estirpes de *K. pneumoniae* e *K. oxytoca*. Em *K. pneumoniae*, a PAI IV536 foi estatisticamente associada com a resistência à gentamicina e ao sulfametoxazole-trimetoprim (SXT). Neste estudo, a PAI IIICF073 foi descrita pela primeira vez em *K. oxytoca*, encontrando-se estatisticamente associada com a resistência à cefalotina, cefotaxima, ceftazidima, ciprofloxacina e gentamicina. Contrariamente ao observado em *E. coli*, não foi detectada uma relação inversa entre virulência e resistência. De facto, em *K. pneumoniae*, detectaram-se maiores prevalências de factores de virulência em isolados resistentes à ciprofloxacina e à gentamicina, e em menor escala à cefotaxima, e ao SXT. Além disso, também foi observado em *K. oxytoca* uma maior prevalência de genes de virulência em isolados resistentes ao SXT.

Finalmente, foram estudados isolados de um surto de *K. pneumoniae* multiresistente numa unidade de transplantes renais. Investigaram-se os factores de virulência, determinantes de resistência e a relação clonal. Foram detectadas duas linhagens genéticas principais, o que permitiu excluir a hipótese da ocorrência de um
surto provocado por apenas um clone interno. Neste estudo, as ilhas PAI III\textsubscript{536} e PAI II\textsubscript{CFT073} foram detectadas, pela primeira vez, em \textit{K. pneumoniae}.

Assim, os resultados apresentados nesta tese indicaram que, apesar de se ter verificado uma relação inversa entre virulência e resistência em \textit{E. coli}, o mesmo não se verificou na generalidade dos isolados do gênero \textit{Klebsiella}. Apesar da relação inversa verificada em \textit{E. coli}, o clone ST131, detectado neste estudo entre isolados portugueses e nigerianos, é um exemplo de como, nesta espécie, podem co-existir traços de virulência e resistência. A relação entre resistência e virulência parece assim ser influenciada pelo gênero e espécie, factor de virulência em causa e fenótipo e genótipo de resistência.
Thesis Outline

With the increase of resistance among *E. coli* and *K. pneumoniae*, concerns about the possible emergence of strains simultaneously resistant and virulent start to emerge. The studies presented in this PhD dissertation are focused on the relation between resistance and virulence among these *Enterobacteriaceae*.

Chapter 1 consists in a general introduction.

Chapter 2 presents a new genotype in the Clermont method, used to identify the phylogenetic group of *E. coli*.

Chapter 3 describes the relation between resistance and virulence among a collection of uropathogenic *E. coli*.

Chapter 4 focuses on the genetic relatedness of CTX-M-15 producers among *E. coli*, and identifies a prevalent combination of virulence factors and resistance determinants in ST131 isolates.

Chapter 5 presents the resistance phenotype and genotype of *E. coli* from a Nigerian collection of bovine and clinical isolates.

Chapter 6 characterizes the virulence and resistance profile of *E. coli* from wastewater treatment plants.

Chapter 7 describes the relation between virulence and resistance among *K. pneumoniae* and *K. oxytoca* from several clinical origins.

Chapter 8 characterizes the virulence and resistance profile and clonal relatedness of *K. pneumoniae* isolates from an outbreak in a Renal Transplant Unit.

Chapter 9 presents a general discussion of all the results from this study, as well as the main concluding remarks.

Chapters 2, 4, 5, and 8 were published, chapter 6 has been accepted for publication and chapters 3 and 7 are submitted for publication.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bla</td>
<td>β-Lactamase encoding gene</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>DAEC</td>
<td>Diffusely Adherent <em>E. coli</em></td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>E. coli</em></td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>E. coli</em></td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>E. coli</em></td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>E. coli</em></td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-Spectrum β-Lactamase</td>
</tr>
<tr>
<td>ExPEC</td>
<td>Extraintestinal Pathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>Is</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>ISCR</td>
<td>Insertion Sequence Common Region</td>
</tr>
<tr>
<td>MNEC</td>
<td>Meningitis-associated <em>E. coli</em></td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-Field Gel Electrophoresis</td>
</tr>
<tr>
<td>PLA</td>
<td>Pyogenic liver abscess</td>
</tr>
<tr>
<td>PMQR</td>
<td>Plasmid mediated quinolone resistance</td>
</tr>
<tr>
<td>SEPE</td>
<td>Sepsis-causing <em>E. coli</em></td>
</tr>
<tr>
<td>SXT</td>
<td>Sulfamethoxazole- trimethoprim</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
</tbody>
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Chapter 1

General Introduction
1. *Escherichia coli* and *Klebsiella* spp. as human pathogens

*Escherichia coli* and *Klebsiella* species are important human pathogens, responsible for a growing number of nosocomial and community infections. Nowadays, the growing resistance among these pathogens is considered a concerning threat to public health. It is responsible not only for the failure of empirical treatment, delaying the administration of the adequate antimicrobial treatment, but also to the reduction of therapeutic options leading to higher morbidity and mortality rates.

It is therefore important to understand if more resistant bacteria from these species, may also be simultaneously more virulent. This will allow the better acknowledging of the real danger that these bacteria may constitute to public health and to the environment.

1.1. *Escherichia coli*

*Escherichia coli* is the most common Gram-negative, non-sporulating facultative anaerobe in the human intestinal tract (Dworkin et al., 2006b).

This member of *Enterobacteriaceae* family generally inhabits the terminal part of the small intestine, as well as the large intestine of mammals. The presence of this bacilli in the environment is attributed to fecal contamination, rather than to presenting the capacity to replicate outside of the intestine (Dworkin et al., 2006b). Nonetheless it has been suggested that *E. coli* is capable of replicating in tropical fresh water (Bermúdez & Hazen, 1988).

*E. coli* was firstly identified by Theodore Escherich in 1886, in the feces of a child (Escherich, 1886) and designated *Bacterium coli commune* (common colon bacterium). Afterwards, Alphonse Lesage inferred that this species was composed by innocuous elements, as well as strains with variable pathogenic potential (Lesage, 1897). It is known that commensal *E. coli* are part of the normal intestinal flora, while other *E. coli* strains developed their pathogenic potential, becoming capable of causing disease in humans and animals (Clements, Young, Constantinou, & Frankel, 2012).
1.1.1. *Escherichia coli* clinical importance

*E. coli* pathogenic strains can cause enteric or extraintestinal infections in humans. Enteric infections are generally divided in six pathotypes: Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC, including *Shigella* sp), Diffusely Adherent *E. coli* (DAEC) and Enterotoxigenic *E. coli* (ETEC). This classification is based in their pathogenicity characteristics including virulence factors, phylogenetic background and clinical pathology (Kaper, Nataro, & Mobley, 2004). Furthermore, *E. coli* strains responsible for infections outside of the gastrointestinal tracts, including uropathogenic strains isolated from the urinary tract (UPEC), sepsis-causing *E. coli* (SEPEC), as well as meningitis-associated *E. coli* (MNEC) have been grouped as extraintestinal pathogenic *E. coli* (ExPEC) (Johnson & Russo, 2002b; Russo & Johnson, 2000). ExPEC are the most common cause of urinary tract infections (UTI), sepsis, community-acquired bacteremia, neonatal sepsis and neonatal meningitis. They can also be pathogenic agents in nosocomial pneumonia, intra-abdominal infections, and less frequently, can be implicated in other infections such as cellulitis, wound infections and osteomyelitis (Johnson & Russo, 2002a).

*E. coli* strains are classically classified into four main phylogenetic groups (A, B1, B2, and D). This classification scheme is based in the simple and rapid detection of two genes (*chuA* and *yjaA*) and a DNA fragment TspE4C2 (Clermont, Bonacorsi, & Bingen, 2000). According to this classification, virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D, while the majority of commensal strains belong to group A.

1.1.2. Uropathogenic *Escherichia coli* and urinary tract infections

UPEC are the most frequent pathotype of ExPEC. They are a heterogeneous group of clones responsible for urinary tract infections (UTIs). UPEC are responsible for 70-90% of community UTIs, as well as 50% of nosocomial urinary infections (Kucheria, Dasgupta, Sacks, Khan, & Sheerin, 2005). It has been estimated that, more than 50% of women will experience an UTI during their lifetime, 25% will be affected for a second urinary infection and, in addition, 3% will suffer a third UTI during the next six months after the primo-infection (Smith, Fratamico, & Gunther, 2007).
According to the site of infection, UTIs may be classified as cystitis (bladder), prostatitis (prostate), pyelonephritis (kidney), or asymptomatic bacteriuria (urine). While in immunocompetent individuals, cystitis is generally resolved without further problems, pyelonephritis cases can be responsible for increased morbidity and can be fatal. In addition, some persons may present asymptomatic bacteriuria, characterized by high concentrations of bacteria in the urine, without having any clinical symptoms (Smith et al., 2007).

Several factors that may predispose to the development of UTIs have been identified. These include: age (infants and elderly); pregnancy; diabetes; multiple sclerosis; persons with spinal cord damages; urinary catheters; HIV infection and abnormal urologic apparatus (Foxman, 2002).

_E. coli_ infections affecting the urinary tract are thought to begin with an UPEC strain harboring several virulence factors colonizing the bowel, and further contaminating the perirectal space. Afterwards, the bacteria ascend throughout the urinary apparatus, up to the urethra and posteriorly to the bladder, using flagella. Once in the bladder, UPEC uses a set of adhesins for colonization, toxins to escape from the host innate immune system, and iron acquisition mechanisms to promote growth. Afterwards, some _E. coli_ can detach and ascend to the kidneys, causing pyelonephritis (Bien, Sokolova, & Bozko, 2012).

### 1.2. Klebsiella spp.

*Klebsiella* genus belongs to _Enterobacteriaceae_ family, and is composed by gram-negative rod-shaped bacteria, generally encapsulated, lysine decarboxylase but not ornithine decarboxylase producers, and commonly positive in the Voges-Proskauer test (Dworkin et al., 2006a).

*Klebsiella* spp. are ubiquitous in the environment, and are often detected in a variety of environmental sources including water, soil, vegetation and sewage (Podschun & Ullmann, 1998). In addition, _Klebsiella_ spp. have also been detected in insects (Dillon, Vennard, & Charnley, 2002) and several mammals (Gordon & FitzGibbon, 1999). In humans, _Klebsiella_ spp. may be a commensal colonizing the nasopharynx and gastrointestinal tract, or may act as an opportunistic human pathogen (Podschun & Ullmann, 1998).
1.2.1. *Klebsiella* spp. clinical importance

*K. pneumoniae* is the most relevant pathogen within genus *Klebsiella*, being responsible for 75% to 86% of *Klebsiella* spp infections. Additionally, *K. oxytoca* is the second most prevalent *Klebsiella* species, being responsible for 13% to 25% of infections (Broberg, Palacios, & Miller, 2014).

*Klebsiella* spp., as human pathogens, may be responsible for both community and nosocomial infections. In the community set, *K. pneumoniae* is associated to several infections. It is responsible for Friedländer's pneumoniae, a community-acquired pulmonary infection, which affects chronic alcoholics, and whose incidence has been decreasing over the years (Carpenter, 1990; Dworkin et al., 2006a). Additionally, three new severe clinical syndromes have been emerging in Asia. First of all, community-acquired primary pyogenic liver abscess (PLA), a *K. pneumoniae* infection that as emerged in the past 20 years and may or may not be associated to septic metastatic complications (Keynan & Rubinstein, 2007). The majorities of PLA cases are generally associated with K1 serotype and have been detected mainly in Taiwan (Chuang, Fang, Lai, Chang, & Wang, 2006). Another clinical manifestation is *K. pneumoniae* endophtalmitis, which may be a secondary complication of PLA and is similarly mainly detected in Asia (Connell, Thomas, Sabharwal, & Gelbard, 2007; Liu, Cheng, & Lin, 1986). Finally, in Taiwan, *K. pneumoniae* is also responsible for community-acquired bacterial meningitis in adults, without having connection to infections in other body locations (Keynan & Rubinstein, 2007; Tang, Chen, Hsu, & Chen, 1997).

Considering nosocomial infections, *Klebsiellae* are considered important opportunistic pathogenic agents, being responsible for infections mainly located in the urinary and respiratory tracts, but which may also affect soft tissues and wounds and cause septicemia (Dworkin et al., 2006a). Several host characteristics such as diabetes mellitus, extremes of age, renal, cardiac or pulmonary chronic diseases, as well as oncologic problems, may predispose to *Klebsiella* spp. infections (Feldman et al., 1990; Hansen, Gottschau, & Kolmos, 1998).

In the hospital setting, *Klebsiella* spp. colonization rates increase in a direct proportion to the duration of the hospitalization, and also seem to be associated with antibiotic therapy (Podschun & Ullmann, 1998).
2. Antimicrobial resistance

Bacteria can present different resistance phenotypes, in which multidrug resistant is defined if bacteria present resistance to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). Thus, considering the progressive risk of occurrence of untreatable infections due to multidrug resistant bacteria, antimicrobial resistance has become a central problem worldwide.

Multidrug resistance in Gram-negative bacteria, particularly Enterobacteriaceae such as E. coli and K. pneumoniae, has become one of the biggest global concerns. Infections with these bacteria lead to prolonged hospital admissions and higher mortality rates (Munoz-Price et al., 2013).

In Portugal, increasing rates of resistance to important antibiotics such as beta-lactams, fluoroquinolones and aminoglycosides have been reported for both E. coli and K. pneumoniae. Data on resistance reported by the European Antimicrobial Resistance Surveillance System (EARSS) for these microorganisms in Portugal between 2003 and 2012 are shown in Table 1.1 (EARSS, 2013).

| Table 1.1 – Annual percentage (%) of antimicrobial resistance reported between 2003 and 2012 in Portugal for Escherichia coli and Klebsiella pneumoniae (adapted from EARSS 2013). |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **E. coli** |  |  |  |  |  |  |  |  |  |
| Aminopenicillins | 53 | 58 | 58 | 59 | 59 | 58 | 58 | 56 | 57 | 59 |
| Third generation Cephalosporins | 7 | 8 | 12 | 10 | 10 | 10 | 9 | 10 | 11 | 14 |
| Fluoroquinolones | 26 | 27 | 29 | 28 | 30 | 29 | 28 | 27 | 27 | 30 |
| Aminoglycosides | 9 | 13 | 12 | 12 | 12 | 14 | 11 | 12 | 16 | 16 |
| **K. pneumoniae** |  |  |  |  |  |  |  |  |  |  |
| Third generation Cephalosporins | - | - | - | 21 | 17 | 26 | 28 | 28 | 35 | 39 |
| Fluoroquinolones | - | - | <1 | 20 | 18 | 22 | 28 | 31 | 36 | 36 |
| Aminoglycosides | - | - | <1 | 13 | 11 | 19 | 20 | 27 | 32 | 32 |
In general, resistance mechanisms are mainly due to increased efflux of the antibiotic, decreased wall penetration, target modification, or enzymatic alteration or inactivation of the antimicrobial agent (Figure 1.1).

![Figure 1.1 – General mechanisms of antimicrobial resistance.](image)

### 2.1. Resistance to beta-lactams

Resistance to β-lactams may be a result of several mechanisms including modification of penicillin-binding proteins, loss of porins, production of β-lactamases or overexpression of efflux pumps (Talbot, 2013). Nonetheless, in Gram-negative bacteria, β-lactams resistance is mainly due to the hydrolytic action of β-lactamases. These enzymes inactivate beta-lactam antibiotics by hydrolyzing the peptide bond of the four-membered beta-lactam ring (Majiduddin, Materon, & Palzkill, 2002).

Classification of β-lactamases has been conventionally made using two different approaches, one based on the primary structure of the enzymes (Ambler, 1980) an other on their functional characteristics (Bush, Jacoby, & Medeiros, 1995; Richmond & Sykes, 1973).

The Ambler scheme classifies β-lactamases into four major classes (A to D) according to their protein homology, not considering their phenotypic characteristics.
In this scheme, β-lactamases from classes A, C, and D are serine β-lactamases, while the class B enzymes are metallo- β-lactamases (Paterson & Bonomo, 2005).

The functional classification is based in functional similarities such as substrate and inhibitor profile. Recently an updated functional classification scheme was published by Bush and Jacoby (Bush & Jacoby, 2010). The main classification schemes for β-lactamases and the new updated classification are represented in Table 1.2.

Four major groups of β-lactamases can be identified considering the substrate characteristics: penicillinases, AmpC-type cephalosporinases, Extended-Spectrum β-lactamases (ESBLs) and carbapenemases, with ESBLs being the largest group (Bush, 2010).

2.1.1. Naturally occurring resistance

Several gram-negative bacteria have an intrinsic chromosomally mediated β-lactamase (Bush & Fisher, 2011). *E. coli* naturally present insignificant levels of a non-inducible chromosomal AmpC β-lactamase. Due to its limited capacity, this AmpC is only responsible for resistance to agents which have a poor capacity of penetration like isoxazolyl penicillins and benzylpenicillin, and to cefsulodin. *E. coli* carrying only this non-inducible AmpC are characteristically susceptible to ampicillin and the narrow-spectrum cephalosporins, including cephalothin and cephalaxin (Livermore, 1995).

*Klebsiella* spp. also presents one chromosomal intrinsic gene belonging to the *bla*SHV, *bla*OKP or *bla*LEN family in *K. pneumoniae*, and *bla*OXY in *K. oxytoca*. Among *K. pneumoniae*, these β-lactamases confer natural resistance to ampicillin and amoxicillin (aminopenicillins); carbenicillin and ticarcillin (carboxypenicillins) and other penicillins, but are unable to confer resistance to the majority of the remaining β-lactam antibiotics (Dworkin et al., 2006a).

Despite of the occurrence of these chromosomally encoded β-lactamases among *E. coli* and *K. pneumoniae*, the transferable β-lactamases including plasmidic ESBLs are of much more concern, and constitute a major threat in antimicrobial resistance.
Table 1.2 – Classification schemes for β-lactamases, adapted from Bush and Jacoby 2010.

<table>
<thead>
<tr>
<th>Bush-Jacoby group (2009)</th>
<th>Molecular class (subclass)</th>
<th>Distinctive substrate(s)</th>
<th>Inhibited by CA or TZB</th>
<th>Inhibited by EDT A</th>
<th>Defining characteristic(s)</th>
<th>Representative enzyme(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>Cephalosporins</td>
<td>No</td>
<td>No</td>
<td>Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins</td>
<td>E. coli AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1</td>
</tr>
<tr>
<td>1e</td>
<td>C</td>
<td>Cephalosporins</td>
<td>No</td>
<td>No</td>
<td>Increased hydrolysis of ceftazidime and other oxyimino-β-lactams</td>
<td>GC1, CMY-37</td>
</tr>
<tr>
<td>2a</td>
<td>A</td>
<td>Penicillins</td>
<td>Yes</td>
<td>No</td>
<td>Greater hydrolysis of benzylpenicillin than cephalosporins</td>
<td>PC1</td>
</tr>
<tr>
<td>2b</td>
<td>A</td>
<td>Penicillins, early cephalosporins</td>
<td>Yes</td>
<td>No</td>
<td>Similar hydrolysis of benzylpenicillin and cephalosporins</td>
<td>TEM-1, TEM-2, SHV-1</td>
</tr>
<tr>
<td>2be</td>
<td>A</td>
<td>Extended-spectrum cephalosporins, monobactams</td>
<td>Yes</td>
<td>No</td>
<td>Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftizidime, ceftriaxone, cefepime, aztreonam)</td>
<td>TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1</td>
</tr>
<tr>
<td>2br</td>
<td>A</td>
<td>Penicillins</td>
<td>No</td>
<td>No</td>
<td>Resistance to clavulanic acid, sublactam, and tazobactam</td>
<td>TEM-30, SHV-10</td>
</tr>
<tr>
<td>2ber</td>
<td>A</td>
<td>Extended-spectrum cephalosporins, monobactams</td>
<td>No</td>
<td>No</td>
<td>Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sublactam, and tazobactam</td>
<td>TEM-50</td>
</tr>
<tr>
<td>2c</td>
<td>A</td>
<td>Carbenicillin</td>
<td>Yes</td>
<td>No</td>
<td>Increased hydrolysis of carbenicillin</td>
<td>PSE-1, CARB-3</td>
</tr>
<tr>
<td>2ce</td>
<td>A</td>
<td>Carbenicillin, cefepime</td>
<td>Yes</td>
<td>No</td>
<td>Increased hydrolysis of carbenicillin, cefepime, and cefpirome</td>
<td>RTG-4</td>
</tr>
<tr>
<td>2d</td>
<td>D</td>
<td>Cloxacillin</td>
<td>Variable</td>
<td>No</td>
<td>Increased hydrolysis of cloxacillin or oxacillin</td>
<td>OXA-1, OXA-10</td>
</tr>
<tr>
<td>2de</td>
<td>D</td>
<td>Extended-spectrum cephalosporins</td>
<td>Variable</td>
<td>No</td>
<td>Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams</td>
<td>OXA-11, OXA-15</td>
</tr>
<tr>
<td>2df</td>
<td>D</td>
<td>Carbapenems</td>
<td>Variable</td>
<td>No</td>
<td>Hydrolyzes cloxacillin or oxacillin and carbapenems</td>
<td>OXA-23, OXA-48</td>
</tr>
<tr>
<td>2e</td>
<td>A</td>
<td>Extended-spectrum cephalosporins</td>
<td>Yes</td>
<td>No</td>
<td>Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam</td>
<td>CepA</td>
</tr>
<tr>
<td>2f</td>
<td>A</td>
<td>Carbapenems</td>
<td>Variable</td>
<td>No</td>
<td>Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephalosporins</td>
<td>KPC-2, IMI-1, SME-1</td>
</tr>
<tr>
<td>3a</td>
<td>B (B1)</td>
<td>Carbapenems</td>
<td>No</td>
<td>Yes</td>
<td>Broad-spectrum hydrolysis including carbapenems but not monobactams</td>
<td>IMP-1, VIM-1, CcrA, IND-1</td>
</tr>
<tr>
<td>3b</td>
<td>B (B2)</td>
<td>Carbapenems</td>
<td>No</td>
<td>Yes</td>
<td>Preferential hydrolysis of carbapenems</td>
<td>CphA, Sfh-1</td>
</tr>
</tbody>
</table>
2.1.2. Extended spectrum β-lactamases

The designation ESBL was used to define β-lactamases with a broad spectrum of hydrolysis, resulting from the occurrence of amino acid substitutions in the structure of the enzymes (Livermore, 2008). ESBLs belong to Class A according to Ambler’s classification, and group 2be functional group of Bush’s classification (Ambler et al., 1991; Bush & Jacoby, 2010). These β-lactamases are active against broad-spectrum cephalosporins (such as ceftazidime, cefotaxime and ceftriaxone) but are inhibited by β-lactamases inhibitors like tazobactam, sulbactam and clavulanic acid (Poirel, Bonnin, & Nordmann, 2012).

The majority of the ESBLs detected among Enterobacteriaceae belong to three main families: TEM, SHV and CTX-M.

2.1.2.1. TEM β-lactamases

The TEM (for Temoneira patient’s name) β-lactamase family is composed of more than 219 variants according to the database of the Lahey Clinic (http://lahey.org/studies/temtable.asp last access on 22 of September 2014).

The first enzyme of this family described, the native TEM-1 β-lactamase is capable of hydrolyzing ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has insignificant activity against extended-spectrum cephalosporins. In addition, it is inhibited by clavulanic acid. TEM-2, was the first variant identified, but it is not considered an ESBL as the hydrolitic profile is identical to TEM-1. Besides an amino acid substitution (Q39K), TEM-2 differs from TEM-1 because it presents a more active promoter and has a different isoelectric point (Paterson & Bonomo, 2005).

The TEM-type ESBLs are derivates of TEM-1 and TEM-2. Amino acid substitutions acting alone or with additional other structural gene mutations, have been detected in over than 90 described TEM-1-or TEM-2-derived enzymes, according to internet site of the Lahey Clinic. Each TEM-derived slightly differs in the hydrolytic profile, and therefore one ESBL may hydrolyze a specific extended-spectrum cephalosporin in a more efficient way than another ESBL (Rupp and Fey 2003).

The most disseminated TEM-type ESBLs among Enterobacteriaceae in Europe in the clinical setting are TEM-24, TEM-4 and TEM-52, while in isolates from
animals TEM-52, TEM-106 and TEM-116 are the most common (Coque, Baquero, & Cantón, 2008a).

2.1.2.2. SHV β-lactamases

The SHV (for Sulfhydryl reagent Variable) β-lactamase family comprises 185 variants according to the internet site of the Lahey Clinic.

The native SHV-1 β-lactamase is an enzyme which may be chromosomally or plasmid encoded, and is responsible for resistance to penicillins (Livermore, 1995).

SHV-type ESBLs are point mutants of both narrow-spectrum β-lactamases SHV-1 or SHV-11 that had origin in the K. pneumoniae chromosome (Poirel et al., 2012). The hydrolytic spectrum of SHV-type ESBLs includes activity against oxyimino-β- lactams such as cefotaxime, ceftazidime, ceftriaxone and aztreonam (Bush & Jacoby, 2010).

According to internet site of the Lahey Clinic, more than 45 SHV-type ESBLs have been described so far. In Europe, SHV-5 is widespread in the clinical setting, while SHV-12 has been reported in both human and animal strains (Coque et al., 2008a).

2.1.2.3. CTX-M β-lactamases

CTX-M-type (for Cefotaximase firstly identified in Munich) β-lactamases are a family of ESBLs which comprise 157 elements, according to the internet site of the Lahey Clinic.

These β-lactamases confer resistance to penicillins and expanded-spectrum cephalosporins, and the majority of the variants present higher rates of hydrolysis to cefotaxime than to ceftazidime (Bonnet, 2004). Nonetheless, some of these enzymes, including CTX-M-15, -16,- 25, -27, -28, -29 and- 32 have an Asp240Gly substitution which enhances the catalytic activity against ceftazidime (Woodford & Ellington, 2007).

CTX-M enzymes are also susceptible to clavulanate and tazobactam β-lactamase inhibitor combinations, with tazobactam presenting greater inhibitory activity compared to clavulanic acid (Bonnet, 2004).
The main CTX-M enzymes may be classified into five clusters according to the amino acid sequences (Bonnet, 2004). Table 1.3 presents the five main groups and corresponding enzymes (Poirel et al., 2012).

**Table 1.3** – Main groups of CTX-M enzymes according to amino acid sequences and corresponding elements.

<table>
<thead>
<tr>
<th>CTX-M group</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M-1</td>
<td>CTX-M-1,3,10,11, 12, 15, 22, 23, 28-30, 32-34, 36, 37, 42, 52-54, 57, 58, 60,61</td>
</tr>
<tr>
<td>CTX-M-2</td>
<td>CTX-M-2, 4-7, 20, 31, 35, 43, 44</td>
</tr>
<tr>
<td>CTX-M-8</td>
<td>CTX-M-8, 40, 63</td>
</tr>
<tr>
<td>CTX-M-9</td>
<td>CTX-M-9, 13, 14, 16, 17-19, 21, 24, 27, 38, 46-51, 55, 65</td>
</tr>
<tr>
<td>CTX-M-25</td>
<td>CTX-M-25, 26, 39, 41</td>
</tr>
</tbody>
</table>

The bla_{CTX-M} genes had origin from the chromossome of different *Kluyvera* species. *Kluyvera georgiana* is though to be the progenitor of bla_{CTX-M-8-like} and bla_{CTX-M-9-like} genes, while *Kluyvera ascorbata* and *Kluyvera cryocrescens* the progenitors of bla_{CTX-M-1-like} and bla_{CTX-M-2-like} genes respectively (Bonnet 2004).

The dissemination of bla_{CTX-M} genes is now a global concern, as they are worldwide disseminated in both nosocomial and community-acquired pathogens (Hawkey & Jones, 2009; Poirel et al., 2012). The main CTX-M-types identified worldwide are represented in Figure 1.2.

The epidemiology of CTX-M β-lactamases is associated to plasmid dissemination on one hand, and clonal success on the other. The most prevalent enzyme, CTX-M-15, is mainly carried by IncF conjugative plasmids (Carattoli, 2009). In *K. pneumoniae*, these plasmids are associated with diverse strains, none of them being a wide case of success (Livermore, 2012). Contrarily, in *E. coli*, IncF plasmids carrying bla_{CTX-M-15} are strongly associated with sequence type (ST)131 (Livermore, 2012). This is a highly successful clone, which is, nowadays, worldwide disseminated and, also, the predominant *E. coli* lineage among ExPEC (Nicolas-Chanoine, Bertrand, & Madec, 2014).
2.2. Resistance to Quinolones

The main and most clinically relevant mechanism of resistance to quinolones is specific amino acid substitutions in the quinolones targets. In addition, other chromosomal mechanisms including under expression of porins or overexpression of efflux pumps have also been described (Aldred, Kerns, & Osheroff, 2014). Several transferable plasmid-mediated mechanisms can also occur, being responsible for different levels of resistance.

2.2.1. Target mediated Quinolone resistance

The main mechanism leading to high-levels of fluoroquinolone resistance is the acquisition of mutations, in one or more genes, that encode the targets of these antibiotics, the type II topoisomerases (Redgrave, Sutton, Webber, & Piddock, 2014). The target genes are DNA gyrase subunits $gyrA$ and $gyrB$, and DNA topoisomerase IV subunits $parC$ and $parE$. The region in which the mutations occur, is a small DNA sequence denominated quinolone resistance determining region (QRDR) (Piddock, 1999). Mutations in this region, lead to amino acid substitutions, and consequent changes of the target protein structure. This alters the fluoroquinolone-binding affinity of the enzyme and causes resistance (Piddock, 1999). The most common mutation in
Gram-negatives involves a substitution at a serine 83 within the gyrA (Redgrave et al., 2014). Alterations in the primary target site may be further followed by secondary mutations in lower affinity binding sites. In fact, highly resistant Gram-negative bacteria characteristically carry a combination of mutations within gyrA and parC genes (Everett, Jin, Ricci, & Piddock, 1996).

2.2.2. Plasmid-mediated quinolone resistance (PMQR)

The PMQR mechanisms include proteins that avoid quinolone-enzyme interaction (Qnr), increase antibiotic efflux by efflux pumps (QepA or OqxAB) or enzymes that alter the drug [AAC (6’)-lb-cr]. Nonetheless, these mechanisms only slightly increase the minimum inhibitory concentration (MIC) of quinolones (Ruiz, Pons, & Gomes, 2012).

2.2.2.1. QNR proteins

Qnr proteins are encoded by the qnr genes and belong to the pentapeptide repeat protein family (Aldred et al., 2014). These proteins interact with DNA gyrase and topoisomerase IV, physically preventing the interaction between the antibiotic and the target enzymes. This lack of contact avoids the action of quinolones, and consequently reduces their inhibitory effect (Ruiz et al., 2012).

Qnr proteins are grouped in five main families according to their DNA homology, each of them composed of one or more alleles: QnrA (seven alleles), QnrB (48 alleles); QnrS (six alleles), QnrC (one allele) and QnrD (one allele) (Ruiz et al., 2012).

The qnr genes commonly confer modest protection against fluoroquinolones. The transfer of the original qnrA plasmid into a receptor increased in the MIC of ciprofloxacin from 0.008µg/ml to 0.25 µg/ml (Martínez-Martínez, Pascual, & Jacoby, 1998). Plasmids carrying qnrS or qnrB genes also confer levels of quinolone resistance similar to the ones conferred by qnrA1 (Strahilevitz, Jacoby, Hooper, & Robicsek, 2009).

2.2.2.2. AAC (6’)-lb-cr

AAC (6’)-lb-cr is a variant of an aminoglycoside acetyltransferase which confers low levels of resistance to quinolones, and maintains its ability to inactivate
aminoglycosides. It acts by acetylating the amino nitrogen on the piperazinyl substituent in ciprofloxacin and norfloxacin, not affecting other quinolones that lack non-substituted piperazinyl nitrogen. This enzyme increases the MICs of ciprofloxacin and norfloxacin in approximately three to fourfold (Robicsek et al., 2006b).

This variant contains two specific point mutations, D179Y which seems to be responsible for increasing the affinity of this enzyme for fluoroquinolones, and W102R which is thought to play a role in stabilizing the interactions, or in interacting with the fluoroquinolone carboxylate (Maurice et al., 2008; Ruiz et al., 2012).

AAC-(6')-Ib-cr, which is either carried by plasmids or chromosomally encoded, has a huge dissemination potential. It has been described worldwide, including in strains from farm animals and environmental bacteria (Ruiz et al., 2012).

2.2.2.3. Efflux pumps

There are different families of efflux pumps with the capacity to pump out quinolones. They present different affinities and affect the MICs in different manners. Three quinolones efflux pumps have been described so far, QepA1, QepA2 and OqxAB. Nonetheless, while QepA efflux pumps have been found mainly in human infections, OqxAB is almost exclusively detected in animal infections (Aldred et al., 2014).

The qepA1 gene encodes a proton-dependent efflux pump. The substrates for this pump are hydrophilic quinolones such as ciprofloxacin and norfloxacin, nonetheless, it has reduced or no effect on more hydrophobic quinolones including nalidixic acid, levofloxacin or moxifloxacin (Ruiz et al., 2012). It increases the MICs of nalidixic acid, ciprofloxacin and norfloxacin in 2-, 32- and 64- fold, respectively (Yamane et al., 2007).

QepA2 is another QepA-like efflux pump which presents a spectrum of substrates similar to QepA1, and only differs from this pump in two of the 511 amino acids and in the genetic environment (Ruiz et al., 2012).

Despite of the fact that QepA-like efflux pumps may act as a factor in favoring the development of full resistance either to ciprofloxacin or norfloxacin, the prevalence of these pumps seems to be very low, with reported frequencies between 0.3- 0.8% (Ruiz et al., 2012).
2.3. Resistance to aminoglycosides

Resistance to aminoglycosides may be mediated by different mechanisms: modification of the 16S RNA of bacterial 30S ribosomal subunit by mutation or methylation of the aminoglycoside binding site; decrease of the intracellular concentration of the antibiotic resulting from the occurrence of modifications in the bacterial outer membrane; increase of the activity of active efflux systems; decrease of the drug transport into the cell; and by enzymatic deactivation of aminoglycosides (Houghton, Green, Chen, & Garneau-Tsodikova, 2010).

The aminoglycosides modifying enzymes are N-acetyltransferases (AAC), which deactivate the aminoglycosides by N-acetylation in positions 3, 29, and 69; O-phosphotransferases (APH) that modify the antibiotic in positions 39 and 20 by O-phosphorylation; and O-nucleotidytransferases (ANT) which cause aminoglycosides deactivation at positions 49 and 20 by O-nucleotidylation (Shakil, Khan, Zarrilli, & Khan, 2008).

2.4. Antimicrobial resistance dissemination

Antimicrobial resistance dissemination has been associated to the elevated use of antimicrobial agents in both human and veterinary medicine, agriculture, higher movement of people and increased industrialization (Figure 1.3) (Cantas et al., 2013).

In addition, wastewater treatment plants (WWTPs) are important reservoirs of antimicrobial resistant bacteria and resistance genes (Figure 1.3), as they contain wastewaters from human and animal origins. The concomitant presence of commensal and pathogenic resistant bacteria, with antibiotics which are not fully degraded in the untreated waters, may favor and select these microorganisms (Cantas et al., 2013). Although the wastewater treatment reduces the concentration of resistant bacteria, treated water still contains antimicrobial resistant bacteria and this may therefore contribute to the selection and further dissemination of multidrug resistant microorganisms (Czekalski, Berthold, Caucci, Egli, & Bürgmann, 2012).

The interaction between bacteria from different backgrounds, may contribute to the dissemination of different resistance traits by horizontal gene transfer, thus contributing to the aggravation of this worldwide concern.
2.4.1. Mechanisms of horizontal gene transfer

Horizontal gene transfer is the stable unidirectional transfer of genetic material from one organism (donor) into another (receptor) without reproduction (Syvanen, 1994). It is driven by three main mechanisms: bacterial transformation, bacterial transduction and bacterial conjugation.

Natural transformation consists in the uptake of foreign extracellular DNA which may be released from living cells by excretion, or from decomposing or disrupted cells or viral particles; its integration and functional expression under natural bacterial growth conditions. It requires that naturally transformable bacteria develop a genetically programmed physiological state of competence, which is achieved as a response to specific environmental conditions, including starvation and changes in the nutrient access, growth conditions or cell density (Thomas & Nielsen, 2005). After the uptake, the DNA can be integrated in the bacterial genome by
homologous recombination, homology-facilitated illegitimate recombination, or may form an autonomous self-replicating element (de Vries & Wackernagel, 2002).

Transduction is another mechanism contributing to antibiotic resistance gene transfer. It occurs when a bacteriophage that has previously infected and replicated in another bacteria (donor), packages a part of the host genome into the phage head, and further transfers the genes to another bacteria (recipient) (Huddleston, 2014).

Finally, conjugation is the process by which a DNA molecule is transferred from a donor to a recipient, mediated by cell-to-cell junctions and a pore through which DNA can pass (Thomas & Nielsen, 2005). This gene transfer mechanism involves cell-to-cell contact, mating pair formation, and finally transfer of plasmid DNA through a conjugative pilus (Huddleston, 2014).

Some plasmids are conjugative as they harbor genes encoding the transfer machinery and thus are self-transmissible. On the other hand, other plasmids are non-conjugative, and thus, are only mobilized when a helper self-transmissible plasmid is present (Huddleston, 2014).

### 2.4.2. Plasmids and the dissemination of resistance

Plasmids constitute one of the most difficult issues to control, in order to avoid the spread of antimicrobial resistance, as these elements contribute to the dissemination of important resistance genes (Carattoli, 2013). They can acquire mobile genetic elements, including transposons and insertion sequences, which can lead to the mobilization of the antimicrobial resistance genes. Furthermore, they may promote the dissemination of these genes among bacteria from different species, genera or kingdoms, depending on their host range (Thomas & Nielsen, 2005).

Plasmids may carry genes that can be responsible for conferring resistance to the major classes of antibiotics including β-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, trimethoprim, macrolides and chloramphenicol (Carattoli, 2009). In fact, regularly, multiple physically linked genetic determinants, conferring resistance to different classes of antibiotics, may be carried in the same plasmid. These plasmids may be responsible for the acquisition of a multidrug resistant phenotype, and thus, may provide selective advantage to the bacteria which carries the plasmid (Carattoli, 2013).
Plasmid identification in Enterobacteriaceae, is standardly performed using PCR-based replicon typing, a method that targets the main families of replicons (Carattoli et al., 2005).

In addition to the typical characterization of the resistance profile of bacteria, the necessity to characterize the bacterial virulence starts to emerge.

3. Virulence characterization

A better knowledge of the virulence factors carried by a strain may help to determine the real pathogenic potential of bacteria, and the possible evolution of an infectious disease. Strains that carry a great armory of virulence factors may be responsible for the development of serious health conditions in a short period of time.

Several virulence factors have been described in bacteria. These include fimbriae and other adhesins, siderophore systems, toxins, capsule, pathogenicity islands and several putative virulence factors.

3.1. Escherichia coli virulence factors

3.1.1. Fimbriae and other adhesins

In the pathogenesis of UTIs, the successful colonization of the urinary tract is a crucial step to the development of infection. The attachment to the host epithelium mediated by fimbriae and other adhesins, avoids that bacteria to be washed out during the urination process. Several adhesins have been described in UPEC.

3.1.1.1. P fimbriae

P fimbria was the first virulence factor of UPEC being described (Edén, Hanson, Jodal, Lindberg, & Akerlund, 1976). Edén and Hansson reported that E. coli involved in acute symptomatic pyelonephritis adhered more frequently to exfoliated uroepithelial cells, compared to E. coli from patients with asymptomatic bacteriuria. Later, the presence of fimbriae, was associated to the ability of UPEC to attach to human uroepithelial cells (Edén & Hansson, 1978).
P fimbria is encoded by the *pap* (pyelonephritis associated pili) operon, comprising 11 genes (Hull, Gill, Hsu, Minshew, & Falkow, 1981). It is considered an important virulence factor, being detected in more than 80% of pyelonephritogenic *E. coli* (Antão, Wieler, & Ewers, 2009). These adhesins are strongly associated with pyelonephritis, and are essential to adhesion to renal tissue, but are unnecessary for bladder colonization in cynomogus monkeys (Roberts et al., 1994). In addition, P pili have a significant role in initiating pyelonephritis in normal urinary tracts, but seem to be less important in the colonization of urinary tracts with obstructions or abnormalities (Tseng, Huang, Ko, Yan, & Wu, 2001). P-fimbriae are positively related to the severity of the infection (Spurbeck et al., 2011). These pili increase the severity of the UTI by allowing a strong attachment to the vascular endothelium as well as to the muscular layer and promoting weak adherence to bladder epithelium (Virkola et al., 1988). It has also been proposed that P-fimbriae may enable UPEC to establish a reservoir in the intestine, allowing the UPEC persistence in the intestinal flora (Goetz et al., 1999).

### 3.1.1.2. S and F1C fimbriae

S fimbriae are a group of fimbriae discovered in pyelonephritogenic *E. coli* which have the capacity to recognize structures containing neuraminic acid (sialic acid), other than P antigens of human red blood cells or mannosides (Parkkinen, Finne, Achtman, Väisänen, & Korhonen, 1983).

S pili are important adhesins and are frequently associated with *E. coli* causing meningitis, sepsis, ascending UTIs, including cystitis and pyelonephritis (Korhonen et al., 1985; Malagolini, Cavallone, Wu, & Serafini-Cessi, 2000; Parkkinen, Korhonen, Pere, Hacker, & Soinila, 1988). In fact, S fimbriae have been associated with several ExPEC strains, being present in 50% of UPEC, 24% of NMEC and 9.2% of APEC strains (Ewers et al., 2007).

Other adhesins genetically homologous to S fimbriae have been described, including F1C; but despite of the genetic similarities, they exhibit differences in the receptors specificity (Ott, Hoschützky, Jann, Van Die, & Hacker, 1988). The binding specificity of F1C pili allied with the fact that these structures are present in approximately 14% of UPEC, indicates that they may be important in the pathogenesis of UTI (Mulvey, 2002).
On *E. coli* Nissle 1917, a probiotic and colonizer of the human intestine, F1C fimbriae are necessary for biofilm formation on inert surfaces, adherence to epithelial cells, as well as persistence during the colonization of an infant mouse (Lasaro et al., 2009).

### 3.1.1.3. Dr/Afa Adhesins

Vaisanen-Rhen firstly described a mannose–resistant P blood group independent haemagglutinin, further named 075X, because it was detected in UPEC from serogroup 075 (Väisänen-Rhen, 1984). Later, 075X was termed Dr haemagglutinin (Nowicki, Moulds, Hull, & Hull, 1988).

Dr adhesins are a family of adhesins from *E. coli*, which target the Dr blood group antigen (also known by decay-accelerating factor) as receptor (Nowicki et al., 1988). This antigen is present in the Bowman’s capsule and the tubular basement membrane of the kidney (Nowicki et al., 1988). Dr adhesin family elements, also have the capacity to bind to carcinoembryonic antigen (CD66e) (Guignot et al., 2000). Furthermore, Dr adhesin contrarily to other members of this family, can also bind to type IV collagen (Nowicki, Selvarangan, & Nowicki, 2001).

The Dr family comprises fimbrial adhesins, such as Dr adhesins and F1845, as well as non-fimbrial adhesins, including AFA-I and AFA-III (Nowicki et al., 1990). The members of this family are referred in Table 1.4.

Elements from Dr adhesins family are thought to have a role in the ascending colonization of the urinary tract, as well as in chronic interstitial infection (Mulvey, 2002). In addition, *E. coli* expressing Dr fimbriae are able of causing tubulointerstitial nephritis and persist in the kidney tissue, thus contributing to recurrent UTIs (Goluszko et al., 1997a).

The expression of Dr adhesins in *E. coli* causing infections in pregnant women may constitute a threat, because of the elevated invasive potential of these bacteria, associated to the up-regulation of the DAF receptor during pregnancy (Goluszko et al., 2001).

Dr fimbriae also mediates cell invasion, which allows bacteria to hide in the intracellular space and, consequently, to escape from the humoral immune response (Das et al., 2005; Goluszko et al., 1997b).
Table 1.4 – Members of the family of Dr adhesins (adapted from Nowicki, Selvarangan at al. 2001).

<table>
<thead>
<tr>
<th>Adhesion factor</th>
<th>Type</th>
<th>Adhesin</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr</td>
<td>Fimbrial</td>
<td>DraE</td>
<td>Pyelonephritis and cystitis</td>
</tr>
<tr>
<td>F1845</td>
<td>Fimbrial</td>
<td>DaaE</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Afa-I</td>
<td>Afimbrial</td>
<td>AfaE1</td>
<td>Cystitis and diarrhea</td>
</tr>
<tr>
<td>Afa-III</td>
<td>Afimbrial</td>
<td>AfaE3</td>
<td>Cystitis and diarrhea</td>
</tr>
<tr>
<td>Dr-II</td>
<td>Nonfimbrial</td>
<td>DraE2</td>
<td>Gestational-pyelonephritis</td>
</tr>
<tr>
<td>Nfa-Ia</td>
<td>Nonfimbrial</td>
<td>NfaA</td>
<td>Cystitis</td>
</tr>
<tr>
<td>Afa-V</td>
<td>Afimbrial</td>
<td>AfaE5</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Afa-II</td>
<td>Afimbrial</td>
<td>AfaE2</td>
<td>Cystitis and diarrhea</td>
</tr>
<tr>
<td>Afa-IV</td>
<td>Afimbrial</td>
<td>AfaE4</td>
<td>Cystitis and diarrhea</td>
</tr>
<tr>
<td>Afa-VII</td>
<td>Afimbrial</td>
<td>AfaE7</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Afa-VIII</td>
<td>Afimbrial</td>
<td>AfaE8</td>
<td>Septicemia</td>
</tr>
<tr>
<td>Aaf-I</td>
<td>Afimbrial</td>
<td>AggA</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Aaf-II</td>
<td>Afimbrial</td>
<td>AafA</td>
<td>Diarrhea</td>
</tr>
</tbody>
</table>

3.1.2. Siderophore systems

Iron is an essential element for bacterial growth. In *E. coli*, iron is required for several biological functions, including DNA replication, oxygen transport and storage, electron transport, as well as peroxides metabolism (Johnson, 1991). Considering the limited iron conditions in hosts, bacteria have mechanisms for scavenging this element, including the production of siderophores. Siderophores are high affinity ferric chelators that have the capacity to capture ferric iron from host sources (Skaar, 2010). In *E. coli*, the siderophore aerobactin is considered the most effective iron chelating mechanism (Johnson, 1991).

3.1.2.1. Aerobactin

The aerobactin system is an important virulence factor, implicated in UTIs and other severe infections in humans and animals, possibly because it allows bacterial growth even under the limiting iron conditions which are found during the infection process (Johnson, 1991).

Aerobactin is more prevalent among *E. coli* from patients suffering from pyelonephritis (73%) cystitis (49%) or bacteremia (58%), compared to strains causing asymptomatic bacteriuria (38%) or fecal isolates (41%), suggesting that aerobactin
pathophysiology is important, not only inside, but also outside of the urinary tract (Johnson, 1991).

The induction of a deficiency in the aerobactin system reduces the virulence of an APEC strain in a chicken systemic infection model (Dozois, Daigle, & Curtiss, 2003). In addition, mutants of the iucD (gene from the aerobactin operon), present decreased capacity of colonization in several organs, suggesting that aerobactin system plays an important role both in APEC and UPEC strains (Gao et al., 2012). Furthermore, the expression of iutA, encoding aerobactin receptor, is higher during infection by ExPEC, or in conditions mimicking the infection (Chouikha, Bree, Moulin-Schouleur, Gilot, & Germon, 2008).

3.1.3. Toxins

Several toxins may be produced by *E. coli*. Hemolysin and cytotoxic necrotizing factor are particularly important in ExPEC. Despite of their differences, they are both responsible for host cells destruction.

3.1.3.1. Hemolysin

Hemolysin (HlyA) is an extracellular pore-forming cytolysin that was originally identified by its ability to lyse erythrocytes, and is considered the prototype of the RTX (Repeats in ToXin) family of bacterial toxins (Cavalieri & Snyder, 1982; Jorgensen, Short, kurz, Mussen, & Wu, 1976; Mackman & Holland, 1984).

The synthesis, maturation and export of hemolysin are determined by a four-gene operon termed hlyCABD (Felmlee, Pellett, & Welch, 1985; Koronakis & Hughes, 1996). This operon may be integrated in the chromosome of *E. coli* or located in large plasmids. Chromosomal hemolysin operons are located within pathogenicity islands in UPEC isolates (Hacker et al., 1990), and may present several differences in 5’ flanking region, as well as in the levels of toxin expression (Hacker et al., 1990; Nagy et al., 2006). On the other hand, plasmidic HlyA genes were found associated with EPEC strains, (Burgos, Pries, Pestana de Castro, & Beutin, 2009), and with ETEC and STEC strains (Prada et al., 1991; Wu et al., 2007). Hemolysin containing plasmids of *E. coli* are different in size, conjugation capacity, as well as incompatibility groups (Burgos et al., 2009; Knapp et al., 1985; Prada et al., 1991).
Considering the environments colonized by *E. coli*, some of them with elevated nutrient depletion, hemolysin is thought to be important in destroying the host cells, with the objective of acquiring their nutrients to support bacterial growth (Wiles, Kulesus, & Mulvey, 2008).

### 3.1.3.2. Cytotoxic Necrotizing Factor 1

Cytotoxic necrotizing factor 1 is a bacterial toxin firstly described by Caprioli and colleagues. It was described as a cytotoxic toxin, because of its capacity to cause multinucleation in cultured cells, and necrotizing because of its ability to originate necrosis in rabbit skin (Caprioli, Falbo, Roda, Ruggeri, & Zona, 1983).

This toxin is chromosomally encoded by *cnf1*, a single open reading frame, comprising 3042-bp (Falbo, Pace, Picci, Pizzi, & Caprioli, 1993).

CNF1 production has been detected in strains causing UTIs, including prostatitis (Andreu et al., 1997), and pyelonephritis (Jacobson, Katouli, Tullus, & Brauner, 1990); bacteremia (Blanco, Alonso, Gonzalez, Blanco, & Garabal, 1990) and meningitis (Wang & Kim, 2013).

### 3.1.4. Group 2 capsules

Bacterial capsule is an important virulence factor of pathogenic bacteria responsible for invasive infections. It allows the bacteria to evade the immunological defenses of the host, permitting the survival and establishment of infection in sites which are generally sterile and hostile, including lungs, blood, kidney and meninges (Moxon & Kroll, 1990).

*E. coli* can express more than 80 different capsular polysaccharides, which consist of linear polymers of repeating carbohydrate subunits, and may include an amino acid or a lipid element (Johnson, 1991).

Epidemiological studies associate group 2 capsules to several pathologies. Some of group 2 capsular antigens, including K1, K2, K3, K5, K12, K13, K20 and K51 are more common among *E. coli* of patients with cystitis and pyelonephritis than in isolates from fecal samples (Johnson, 1991). Capsular antigens K1 and K5 are present in 63% of women suffering from pyelonephritis, and in addition, K1, K2, K3, K12 and K13 are detected in 70% of isolates from pyelonephritis affecting girls (Johnson, 1991). K1 capsule is prevalently associated to meningitis, being detected
in more than 79% of *E. coli* isolates from neonatal meningitis, as well as most of the isolates from neonatal sepsis (Johnson, 1991). In addition, K2 capsule is necessary for serum resistance and in competitive colonization experiments in a murine model of ascending UTI, a mutant lacking the capacity to synthesize K2 capsule was outcompeted by the wild type strain. This indicates that expression of the K2 capsule is relevant in the pathogenesis of UTI (Buckles et al., 2009).

### 3.1.5. Virulence factors associated with Shiga toxin-producing *E. coli*

The crescent identification of food borne ExPEC infections, namely UTIs (Bélanger et al., 2011; Manges & Johnson, 2012; Vincent et al., 2010), raises the necessity of studying the presence of virulence traits generally associated to animal and food backgrounds. This knowledge will allow understanding the possible capacity of other *E. coli* pathotypes, including STEC, to cause extraintestinal disease.

STEC are responsible for hemorrhagic colitis, characterized by bloody diarrhea, as well as hemolytic uremic syndrome (HUS), potentially fatal in children, and responsible for acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia (Bergan, Dyve Lingelem, Simm, Skotland, & Sandvig, 2012).

In the pathophysiology of STEC infections, there are two main classes of virulence factors: toxins, including Shiga-like toxins, and adhesins, such as intimin (Serna & Boedeker, 2008).

#### 3.1.5.1. Shiga toxins

Shiga toxins are proteins which belong to the family of AB5 toxins. These toxins contain one subunit A, enzymatically active, and 5 subunits B, that bind to the glycosphingolipid globotriosylceramide, Gb3 receptor, expressed in various organs, including brain, kidney, liver and pancreas (Etcheverría & Padola, 2013).

Two types of shiga-toxin have been identified in STEC, shiga toxin type 1 and shiga toxin type 2. These toxins are respectively encoded by *stx1* and *stx2* genes, inserted in the genome of defective or functional lambdoid bacteriophages, the Stx-phages (Bergan et al., 2012; O'Brien et al., 1984).

The toxic effects of Shiga toxins are exerted by catalytic inactivation of the 60S ribosomal subunit and consequent inhibition of protein synthesis in the target cells.
(Bergan et al., 2012). In addition, these toxins are also able to induce apoptosis in several cellular types (Bergan et al., 2012).

3.1.5.2. Intimin

Intimin is an outer-membrane adhesion protein present in a group of pathogenic *E. coli*, called Attaching and Effacing *Escherichia coli* (AEEC). AEEC include EPEC, responsible for epidemic and sporadic infantile diarrhoea, and STEC that can cause acute gastroenteritis and haemorrhagic colitis. These pathogenic *E. coli* are capable of causing disease in animals and humans, by forming attaching and effacing (A/E) lesions in the intestine (Blanco et al., 2004; Frankel et al., 1998). These lesions lead to the destruction of microvillus border, by restructuring the underlying cytoskeleton through signal transduction mechanisms between bacteria and the host cells; intimate adherence to the intestinal epithelium, as well as aggregation of polymerized actin at the local and pedestal formation (Frankel et al., 1998). Intimin is encoded by the *eae* gene, which is integrated in a 35-kb pathogenicity island designated LEE (locus of enterocyte effacement) (Jerse, Yu, Tall, & Kaper, 1990; McDaniel, Jarvis, Donnenberg, & Kaper, 1995).

3.1.6. Pathogenicity Islands

In the 1980s, Hacker and colleagues investigated the production of virulence factors in UPEC *E. coli* strains, including α-hemolysin (*hly*) (Hacker, Knapp, & Goebel, 1983; Knapp et al., 1985). Afterwards, the term “hemolysin islands” emerged to describe large chromosomal DNA regions in which *hly* genes were found to be located in (Knapp, Hacker, Jarchau, & Goebel, 1986).

The designation pathogenicity islands (PAIs), was firstly introduced by Jörg Hacker and colleagues, while they were investigating the genetic base of the virulence of two uropathogenic *E. coli* strains, 536 and J96. They observed a close linkage between genes coding for P, P-related fimbriae and hemolysin; as well as the co-deletion of these linked gene clusters (Hacker et al., 1990).
PAIs were further defined by Hacker and colleagues (Hacker, Blum-Oehler, Mühldorfer, & Tschäpe, 1997) using a set of characteristics:

a) PAIs carry genes coding for one or more virulence determinants, including toxins, adhesins, invasins, iron uptake systems, as well as protein secretion systems, among others.

b) PAIs are present in pathogenic strains but absent or sporadically distributed in the genome of less pathogenic members of the same, or closely related species.

c) PAIs occupy relatively extensive chromosomal regions, frequently with more than 30Kb.

d) PAIs are frequently flanked by direct repeats (DR), small DNA sequences which may be generated during the integration of the PAI in the host genome through recombination.

e) PAIs are frequently composed of DNA sequences which differ from the remaining genome of the host in both G+C content and in codon usage, indicating the possible foreign origin of the fragment. This feature will not be pronounced or may also be absent if the donor and receptor present similar G+C content.

f) PAIs are usually associated with tRNA genes, which may act as target sites for the integration of external DNA, including pathogenicity islands. Furthermore, the 3’ regions of the tRNA loci are usually similar to the attachment sites of bacteriophages (Cheetham & Katz, 1995; Schmidt, Scheef, Janetzki-Mittmann, Datz, & Karch, 1997). The association of PAIs and tRNA genes and additionally the presence of even cryptic genes coding for phage integrases next to the tRNA loci, may point to the possibility that PAIs, or PAIs components, are bacteriophage-derived elements.

g) PAIs are frequently unstable, and moreover may be deleted via the insertion sequences (IS) elements, DR sequences inserted at their ends, or throughout other homologous sequences from the PAIs.

h) PAIs are usually carriers of cryptic or functional genes coding mobility elements, namely transposases, integrases or parts of insertion sequences.

The main PAIs detected among uropathogenic *E. coli* and corresponding characteristics are presented in Table 1.5.
<table>
<thead>
<tr>
<th>PAI</th>
<th>Size</th>
<th>Insertion site</th>
<th>GC content</th>
<th>Encoded virulence factors</th>
<th>Deletion frequencies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI I&lt;sub&gt;536&lt;/sub&gt;</td>
<td>76,843</td>
<td>selC</td>
<td>46%</td>
<td>alpha-hemolysin, F17- and CS12-like adhesins, ORF with homology to the modification methylase NgoFVII</td>
<td>$2 \times 10^{-6}$</td>
<td>(Blum et al., 1994; Dobrindt et al., 2002; Middendorf et al., 2004)</td>
</tr>
<tr>
<td>PAI II&lt;sub&gt;536&lt;/sub&gt;</td>
<td>102,200</td>
<td>leuX</td>
<td>46%</td>
<td>alpha-hemolysin, P-related fimbrial adhesin, Hek adhesin, and haemagglutinin-like adhesins</td>
<td>$2 \times 10^{-5}$</td>
<td>(Blum et al., 1994; Dobrindt et al., 2002; Middendorf et al., 2004; Ritter et al., 1995)</td>
</tr>
<tr>
<td>PAI III&lt;sub&gt;536&lt;/sub&gt;</td>
<td>68,124</td>
<td>thrW</td>
<td>47%</td>
<td>S-fimbriae, HmuR-like heme receptor, TSH-like hemoglobin protease, siderophore system iro and autotransporter-adhesin Sap</td>
<td>$5 \times 10^{-5}$</td>
<td>(Dobrindt et al., 2002; Middendorf et al., 2004)</td>
</tr>
<tr>
<td>PAI IV&lt;sub&gt;536&lt;/sub&gt;</td>
<td>30,200</td>
<td>asnT</td>
<td>57%</td>
<td>yersiniabactin siderophore system stable</td>
<td>(Dobrindt et al., 2002; Middendorf et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>PAI I&lt;sub&gt;CFT073&lt;/sub&gt;</td>
<td>123,000</td>
<td>pheV</td>
<td>47%</td>
<td>Alpha- hemolysin, P-fimbriae, aerobactin and aerobactin receptor, secreted autotransporter toxin sat, IrgA homologue adhesion iha and kps TM</td>
<td>?</td>
<td>(Lloyd, Rasko, &amp; Mobley, 2007)</td>
</tr>
<tr>
<td>PAI II&lt;sub&gt;CFT073&lt;/sub&gt;</td>
<td>52,000</td>
<td>pheU</td>
<td>48%</td>
<td>P fimbriae, iron-related genes</td>
<td>?</td>
<td>(Lloyd et al., 2007; Rasko, Phillips, Li, &amp; Mobley, 2001)</td>
</tr>
<tr>
<td>PAI I&lt;sub&gt;J96&lt;/sub&gt;</td>
<td>&gt;170,000</td>
<td>pheV</td>
<td>?</td>
<td>Alpha-hemolysin, P-fimbriae</td>
<td>?</td>
<td>(Swenson, Bukanov, Berg, &amp; Welch, 1996).</td>
</tr>
<tr>
<td>PAI II&lt;sub&gt;J96&lt;/sub&gt;</td>
<td>110,000</td>
<td>pheU</td>
<td>?</td>
<td>prs operon, alpha-hemolysin, cytotoxic necrotizing factor-1</td>
<td>?</td>
<td>(Blum, Falbo, Caprioli, &amp; Hacker, 1995; Swenson et al., 1996).</td>
</tr>
</tbody>
</table>
Pathogenicity islands are inserted in the host chromosome by a site-specific event. Although not fully understood, it is thought that tRNA genes are used as integration sites for PAIs. This hypothesis is consistent with the observation that the majority of the islands are inserted in the 3’ end of the loci of these tRNA genes. Furthermore, these are the regions in which phage attachments sites are usually positioned (Schmidt & Hensel, 2004).

The loss of PAIs plays an important role in the bacterial genome plasticity and consequently in microbial evolution. In addition, it is important in the transition between the stages of acute and chronic infection (Blum et al., 1994).

Excision of pathogenicity islands, mediated by site-specific recombination between the DRs flanking the islands, has been firstly described for PAI I536 and PAI II536 (Blum et al., 1994). The DRs associated to the pathogenicity islands are considered to correspond to the sequences located at the left and right end of prophages (attL and attR) (Hochhut et al., 2006).

With the exception of PAI IV536, which has one degenerate DR, all other islands of strain 536 were found to have the capacity to excise from the chromosome, in frequencies from $10^{-6}$ to $10^{-5}$. In addition, the island PAI III536 was determined to be the most unstable (Middendorf et al., 2004). Two types of excision were identified for PAIs of strain 536: site-specific recombination between the flanking DRs for PAI I536, PAI II536, PAI III536 and PAI V536; and an additional partial deletion mechanism for PAI III536, involving homologous recombination between two incomplete IS100 copies, which was called type II deletion (Middendorf et al., 2004).

With the exception of this referred deletion, excision mechanisms in *E. coli* strain 536, are not *RecA*-dependent (Blum et al., 1994; Middendorf et al., 2004), but alternatively, appear to be mediated by the respective PAI-encoded integrases, similarly what happens in the bacteriophages (Hochhut et al., 2006).

The excision of pathogenicity islands has been associated with several contributing factors, including environmental stimuli. Low temperature and high cell density were found to induce the excision of PAI II536. In contrast, growth in artificial urine, high temperature, depletion of nutrients, and salt stress, do not affect the excision rate of this island (Middendorf et al., 2001; Middendorf et al., 2004). PAI III536 excision rate is not significantly changed by these environmental factors, but low temperature shifts the deletion types of this island to site specific recombination, the type 1 deletion. This also occurs under nutrient depletion, salt stress or at high
temperature, and is most potentiated in artificial urine. Contrarily to these referred islands, PAI 1536 excision, is not affected by environmental factors. This is another indication that this island is reasonably stable in the bacterial host chromosome (Middendorf et al., 2004).

In addition to environmental factors affecting the loss of pathogenicity islands, quinolones also increase the frequency of excision of some PAIs (Soto, Jimenez de Anta, & Vila, 2006). The exposure of strains to sub-inhibitory concentrations of quinolones induces total or partial loss of PAI 1536, as well as PAI IIJ96. Contrarily, the excision of PAI III1536 and PAI IV1536 under sub-inhibitory concentrations of quinolones is not verified (Soto et al., 2006).

3.2. *Klebsiella* virulence factors

Although virulence has been more extensively studied in *E. coli*, several virulence factors have also been associated to the pathogenesis of *Klebsiella* spp. infections. Additionally to the common virulence factors, like adhesins and capsule, other virulence factors have been described among the genus *Klebsiella*.

3.2.1. Adhesins

In *Klebsiella* spp., adhesins like type 1 and type 3 fimbriae are important virulence factors, which contribute to the development of urinary and respiratory tract infections.

3.2.1.1. Type 1 fimbriae

Type 1 fimbriae are mannose-sensitive hemagglutinins that have the capacity to agglutinate guinea pig erythrocytes, and can be detected on virtually all *Enterobacteriaceae* members (Jones et al., 1995; Podschun & Ullmann, 1998). They are composed of cylindrical pilus rods constituted by subunits of FimA pilin, as well as small tip fibrillum which integrates FimF, FimG and the adhesin FimH (Jones et al., 1995). FimC and FimD are not part of the final structure but are necessary for the type 1 assembly (Jones et al., 1995).
Similarly to *E. coli*, the expression of *fim* operon is phase variable. During colonization and infection in lungs and in the intestine the switch is “off” while when in the urinary tract it is on the position “on” (Struve, Bojer, & Krogfelt, 2008).

The majority of studies focusing the role of type 1 pili in the UTI pathogenesis were performed using *E. coli* strains, in which this fimbriae has been associated with lower UTI, as well as pyelonephritis (Podschun & Ullmann, 1998). Nonetheless, the role of type 1 fimbriae in UTI has also been reported in *K. pneumoniae* using animal models (Fader & Davis, 1980, 1982; Maayan, Ofek, Medalia, & Aronson, 1985). In addition, type 1 pili also participates in the colonization of the urogenital and respiratory tracts, contributing not only to the development of UTI, but also for development of pneumonia (Podschun & Ullmann, 1998). Type 1 fimbriae are also expressed in biofilm-like intracellular bacterial communities both in UPEC and in *K. pneumoniae* strains (Rosen et al., 2008).

### 3.2.1.2. Type 3 fimbriae

Type 3 fimbriae belong to the fimbrial group of mannose-resistant hemagglutinins, and have the capacity to cause mannose-resistant agglutination of erythrocytes treated with tannic acid (Hornick, Allen, Horn, & Clegg, 1992).

These hemagglutinins are adhesins which are expressed by the majority of the isolates associated to human infections, in both the respiratory and urinary tracts (Hornick, Thommandru, Smits, & Clegg, 1995; Huang, Liao, Wu, & Peng, 2009).

Type 3 fimbriae mediate the connection to the type V collagen, basolateral surfaces of different types of cells, including trypsinized human buccal cells, renal tubular cells, bronchial cells, tracheal epithelial cells, extracellular matrix proteins, as well as basement membrane of lung tissue (Hornick et al., 1992; Hornick et al., 1995; Sebghati, Korhonen, Hornick, & Clegg, 1998). In addition, this adhesin has been shown to participate in biofilm formation on plastic surfaces (Jagnow & Clegg, 2003; Langstraat, Bohse, & Clegg, 2001). The formation of bacterial biofilms plays an important role in bacterial cells communication, escape from host defenses mechanisms, as well protection against antibiotics, thus contributing to bacterial virulence (Jagnow & Clegg, 2003).
3.2.1.3. Adhesin CF29K

CF29K is a non fimbrial protein of 29kDa, from the K88 adhesin family. This adhesin is expressed in *K. pneumoniae* and is related to CS31A surface protein in *E. coli* (Di Martino et al., 1995). The gene encoding CF29K, termed *cf29A*, was found inserted on a 185-kb conjugative R plasmid, and is 100% homologue to the structural gene encoding CS31A. In fact, CF29K is considered an antigenic subtype of CS31A (Di Martino et al., 1995).

Despite of the fact that no *in vivo* studies support the role of this adhesin in virulence, the presence of this determinant is more strongly associated to hipervirulent strains. Nonetheless, *cf29A* gene has also been detected in non-hipervirulent *K. pneumoniae* (Brisse et al., 2009; Di Martino et al., 1995; Shon, Bajwa, & Russo, 2013).

3.2.2. Capsule

*Klebsiella* spp. are covered by a hydrophilic polysaccharide capsule which confers the mucoid aspect of the colonies on agar plates (Dworkin et al., 2006a). There are as a minimum, 78 capsular polysaccharide distinct serotypes (Pan et al., 2008); eight of which are associated with hypervirulent strains (K1, K2, K5, K16, K20, K54, K57 and KN1) (Shon et al., 2013).

The capsule is an important virulence factor, as it protects bacteria against phagocytosis by polymorphonuclear leukocytes, and against serum killing (Dworkin et al., 2006a). In addition, the capsule was found to be an important virulence factor in several animal infection models. Strains from serogroups K1 and K2 were found to be particularly virulent in a mouse peritonitis model, compared to strains from other capsular antigens (Mizuta et al., 1983). In experimentally induced skin lesions in mice, strains from K1, K2, K4 and K5 were found to be more virulent than other capsular antigens types (Simoons-Smit, Verwey-van Vught, Kanis, & MacLaren, 1984). Furthermore, in an experimental murine burn wound sepsis model, strains from K1 serotype were found to be extremely virulent (Cryz, Fürer, & Germanier, 1984). Capsular polysaccharides were also found to be crucial for virulence in a murine model of pneumonia (Cortés et al., 2002). Additionally, capsule expression was shown to be an important virulence factor in UTI, but not in intestinal colonization in animal models (Struve & Krogfelt, 2003).
3.2.3. Other virulence factors

3.2.3.1. Regulator of the mucoid phenotype A

The regulator of the mucoid phenotype A (RmpA) is a mucoid factor which is responsible for the regulation of the capsular polysaccharide biosynthesis (Cheng et al., 2010). RmpA protein is encoded by the *rmpA* gene which was first detected in a 180 kb plasmid of a K2 strain (Nassif, Fournier, Arondel, & Sansonetti, 1989). Later, gene *rmpA2* sharing 80% of identity with *rmpA* was also identified in a large plasmid (Wacharotayankun et al., 1993), and in addition a chromosomal gene, c-*rmpA* was also identified (Hsu, Lin, Chen, Chou, & Wang, 2011).

The presence of *rmpA* gene has been associated with the hypermuscoviscosity phenotype in *K. pneumoniae*, and was found to be more prevalent in liver abscess strains than in bacteremia isolates (Yu et al., 2006). In addition, this gene is more frequently detected among strains from K1/K2 serotypes (Cheng et al., 2010), in which it has been linked to virulence in a mouse model of infection (Brisse et al., 2009). Contrarily, in another study, after intraperitoneal and intragastric inoculation and *in vivo* competition assays in a K1 isolate, *rmpA* was not responsible for enhancing the virulence, but the authors suggest that the effects of this gene in virulence may differ according to the strain (Hsu et al., 2011). Thus, it has been suggested that *rmpA* may be a marker of *Klebsiella* PLA rather than being directly related to virulence (Hsu et al., 2011).

3.2.3.2. Activator of the allantoin regulon

Some *Enterobacteriaceae* members have the capacity to use allantoin as a nitrogen source (Cusa, Obradors, Baldomà, Badia, & Aguilar, 1999). The allantoin regulon comprises several genes involved in the allantoin utilization, and in addition, it carries two regulator genes: *allR*, a repressor gene; and *alls*, an activator of the regulon (Rintoul et al., 2002).

Contrarily to *E. coli*, which is only capable of using allantoin anaerobically as a sole nitrogen source but not as sole carbon source, *K. pneumoniae* can use allantoin as unique source of carbon, nitrogen and energy, under both aerobic and anaerobic conditions (Chou et al., 2004). Thus, the utilization of allantoin may be extremely useful for *Klebsiella* during competition for nitrogen supplies.
In *K. pneumoniae*, a 22kb region containing the *alls* gene has been associated to liver infection. Furthermore, in a mouse model of intragastric infection, the wild type strain showed increased virulence compared to a mutant without this region (Chou et al., 2004). Considering that primary liver abscess is more prevalent in patients with diabetes mellitus, in whom allantoin levels are increased, the presence of this region may constitute an important benefit for bacteria (Chou et al., 2004).

Epidemiological studies associate the presence of *alls* gene with K1 isolates from PLA (Brisse et al., 2009; Yu et al., 2008), although this determinant is not present within all K1 strains (Brisse et al., 2009). In addition, *alls* has also been detected in non-K1 isolates (Turton, Perry, Elgohari, & Hampton, 2010).

### 3.2.3.3. Uridine phosphate galacturonate 4- epimerase

In several members of the *Enterobacteriaceae* family, the lipopolysaccharide (LPS) core oligosaccharide backbone is altered by phosphoryl groups, which are known to be important in the maintenance of the barrier function provided by the outer membrane (Fridrich, Bouwman, Vinogradov, & Whitfield, 2005). These groups are absent in *K. pneumoniae*, in which this function is achieved by the presence of galacturonic acid (Fridrich et al., 2005).

Uridine phosphate galacturonate 4- epimerase is an enzyme responsible for the conversion of the UDP - GlcUA to UDP-GalUA (UDP galacturonic acid), which is essential for the synthesis of the core oligosaccharide of LPS, a major virulence determinant in *Enterobacteriaceae* (Fridrich & Whitfield, 2005). This enzyme is encoded by *uge* gene, (Regué et al., 2004), further designated *glaKP* (Fridrich & Whitfield, 2005).

### 3.2.3.4. Glucosyltransferase

Glucosyltransferase is a protein responsible for the attachment of α-L-glycero-D-manno-heptopyranose II (L,DHeppII) to the O-3 position of an α-D-galactopyranosyluronic acid (α-D-GalAp) in the outer core lipopolysaccharides (LPS) (Izquierdo et al., 2003). This enzyme is encoded by the gene *wabG* (Izquierdo et al., 2003), inserted in the *wa* region of the *K. pneumoniae* chromosome, responsible for LPS core synthesis (Regué et al., 2001; Regué et al., 2005).
*K. pneumoniae* wabG mutants loose the capacity to synthetize the outer core LPS, including O-antigen, and thus totally lack the outer core LPS. Furthermore, mutants are also non-capsulated, consequently being more sensitive to polymyxinB as well as to SDS (Izquierdo et al., 2003).

In experimental UTIs, the mutation of this gene reduces radically the colonization capacity of *K. pneumoniae* (Izquierdo et al., 2003) and in an experimental model of pneumonia, wabG mutants are totally avirulent (Fresno et al., 2007). The reintroduction of the gene in the mutants recovers the pathogenic capacity of strains (Fresno et al., 2007; Izquierdo et al., 2003).

### 3.2.3.5. Urease

Urease is a metalloenzyme which contains nickel and has the capacity to catalyse the hydrolysis of urea, originating ammonia and carbamate. Furthermore, carbamate suffers spontaneous decomposition creating a second molecule of ammonia and carbonic acid (Pearson, Michel, Hausinger, & Karplus, 1997). Once released by the action of urease, the extremely alkaline agent ammonia may become protonated originating ammonium ion. Ammonium is responsible for alkalinizing the cytoplasm, as well as the extracellular environment (Maroncle, Rich, & Forestier, 2006). The release of ammonia leads to tissue damage, and in some circumstances it is also an important factor contributing for the persistence of pathogens (Burne & Chen, 2000).

Urease is a recognized virulence factor in several animal and human pathogens, being associated to a variety of pathologies including pyelonephritis, urolithiasis and peptic ulcers (Mobley, Island, & Hausinger, 1995). Furthermore, the expression of urease is thought to be important to the infective capacity of bacteria, the severity of the disease, and in addition, to the persistence in the host (Maroncle et al., 2006).

It has been shown that *K. pneumoniae ΔureA* mutants have an attenuated capacity to colonize the mouse intestine (Maroncle et al., 2006). Considering that nosocomial infections caused by *K. pneumoniae*, are generally preceded by gastrointestinal colonization despite of the infection localization (Maroncle et al., 2006), the contribution of urease for enhancing the colonization capacity in this tract makes it an important virulence factor.
3.2.3.6. *Klebsiella ferric iron uptake*

*Klebsiella ferric iron uptake* (kfu) is a virulence factor responsible for iron uptake. It allows bacteria to acquire iron, even under the limited iron conditions provided by the human host (Ma, Fang, Lee, Shun, & Wang, 2005).

The *kfu* operon is present in the majority of the genomes of tissue-invasive *K. pneumoniae* and absent in noninvasive isolates, suggesting that this operon may be implicated in the modulation of *in vivo* virulence, and constitute a competitive advantage to strains that carry it (Ma et al., 2005). In addition, mutants lacking this operon showed decreased virulence *in vivo*, confirming the role of this system in virulence (Ma et al., 2005).

In patients with PLA, *kfu* has been correlated with *magA* and *alls*. The association of these genes relates with the invasiveness capacity of bacteria (Ma et al., 2005).

4. **Virulence versus resistance**

Along with the global concern about the rise of resistance among human pathogens, fears about the simultaneously enhancement of the pathogenic capacity of bacteria started to emerge. The possibility of acquisition of both resistance and virulence traits via horizontal gene transfer could be responsible for the appearance of strains simultaneously virulent and resistant. Furthermore, it is also possible that antimicrobial agents may select strains with more virulence factors. This could lead to serious public health problems. Multidrug resistant and virulent strains could be responsible for severe infections and antibioterapy would be inefficient to treat these infections, increasing morbidity and mortality rates. Despite of the realization of some studies focusing on the relation between resistance and virulence, this interplay is still not completely elucidated.

4.1. **Resistance to beta-lactams versus virulence**

Studies focusing on the interplay between resistance to beta-lactams and virulence are based either on phenotypic resistance profile or in the presence of specific beta-lactamases.
Resistance to extended-spectrum cephalosporin plus cephemycin is associated to non-B2 groups, fewer virulence factors and, in addition, to depletion for hlyD, pap, sfa/foc and kpsM II in E. coli human isolates. It is also associated to lower prevalence of kpsM II and malx (marker for a pathogenicity island of strain CFT073) among ExPEC animal isolates (Johnson, Kuskowski, Owens, Gajewski, & Winokur, 2003).

In a group of E. coli urinary isolates, few differences were found between the prevalence of virulence factors among ampicillin resistant and susceptible strains. Only K1 kpsM (group 2 capsule variant K1) was found to be more prevalent among isolates susceptible to ampicillin (Horcajada et al., 2005).

A study among E. coli from women with acute uncomplicated cystitis revealed that the differences on the prevalence of virulence factors, aggregate virulence factors scores and phylogenetic distribution, according to susceptibility versus resistance to ampicillin were minimal. Exceptions were F12 papA allele (variant F12 from papA), more prevalent among susceptible isolates, and traT (serum resistance associated) gene, often associated with plasmids, which was more prevalent among strains resistant to ampicillin (Johnson, Kuskowski, O'Bryan T, Colodner, & Raz, 2005b).

According to Branger et al. (2005), the production of SHV, and to a lesser extent, of TEM type beta-lactamases, are preferentially associated to the B2 phylogenetic group, whose strains carry more VFs but are fluoroquinolone-susceptible. Contrarily, the CTX-M type is associated with the D phylogenetic group in which strains present less virulence factors but are fluoroquinolone-resistant (Branger et al., 2005). Nonetheless, other studies indicate that E. coli CTX-M-producers generally belong to the virulent phylogenetic groups, especially the B2 phylogroup (Carattoli et al., 2008; Karisik, Ellington, Livermore, & Woodford, 2008; Pitout, Laupland, Church, Menard, & Johnson, 2005).

According to Lee et al. (2010), among commensal E. coli isolated from faeces of healthy individuals, and pathogenic strains from blood and urine isolates, the prevalence of nine virulence factors, except S fimbrial adhesin, was significantly higher in pathogenic strains compared with commensal strains. Phylogenetic group B2 presented more virulence genes, and the phylogenetic distribution among CTX-M-producers was similar to that observed among the total pathogenic strains, with a higher prevalence of phylogroup B2. Nonetheless, blaCTX-M-1 group strains belonged
mainly to phylogenetic group B2 and A, while $bla_{CTX-M-9}$ group strains belonged to phylogenetic group D and B2. No differences were found in aggregate virulence factor scores between CTX-M-producers and non-CTX-M-producers in the pathogenic strains. Despite of the similarities between the prevalence of each individual virulence factor regardless of CTX-M production, $iutA$ and $traT$ were significantly more frequent in $bla_{CTX-M-1}$ group and in $bla_{CTX-M-9}$ group, respectively, than in CTX-M-non-producers. These virulence genes, along with α-haemolysin and yersiniabactin receptor, were found to be predictors of pathogenicity (Lee et al., 2010). The higher prevalence of phylogroup B2 among CTX-M-type producers reported by these authors, is in agreement with previous reports (Carattoli et al., 2008; Karisik et al., 2008; Pitout et al., 2005), but contradicts the observations of Branger et al. (2005), which reported an association to phylogroup D (Branger et al., 2005).

Additionally, among a collection of ESBL producing $E. coli$ isolates from North America, the majority of CTX-M-14 and SHV producers were from phylogroup D, while CTX-M-15 and TEM producers were mainly from group B2. Substantial prevalence differences were found between CTX-M producers and non-producers, for individual virulence factors. CTX-M producers were more commonly carriers of $afa/dra$, $iha$ (putative adhesin-siderophore receptor), $sat$ and $kpsM$ II. Contrarily, among non-CTX-M producers, $ireA$ (iron-regulated element) and $cvaC$ (colicin [microcin] V) genes were more frequently found. The $usp$ gene (uropathogenic-specific protein) was detected in both CTX-M-15 β-lactamase producers and non CTX-M producers, but not among CTX-M-14 producers. Even so, aggregate virulence factor scores were similar (Pitout et al., 2005).

According to a different study, the epidemic lineages from B2 group with CTX-M-15 enzyme in United Kingdom, did not produce more virulence factors, neither appeared to be more virulent than the non-epidemic isolates of the same phylogroup, regardless of the ESBL produced. However, $iutA$ and $fyuA$ (yersiniabactin receptor) were significantly more prevalent among epidemic strains than in non-clonal isolates also belonging to phylogroup B2. Similarly to the finding of Pitout et al. (2005), the $afa/dra$BC gene pair was associated to the production of CTX-M β-lactamases (Karisik et al., 2008).

The presence of PAIs from UPEC has also been reported among CTX-M producers. Among these ESBL producers, three PAIs were detected, with PAI $IV_{536}$
being the most prevalent, as it was identified in all isolates. The majority of the strains were CTX-M-15 producers, from B2 phylogroup and carried PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073} (Carattoli et al., 2008).

In a study with Caenorhabditis elegans infection assays, using susceptible and ESBL-producing E. coli, it was determined that the ability to kill these nematodes correlated with the presence of virulence factors. As susceptible strains presented higher prevalence of virulence factors than ESBL producers, they were more virulent than the resistant isolates. The number of virulence factors, contrarily to the reported by Lee et al. (2010) was found to be higher among TEM-type producers than in CTX-M-type, and thus, CTX-M-producing isolates had lower virulence \textit{in vivo} than TEM-producers. As it was previously reported, \textit{traT} and \textit{iutA} were also more prevalent among ESBL producers than in susceptible strains (Lavigne et al., 2006).

### 4.2. Resistance to quinolones versus virulence

An inverted relation between virulence and resistance to quinolones has been documented in several studies. Generally, quinolones and fluoroquinolones resistant strains show reduced virulence and invade immunocompromised hosts, while on the contrary, susceptible strains are more virulent, and affect uncompromised patients (Piatti, Mannini et al. 2008). The investigations that leaded to this observation generally have taken in account the phylogroup of the strains, number of virulence factors carried, and the presence or absence of specific virulence factors, as well as their expression.

#### 4.2.1. Resistance to quinolones and phylogroups

Resistance to quinolones and particularly to fluoroquinolones has been associated to shifts in the phylogenetic groups towards phylogenetic groups A, B1 and D, and away from phylogroups B2.

In a study performed in E. coli mainly from extraintestinal human infections, cattle and swine isolates, differences were found between the human and animal isolates, considering the relation between resistance to fluoroquinolones and phylogroups (Johnson, Kuskowski et al. 2003). Among human isolates, despite of the general higher prevalence of phylogenetic B2 group, it was observed that the resistant isolates, in comparison with the susceptible counterparts, were considerably
depleted for phylogroup B2, and presented higher prevalence of group D. In contrast with human isolates, the resistant animal isolates did not present significant shifts in overall virulence.

Moreno et al. reported that among UPEC, resistance to quinolones or fluoroquinolones was also associated with shifts away from phylogenetic group B2 to phylogroups A, B1 or D. The magnitude of this shift depended on antimicrobial resistance profile, being greater for resistance to fluoroquinolones in comparison with resistance to quinolones or trimethoprim-sulfamethoxazole. In addition, it changed among fluoroquinolone-resistant isolates according to the trimethoprim-sulfamethoxazole phenotype (Moreno, Prats et al. 2006).

Other studies, within strains from urinary origin (Piatti, Mannini et al. 2008) and strains causing acute uncomplicated cystitis (Johnson, Kuskowski et al. 2005, Kim, Kim et al. 2010), also reported that ciprofloxacin resistance was associated to shifts away from phylogroup B2. This observation was also verified for urosepsis isolates but concerning resistance to nalidixic acid (Houdouin, Bonacorsi et al. 2006). In addition, the shift towards non-B2 phylogroups was also reported among isolates from human and animal wastewaters (Sabaté, Prats et al. 2008).

In opposition to the referred studies, Kawamura-Sato et al. reported that among a collection of UPEC from Japan, the shift of quinolone and fluoroquinolone resistant strains to non-B2 groups of was not observed, as this group was the most prevalent among either susceptible and resistant isolates (Kawamura-Sato, Yoshida et al. 2010). Therefore, despite of the apparent link between phylogenetic background and resistance to quinolones and fluoroquinolones reported by the majority of studies, some contradictory results may indicate that this relationship is more complex.

4.2.2. Resistance to quinolones and virulence genes

Resistance to quinolones and fluoroquinolones also seems to be associated to the presence of less virulence genes. Several studies reported that among human UPEC and ExPEC, resistance to nalidixic acid or ciprofloxacin was accompanied by reduced virulence scores (Graziani et al., 2009; Johnson et al., 2006; Johnson et al., 2005b; Johnson et al., 2003; Moreno et al., 2006).
Furthermore, another study reported that there was no correlation between the number of virulence factors carried, and the number of agents to which the isolates were resistant to, among fluoroquinolone resistant E. coli from urinary origin (Grude et al., 2008).

Among E. coli strains from animal origin, studies seem to be more contradictory. A study performed with cattle and swine intestinal E. coli, reported that aggregate virulence factor scores differed minimally between resistant and susceptible isolates (Johnson et al., 2003). Nonetheless, studies performed in E. coli from chickens indicate that similarly to the human strains, resistance to quinolones and fluoroquinolones is associated to lower virulence scores (Graziani et al., 2009; Johnson et al., 2006).

In addition to the connection between resistance to quinolones and the number of virulence factors carried, the presence and expression of specific virulence factors and their relation to resistance has also been studied. However, this question seems to be more complex and depend on the specific virulence factor.

Vila et al. (2002) investigated if quinolone resistant UPEC were less virulent than susceptible isolates. They suggested that resistance to quinolones may be associated to a diminishing in the carriage or expression of virulence factors (Vila et al., 2002). They reported that the expression of type1-fimbriae and hemolysin, and, in addition, the carriage of genes hlyA, cnf1 and sat, was significantly lower among nalidixic acid- resistant isolates compared to susceptible isolates. At a lesser extent, genes fimA and papC were also less prevalent among resistant isolates. Considering these findings, they first hypothesised that these observations were a result of the acquisition of resistance to quinolone and further spread in a clonal fashion, among strains that naturally do not carry these virulence factors. However, clonality was not demonstrated in the genetic relationship studies they performed, and they suggested two other hypotheses which do not exclude each other. The first theory is based on the detection of a mutation at the codon 83 of the gyrA gene, which may be responsible for the reduction of the degree of DNA supercoiling catalyzed by DNA gyrase, and consequently affect some gene expression. This may explain the reduced expression of type-1-fimbriae. The second hypothesis relies on the fact that the genes which were significantly depleted among resistant strains may be carried by pathogenicity islands. Therefore, during the development of quinolone resistance, possibly aided by quinolone exposure, these antimicrobial agents can lead to the
excision of these elements mediated by SOS response (Vila et al., 2002). Contrarily to the observed for the remaining genes, $iucD$ (a gene from aerobactin operon) was found to be more prevalent among resistant isolates, but the authors were unable to explain this observation.

A set of 35 virulence markers was studied by Johnson and colleagues in a Dutch collection of fluoroquinolone resistant $E. coli$, and was compared to fluoroquinolone susceptible $E. coli$ isolates (Johnson, van der Schee, Kuskowski, Goessens, & van Belkum, 2002). The fluoroquinolone resistant $E. coli$ were found to be extremely depleted in virulence factors, and the classic ExPEC virulence determinants $pap$, $sfa/foc$, $hly$ and $cnf$ were remarkably absent in these isolates. The fluoroquinolone susceptible $E. coli$ presented higher prevalence for 25 of the 35 genes analysed, being that, for $fuyA$, $kpsMT$ II and $ompT$ the differences observed were statistically significant. On the opposite, $iutA$, $ibeA$ and $traT$ were found to be more prevalent among fluoroquinolone resistant $E. coli$, although this was not statistically significant. According to the authors, the phylogenetic background and virulence profiles of fluoroquinolone resistant $E. coli$ are more similar to animal isolates. This observation gives strength to previous suggestions according to which fluoroquinolone resistant $E. coli$ are derived from animal sources and may be transmitted to humans by foodborne transmission (Garau et al., 1999).

Another study was performed by Horcajada et al, comparing the prevalence of 31 virulence genes among phylogroup B2 $E. coli$ isolates susceptible or resistant to nalidixic acid (Horcajada et al., 2005). The findings were similar to the reports by Johnson et al. and Vila et al. (Johnson et al., 2002; Vila et al., 2002), but added the information that there was a significant increase in the prevalence of $bmaE$ (M fimbriae) and $gafD$ (G fimbriae) among nalidixic acid resistant strains from phylogroup B2.

Regarding studies from animal origin, Johnson et al. reported that among swine and cattle $E. coli$ isolates, the five genes which are used to define an ExPEC and $hlyD$ exhibited minimal shifts according to the resistance profile. With the exception of $iutA$, $papA$ and $papC$, which were more prevalent among fluoroquinolone resistant isolates, the remaining virulence genes were more prevalent among fluoroquinolone susceptible isolates. Nonetheless, only for $kpsMTII$ the differences were considered statistically significant, as fluoroquinolone resistant isolates were totally depleted of this virulence factor (Johnson et al., 2003). Contrarily
to the observed in this study, among *E. coli* isolated from chicken, *kps*MTII was found to be more prevalent among fluoroquinolone resistant *E. coli* than in fluoroquinolone susceptible isolates. Nonetheless, this was not statistically relevant, as the only data with statistical significance was the higher prevalence of *ireA* and *iutA* among fluoroquinolone resistant *E. coli* (Johnson et al., 2006).

As it was demonstrated by the referred studies, although controversial, quinolone- and fluoroquinolone-resistant *E. coli* isolates typically seem to be less virulent than their susceptible counterparts. Quinolone and fluoroquinolone resistant isolates generally lack characteristic ExPEC virulence genes, such as *pap* and *hly*, which may indicate that mutation to quinolone or fluoroquinolone resistance may be accompanied by the loss of virulence genes (Vila et al., 2002). Reports indicating that exposition to sub-inhibitory concentrations of quinolones, leaded to the loss of virulence genes in a small subset of the studied population support this theory (Soto et al., 2006). Nonetheless, the reported absence of detectable alterations on the virulence factors profile in two wild-type *E. coli* strains, after experimental selection for quinolone resistance contradicts this theory (Johnson et al., 2005b; Martínez-Martínez, Fernández, & Perea, 1999). Also opposing to this theory, is the consistent epidemiological observation that quinolone and fluoroquinolone-resistant human clinical isolates present a clearly distinct phylogenetic group distribution, compared to their susceptible counterparts (Houdouin et al., 2006; Johnson et al., 2005b; Johnson et al., 2003; Moreno et al., 2006; Piatti, Mannini, Balistreri, & Schito, 2008). A possible alternative hypothesis explaining these observations may be that the conversion to resistance occurs more easily in non-B2 strains or in isolates harbouring fewer virulence factors (Johnson et al., 2005b).

In order to elucidate this question, Johnson et al. performed the molecular analysis of 40 diverse wild-type and reference *E. coli*. The isolates were submitted to selective passages on antimicrobial-supplemented agar plate, to induce spontaneous transition to nalidixic acid or ciprofloxacin resistance (Johnson, Johnston, Kuskowski, Colodner, & Raz, 2005a). They reported that this transition occurred independently of strain characteristics, including phylogenetic group, collection of origin, and virulence profile, and, in addition, it was accompanied by negligible alterations in the inferred phylogenetic group or virulence genetic content of the strains. In view of these results, the authors hypothesise that resistant strains may be acquired from an external selection source. The strains reservoir could be
food animals which carry a susceptible source population exhibiting the same low virulence and non-group-B2 profile, similar to resistant human clinical isolates. This would explain the inverted relation between resistance and virulence. Therefore, this study contradicts the theories that defend the direct loss of virulence genes during the acquisition of resistance, and the higher capacity of acquisition of resistance among non-B2 strains (Vila et al., 2002).

4.3. Resistance to sulfamethoxazole-trimethoprim and to aminoglycosides

The relation between resistance to sulfamethoxazole-trimethoprim and virulence has also been studied. Resistance to this combination of agents is associated with borderline or moderate shifts away from B2 phylogroup (Houdouin et al., 2006; Johnson et al., 2005b; Moreno et al., 2006). In fact, resistant strains have been shown to be mainly associated with phylogenetic group D (Houdouin et al., 2006; Moreno et al., 2006). Differences were also observed in the prevalence of virulence genes considering the resistance phenotype. Genes papGIII (Johnson et al., 2005b); iroN (Horcajada et al., 2005); sfa/focDE, hlyA and fyuA (Moreno et al., 2006); as well as malX (Horcajada et al., 2005; Johnson et al., 2005b; Moreno et al., 2006), were shown to be statistically significant less prevalent among resistant isolates. In opposition, afa/dra, iutA, traT, which are often associated with plasmids (Johnson et al., 2005b); as well as bmaE and gafD (Horcajada et al., 2005), were found to be significantly more prevalent among isolates resistant to this antimicrobial combination.

Considering the virulence scores of the isolates, contradictory findings have been reported, as on one hand Johnson et al. reported that no net changes were observed in the virulence scores of resistant isolates compared to the susceptible ones (Johnson et al., 2005b); and on the other, Moreno et al. reported that the aggregate virulence scores of sulfamethoxazole-trimethoprim- susceptible isolates were significantly higher (Moreno et al., 2005).

Contrarily to the other antibiotics referred before, the relation between resistance to aminoglycosides and virulence is still largely unknown. Resistance to gentamicin in B2 E. coli isolates, was reported to be associated with a reduction in the prevalence of fimH and an increase in iutA prevalence, in comparison to
susceptible isolates (Horcajada et al., 2005). Nonetheless no further studies were performed that supported this observation.

5. Objectives

The interplay between resistance and virulence is a complex phenomenon which seems to depend on diverse factors. Moreover, several contradictory evidences have been reported, which make this matter even more complicated. Studies focusing on this relation among Enterobacteriaceae have been mainly performed in E. coli strains, disregarding other clinically important pathogens, including Klebsiella spp. that is emerging in the nosocomial setting. Additionally, the presence of the different pathogenicity islands, which are important virulence gene clusters that may be responsible for enhancing the virulence potential of bacteria, are generally not considered.

With the worldwide dissemination of resistance, and facing the possibility of the emergence of strains simultaneous highly virulent and resistant, it is therefore crucial to elucidate if resistance can be accompanied of more virulence, or if a trade-off between these characteristics is verified.

Therefore, the main objective of this study is to understand the relation between virulence and resistance in E. coli and Klebsiella spp. from clinical and non-clinical sources, by investigating the distribution of virulence factors; determining their resistance profile, and characterizing the plasmids and resistance determinants carried by the strains. This will allow understanding if resistant strains, both from clinical and non-clinical origins, also carry more virulence determinants, and to ascertain if the use of antibiotics may also be selecting for more virulent strains. Additionally, we also aim to assess if there is a significant fitness cost in the cell, associated to the maintenance of pathogenicity islands and a plasmid carrying \textit{bla}\textsubscript{CTX-M-15}. 


Chapter 2

Unusual Genotype of an Uropathogenic *Escherichia coli* Strain Assigned to the B2 Phylogenetic Group

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains cause infections such as urinary tract infections, septicaemia, and meningitis. Knowledge of the phylogeny of the strains contributes to the recognition of their virulence potential and clinical outcome. ExPEC virulence factors are often clustered in pathogenicity-associated islands (PAIs) (Diard et al., 2010). Pathogenic ExPEC strains belong to the B2 and, to a lesser extent, D phylogenetic groups, whereas commensal isolates are assigned to groups A and B1. The triplex PCR method is often used to assess phylogenetic groups (Clermont et al., 2000). The accuracy with which this method assigns strains to their correct multilocus sequence typing (MLST)-based phylogroups is good (Gordon, Clermont, Tolley, & Denamur, 2008).

As part of a project investigating antimicrobial resistance, virulence, and phylogeny, we used this method (Clermont et al., 2000) to determine the phylogroup of *E. coli* isolates collected at a university hospital. *E. coli* strain HUC270 was isolated in 2007 from the urine of a 52-year-old man hospitalized in a medical ward. The antimicrobial susceptibility testing was performed with the Vitek 2 Advanced Expert System (bioMérieux, France). Eight PAIs were screened by a multiplex PCR (Sabaté, Moreno, Pérez, Andreu, & Prats, 2006). The amplification products were sequenced (Macrogen). MLST was used to determine the sequence type (Wirth et al., 2006).

*E. coli* HUC270 was resistant only to ampicillin and cephalothin, being susceptible to amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftaxime, ceftazidime, levofloxacain, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and nitrofurantoin. An inverted relationship between the presence of virulence factors and susceptibility to quinolones has been reported in uropathogenic *E. coli*, which may suggest a possible association of *E. coli* HUC270 with a virulent phylogenetic group like B2 (Moreno et al., 2006). The majority of uropathogenic strains belonging to the B2 phylogroup contain three or more PAIs (Sabaté et al., 2006). Indeed, *E. coli* HUC270 encoded different virulence determinants in PAIs: in PAI III\textsubscript{536}, S-fimbriae and an iron siderophore system; in PAI IV\textsubscript{536}, the yersiniabactin siderophore system; and in PAI II\textsubscript{cft073}, P-fimbriae and iron-regulated genes. However, it was not possible to cluster the strain in the major phylogenetic groups or subgroups (Clermont et al., 2000; Escobar-Páramo et al., 2004b). The chuA gene was not amplified, but the yjaA and DNA fragment tspE4.C2 genes were present in the genome (Figure 2.1). The result was reproducible and consistent for six colonies submitted to triplex PCR to
avoid the hypothesis of a mix of strains belonging to the A0 or A1 (Escobar-Páramo et al., 2004b) and B1 phylogroups.

![Figure 2.1 – Triplex PCR profiles specific for E. coli phylogenetic groups. Lanes 1 and 2, group A; lane 3, group B1; Lanes 4 and 5, group D; lanes 6 and 7, group B2; lane 8, E. coli HUC270 profile. Lane M contained 100bp marker.](image)

According to the MLST scheme (MLST databases at the Environmental Research Institute [ERI], University College Cork), E. coli HUC270 revealed a new sequence type (ST2084), which was related to the B2 reference E. coli strain (ST127) by five housekeeping genes (adk, gyrB, icd, mdh, and recA). These findings suggested that this unusual genotype profile may be included in phylogroup B2 and not considered as a “hybrid” strain like E. coli reference strain 70 (ECOR 70), in which some housekeeping genes exhibit nucleotide sequences shared by group A ECOR strains and some show sequences from B1 ECOR strains (Clermont et al., 2000).

To our knowledge, the combination of yjaA gene and DNA fragment tspE4.C2 reported here has not been described previously. A study correlating phylogroup with MSLT results for 600 E. coli strains did not report this phylogenetic profile (Gordon et al., 2008), suggesting that it is uncommon. Also, it could not be assigned to one of the seven groups defined by Escobar-Páramo et al. (Escobar-Páramo et al., 2004b). On the basis of the results of PAI determination, MLST profile, and susceptibility to quinolones, we suggest that E. coli strains showing this combination of genes by the triplex PCR method should be assigned to the major phylogenetic group B2 and considered potentially pathogenic.
Chapter 3

Interplay between pathogenicity islands carriage, resistance profile and plasmid acquisition in uropathogenic *Escherichia coli*.

Submitted for publication.
ABSTRACT

This study aimed to characterize the relation 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 ESBL-production were determined with Vitek 2 AES. Phylogroup determination, detection of PAIs, virulence genes *pap*AH, *pap*C, *sfa/foc, afa/dra, iut*A, *kps*M II, *cnf*1, *eae*A, *hly*A, *stx*1 and *stx*2; plasmid replicon typing and screening for plasmidic resistance determinants *qnr*, aac(6’)-Ib-cr, *qep*A and *bla*CTX-M were done by PCR. Conjugation was performed between a donor carrying IncF, IncK and *bla*CTX-M-15, and receptors carrying one to six PAIs. The relative fitness of transconjugants was estimated by pairwise competition experiments. PAI IV\textsubscript{536} (68%), gene *iut*A (57%) and resistance to ampicillin were the most prevalent traits. PAI I\textsubscript{536}, PAI II\textsubscript{536}, PAI III\textsubscript{536} and PAI II\textsubscript{J96} were exclusively associated to susceptibility to amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole and were more prevalent in strains susceptible to ampicillin and cephalothin. PAI IV\textsubscript{536}, PAI II\textsubscript{CFT073} and PAI I\textsubscript{CFT073} were more prevalent among resistance to amoxicillin-clavulanic acid, cephalothin, cefotaxime, ceftazidime, and gentamicin. An inverted relation was observed between the number of plasmids and number of PAIs carried. Transconjugants were obtained for receptors carrying three or less PAIs. The mean relative fitness rates obtained were 0.87 (two PAIs), 1.00 (one PAI) and 1.09 (three PAIs). The interplay between resistance, PAIs carriage and fitness cost of plasmid acquisition could be considered PAI specific, and not necessarily associated to the number of PAIs.

INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) are the most frequent cause of community and hospital acquired urinary tract infections. They express several virulence factors that contribute to the efficient colonization and infection of the host urinary tract (Wiles et al., 2008), such as adhesins, siderophores and toxins. 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 (Gal-Mor & Finlay, 2006; Hacker et al., 1997).
The antimicrobial resistance reported in UPEC during the last 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 the clinical management, it is usually accepted that resistance, especially multidrug resistance, equates the virulence of the strain. However, molecular studies indicated an inverted relationship between the distribution of virulence factors and antimicrobial resistance determinants, and particularly with quinolones (Johnson et al., 2003; Moreno et al., 2006; Piatti et al., 2008; Vila et al., 2002).

Phylogenetic analysis of extra-intestinal pathogenic E. coli (ExPEC) strains showed that they cluster into four main groups, in which virulent strains belong mainly to phylogroup B2 and, to a lesser extent, to group D, and most of the commensal strains belong to groups A and B1 (Clermont et al., 2000). Eventually, the less virulent strains maybe more prone to acquire resistance determinants, and 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 pathogenicity islands 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 to the host cell. In fact, most of the studies show a fitness burden associated with the presence of resistant plasmids (Björkman & Andersson, 2000). Therefore, the main objectives of this study were to characterize the relation 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 harboring different number of diverse PAIs.
MATERIALS AND 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), Portugal.

The antimicrobial susceptibility profiles and the preliminary identification of ESBL-producing isolates were determined at the Service of Clinical Pathology using the Vitek 2 Advanced Expert System (BioMérieux, Marcy l’Etoile, France). The results were interpreted according Clinical and Laboratory Standards Institute guidelines (C.L.S.I., 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 *chuA*, *yjaA* and DNA fragment tspE4.C2 genes (Clermont et al., 2000; Mendonça et al., 2011).

**Detection of pathogenicity island markers and virulence genes**

PAIs markers were detected using the Bronowski *et al.* method (Bronowski et al., 2008), based in the technique described by Sabaté *et al.* (Sabaté et al., 2006). The method consists in three multiplex-PCR which allow the detection of eight PAIs, encoding different virulence genes: PAI I$_{536}$, α-haemolysin, CS12 fimbriae and F17-like fimbrial adhesin; PAI II$_{536}$, α-haemolysin and P-related fimbriae; PAI III$_{536}$, S-fimbriae and an iron siderophore system; PAI IV$_{536}$, yersiniabactin siderophore system; PAI I$_{96}$, α-haemolysin and P-fimbriae; PAI II$_{96}$, α-haemolysin, Prs-fimbriae and cytotoxic necrotising factor 1; PAI I$_{CFT073}$, α-haemolysin, P-fimbriae and aerobactin; and finally PAI II$_{CFT073}$ coding for P-fimbriae and iron-regulated genes.

Other virulence genes that maybe present in ExPEC such as *papAH, papC* (P fimbriae structural subunit and assembly), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *iutA* (aerobactin receptor), *kpsM II* (group 2 capsules) and *cnf1* (cytotoxic necrotizing factor 1) were screened by PCR (Johnson & Stell, 2000). Additionally, specific genes of intestinal pathogenic *E. coli* (IPEC), namely the Enterohemorrhagic *E. coli* associated virulence genes *eaeA* ( intimin), *hlyA* (pore-
forming cytolysin), \textit{stx}1 and \textit{stx}2 (shiga-like toxins) were also screened (Ram, Vajpayee, & Shanker, 2008).

PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Izasa, Portugal), according to the manufacturer's instructions and amplicons were further sequenced at Macrogen, Amsterdam, Netherlands.

**Antimicrobial resistance determinants detection**

The \textit{bla}\textsubscript{CTX-M}, \textit{bla}\textsubscript{TEM} and \textit{bla}\textsubscript{SHV} genes encoding for β-lactamases and the plasmid mediated quinolone resistance (PMQR) determinants \textit{aac(6')-Ib-variant}, \textit{qnrA}, \textit{B} and \textit{S}, and \textit{qepA} were screened with specific primers by PCR (Cattoir, Poirel, Rotimi, Soussy, & Nordmann, 2007; Ma et al., 2009; Mendonça, Leitão, Manageiro, Ferreira, & Caniça, 2007; Park, Robicsek, Jacoby, Sahm, & Hooper, 2006). All the amplicons were purified with ExoSAP-IT and further sequenced at Macrogen, Amsterdam, Netherlands.

**Plasmid Replicon typing**

Plasmid replicons were identified by a PCR-based replicon typing scheme described by Carattoli \textit{et al}. (Carattoli \textit{et al}., 2005), that consists of five different multiplex and three simplex, and allows the detection of the major replicon families in \textit{Enterobacteriaceae}: HI2, HI1, I1-Y, 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, Ribeiro, Mendonça, & Da Silva, 2013) was used as a plasmid donor. \textit{E. coli} strains with one to six PAIs were used as receptors: Ec396 (PAI \textit{IV}\textsubscript{536}, Ec93 (PAI \textit{IV}\textsubscript{536}, PAI \textit{II}\textsubscript{CFT073}), Ec107 (PAI \textit{IV}\textsubscript{536}, PAI \textit{I}\textsubscript{CFT073} and PAI \textit{II}\textsubscript{CFT073}) Ec 112 (PAI \textit{I}\textsubscript{536} , PAI \textit{II}\textsubscript{536} , PAI \textit{IV}\textsubscript{536} and PAI \textit{II}\textsubscript{J96}), Ec270 (PAI \textit{I}\textsubscript{536} , PAI \textit{III}\textsubscript{536}, PAI \textit{IV}\textsubscript{536}, PAI \textit{II}\textsubscript{CFT073} and PAI \textit{II}\textsubscript{J96}) and Ec91(PAI \textit{I}\textsubscript{536} , PAI \textit{II}\textsubscript{536}, PAI \textit{IV}\textsubscript{536}, PAI \textit{I}\textsubscript{CFT073}, PAI \textit{II}\textsubscript{CFT073} and PAI \textit{II}\textsubscript{J96}). Briefly, donor and recipient strains were grown in Luria Bertani (LB) broth overnight and mating assays were performed in LB agar using 100 µl of receptor and 50 µl of the donor, and incubated at 37°C overnight. A suspension of the grown culture was made in 500 µl of physiological serum and 25 µl were dispersed into selective plates. The transconjugants were selected in LB agar plates containing 10
µg/ml of tetracycline plus 1 µg/ml of cefotaxime or 50 µg/ml of nalidixic acid plus 1 µg/ml of cefotaxime, 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 previously described (Ray et al., 2009), with the exception that 25 mg/ml glucose was added to the medium (Enne et al., 2005). Briefly, a single CFU was inoculated in the medium and incubated at 37 °C overnight with good aeration (225 rpm) (Day -1). On the next day (Day 0), cultures were diluted 1:10 in 0.9 % NaCl, and 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 in antibiotic-free LB and in LB supplemented with 1 µg/ml of cefotaxime, respectively. The competition culture was incubated at 37 °C with agitation at 225 rpm. After 24 hrs, the competition assay was stopped (Day 1). The culture was diluted and different dilutions were plated in LB with antibiotic selection and without 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: \[ W_{AB} = \frac{M_A}{M_B}, \] where \[ M_A = \ln \frac{A(1)}{A(0)}, \] \[ M_B = \ln \frac{B(1)}{B(0)}, \] A or B(0) = estimated density of A or B at Day 0 (cells per ml), and A or B(1) = estimated density of A or B at Day 1 (cells per ml).

**Statistical analysis**

Chi-square test and t-test were applied using IBM SPSS statistics version 22.0. A \( P \leq 0.05 \) was considered statistically significant for the chi-square test and the variables considered statistically associated.

**RESULTS**

**Strains origin and phylogeny**

*E. coli* strains were obtained from patients suffering from UTIs presenting ages from 14 to 98 years, with the majority being isolated from female gender (72%). Fifty-seven percent of the isolates were from 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 3.1).

**Table 3.1 – Prevalence of Pathogenicity Islands, virulence genes and plasmid groups in the different phylogroups.**

<table>
<thead>
<tr>
<th>Phylogenetic groups</th>
<th>A (n=18)</th>
<th>B1 (n=10)</th>
<th>B2 (n=30)</th>
<th>D (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pathogenicity islands</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI I</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>20 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI II</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI III</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PAI IV</td>
<td>39 (7)</td>
<td>10 (1)</td>
<td>100 (30)</td>
<td>86 (6)</td>
</tr>
<tr>
<td>PAI CFT073</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>80 (24)</td>
<td>43 (3)</td>
</tr>
<tr>
<td>PAI II J966</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>90 (27)</td>
<td>14 (1)</td>
</tr>
<tr>
<td><strong>Virulence genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>papAH</em></td>
<td>6 (1)</td>
<td>20 (2)</td>
<td>27 (8)</td>
<td>43 (3)</td>
</tr>
<tr>
<td><em>papC</em></td>
<td>6 (1)</td>
<td>20 (2)</td>
<td>40 (12)</td>
<td>57 (4)</td>
</tr>
<tr>
<td><em>afa/draBC</em></td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (1)</td>
</tr>
<tr>
<td><em>sfa/focDE</em></td>
<td>11 (2)</td>
<td>10 (1)</td>
<td>20 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>kpsMTI</em></td>
<td>22 (4)</td>
<td>40 (4)</td>
<td>73 (22)</td>
<td>57 (4)</td>
</tr>
<tr>
<td><em>iutA</em></td>
<td>22 (4)</td>
<td>40 (4)</td>
<td>87 (26)</td>
<td>43 (3)</td>
</tr>
<tr>
<td><em>cnf1</em></td>
<td>11 (2)</td>
<td>10 (1)</td>
<td>43 (13)</td>
<td>14 (1)</td>
</tr>
<tr>
<td><em>eae</em></td>
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<td>0 (0)</td>
<td>7 (2)</td>
<td>0 (0)</td>
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<tr>
<td><em>stx1</em></td>
<td>6 (1)</td>
<td>0 (0)</td>
<td>3 (1)</td>
<td>0 (0)</td>
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<tr>
<td><strong>Plasmid Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IncF</td>
<td>78 (14)</td>
<td>70 (7)</td>
<td>90 (27)</td>
<td>100 (7)</td>
</tr>
<tr>
<td>Inc K</td>
<td>83 (15)</td>
<td>90 (9)</td>
<td>43 (13)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>IncB/O</td>
<td>11 (2)</td>
<td>10 (1)</td>
<td>10 (3)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>IncHI</td>
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<td>20 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IncI</td>
<td>28 (5)</td>
<td>30 (3)</td>
<td>13 (4)</td>
<td>43 (3)</td>
</tr>
<tr>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IncL/M</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IncN</td>
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<td>0 (0)</td>
<td>13 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
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<td>22 (4)</td>
<td>30 (3)</td>
<td>7 (2)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>IncY</td>
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<td>20 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IncP</td>
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<td>10 (1)</td>
<td>0 (0)</td>
<td>29 (2)</td>
</tr>
<tr>
<td>IncA/C</td>
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<tr>
<td>IncT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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</tr>
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</table>
Virulence characterization

The pathogenicity islands most frequently detected were PAI IV\textsubscript{536} (68%) followed by PAI II\textsubscript{CFT073} (42%) and PAI I\textsubscript{CFT073} (42%) (Table 3.1). PAI I\textsubscript{536} and PAI II\textsubscript{J96} were detected in 9% of the isolates, PAI II\textsubscript{536} in 6% and PAI III\textsubscript{536} in 3% (n=2). PAI I\textsubscript{J96} was not detected. PAI I\textsubscript{536}, PAI II\textsubscript{536}, PAI III\textsubscript{536} and PAI II\textsubscript{J96} were exclusively detected in phylogroup B2 and PAI IV\textsubscript{536} was detected among all the phylogroups but was more prevalent among phylogroups B2 (100%) and D (86%). PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073} were only detected in phylogroups B2 and D (80% of B2 and 43% of group D isolates; and 90% of B2 and 14% of phylogroups D strains, respectively).

All the virulence genes were identified among the isolates, with exception of hlyA and stx\textsubscript{2} (Table1). The most frequently detected was iutA (57%), followed by kpsMTII (52%), papC (29%), cnf1 (26%) and papAH (22%). The least frequent 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 B2 phylogroup. Exceptions were genes papAH (43%), papC (57%) and afa/dra (14%), more prevalent among isolates from phylogenetic group D, and stx\textsubscript{1} gene, detected in one isolate from phylogroup A and in other from B2 group.

Phenotypic and genotypic resistance profile

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

The plasmidic resistance determinants, aac (6')-lb-cr (49%) and bla\textsubscript{CTX-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. Gene aac (6')-lb-cr was detected in 44% of strains resistant to ciprofloxacin.

Phylogenetic background and virulence genes in relation to phenotypic resistance

The distribution of the phylogroups and virulence traits among susceptible and resistant strains to different antibiotics are shown Table 3.2. Phylogroups A, B1 and D were more prevalent among susceptible isolates to amoxicillin-clavulanic acid, cefotaxime, ceftazidime and gentamicin, while group B2 was more prevalent in the resistant isolates. The reverse was observed for trimethoprim-sulfamethoxazole.
Phylogroups A, B1, and D were more prevalent in resistant isolates (42% vs 15%, 23% vs 9% and 16% vs 6%, respectively) while isolates from phylogroup B2 were more susceptible (71% vs 19%).

Considering resistance to ampicillin and ciprofloxacin, phylogenetic group A was more prevalent among resistant strains, compared to the susceptible isolates (31% vs 18% for ampicillin and 33% vs 15% for ciprofloxacin). The same was observed for phylogroup B1 (17% vs 12% and 20% vs 5%, respectively). On the opposite, 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 susceptible strains.

The majority of virulence genes were more prevalent among susceptible isolates (Table 3.2). Exceptions were observed for iutA, kpsMTII, afa/dra and eae genes: iutA gene was more common in resistant strains except for trimethoprim-sulfamethoxazole (71% of susceptible vs 42% of resistant); kpsMTII was more frequent among resistant strains except for ciprofloxacin (60% in susceptible vs 49% in resistant) and trimethoprim-sulfamethoxazole (62% in susceptible vs 42% in resistant strains); 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%), cephalothin (5% vs 0%), ciprofloxacin (4% vs 0%), gentamicin (11% vs 0%) and displayed no differences between susceptible and resistant trimethoprim-sulfamethoxazole isolates.
### Table 3.2 – Distribution of phylogroups, PAIs and virulence genes among susceptible and resistant strains.

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<th>CIP</th>
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AMP - Ampicillin; KF - Cephalothin; CTX - Cefotaxime; CAZ - Cefazidine; AMC - Amoxicillin-Clavulanic Acid; CIP - Ciprofloxacin; GEN - Gentamicin; SXT - Trimethoprim-sulfamethoxazole
Distribution of PAIs according to resistance profile

Pathogenicity islands PAI I536, PAI II536, PAI III536 and PAI IIJ96 were exclusively found among strains susceptible for amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole and were more prevalent in strains susceptible to ampicillin and cephalothin (Table 3.2). Nonetheless, only the absence of PAI I536, PAI II536 and PAI IIJ96 in isolates resistant to ciprofloxacin (p<0.001 each), and of PAI I536 and PAI IIJ96 in isolates resistant to trimethoprim-sulfamethoxazole (p=0.014 each) were considered statistically significant. The remaining PAIs detected were found both in resistant and susceptible isolates for all antibiotics studied. PAI ICFT073 was more prevalent in strains susceptible for ampicillin (52% vs 38%), ciprofloxacin (50% vs 38%) and trimethoprim-sulfamethoxazole (59% vs 23%, p= 0.003) and in strains resistant to amoxicillin-clavulanic acid (44% vs 40%), cefotaxime, (75% vs 34%, p=0.009) ceftazidime (75% vs 34%, p=0.029), cephalothin (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), cephalothin (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%), cephalothin (76% vs 57%) and gentamicin (79% vs 63%) (Table 3.2).

All isolates carried from none to six PAIs. Isolates resistant to amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole did not presented more than three PAIs (Table 3.2).

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 only detected in strains from phylogroups B1, IncN in phylogenetic group B2 and IncA/C was exclusively found in isolates from phylogroups A (Table 3.1).
The number of PAIs versus plasmids carriage is shown in Table 3.3. Strains carrying more than three plasmids contained three or less PAIs, while strains with three or less identified plasmids harbored from none to six PAIs (Table 3.3).

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

Considering the results obtained, which seem to indicate an inverted relation between the amount of plasmids and number of pathogenicity islands simultaneously carried in the same isolates, fitness studies were performed. Transconjugants carrying a plasmid were only obtained in three receptors harboring three or less PAIs. PCR reactions confirmed the presence of IncK and \( \text{bla}_{\text{CTX-M-15}} \) in the transconjugants. The fitness cost of the same plasmid in the transconjugants was evaluated and the mean relative fitness ranged from 0.87 (CI 0.61-0.87) to strain 93 (2 PAIs); 1.00 (CI 0.86-1.12) to strain 396 (1 PAI) and 1.09 (CI 1.09-1.29) for strain 107 (3 PAIs).

<table>
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<th>3 (n=15)</th>
<th>( \geq 4 ) (n=5)</th>
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**DISCUSSION**

*E. coli* is one of the most versatile bacterial species and the diversity of its lifestyles is achieved through a high degree of genomic plasticity, via gene loss or gain, through lateral gene transfer (Rasko et al., 2008). Pathogenicity islands are important mobile genetic elements which allow the simultaneous transmission of several virulence
determinants in one single horizontal gene transfer event, leading to important changes in the virulence phenotype of bacteria. Despite of the importance of PAIs in the bacterial genome, generally these elements have not been included in studies focusing in the interplay between resistance and virulence. Thus the main goal of this study was to investigate the association between the main PAIs found in UPEC, virulence genes, phylogenetic background and resistance, as well as evaluate the possible influence of the presence of PAIs in the acquisition of a resistance plasmid.

A link between phylogeny and virulence has been previously reported in *E. coli* strains. The most virulent strains belong mainly to group B2, and in lesser extent to group D, while the commensal strains are mostly associated with groups A and B1 (Clermont et al., 2000; Picard et al., 1999). In this UPEC collection, phylogroup B2 was the most prevalent and was the only group in which PAI I$_{536}$, PAI II$_{536}$, PAI III$_{536}$ and PAI II$_{J96}$ were detected. The remaining PAIs were also more prevalent among strains from this group. Sabaté et al. similarly reported the occurrence of PAI II$_{536}$ and III$_{536}$ only among B2 strains, and PAI I$_{536}$ and II$_{J96}$ among B2 and B1 strains (Sabaté et al., 2006). There is evidence that PAI II$_{536}$ and PAI III$_{536}$ are the most unstable islands, and thus more prone to be excised, which may explain the low prevalence found in this study (Middendorf et al., 2004). 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 which may somehow contribute to enhance the stability of this islands in the bacterial genome. In addition, the virulence genes detected were also more frequent in B2 phylogroup with the exception of *pap*AH and *pap*C and *afa/dra*, more common in phylogroup D, considered to be the second more virulent. Nonetheless the reduced number of strains from this phylogenetic group compared to the remaining phylogroups may be influencing the results, as this was the less prevalent group among our strains. The concentration of virulence factors within group B2 isolates is mainly due by a genetic background that allows numerous horizontal gene transfer events (Escobar-Páramo et al., 2004a). This may explain the higher prevalence of virulence factors and PAIs among this group.

Genes *eae* and *stx$_1$* though rare, were 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 *iut*A gene, encoding the aerobactin receptor, part of a siderophore system was the most detected virulence gene. The production of aerobactin was shown to
facilitate the growth in urine (Johnson, Moseley, Roberts, & Stamm, 1988), a medium with limited iron conditions, in which siderophores may be extremely useful and constitute an important virulence factor in UTIs development.

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

Higher prevalence of phylogroup B2 was observed in strains susceptible to ampicillin, ciprofloxacin and trimethoprim/sulfamethoxazole, which may account for the old paradigm of a tradeoff between resistance and virulence (Houdouin et al., 2006; Johnson et al., 2005b; Johnson et al., 2003; Moreno et al., 2006; Piatti et al., 2008). Nonetheless, considering resistance to ampicillin and ciprofloxacin, though the susceptible isolates presented higher prevalence's of B2 group compared to the resistant ones, B2 was still the most prevalent phygroup among resistant strains. Thus, a shift towards non-B2 phylogroups was only observed for sulfamethoxazole-trimethoprim.

A positive relation was observed for phylogroup B2 and the most frequent PAIs, PAI IV\textsubscript{536}, PAI II\textsubscript{536}CFT073 and PAI I\textsubscript{CFT073} and resistance to amoxicillin-clavulanic acid, cephalothin, cefotaxime, ceftazidime, and gentamicin. It has been suggested that some plasmids, including from FIB replicon may be carriers of PAIs (Johnson & Nolan, 2009). PAI IV\textsubscript{536}, also known as the High Pathogenicity Island (HPI), may be transferred by a conjugative F plasmid (Schubert et al., 2009), and PAI II\textsubscript{536} was also previously transferred using conjugation (Schneider et al., 2011). The higher prevalence of the most common islands, including PAI IV\textsubscript{536}, among strains resistant to antibiotics, which are mainly associated to plasmidic resistance mechanisms, may suggest that these islands may be using resistance plasmids to potentiate their dissemination.

The remaining PAIs were only detected among strains susceptible to all the antibiotics tested, with the exception of ampicillin and cephalothin. For these antibiotics, although the PAIs were also present in resistant strains, they were more prevalent in susceptible strains. This confirms that the trade-off between resistance and virulence is also verified for the PAIs considered to be more unstable. Previous
studies have already demonstrated that the resistance to quinolones is associated to a lower prevalence of PAI II\textsubscript{J96}-like domains (Houdouin et al., 2006; Piatti et al., 2008).

The presence of virulence genes was generally more frequent among strains susceptible to the tested antibiotics, which was in agreement with previous studies, particularly with quinolone susceptible strains (Johnson & Stell, 2000; Moreno et al., 2006; Piatti et al., 2008; Vila et al., 2002). Nonetheless this tendency was not observed for \textit{iutA}, \textit{afa/dra} and \textit{eae} genes. This could be associated to the fact that these genes may be carried by plasmids which also contain resistance determinants.

The results seemed to indicate an inverse tendency between the number of plasmids and the number of pathogenicity islands. This led us to the hypothesis that the presence of more PAIs may be a factor that could affect the acquisition of plasmids. The fact that we could not obtain transconjugants using strains with more than three PAIs as receptors seemed to confirm this hypothesis. Nonetheless, the \textit{in vitro} fitness studies performed between transconjugants and the wild-type receptor indicated that, at least for strains harboring three or less PAIs, the carriage of higher number of islands was not directly associated to 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, Conceição, Marques, Fernandes, & Gordo, 2005; Enne et al., 2005). Thus, other factors possibly related to the genetic background of bacteria may be responsible for this tendency. Nonetheless, the transconjugants were only carriers of the PAIs considered to be more prevalent, and as we have seen which seem to be positively associated to the presence of resistance mediated by plasmidic determinants, including to third generation cephalosporins. In fact, the combination of three PAIs carried by the transconjugant with a fitness advantage compared to the receptor strain has already been reported as being the prevalent combination of PAIs for clone ST131 (Calhau et al., 2013). Therefore, the trade-off between the plasmid number and the number of PAIs may be related to the presence or absence of specific PAIs 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 pathogenicity islands.
Chapter 4

Prevalent combination of virulence and plasmidic-encoded resistance in ST 131 *Escherichia coli* strains

ABSTRACT

*Escherichia coli* ST131, is an important cause of multidrug-resistant infections. Thus, the aim of this study was to evaluate the concomitant presence of resistance plasmids and pathogenicity islands (PAIs) in ST131 *E. coli*. From 97 extra-intestinal *E. coli* characterized for antimicrobial susceptibility and extended-spectrum beta-lactamase production, 16% of isolates were identified as CTX-M-15 producers. These strains were studied by PFGE, MLST and phylogroups, plasmid groups, PAIs and plasmid-mediated quinolone-resistance determinants. MLST identified one ST10 strain from phylogroup A and the remaining isolates were ST131, from group B2. Despite the genetic variability, 64% of ST131 strains presented a profile composed by PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073}, IncF plasmid, *bla\textsubscript{CTX-M-15}* and *aac(6')-lb-cr* genes. The prevalent virulence and resistance profile detected among the strains, may constitute an optimal combination of factors which allow *E. coli* ST131 to maintain both features becoming concomitantly virulent and extremely resistant.

INTRODUCTION

The high levels of resistance observed in *Escherichia coli*, a pathogen responsible for several infections in the human host, to several important antibiotics groups, such as cephalosporins and quinolones, have become a main concern. One of the most successful *E. coli* clone, ST131, has emerged as very important cause of multidrug-resistant infections worldwide (Totsika et al., 2011).

The *E. coli* ST131 clone has been usually associated with resistance to β-lactams, mostly due to the production of extended-spectrum beta-lactamase (ESBL) CTX-M-15, which confers resistance to penicillins, cephalosporins and monobactams except to cephamycins (Rogers, Sidjabat, & Paterson, 2011). Resistance to fluoroquinolones is also frequently found in *E. coli* ST131 clones, probably potentiated by the presence of plasmid-mediated quinolone resistance (PMQR), like *qnr* encoding genes or aminoglycoside modifying enzyme AAC(6')-lb-cr, usually present in ST131 strains (Rogers et al., 2011). These mobile resistance determinants (ESBLs and PMQRs), responsible for antimicrobial resistance, can be carried and disseminated through plasmids. In *Enterobacteriaceae* family, plasmids are gathered into major replicon families HI2, HI1, I1-Y, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O (Carattoli, 2009).
Additionally to the resistance capacities, *E. coli* ST131 strains have been described to harbor several virulence factors, contributing to the pathogenicity and invasion of the hosts. These determinants are generally involved in escaping the host immune system, adhesion, collection of nutrients under limited conditions and induction of inflammation, instigating the development of extraintestinal pathology. Virulence factors in this clone include toxins, adhesins, and siderophore systems among others (Rogers et al., 2011). Furthermore, the virulence factors can be carried and disseminated by horizontal gene transfer through pathogenicity islands (Sabaté et al., 2006). Although several virulence factors have been identified in *E. coli* ST131 strains (Lavigne et al., 2012), the lack of pathogenicity islands could make it less virulent than other uropathogenic bacteria.

Regardless of the general assumption that the acquisition of resistance may have a fitness cost which leads to decreased virulence, *E. coli* ST131 has proven to be able to maintain both features (Clark et al., 2010). Thus, the aim of this work was to evaluate the concomitant presence of plasmids as resistance determinants transporters and pathogenicity islands carrying several virulence factors in multidrug resistant ST131 *E. coli* clinical isolates.

**MATERIALS AND METHODS**

From a total group of 97 non-duplicate isolates of *E. coli* collected between November and December 2007 from different clinical samples and wards of the University Hospitals of Coimbra (HUC), located in the Centre region of Portugal, presumable ESBL producers were selected based in the antimicrobial susceptibility tests. ESBL production was further confirmed with the double disk synergy test using the antimicrobials disks of amoxicillin-clavulanic acid, cefotaxime, ceftazidime, cefepime and aztreonam (OXOID). Results interpretation was done according Clinical and Laboratory Standards Institute guidelines (C.L.S.I., 2010). *E. coli* ATCC 25922 was used as a quality control strain.

Initially, multi-locus sequence typing (MLST) was performed based in the University of College Cork (Cork, Ireland) scheme for *E. coli* (Wirth et al., 2006). To strengthen the phylogenetic relationship of the strains, pulsed-field gel electrophoresis (PFGE) was performed as previously described (Mendonça et al., 2007), and PFGE images were analyzed with Bionumerics 6.6. Clustering was performed using the Dice band-based similarity coefficient, with a band position
tolerance of 1.0% and an optimization of 1.8. A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software. Isolates with a Dice band-based similarity coefficient value > 80% were assigned to the same cluster.

In order to evaluate the presence of resistance determinants, PMQR and ESBL encoding genes (qnrA, B and S, aac[6’]-lb-variant, qepA and bla<sub>CTX-M</sub>) were screened by PCR (Cattoir et al., 2007; Ma et al., 2009; Park et al., 2006). E. coli 12HUC carrying the bla<sub>CTX-M-15</sub> and aac(6’)-lb-cr determinants, Klebsiella pneumoniae5, carrying qnrA and qnrB, and the plasmid pSTV that carries qepA, were used as positive controls in PCR. Resulting PCR products were submitted to purification using the QIAquick PCR Purification kit (QIAGEN, Izasa, Portugal), according to the producer's instructions and were sequenced at Macrogen, South Korea.

The detection of plasmid mediated resistance mechanisms implied the plasmid replicon typing. For this, a PCR-based replicon typing scheme described by Carattoli et al. detecting the principal incompatibility groups in enterobacteria: HI2, HI1, I1-Y, X, L/M, N, FIA, FIB, FIC,W, Y, P, A/C, T, K and B/O was performed (Carattoli et al., 2005). When needed, plasmid extraction was performed using QIAGEN Plasmid Midi Kit.

Finally, to evaluate the pathogenicity potential of these E. coli strains, pathogenicity island (PAI) markers were detected using the method of Sabaté et al. consisting in three multiplex-PCR which allow the detection of eight PAIs (Sabaté et al., 2006), reinforced by the determination of the major E. coli phylogenetic groups (A, B1, B2 and D), according to the Clermont’s method (Clermont et al., 2000).

RESULTS AND DISCUSSION

ST131 clone is a highly adapted E. coli with a genetic structure with several virulence and resistance genes which contribute to the efficient and global spread of this pathogen. The virtual omnipresence of ST131 E. coli strains, normally associated with CTX-M-15 ESBL production, impelled us to investigate the relationship between the presence of plasmids and PAIs within the cell. Thus, the present study aimed the investigation of the optimal relationship between these two mobile elements, within multidrug resistant ST131 E. coli strains presenting PMQRs and ESBL.
From the total of 97 isolates, the co-analysis of the antibiogram performed by Vitek2 Advanced Expert System and the double disk synergy results indicated the expression of ESBL in 15 strains, and suggested the production of CTX-M enzymes. This was further confirmed using PCR and sequencing techniques which identified the bla\textsubscript{CTX-M-15} gene, whose expression was responsible for the ESBL phenotype in all strains.

In order to establish a phylogenetic relationship between the isolates, MLST was first executed, which allowed the identification of ST131 clone in 93% of the strains, all belonging to phylogroup B2. The remaining strain was integrated in the phylogenetic group A and presented a MLST corresponding to the ST10. ST131 has been mainly known as a worldwide extraintestinal pathogenic E. coli from phylogroup B2 with the capacity to acquire simultaneously virulence and resistance, thus having the capacity to cause severe antimicrobial-resistant infections (Rogers, Sidjabat, & Paterson, 2011). This clone contradicts the hypothesis that bacteria displaying high levels of resistance have a high fitness cost which results in decreased pathogenic capacities (Clark et al., 2010).

In this study, despite of the genetic variability observed in the PFGE profiles for the ST131 isolates, it was possible to observe that 64% of the strains presented the same characteristics in terms of virulence and resistance determinants (Figure 4.1). These strains presented one IncF plasmid, containing a bla\textsubscript{CTX-M-15} and aac(6')-\textit{lb}-\textit{cr} genes and three PAIs (PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073}). This may constitute a favorable and equilibrated combination of resistance and virulence factors for E. coli ST131 clones which may enhance its fitness and adaptation capacity, contributing to the dissemination of this clone.
### Figure 4.1 – Clonal relatedness of strains and characterization of phylogeny, virulence and resistance determinants, and plasmid incompatibility groups.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phylogenetic group</th>
<th>MLST</th>
<th>PAIs</th>
<th>Plasmids</th>
<th>ESBL</th>
<th>PMQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>467</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;CFT073&lt;/sub&gt;, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>138</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;CFT073&lt;/sub&gt;, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>47</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>qnrS, aac(6')-Ib-Or</td>
</tr>
<tr>
<td>90</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>305</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>155</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>89B</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536</td>
<td>F, K</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>85</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>149</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F, K</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>115</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F, II/Ig</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>161B</td>
<td>A</td>
<td>ST10</td>
<td>-</td>
<td>F, K</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>144A</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>94</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>378</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
<tr>
<td>104</td>
<td>B2</td>
<td>ST131</td>
<td>PAI&lt;sub&gt;I&lt;/sub&gt;CFT073, PAI&lt;sub&gt;IV&lt;/sub&gt;536, PAI&lt;sub&gt;ICF073&lt;/sub&gt;</td>
<td>F</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>aac(6')-Ib-Or</td>
</tr>
</tbody>
</table>
The resistance determinants \( \textit{bla}_{\text{CTX-M-15}} \), and the \( \textit{aac(6')-lb-cr} \) enhance the survival of \( \textit{E. coli} \) ST131 strains regarding the action of the main antibiotics used in clinical practice such as cephalosporins and quinolones. In fact, CTX-M-15 enzyme is now worldwide disseminated mostly by \( \textit{E. coli} \) strains, and it has been previously detected among portuguese clinical strains (Mendonça et al., 2007). The association between \( \textit{bla}_{\text{CTX-M-15}} \) and \( \textit{aac(6')-lb-cr} \) genes is common, because they are usually found in the same plasmid, namely in IncF and in IncI1 plasmids, since they share the same genetic platform (Carattoli, 2009). Actually, in all the strains we have detected one plasmid from the group IncF. Moreover, in one ST131 strain an IncI1/Iγ was also detected and in two ST131 strains and one ST10 isolate the IncK was also identified. After plasmid extraction we detected one plasmid carrying \( \textit{bla}_{\text{CTX-M-15}}, \textit{aac(6')-lb-cr} \) as well as PAI IV\(_{536}\) which was assigned by replicon typing to IncF group.

Regardless of the high dissemination of \( \textit{bla}_{\text{CTX-M-15}} \) associated to \( \textit{aac(6')-lb-cr} \), the acquisition of the mobile element containing these determinants may have an implicated fitness cost. In fact Sandegren and colleagues have studied the fitness cost of the acquisition of a plasmid containing the multiresistance gene cassette associated with ST131 clone composed namely by these two resistance elements and found that it originated a reduction of 3-4% of exponential growth rate (Sandegren, Linkevicius, Lytsy, Melhus, & Andersson, 2012).

The virulence factors encoded in the prevalent combination of PAIs, PAI IV\(_{536}\), PAI I\(_{\text{CFT073}}\) and PAI II\(_{\text{CFT073}}\) include P-fimbriae, siderophore systems and hemolysin and may be responsible for the enhancement of the virulence potential of \( \textit{E. coli} \) strains and thus the development of more invasive disease (Johnson, 1991; Sabaté et al., 2006). In fact, 80% of the strains were isolated from urinary tract, where P-fimbriae mediates bacterial adherence to human epithelial cells through digalactoside-specific binding to the P-blood group antigens, which are present all over the urinary tract and enable ascending infection of the ureter and kidney (Johnson, 1991). Furthermore, siderophores systems, as efficient methods to acquire iron, have been described to be essential to bacterial growth especially under limiting conditions such as the ones found in the urinary tract (Johnson, 1991). These virulence factors present in the detected islands increase the \( \textit{E. coli} \) ST131 capacity to subsist in the host and ascend in the urinary tract and may therefore contribute to the survival of this clone and to the evolution of its pathogenesis.
Regardless of the presence of PAIs carrying important virulent factors, we have not detected other islands which are present in more virulent uropathogenic strains. The results are supported by a genomic mapping performed by Lavigne and colleagues in which they have not detected typical extraintestinal PAIs such as PAI \textsubscript{I536}, PAI \textsubscript{II536} and PAI \textsubscript{III536} (Lavigne et al., 2012).

In conclusion, the \textit{E. coli} ST131 clone despite not being an extremely virulent clone presents an efficient organization of virulence and resistance determinants. The predominant arrangement of virulence and resistance determinants found in the ST131 isolates may therefore constitute an optimal and balanced combination, providing important resistance and virulence capacities without having an excessive fitness cost to bacteria. This may allow the dissemination and survival of these clonal strains among the clinical and communitarian sets.
Chapter 5

Occurrence of extended-spectrum beta-lactamases in human and bovine isolates of *Escherichia coli* from Oyo state, Nigeria

ABSTRACT

Introduction: The main objective of the study was the molecular characterization of extended spectrum β-lactamases (ESBL) in *Escherichia coli* isolates collected from human and bovine samples in Oyo state, Nigeria.

Methodology: Between August 2010-2011, 114 *E. coli* isolates were collected from hospitals (n = 57) and bovine (n = 57). PCR and sequencing were used for identification of ESBLs, upstream sequences, plasmid-mediated quinolone resistance (PMQR) genes and class 1 integrons. Plasmid incompatibility groups were identified among ESBL-positive isolates by PCR. Genetic relatedness was assessed by rep-PCR and MLST. Transfer of ESBL determinants to the recipient strain *E. coli* J53 was performed by broth mating assays.

Results: CTX-M-15 was the unique ESBL found in eight human isolates. Six CTX-M-15 producers also carry the *aac(6′)-lb-cr* gene and/or *qnrB* gene, and class 1 integrons. FIA, FIB, H11, H12, F, Y and K were the plasmid replicon types found. CTX-M-15 and PMQR determinants were transferred by conjugation in two *E. coli* assigned by MLST to ST131 and ST2695, a new allele.

Conclusions: The study highlights the dissemination ability of CTX-M-15 associated with PMQR, and the presence of class 1 integrons, able to capture additional genes, justifying the urgent need of antimicrobial resistance surveillance in Nigeria.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are plasmid borne, and are harboured by Gram-negative bacteria. They can be divided into three major groups: TEM, SHV, and CTX-M types (Pitout & Laupland, 2008). ESBL-producing strains can increase morbidity and mortality rates, in part as a result of associated resistance to other antibiotic families, which limits therapeutic options and raises healthcare costs (Perez, Endimiani, Hujer, & Bonomo, 2007). Plasmid-mediated quinolone resistance genes (PMQRs) have been reported to be associated with ESBLs (and other β-lactamases) (Robicsek, Jacoby, & Hooper, 2006a). Many reports have described and characterized ESBLs in *Klebsiella* spp. and *Escherichia coli*, including reports from African countries (Blomberg et al., 2005; Gangoué-Piéboji et al., 2005). However, few reports on the prevalence of ESBL and PMQR determinants in both human and
bovine isolates in Nigeria have been documented. A recent study reported high rates of resistance to quinolones in association with β-lactams among *E. coli* isolates from healthy animals in Nigeria (Fortini, Fashae, García-Fernández, Villa, & Carattoli, 2011). Thus, the objectives of this study were to determine the prevalence of ESBL in *E. coli* clinical isolates from human and bovine origins collected in Oyo state, Nigeria, to perform their molecular characterizations, and to assess their potential dissemination.

**MATERIALS AND METHODS**

**Bacterial isolates**

Between August 2010 and August 2011, 57 *E. coli* isolates were collected from different inpatients from four hospitals located in Oyo state, Nigeria: two tertiary care hospitals (University College Hospital Ibadan and Bowen Teaching Hospital Ogbomoso), and two secondary hospitals (Oluyoro Catholic Hospital Ibadan and General Hospital Adeoyo Ibadan). During the same period, 57 *E. coli* isolates were also isolated from fecal samples of different healthy bovine animals from diverse farms at slaughter in Oyo state, Nigeria. Farms from three different locations (Ibadan, Ogbomoso, and Iseyin) were included in the study.

**Susceptibility testing and phenotypic ESBL detection**

Susceptibility testing for 12 antibiotics was performed using the Kirby-Bauer method. Results were interpreted using Clinical Laboratory and Standards Institute criteria (C.L.S.I., 2010). The double disk synergy test was used to screen the production of ESBLs in all isolates (Jarlier, Nicolas, Fournier, & Philippon, 1988).

**Identification of ESBL genes and upstream sequences, PMQR genes, and class 1 integrons**

PCR was used to screen for *bla*\textsubscript{TEM}, *bla*\textsubscript{CTX-M}, and *bla*\textsubscript{SHV} types of β-lactamases using specific primers (Mendonça et al., 2007) in presumptive ESBL producers. *E. coli* 39FFC (*bla*\textsubscript{TEM}), *E. coli* 144FFC (*bla*\textsubscript{CTX-M}), and *Klebsiella pneumoniae* 339FFC (*bla*\textsubscript{SHV}) were used as positive controls. PCR products were purified with Exosap IT (Affymetrix, Santa Clara, USA), and sequenced in both strands (Macrogen, Seoul, Korea). Nucleotide sequences were analysed with BioEdit software, and database searches were performed using the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).
All isolates were screened by multiplex PCR for the most common *qnr* genes: *qnrA*, *qnrB*, and *qnrS* (Cattoir et al., 2007). Two simplex PCRs were carried out to detect *aac(6’)-lb* and *qepA* genes (Fortini et al., 2011; Park et al., 2006). Human isolates positive for the *aac(6’)-lb* gene were further analyzed by digestion with a *BtsCl* enzyme (New England Biolabs Ipswich, USA) to identify *aac(6’)-lb-cr*, which lacks the *BtsCl* restriction site present in the wild type gene (Park et al., 2006). *Isep*, *Is26*, and *Is903* elements were investigated in the ESBL-positive isolates (Mendonça et al., 2007).

Integrase gene, *intI1*, was screened by PCR in ESBL-positive and Qnr-positive isolates (Barlow, Pemberton, Desmarchelier, & Gobius, 2004). Isolates positive for *intI1* were subjected to amplification with 5’-CS and 3’-CS primers (Lévesque, Piché, Larose, & Roy, 1995) to amplify the variable region of class 1 integrons. PCR products were purified and sequenced as previously described.

**Plasmid replicon typing**

Plasmid incompatibility groups were identified among positive ESBL isolates. The PCR-based Inc/rep typing method consists of five multiplex-PCRs recognizing three different replicon types and three simplex PCRs for K, F, and B/O replicon types (Carattoli et al., 2005).

**Conjugation experiments**

The transfer of ESBL determinants to the recipient strain *E. coli* J53 (sodium azide resistant) was performed by broth mating assays, at a bacterial cell ratio of 1:10. Overnight cultures were centrifuged, the sediment suspended in 50 μL of PBS buffer, and 20 μL were plated in duplicates of Luria-Bertani agar containing sodium azide (100 mg/L) and cefotaxime (1 mg/L). The antibiotic susceptibility profile was determined for transconjugants by the disk diffusion method. PCR amplification of the ESBL determinants and plasmid replicon types was also performed to confirm the transfer of resistance determinants and to identify the plasmid replicon type involved in the transfer of resistance, using the methodology described.

**Genetic relatedness and sequence typing by MLST**

The rep-PCR genomic fingerprinting method was performed on the ESBL producers to assess their genetic relatedness (Mohapatra, Broersma, & Mazumder, 2007). Multilocus sequence typing (MLST) analysis was performed (Wirth et al., 2006) on the two ESBL-positive strains that were successful on conjugation assays.
RESULTS

Origin of bacterial isolates, antibiotic susceptibility, and phenotypic detection of ESBLs

Of the isolates from human clinical samples, 34 were from urine, 10 were from high vaginal swabs, 9 were from stool, 3 were from sputum, and 1 was from an endocervical swab. The majority of the human samples (54%) were isolated from the University College Hospital (UCH), the first teaching hospital in Nigeria. Seventy-four percent of the bovine samples (n = 42) were obtained from farms in Ibadan, which is the capital city of Oyo state and the third-largest metropolitan area in Nigeria.

Table 5.1 shows the susceptibility profiles obtained for the isolates. As shown, the bovine isolates were more susceptible to the antimicrobials tested than were the human isolates. ESBLs were only detected in the human *E. coli* isolates. ESBL-positive strains were also resistant to gentamicin (87.5%), nalidixic acid (62.5%), and ciprofloxacin (50%), but susceptible to cefoxitin and imipenem (100%). The ESBL-producing isolates were predominantly isolated from urine samples, followed by high vaginal swabs.

Table 5.1 – Antibiotic susceptibility pattern of the human and bovine *E. coli* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Human (%)</th>
<th></th>
<th></th>
<th>Bovine (%)</th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>88</td>
<td>0</td>
<td>12</td>
<td>18</td>
<td>5</td>
<td>77</td>
</tr>
<tr>
<td>Amoxicillin plus clavulanic acid</td>
<td>30</td>
<td>47</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>9</td>
<td>4</td>
<td>88</td>
<td>0</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>14</td>
<td>5</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>5</td>
<td>9</td>
<td>81</td>
<td>0</td>
<td>0</td>
<td>100</td>
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<td>Cefepime</td>
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<td>97</td>
<td>0</td>
<td>0</td>
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<td>Imipenem</td>
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<td>0</td>
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<td>Cefoxitine</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>100</td>
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<tr>
<td>Nalidixic acid</td>
<td>47</td>
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<td>49</td>
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<td>60</td>
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<td>18</td>
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<tr>
<td>Gentamicin</td>
<td>46</td>
<td>0</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Characterization of ESBL and PMQR

Table 5.2 summarizes the molecular characterization of ESBL producers and a bovine isolate. CTX-M-15 enzyme was identified in eight human *E. coli* isolates, confirming the resistance phenotype. All isolates also carried the non-ESBL *bla*TEM-1. Two and six *E. coli* *bla*CTX-M-15 producers showed the *qnrB* and *aac(6')-lb-cr*-variant genes, respectively. Two isolates carried both the *qnrB* and *aac (6')-lb-cr* genes. A single bovine strain carried the *qnrS* gene. ESBLs and other PMQRs were not found in the bovine isolates.

An *ISEcp1* element was detected upstream of the *bla*CTX-M-15 gene. The *IS26* element was located in three of the ESBL producers, and one isolate had both the *IS26* and *IS903* elements.

Rep-PCR identified three DNA fingerprint patterns among the *bla*CTX-M-15 producers. They were from two hospitals in Oyo state; UCH had the highest number of isolates (*n* = 7). Most of the isolates shared a common fingerprinting pattern (data not shown).

Plasmid identification

Plasmids carrying the CTX-M-15 β-lactamase and the PMQRs determinants were assigned to the FIA, FIB, HI2, F, and K replicon types (Table 2). In the bovine strain, plasmids were assigned to the HII, FIB, and Y incompatibility groups.

Conjugation assays

Transfer by conjugation of the ESBL and PMQR phenotypes to *E. coli* J53 was successful for two out of the eight *bla*CTX-M-5*-positive strains. The transconjugants showed resistance profiles identical to the donors. The *bla*TEM-1, *bla*CTX-M-15, *aac(6')-lb-cr*-variant, and *qnrB* genes were confirmed to be present in transconjugants. The transferred plasmids were assigned to the FIB and HI2 replicon types (Table 2). *E. coli* with conjugative plasmids belonged to different sequence types; H15, carrying *aac(6')-lb-cr*, *bla*TEM-1, and *bla*CTX-M-15, was identified as ST131, while *E. coli* H1, harboring *qnrB*, *aac(6')-lb-cr*-variant, *bla*TEM-1, and *bla*CTX-M-15, was assigned to ST2695, a new allele.
### Table 5.2 – Origin, antimicrobial resistance pattern, and genetic characteristics of human and bovine *E. coli* isolates.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hospital/farm location</th>
<th>Specimen</th>
<th>Antimicrobial resistance pattern</th>
<th>PMQR gene</th>
<th>β-lactamase gene</th>
<th>Transferred genes</th>
<th>Plasmid replicon</th>
<th>Is elements</th>
<th>Gene cassette(s) on integrons</th>
<th>ST*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV36</td>
<td>Ibadan</td>
<td>Feces</td>
<td>W, AML</td>
<td>QnrS</td>
<td>-</td>
<td>-</td>
<td>HI1, Y, FIB</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>UCH</td>
<td>Urine</td>
<td>CZ, CT, AT, W, CN, A, ML</td>
<td>QnrB, aac(6')-ib-cr</td>
<td>blaTEM1, blaCTX-M15</td>
<td>QnrB, aac(6')-ib-cr, blaTEM, blaCTX-M15</td>
<td>FIB, H12, K</td>
<td>IsEcp1</td>
<td>aadA1</td>
<td>2695</td>
</tr>
<tr>
<td>H6</td>
<td>UCH</td>
<td>Urine</td>
<td>CZ, CT, NA, W, AML</td>
<td>-</td>
<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>FIB</td>
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<td>aadA5, dfrA17</td>
<td></td>
</tr>
<tr>
<td>H12</td>
<td>UCH</td>
<td>HVS</td>
<td>CZ, CT, AT, CIP, NA, W, CN, AML</td>
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<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>FIA, FIB</td>
<td>IsEcp1</td>
<td>aadA5, dfrA17</td>
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<tr>
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<td>UCH</td>
<td>HVS</td>
<td>CZ, CT, CIP, NA, W, CN, AML</td>
<td>-</td>
<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>FIA, FIB</td>
<td>IsEcp1, IS26</td>
<td>-</td>
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</tr>
<tr>
<td>H15</td>
<td>GHA</td>
<td>HVS</td>
<td>CZ, CT, AMC, AT, CIP, NA, W, CN, AML</td>
<td>aac(6')-ib-cr</td>
<td>blaTEM1, blaCTX-M15</td>
<td>aac(6')-ib-cr, blaTEM, blaCTX-M15</td>
<td>FIB</td>
<td>IsEcp1</td>
<td>-</td>
<td>131</td>
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<tr>
<td>H22</td>
<td>UCH</td>
<td>Urine</td>
<td>CZ, CT, AMC, CIP, NA, W, CN, AML</td>
<td>aac(6')-ib-cr</td>
<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>FIA, FIB</td>
<td>IsEcp1</td>
<td>aadA5, dfrA17</td>
<td></td>
</tr>
<tr>
<td>H45</td>
<td>UCH</td>
<td>Urine</td>
<td>CT, W, CN, AML</td>
<td>aac(6')-ib-cr</td>
<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>H12, K, FREPB</td>
<td>IsEcp1, IS26</td>
<td>aadA1</td>
<td></td>
</tr>
<tr>
<td>H47</td>
<td>UCH</td>
<td>Urine</td>
<td>CT, AMC, W, CN, AML</td>
<td>QnrB, aac(6')-ib-cr</td>
<td>blaTEM1, blaCTX-M15</td>
<td>-</td>
<td>H12, FREPB</td>
<td>IsEcp1, IS26, Is903</td>
<td>aadA1</td>
<td></td>
</tr>
</tbody>
</table>

* UCH: University College Hospital, Ibadan; GHA: General Hospital Adeoyo, Ibadan; HVS: High Vaginal Swabs
* CZ - Ceftazidime; CT - Cefotaxime; AT - Aztreonam; W - Trimethoprim; CN - Gentamicin; AML - Amoxicillin; NA - Nalidixic acid; CIP - Ciprofloxacin; AMC - Amoxicillin plus clavulanic acid
* No transferred gene. Conjugation not successful.
* Sequence Type
Characterization of class 1 integrons

The \textit{intI1} gene was present in the \textit{qnrS}-borne bovine strain (no gene cassettes inserted in the variable region) and in 87.5\% of the human ESBL-positive isolates. Two different gene cassette arrays were identified in six ESBL-positive isolates: \textit{aadA1}, encoding resistance to streptomycin and spectinomycin, and \textit{dfrA17-aadA5}, encoding resistance to streptomycin/pectinomycin and trimethoprim (Table 2).

DISCUSSION

ESBLs, especially CTX-M-15 \textit{β}-lactamase, usually located in conjugative plasmids, are disseminated worldwide. This study confirmed the presence of CTX-M-15 in human clinical isolates in Nigeria, though at a moderate prevalence. A strong association between ESBL production and quinolone resistance has been reported in \textit{Enterobacteriaceae} (Perez et al., 2007). In this study, the resistance to nalidixic acid and ciprofloxacin shown by the CTX-M-15 producers could be linked partially to the simultaneous carriage of PMQRs. The \textit{aac(6')-lb-cr}-variant was shown to be prevalent; \textit{qnrB} was found in two isolates. Five different plasmid replicon types (H12, FIA, FIB, F, and K) were identified, but only FIB, H12, and K were shown to conjugate. Both CTX-M-15 and TEM-1 determinants, as well as the \textit{qnrB} and \textit{aac(6')-lb-cr}-variant, could be transferred (Table 2), demonstrating the potential of co-dissemination by horizontal gene transfer of resistance to \textit{β}-lactams and quinolones. Recently, a study reported high rates of resistance to \textit{β}-lactams and quinolones among Gram-negative isolates from different hospitals in Nigeria (Ogbolu, Daini, Ogunledun, Alli, & Webber, 2011), which may support our findings at a molecular level.

Nevertheless, only two \textit{E. coli} were able to conjugate; these belonged to distinct sequence types, as determined by MLST. ST131 was identified as carrying \textit{bla}_{CTX-M-15} and \textit{aac(6')-lb-cr}. The first report recently made in West Africa of \textit{E. coli} ST131 harboring \textit{bla}_{CTX-M-15} and \textit{aac(6')-lb-cr}, found predominantly among hospital isolates, was from Lagos, Nigeria (Aibinu, Odugbemi, Koenig, & Ghebremedhin, 2012). Our results support the dissemination of this strain in the country, since they were collected in a different state. The other \textit{bla}_{CTX-M-15} \textit{E. coli}, harboring the \textit{qnrB} and \textit{aac(6')-lb-cr} genes, was assigned to ST2695, a new allele, not previously reported to be associated with CTX-M-15.
The IncF plasmidic family was the most common found in this study; it includes the F1A and F1B groups frequently reported to be associated with CTX-M and aac(6')-lb-cr resistance genes (Carattoli et al., 2005). However, reports about the association of the qnrB with the plasmid replicon types found in this study are limited, as are reports about the qnrS association with HII, Y, and/or F1B plasmid types detected in bovine isolates. The lack of conjugative transfer of the CTX-M-15 determinant (and PMQR associated genes) may indicate that in the other E. coli isolates, the resistance determinants were encoded on non-conjugative plasmids.

Class 1 integrons coding for resistance to other antibiotic families were also associated with CTX-M-15 producers, though their genetic location was not investigated. However, this finding shows the higher ability of the strains to capture additional resistance genes.

E. coli is commonly associated with urinary tract infections, and the majority of CTX-M-15 strains were found in urine, followed by high vaginal swab samples. Samples were mainly collected at the UCH. This may be due to the fact that most patients in this tertiary care hospital are referred from other hospitals, where they have been started on antibiotherapy. Also, in developing countries like Nigeria, resources are limited and antibiotics are indiscriminately used both in humans and animals, which could result in dissemination of ESBL producers.

In contrast with other Nigerian reports (Ajayi, Oluyege, Olowe, & Famurewa, 2011; Chah & Oboegbulem, 2007; Fortini et al., 2011), bovine E. coli isolates did not show considerable resistance, which may be attributed to less bovine antibiotic dosing in this geographic location. The predominant resistance rate was seen for tetracycline (44%), followed by trimethoprim (25%). Resistance to amoxicillin was detected in 23% of the strains, correlating with data obtained from reports on bacteria isolated from meat tables in the geographical location where the study was conducted (Ajayi et al., 2011).

The use of cephalosporins and fluoroquinolones for prophylaxis and treatment of bovine animals is rare in Nigeria, which supports the high rates of susceptibility of E. coli bovine isolates. However, these resistance determinants may be selected by other drugs frequently used in food animals. Chah et al. (Chah & Oboegbulem, 2007) reported the wide use of ampicillin in poultry production in Nigeria, which may provide a selective pressure favouring the emergence of E. coli strains that carry plasmids with TEM-1/2 and ESBL determinants. These other food animals may serve as
reservoirs of ESBL-producing *E. coli* strains that could be transferred to humans and other animals. However, in this study, we could not find an association between *E. coli* from human and bovine origins.

The study reports the prevalence of CTX-M-15 in human clinical Nigerian *E. coli* strains carrying genetic mobile elements such as conjugative plasmids and class 1 integrons that can amplify the spread of resistance in the country, reinforcing the crucial need for antimicrobial surveillance in a country where over-the-counter antibiotic sales and indiscriminate use of antibiotic are common.
Chapter 6

Virulence and plasmidic resistance determinants of *Escherichia coli* isolated from municipal and hospital wastewater treatment plants

ABSTRACT

Escherichia coli is simultaneously an indicator of water contamination and a human pathogen. This study aimed to characterize the virulence and resistance of Escherichia coli from municipal and hospital wastewater treatment plants (WWTPs) in central Portugal. From a total of 193 isolates showing reduced susceptibility to cefotaxime and/or nalidixic acid, twenty E. coli with genetically distinct fingerprint profiles were selected and characterized. Resistance to antimicrobials was determined using disc diffusion method. ESBL and PMQR genes, phylogroups, pathogenicity islands (PAIs) and virulence genes were screened by PCR. CTX-M producers were typed by multilocus sequence typing. Resistance to beta-lactams was associated to the presence of \textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{CTX-M-15} and \textit{bla}\textsubscript{CTX-M-32}. Plasmid-mediated quinolone resistance was associated to \textit{qnrA}, \textit{qnrS} and \textit{aac(6')-lb-cr}. Aminoglycosides resistance and multidrug resistant phenotype were also detected. PAI IV\textsubscript{536}, PAI II\textsubscript{CFT073}, PAI II\textsubscript{536} and PAI I\textsubscript{CFT073}, and uropathogenic genes \textit{iutA}, \textit{papAH} and \textit{sfa/foc} were detected. The concerning clinical ST131 clone carrying \textit{bla}\textsubscript{CTX-M-15}, \textit{bla}\textsubscript{TEM-type}, \textit{qnrS} and \textit{aac(6')-lb-cr}, IncF and IncP plasmids, and virulence factors PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073}, PAI II\textsubscript{CFT073}, \textit{iutA}, \textit{sfa/foc} and \textit{papAH} was identified in the effluent of a hospital plant. WWTPs contribute to the dissemination of virulent and resistant bacteria into water ecosystems, constituting an environmental and public health risk.

INTRODUCTION

Escherichia coli is mutually a biological indicator of water treatment safety, as well as an important human pathogen responsible for several diseases (Edberg, Rice, Karlin, & Allen, 2000; Kaper et al., 2004). E. coli presents several virulence and antimicrobial resistance genes which contribute to its success as human pathogen (Pitout, 2012). These genes may be disseminated by mobile genetic elements such as pathogenicity islands, carriers of virulence factors, or plasmids with genes coding for both resistance and virulence determinants (Carattoli, 2009; Hacker et al., 1997). Water constitutes a good matrix for the lateral transfer of mobile genetic elements (Taylor, Verner-Jeffreys, & Baker-Austin, 2011) which are responsible for the dissemination of virulence or resistance traits between bacteria from different sources, contributing to the modification of the natural bacterial ecosystems (Baquero, Martinez, & Canton, 2008).
Currently, the inverse relation between antimicrobial resistance and virulence has been of consensus (Moreno et al., 2006). However, recently it has been showed that these two features may co-exist in the same genotype perpetuating the bacterial lineage and highlighting the concern due to its dissemination (Colomer-Lluch et al., 2013; Dolejska et al., 2011).

Wastewater treatment plants (WWTP) are designed to significantly reduce the biological contamination of water. Nevertheless, studies report resistant bacteria in effluents of treated water, and suggested that the conditions in WWTPs favor the proliferation of antibiotic resistant bacteria and the exchange of genetic elements (Dolejska et al., 2011; Korzeniewska, Korzeniewska, & Harnisz, 2013; Moura, Henriques, Ribeiro, & Correia, 2007). The emergence and dissemination of antimicrobial resistant bacteria has led to an increasing concern on potential environmental and public health risks. Moreover, the carriage of specific virulence genes, especially those located in mobile genetic elements, are important to evaluate the public health risks.

The main objectives of this study were to characterize the virulence and antimicrobial resistance profiles of E. coli collected in waters from municipal and hospital WWTPs from central Portugal and to screen for the presence of mobile genetic elements.

MATERIALS AND METHODS

Bacterial isolates

Between April and May 2011, water samples were collected from four hospitals and three municipal WWTPs located in the central region of Portugal:

- University hospital: reference hospital for the central region of Portugal. A large hospital harboring 1456 beds, with an extended set of medical specialties and clinical services, as well as a center of research, serving a population of approximately 430,000 inhabitants;

- General hospital: medium-sized hospital with thirteen main wards and composed by 350 beds. It serves a population of approximately 369,000 inhabitants.

- Pediatric hospital: small reference hospital in the center of Portugal that supports pediatric units. It is composed by nine mainwards and 110 beds serving a population of about 90,000 inhabitants.
- Maternity: small hospital with 96 beds and three main wards - gynecology, obstetrics and neonatology, not including the baby unit. It serves a population of approximately 507,000 women.

- Municipal WWTP1: serves 14,000 population equivalent.

- Municipal WWTP2: serves a 213,000 population equivalent. It receives urban wastewaters which include domestic wastewaters and hospital effluents (namely from the four mentioned hospitals).

- Municipal WWTP3: serves 1,500 population equivalent.

Municipal WWTP sampling was performed at the entrance and exit of the station at two occasions and Hospital samples were collected on three different dates at the exit of the station. Wastewater samples (250mL) were collected in amber glass bottles and further vacuum filtered through 1.0 μm glass microfiber filters (GF/C, Whatman, UK), followed by 0.45 μm nylon membrane filters (Whatman, UK). The filters were placed in MacConkey Agar supplemented with 0.5 mg/L of cefotaxime or 10 mg/L of nalidixic acid. A bacterial suspension was prepared with the inoculum and cultured in MacConkey Agar. A maximum of eight presumptive colonies of *E. coli* per plate were further cultured in Eosin Methylene Blue Agar (EMB), and lactose fermenter colonies with a green metallic sheen were selected. The citrate test was used to distinguish *E. coli* from *Citrobacter* spp. The identification was confirmed using a PCR-based technique with specific primers set targeting *uidA* gene (Heijnen & Medema, 2006).

The genetic relationship was evaluated by BOX-PCR (Versalovic, Schneider, de Bruijn, & Lupski, 1994), and only non-duplicate isolates were further analyzed.

**Susceptibility testing and phenotypic extended spectrum β-lactamase detection**

The antimicrobial susceptibility profile for ampicillin (10µg), amoxicillin- clavulanic acid (20/10µg), cefoxitin (30µg), cefotaxime (30µg), ceftazidime (30µg), nalidixic acid (30µg), ciprofloxacin (10µg) and gentamicin (10µg) was determined using a disc diffusion test (OXOID). Extended spectrum β-lactamase (ESBL) producers were detected with the double disk synergy test (Jarlier, Nicolas, Fournier & Philippon, 1988). The methods were performed and the results were interpreted based on the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010). Multidrug
resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

**Antimicrobial resistance determinants detection**

The \( \text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) genes coding for \( \beta \)-lactamases and plasmid mediated quinolone resistance (PMQR) determinants \( qnrA, B \) and \( S \), and \( qepA \) were screened with specific primers by PCR (Cattoir et al., 2007; Ma et al., 2009; Mendonça et al., 2007). For the samples with positive result for the screening of \( \text{bla}_{\text{CTX-M}} \), the full gene was further amplified using previously described primers (Conceição et al., 2005) and amplicons were purified with ExoSAP-IT (Affymetrix, USB products). The whole genes were sequenced at Macrogen, Amsterdam, Netherlands.

\( aac(6')-lb \) was screened by PCR and isolates positive for the \( aac(6')-lb \) gene were further digested with BtsCl enzyme (New England Biolabs) to identify \( aac(6')-lb\text{-cr} \) which lacks the BtsCl restriction site present in the wild type gene (Park et al., 2006).

**Plasmid Replicon typing**

Plasmid replicon identification was performed according to the PCR-based replicon typing scheme (Carattoli et al., 2005), detecting the main replicon families in *Enterobacteriaceae*.

**Detection of pathogenicity islands and other virulence markers**

Pathogenicity island (PAI) markers were screened according to the Bronowski *et al.* scheme (Bronowski et al., 2008), based on the technique first described by Sabaté *et al.* (Sabaté et al., 2006). This method allows the detection of eight PAIs, encoding several virulence determinants: PAI I\(_{536}\), PAI II\(_{536}\), PAI I\(_{536}\), PAI IV\(_{536}\), PAI I\(_{96}\), PAI II\(_{96}\); PAI I\(_{CFT073}\), and finally PAI II\(_{CFT073}\) (Sabaté et al., 2006).

Other virulence genes that maybe present in extraintestinal *E. coli* (ExPEC) such as *papAH*, *papC* (P fimbriae structural subunit and assembly), *sfa/foc* (S and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *iutA* (aerobactin receptor), *kpsM II* (group 2 capsules) and *cnf1* (cytotoxic necrotizing factor 1) were screened by PCR (Johnson & Stell, 2000), as well as the Enterohemorrhagic *E. coli* associated virulence genes *eaeA* (intimin), *hlyA* (pore-forming cytolsin), *stx* 1 and 2 (shiga-like toxins) (Ram et al., 2008).
Chapter 6

Phylogenetic analysis

The determination of *E. coli* major phylogroups (A, B1, B2 and D) was performed with a PCR-multiplex detecting *chuA*, *yjaA* and DNA fragment tspE4.C2 genes (Clermont et al., 2000; Mendonça et al., 2011).

Multilocus sequence typing (MLST)

MLST of the CTX-M producers was performed based on the PCR amplification and sequencing of seven housekeeping genes, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*, according to the University of College Cork (Cork, Ireland) scheme for *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli).

RESULTS

Bacterial isolates

A total of 193 presumably *E. coli* with reduced susceptibility to cefotaxime and/or nalidixic acid were obtained from WWTPs. The majority of the isolates showed an identical genetic profile and only twenty isolates with distinct profiles were selected (non-duplicate isolates) and further characterized for resistance and virulence profiles (Table 1). Fourteen of the non-duplicate isolates were from municipal WWTPs, while the remaining six were recovered from hospital water samples. The municipal isolates were recovered from WWTP2 (n=7), followed by WWTP3 (n=4) and WWTP1 (n=3). Isolate W4 and W12 were detected in both the affluent and effluent of the respective WWTPs, and in addition W12 isolate was detected in two different sampling occasions. From the hospital WWTPs, three strains were recovered from the general hospital, two from the Maternity hospital and one from the University Hospital. *E. coli* isolates with reduced susceptibility to CTX or NAL were not detected in the outflow of the Pediatric hospital.

Resistance profile characterization

The majority of the isolates were resistant to nalidixic acid (85%), followed by resistance to ampicillin (50%), amoxicillin-clavulanic acid (35%), cefoxitin (35%), cefotaxime (35%), ciprofloxacin (30%), ceftazidime (25%) and gentamicin (15%). Strains W4 and W5 from Municipal WWTP1 were susceptible to all the antibiotics tested, and strains W3 from the University hospital, strain W16 from the General Hospital and strain W7 from the Municipal WWTP2 were multidrug resistant. Among
the antimicrobial resistance determinants screened, \( \text{bla}_{\text{TEM}} \) was the most detected (n=7) followed by \( qnrA \) (n=3), \( qnrS \) (n=2) and \( \text{bla}_{\text{CTX-M}} \) (n=2). The studied resistance determinants were not detected in nine isolates.

Only two strains carried \( \text{bla}_{\text{CTX-M}} \) genes: \( \text{bla}_{\text{CTX-M-15}} \) (W3) collected from the University hospital outflow and \( \text{bla}_{\text{CTX-M-32}} \) (W15) from the Municipal WWTP3 inflow water. Isolate W3 was assigned by MLST to ST131 and isolate W15 to ST34. The strain W3 ST131 was multidrug resistant and showed the higher diversity of plasmidic determinants, carrying \( \text{bla}_{\text{CTX-M-15}}, \text{bla}_{\text{TEM-type}}, qnrS \) and \( \text{aac(6')-lb-cr} \).

The main plasmid groups detected in \( \text{Enterobacteriaceae} \) family members were also investigated. Four plasmid groups: IncF, IncK, IncI1/I\( \gamma \) and IncP were detected. IncF was the most prevalent group (n=11) found in both hospital and municipal WWTPs waters, while in 25% of the isolates no plasmid was identified.

**Virulence profile description**

The pathogenicity island more frequently detected was PAI IV\(_{536}\) (n=13) followed by PAI II\(_{CFT073}\) (n=5), PAI I\(_{CFT073}\) (n=2) and PAI II\(_{536}\) (n=1). PAI I\(_536\), PAI III\(_{536}\), PAI I\(_{J96}\) and PAI II\(_{J96}\) were not detected. PAI IV\(_{536}\) and PAI II\(_{CFT073}\) were more prevalent in municipal isolates. PAI II\(_{536}\) was exclusively detected in a strain from a municipal WWTP. Different combinations of pathogenicity islands were identified (Table 6.1).
Table 6.1 – Distribution of strains and characterization of phylogeny, virulence and resistance determinants and plasmid incompatibility groups.

<table>
<thead>
<tr>
<th>WWTP</th>
<th>Strain</th>
<th>Collection date (day/month)</th>
<th>Sampling</th>
<th>Phylogroup</th>
<th>Virulence determinants</th>
<th>Resistance profile</th>
<th>Plasmidic resistance determinants</th>
<th>Replicon type</th>
<th>ST</th>
</tr>
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<tbody>
<tr>
<td><strong>Hospital WWTP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternity</td>
<td>W1</td>
<td>19/4</td>
<td>Outflow</td>
<td>A</td>
<td>PAI IV, iutA</td>
<td>NAL</td>
<td>-</td>
<td>F, FIA, FIB, K, 11/1Y, P</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W2</td>
<td>9/5</td>
<td>Outflow</td>
<td>D</td>
<td>iutA</td>
<td>AMP, NAL</td>
<td>blaCTX, CMDB</td>
<td>11/1Y, P</td>
<td>ND</td>
</tr>
<tr>
<td>University</td>
<td>W3</td>
<td>18/4</td>
<td>Outflow</td>
<td>B2</td>
<td>PAI IV, PAI II, PAI IIIb, iutA, stafoc, papAIV</td>
<td>AMP, CAZ, CTX, ON, NAL, CIP</td>
<td>blaciTXAM-15, blaTEM, qnrS, aac(6')-Ib-cr</td>
<td>F, FIB, P</td>
<td>ST131</td>
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<td>General</td>
<td>W16</td>
<td>19/4</td>
<td>Outflow</td>
<td>D</td>
<td>iutA</td>
<td>AMP, FOX, CAZ, CTX, AMG, GEN, NAL, CIP</td>
<td>blar2, qnrA</td>
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<td>W17</td>
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<td>-</td>
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<td>blaTEM</td>
<td>-</td>
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<td></td>
<td>W18</td>
<td>2/5</td>
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<td>A</td>
<td>-</td>
<td>AMP, FOX, CAZ, CTX, AMG, NAL</td>
<td>blaTEM</td>
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<td>ND</td>
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<tr>
<td><strong>Municipal WWTP</strong></td>
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<td>WWTP1</td>
<td>W4</td>
<td>19/4</td>
<td>Inflow/Outflow</td>
<td>D</td>
<td>PAI IV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>W5</td>
<td>19/4</td>
<td>Outflow</td>
<td>B1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W6</td>
<td>19/4</td>
<td>Outflow</td>
<td>B2</td>
<td>PAI IV, PAI IIIb, PAI IIIb, iutA</td>
<td>AMP, AMG</td>
<td>blaTEM</td>
<td>F, FIA, 11/1Y</td>
<td>ND</td>
</tr>
<tr>
<td>WWTP2</td>
<td>W7</td>
<td>19/4</td>
<td>Inflow</td>
<td>B2</td>
<td>PAI IV, PAI II, iutA, papAIV</td>
<td>AMG, GEN, NAL, CIP</td>
<td>blaTEM, qnrS</td>
<td>F, FIA</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W8</td>
<td>19/4</td>
<td>Inflow</td>
<td>A</td>
<td>-</td>
<td>AMP, CAZ, CTX, NAL, CIP</td>
<td>blaTEM</td>
<td>11/1Y</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W9</td>
<td>19/4</td>
<td>Outflow</td>
<td>B1</td>
<td>PAI IV, iutA</td>
<td>AMP, FOX, CAZ, CTX, AMG, NAL, CIP</td>
<td>qnrA</td>
<td>F, FIA, FIB, K, 11/1Y, P</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W10</td>
<td>19/4</td>
<td>Outflow</td>
<td>B1</td>
<td>PAI IV, iutA</td>
<td>NAL</td>
<td>-</td>
<td>F, K</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W11</td>
<td>19/4</td>
<td>Outflow</td>
<td>A</td>
<td>PAI IV, iutA</td>
<td>FOX, AMG, NAL</td>
<td>-</td>
<td>F</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W12</td>
<td>19/4; 5/5</td>
<td>Inflow/Outflow</td>
<td>B2</td>
<td>PAI IV, PAI IIIb, PAI IIIb, iutA</td>
<td>NAL</td>
<td>-</td>
<td>F</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W13</td>
<td>5/5</td>
<td>Inflow</td>
<td>D</td>
<td>PAI IV, iutA</td>
<td>FOX, NAL</td>
<td>-</td>
<td>P</td>
<td>ND</td>
</tr>
<tr>
<td>WWTP3</td>
<td>W14</td>
<td>19/4</td>
<td>Outflow</td>
<td>A</td>
<td>iutA</td>
<td>NAL</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W15</td>
<td>5/5</td>
<td>Inflow</td>
<td>A</td>
<td>PAI IV</td>
<td>AMP, CTX, NAL,</td>
<td>blaciTXAM-102</td>
<td>F, K</td>
<td>ST34</td>
</tr>
<tr>
<td></td>
<td>W19</td>
<td>5/5</td>
<td>Inflow</td>
<td>B2</td>
<td>PAI IV</td>
<td>FOX, NAL</td>
<td>-</td>
<td>F, FIB</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W20</td>
<td>5/5</td>
<td>Inflow</td>
<td>B1</td>
<td>PAI IV</td>
<td>NAL</td>
<td>qnrA</td>
<td>K</td>
<td>ND</td>
</tr>
</tbody>
</table>

AMP - Ampicillin; CAZ - Ceftazidime; FOX - Cefoxitin; CTX - Cefotaxime; GEN - Gentamicin; CIP - Ciprofloxacin; NAL - Nalidixic Acid; AMC - Amoxicillin-Clavulanic Acid; ND - Not determined
Considering individual virulence genes, the most frequently detected was *iut*A (n=13), followed by *pap*AH (n=2), more common among hospital isolates *sfa/foc* and *eae*A were less prevalent, each of them being detected in one isolate; the former found in a hospital source and *eae*A detected in a municipal WWTP. The genes *afa/dra*, *kpsM* II, *cnt*, *hly*A, *stx*1 and 2 were not detected. The most prevalent phylogenetic group was group A (n=7) followed by B2 (n=5), and finally B1 and D (n=4, each). Strains from phylogroup B2 from both municipal and hospital WWTPs carried more virulence factors, including the ST131 isolate (Table 1). All the other isolates presented virulence determinants regardless of the phylogroup, with the exception of W17 and W8 from phylogroup A, and W5 from phylogroup B1.

**Discussion**

This study aimed to characterize the virulence and resistance profiles of *E. coli* selected from municipal and hospital WWTPs from a central region of Portugal, evaluating the possibility of environmental dissemination of pathogenic and/or resistant bacteria from these sources. Several studies indicate the potential dissemination of resistant and/or virulent bacteria from WWTPs into the environment (Biswal, Mazza, Masson, Gehr, & Frigon, 2014; Chagas et al., 2011; Colomer-Lluch et al., 2013; Dolejska et al., 2011; Jakobsen et al., 2008; Sabaté et al., 2008). Nonetheless, only one study concomitantly studied virulence factors and resistance determinants in hospital WWTPs (Jakobsen et al., 2008), and it only focused on gentamicin resistance determinants and in single virulence factors. Here, we extended the study to the identification of PAIs, clusters of virulence genes with the potential to be mobile.

*E. coli* strains showed resistance to important groups of antibiotics such as beta-lactams, quinolones, and aminoglycosides, with multidrug resistance being detected in both municipal and hospital strains, indicating that WWTPs may be responsible for the introduction of multidrug resistant bacteria into the environment. In the Portuguese Mondego river, where the effluents of the studied WWTPs are discharged, several types of antibiotics, including fluoroquinolones, were recently detected (Santos et al., 2013), which may exert a selective pressure in the dissemination of resistant bacteria in environmental waters (Kümmerer & Henninger, 2003). Plasmidic resistance determinants are important vehicles of transmission of resistance genes. Several resistance determinants were detected in this study, including *bla*TEM, *bla*SHV, *bla*CTX-M-15 and *bla*CTX-M-32, responsible for resistance to
several beta-lactams, as well as PMQR genes, including qnrA, qnrS and aac(6’)-Ib-cr. Different beta-lactamase genes were already detected, including bla_{CTX-M-group 1}, bla_{CTX-M-group 9}, bla_{SHV} and bla_{TEM} genes, in hospital and municipal effluents (Korzeniewska & Harnisz, 2013; Korzeniewska et al., 2013). In addition, CTX-M-15 and CTX-M-32 producers were already detected in river waters in Portugal, with unknown origin, indicating that these determinants may be spreading among water systems (Tacão, Correia, & Henriques, 2012).

Several virulence factors responsible for enhancing the pathogenic potential of bacteria have been detected in *E. coli* (Johnson, 1991; Johnson & Stell, 2000), and some of them are clustered in pathogenicity islands, mobile genetic platforms capable of dissemination throughout horizontal gene transfer (Hacker et al., 1997). Virulence profiles were characterized in the isolates. Results show that PAI IV_{536} was the most prevalent island, likewise other studies performed in clinical samples and in waters from several origins, but none of them from WWTPs (Mendonça, Ramalho, Vieira, & Da Silva, 2012; Sabaté et al., 2006). The association of PAI IV_{536} to virulence is controversial, as some studies indicate that this island contributes to the virulence of EXPEC (Schubert, Picard, Gourion, Heesemann, & Denamur, 2002) but other authors suggest that this is rather a fitness element (Oelschlaeger, Dobrindt, & Hacker, 2002). Several Uropathogenic *E. coli* (UPEC) virulence genes were identified in the isolates, including *iutA*, involved in the uptake of iron, *papAH* coding for P-Fimbriae associated to pyelonephritis (Dowling, Roberts, & Kaack, 1987; Källenius et al., 1981) and *sfa/foc* encoding S-Fimbriae/F1C fimbriae, involved in urinary infections, neonatal sepsis as well as meningitis (Antão et al., 2009). In addition, *eae* usually detected in Enteropathogenic (EPEC) and Enterohemorrhagic *E. coli* (EHEC) was also detected in a municipal isolate. This fact may be related to the possible association of animal farms to the municipal WWTP where W12 isolate was detected, as ruminants are known to be important reservoirs of *E. coli* carrying intimin gene (Blanco et al., 2005). This isolate also carried other virulence determinants and was detected in both the affluent and effluent of the WWTP in different collection dates indicating that WWTPs are not only inefficient on the elimination of virulent bacteria, but are also contributing to the dissemination of strains carrying virulence-associated genes in the environment.

Phylogenetic background of the strains was studied as an indicator of the virulence potential of the isolates. *E. coli* strains have been grouped into four different
phylogroups (A, B1, B2, and D) according to their virulence features. Virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D, while commensal strains belong to groups A and B1 (Clermont et al., 2000). Despite group A and B1 are considered less virulent, strains from these phylogroups harboring virulence factors were detected in both municipal and hospital isolates. This observation may indicate that even the considered less virulent bacteria may be enhancing their virulent potential, possibly due to horizontal gene transfer of virulence traits.

In this study the international clone *E. coli* ST131 was detected in the effluent of a hospital. The ST131 isolate carried several pathogenic factors including PAI IV$_{536}$, PAI ICFT073, PAI II$_{CFT073}$, iutA, *sfa/foc* and *papAH* as well as resistance determinants *bla$_{CTX}$*, *bla$_{TEM}$*, *qnrS*, and *aac(6\prime)-lb-cr* and the IncF plasmid, a conjugative plasmid which can easily be spread to other bacteria and known for dissemination of *bla$_{CTX}$* and *aac(6\prime)-lb-cr* (Carattoli, 2009; Partridge, Zong, & Iredell, 2011). ST131 displays both resistance and virulence features which contribute to the success of this international clone, which today is one of the most adapted and efficient human pathogens (Johnson, Johnston, Clabots, Kuskowski, & Castanheira, 2010a; Johnson et al., 2010b). This clone was previously detected in the effluent of a municipal WWTP in the Czech Republic (Dolejska et al., 2011) and in the influent of a WWTP in Catalonia, Spain (Colomer-Lluch et al., 2013). But to our knowledge, this is the first finding in hospital WWTPs, highlighting the crucial need of monitoring the efficiency of hospital WWTPs.

The dissemination of bacteria carrying both resistance and virulence determinants, such as ST131, constitutes an important threat to public health and to the environment. Resistant or pathogenic isolates when in contact with autochthonous bacteria may be responsible for the dissemination of resistance and virulence determinants among natural ecosystems by horizontal gene transfer.

**Conclusions**

WWTPs constitute a potential mechanism of propagation of resistant and pathogenic bacteria from sewage of diverse origin, into the environment, and may thus contribute to the environmental dissemination of virulence and resistance determinants which constitutes an important public health concern.
Chapter 7

Distribution of virulence determinants and pathogenicity islands in clinical *Klebsiella* spp., according to their resistance profile

Submitted for publication.
ABSTRACT

*Klebsiella* spp. are nosocomial opportunistic pathogens often resistant to multiple antibiotics. The virulence of resistant strains is barely known. Thus, the interplay between resistance and virulence in *K. pneumoniae* and *K. oxytoca* was investigated. Resistance phenotype, presence of β-lactamases and plasmid-mediated quinolone resistant genes and plasmid replicon typing were determined and the carriage of virulence genes and pathogenicity islands identified. Only two PAIs were detected, PAI IV$_{536}$ and PAI II$_{CFT073}$. Carriage of PAI II$_{CFT073}$ by *K. oxytoca* was firstly reported. This PAI was statistically associated to resistance to cephalothin, cefotaxime, ceftazidime, ciprofloxacin and gentamicin, while in *K. pneumoniae* PAI IV$_{536}$ was statistically associated to resistance to gentamicin and sulfamethoxazole-trimethoprim (SXT). In general, higher prevalence of virulence factors was detected among strains resistant to ciprofloxacin and gentamicin and, to a lesser extent, to cefotaxime and SXT in *K. pneumoniae*. Similarly, in *K. oxytoca*, higher prevalence of virulence genes was generally observed among strains resistant to SXT. This study confirms that virulence and resistance are compatible features among *Klebsiella* spp.

INTRODUCTION

*Klebsiella* spp. are opportunistic pathogens mostly associated with nosocomial infections including pneumonia, septicaemia, soft tissue and urinary tract infections. Antibiotic β-lactams have been the choice for treating *Klebsiella* spp. infections. Nonetheless, the use of these antibiotics has become problematic because of the emergence of diverse classes of β-lactamases among clinical *Klebsiella* spp. isolates (Broberg et al., 2014; Lowe et al., 2012).

Several virulence factors are carried by *K. pneumoniae* strains, including capsule, lipopolysaccharide, siderophore systems, and fimbrial and non-fimbrial adhesins (Brisse et al., 2009). Pathogenicity islands (PAIs), large DNA fragments carrying several virulence factors with the ability to disseminate by horizontal gene transfer, and usually present in uropathogenic *Escherichia coli*, have also been detected in *K. pneumoniae* strains (Calhau, Boaventura, Ribeiro, Mendonça, & da Silva, 2014).

The studies focusing on the interplay between resistance and virulence in *Klebsiella* spp. are scarce. One reported that in *K. pneumoniae* a significant high proportion of strains expressing type 1 and type 3 fimbrial adhesins were detected
among extended-spectrum beta-lactamases (ESBL) producers (Sahly et al., 2008). Recently, virulence genes _irp2_, _mrkD_ and _fimH_ were more frequently found in _K. pneumoniae_ KPC-producers (de Cássia Andrade Melo et al., 2014). These studies suggest that some virulence genes might be associated with specific resistance determinants.

Thus, the main objective of this work was to assess the relation between virulence genes and PAIs, and the resistance profile of _Klebsiella_ spp. clinical isolates.

**Materials and Methods**

A total of 42 _K. pneumoniae_ and _K. oxytoca_ isolates were collected during 2007 from different wards of the Coimbra University Hospital Centre (CHUC), Portugal.

Identification and susceptibility testing were performed by using Vitek 2 Advanced Expert System (BioMérieux, Marcy l’Etoile, France).

PAIs markers were screened according to the Bronowski _et al._ method (Bronowski et al., 2008). Virulence factors _allS_, _rmpA_, the capsular antigen genes K1, K2, K5, K20, K54, and K57, adhesin genes _fimH_, _mrkD_ and _cf29A_; and _kfu_, _uge_, _wabG_ and _ureA_ genes were detected as described elsewhere (Brisse et al., 2009). Plasmid replicon typing was performed using a PCR-based replicon typing (Carattoli _et al._, 2005). Statistical tests were performed using IBM SPSS statistics version 22.0. A _P_≤0.05 was considered statistically significant.

**Results and Discussion**

From the 42 isolates, 33 were identified as _K. pneumoniae_ and nine were _K. oxytoca_. Isolates were mainly collected from urine (50%) and blood (14%).

All the isolates were resistant to ampicillin. Resistance to cephalothin was the second most prevalent (45%) followed by amoxicillin-clavulanic acid (41%), cefotaxime, ceftazidime and sulfamethoxazole-trimethoprim (SXT) (all of them with 38%). Resistance to ciprofloxacin was observed in 24% of the isolates.

The majority of studies on _Klebsiella_ spp. virulence are mainly performed with _K. pneumoniae_ strains, not considering the opportunistic pathogen _K. oxytoca_. The strains studied are often from pyogenic liver abscess, disregarding other clinical sources that have become more important due to the increase isolation of multidrug
resistant *Klebsiella* spp. strains. In addition, the presence of PAIs usually associated with uropathogenic *E. coli* (UPEC) is generally not considered.

The *ureA* gene was detected in all the isolates in accordance to previous findings (Brisse et al., 2009) and *mrkD*, encoding type 3 fimbriae, was the second most prevalent (88%). The genes *rmpA*, *clf29A* and the capsular antigen genes K1, K5, K20, K54, and K57 were not detected. The *allS* gene, involved in allantoin metabolism and associated with liver infection caused by *K. pneumoniae* (Chou et al., 2004), was found in six isolates, four of which were from urinary samples. In fact, it is known that allantoin is one of the products of the metabolism of uric acid (Kim et al., 2009), and thus it is excreted in the urine. *Klebsiella* spp. may thus be using allantoin as a nitrogen energy source, and therefore, *allS* gene may be considered an important virulence factor on urinary tract infections.

In this study, only two PAIs were detected, PAI IV\textsubscript{536} and PAI II\textsubscript{CFT073}, which have been previously reported in *K. pneumoniae* (Calhau et al., 2014). PAI IV\textsubscript{536} was detected in eleven (33%) *K. pneumoniae* strains and PAI II\textsubscript{CFT073} was exclusively detected in six (66%) *K. oxytoca* strains. To our knowledge, this is the first report of the carriage of this PAI among *K. oxytoca* strains.

So far, the knowledge of the relation between resistance and virulence traits in *Klebsiella* spp. compared to UPEC is limited. The prevalence of the different virulence genes was compared between resistant and susceptible isolates from *K. pneumoniae* and *K. oxytoca*, and results are shown in Table 7.1 and Table 7.2, respectively.
Table 7.1 – Distribution of virulence genes among *Klebsiella pneumoniae* according to the antimicrobial susceptibility profile.

<table>
<thead>
<tr>
<th>Virulence genes</th>
<th>Prevalence % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KF</td>
</tr>
<tr>
<td></td>
<td>S (n=21)</td>
</tr>
<tr>
<td>allS</td>
<td>5 (1)</td>
</tr>
<tr>
<td>fimH</td>
<td>86 (18)</td>
</tr>
<tr>
<td>mrkD</td>
<td>91 (19)</td>
</tr>
<tr>
<td>kfu</td>
<td>5 (1)</td>
</tr>
<tr>
<td>uge</td>
<td>100 (21)</td>
</tr>
<tr>
<td>wabG</td>
<td>95 (20)</td>
</tr>
<tr>
<td>PAI IV&lt;sub&gt;536&lt;/sub&gt;</td>
<td>33 (7)</td>
</tr>
</tbody>
</table>

*AMP - Ampicillin; KF - Cephalothin; CTX - Cefotaxime; CAZ - Cefazidime; AMC - Amoxicillin-clavulanic Acid; PTZ - Piperacillin-tazobactam; CIP - Ciprofloxacin; GEN - Gentamicin; SXT - Trimethoprim-sulfamethoxazole*

*Prevalence % (n) - Susceptible; R - Resistant*

*Encoded virulence factors: allS - Activator of the allantoin regulon; fimH - Type 1 fimbriae; mrkD - Type 3 fimbriae; kfu - Klebsiella iron uptake system; uge - Uridine phosphate galacturonate 4-epimerase; wabG - Glucosyltransferase; PAI IV<sub>536</sub> - Yersiniabactin siderophore system.*
Table 7.2 – Distribution of virulence genes in *Klebsiella oxytoca* according to the antimicrobial susceptibility profile.

<table>
<thead>
<tr>
<th></th>
<th>Prevalence % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KF</td>
</tr>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
</tr>
</tbody>
</table>

**Virulence genes**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AhaS</em></td>
<td>0 (0) 57 (4)  0 (0) 57 (4)  0 (0) 57 (4)  33 (1) 50 (3)  50 (4)  0 (0) 0 (0) 57 (4)  25 (1)  60 (3)  20 (1)  75 (3)</td>
</tr>
<tr>
<td><em>fimH</em></td>
<td>0 (0) 14 (1)  0 (0) 14 (1)  0 (0) 14 (1)  0 (0) 17 (1)  0 (0) 100 (1)  0 (0) 14 (1)  25 (1)  0 (0) 0 (0) 25 (1)</td>
</tr>
<tr>
<td><em>mrkD</em></td>
<td>2 (100) 71 (5)  100 (2) 71 (5)  100 (2) 71 (5)  67 (2) 83 (5)  75 (6) 100 (1)  100 (2) 71 (5)  75 (3) 80 (4)  100 (5) 50 (2)</td>
</tr>
<tr>
<td><em>uge</em></td>
<td>1 (50) 14 (1)  50 (1) 15 (1)  50 (1) 14 (1)  33 (1) 17 (1)  13 (1) 100 (1)  50 (1) 14 (1)  50 (2)  0 (0) 20 (1) 25 (1)</td>
</tr>
<tr>
<td><em>wabG</em></td>
<td>1 (50) 14 (1)  50 (1) 14 (1)  50 (1) 14 (1)  33 (1) 17 (1)  13 (1) 100 (1)  50 (1) 14 (1)  50 (2)  0 (0) 20 (1) 25 (1)</td>
</tr>
<tr>
<td>PAI II&lt;sub&gt;CFT073&lt;/sub&gt;</td>
<td>0 (0) 86 (6)  0 (0) 86 (6)  0 (0) 86 (6)  33 (1) 83 (5)  75 (6)  0 (0) 0 (0) 86 (6)  25 (1) 100 (5)  60 (3) 75 (3)</td>
</tr>
</tbody>
</table>

**Encoded virulence factors:**

- *AhaS* - Activator of the allantoin regulon; *fimH* - Type 1 fimbriae; *mrkD* - Type 3 fimbriae; *uge* - Uridine phosphate galacturonate 4-epimerase; *wabG* - Glucosyltransferase; PAI II<sub>CFT073</sub> - P fimbriae, iron-related genes.
For the majority of *K. pneumoniae* strains, resistance to ciprofloxacin, gentamicin and SXT, was associated to higher prevalence of the virulence genes. Exceptions were gene *allS*, more prevalent among strains susceptible to these antibiotics, and additionally, *wabG* which was also more prevalent in strains susceptible to SXT. Statistically significant differences were only observed for resistance to ciprofloxacin and gene *kfu*, encoding *Klebsiella* ferric iron uptake (38% in resistant vs 4% in susceptible, p= 0.012); resistance to gentamicin and *kfu* (60% in resistant vs 4% in susceptible, p<0.001) and PAI IV<sub>536</sub> (80% in resistant vs 25% in susceptible, p= 0.016); and finally resistance to SXT and PAI IV<sub>536</sub> (58% in resistant vs 19% in susceptible, p=0.021). For the beta-lactams, the distribution depended on the genes and specific antibiotics, however, in the majority of cases a higher tendency of carriage of virulence genes was detected among resistant strains. Statistically significant differences were only observed for the gene *kfu* and resistance to cefotaxime (33% in resistant vs 4% in susceptible, p=0.022) and ceftazidime (30% in resistant and 4% in susceptible, p= 0.038).

Overall, for *Klebsiella* spp. an inverted relation between resistance and virulence does not seem to be verified. Contrarily to the observed for *E. coli*, particularly in quinolones, where a trade-off between resistance and virulence has been reported (Vila et al., 2002), in *K. pneumoniae* the opposite seems to occur. In fact, a higher prevalence of the virulence factors was detected among resistance to ciprofloxacin and gentamicin and at a lesser extent to cefotaxime and SXT.

In the case of *K. oxytoca*, strains resistant to SXT presented more prevalence of virulence genes. Noteworthy, the gene *mrkD*, encoding type 3 fimbria, was the only one in which a higher prevalence was detected in strains susceptible to SXT compared with resistant strains. Considering resistance to cephalothin, cefotaxime, ceftazidime and ciprofloxacin, higher prevalence of genes *allS*, *fimH* (encoding type 1 fimbria), and PAI II<sub>CFT073</sub> were detected in resistant isolates compared to the susceptible counterparts. In contrast, genes *mrkD*, *uge* (encoding uridine phosphate galacturonate), and *wabG* (encoding glucosyltransferase) were more prevalent among susceptible isolates. Additionally, the genes *allS*, *mrkD* and PAI II<sub>CFT073</sub> were more prevalent among isolates resistant to gentamicin, while *fimH*, *uge* and *wabG* were more prevalent among isolates susceptible to this antibiotic. Despite of the differences observed, only the relation between PAI II<sub>CFT073</sub> and resistance to
cephalothin (p= 0.023), cefotaxime (p= 0.023), ceftazidime (p= 0.023), ciprofloxacin (p= 0.023) and gentamicin (p= 0.018) were considered statistically significant.

Similarly to the observed for *K. pneumoniae*, in *K. oxytoca*, higher prevalence of virulence genes were generally observed in SXT resistant strains. Nonetheless, for the remaining antibiotics the tendency observed for the genes prevalence was dependent on the antibiotic and virulence gene considered.

Furthermore, the presence of PAIs among *Klebsiella* spp. was generally more prevalent among resistant strains indicating a possible relation between these elements and antimicrobial resistance. Previously, PAI IV\textsubscript{536} has been reported to be carried in a resistance plasmid in *E. coli* (Calhau et al., 2013).

This study reports for the first time the carriage of PAI II\textsubscript{CFT073} among *K. oxytoca* and demonstrates that in a general way, among *Klebsiella* spp., virulence and resistance features seem to be compatible. Furthermore, resistance to SXT in *Klebsiella* spp. seems to be associated with more virulence factors, thus could be consider as a possible indicator of pathogenity among these species.
Chapter 8

Molecular characterization of *Klebsiella pneumoniae* isolated from renal transplanted patients: virulence markers, extended-spectrum \( \beta \)-lactamases and genetic relatedness

ABSTRACT

The objective was to characterize virulence markers and β-lactam resistance in *Klebsiella pneumoniae* isolates from renal transplant patients and to evaluate their genetic relatedness. Two main genetic lineages were detected: one carried *bla*<sub>CTX-M-15</sub> not associated to IncFIIA plasmid replicon, which was found on the other lineage not expressing CTX-M-type enzyme. PAI III<sub>536</sub> and PAI II<sub>CFT073</sub> were detected for the first time in *K. pneumoniae* in one clone, while the siderophore *kfu* was carried by the other, with only PAI IV<sub>536</sub>. The molecular data indicates colonization before admission, and fuels the discussion on implementation of antibiotherapy before surgery.

TEXT

Urinary tract infection (UTI) constitutes the most common infection among renal transplant patients. *Klebsiella pneumoniae* has been increasingly isolated and it has assumed clinical relevance in some hospitals due to the emergence of multidrug-resistant strains. Antimicrobial resistance limits therapeutic options, increasing the rates of treatment failure and infection-related mortality, also extending hospitalization and health-care related costs. *K. pneumoniae* strains producing extended-spectrum beta-lactamases (ESBLs) have been reported in renal transplant units. However, in contrast with antimicrobial resistance, little is known about the virulence traits of *K. pneumoniae* strains isolated from these particular patients. For example, pathogenicity islands (PAIs) have been extensively described in urinary pathogenic *Escherichia coli* (UPEC) (Kao, Stucker, Warren, & Mobley, 1997; Middendorf et al., 2004; Sabaté et al., 2006), but to our knowledge, only PAI IV<sub>536</sub> has been identified so far in *K. pneumoniae*.

In 2011, an outbreak of multidrug resistant *K. pneumoniae* isolates was identified at the University hospital among renal transplant patients, raising the hypothesis of an emergent clone and its possible dissemination among wards. The goal of this study was to evaluate the clonal relatedness of these isolates, and to characterize at molecular level the virulence features and antimicrobial resistance of the isolates.

From May until July of 2011, 25 *K. pneumoniae* isolates were collected from 22 inpatients submitted to a renal transplant at Coimbra University Hospital a tertiary care 1,200 bed hospital. Identification and susceptibility testing was performed by
using Vitek 2 System (BioMérieux). ESBL production was additionally confirmed by using the double disk synergy test. The genetic relatedness of *K. pneumoniae* isolates was analyzed by pulsed-field gel electrophoresis (PFGE), as previously described (Mendonça, Ferreira, Louro, & Caniça, 2009). A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software. Isolates with a Dice band-based similarity coefficient value > 80% were considered to belong to the same clone. The *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> determinants were screened and sequenced at Stabvida, Portugal. PAIs markers were screened using the Bronowski et al. method (Bronowski et al., 2008). Other virulence factors such as *allS*, *rmpA* gene, the capsular antigen genes K1, K2, K5, K20, K54, and K57, adhesin genes *fimH* coding for type 1 fimbriae, *mrkD* coding for type 3 fimbriae and *clf29A* coding for non fimbrial adhesin CF29K; and *ktf*, *uge*, *wabG* and *urea* were detected as described elsewhere (Brisse et al., 2009). Plasmid replicon typing was performed using a PCR-based scheme described by Carattoli et al. (Carattoli et al., 2005).

The majority of isolates were collected from urine (76%), 12% from blood and 12% from exsudates. All the isolates were resistant to ampicillin and cefotaxime. High frequency levels of resistance were also observed for ceftazidime (96%), ciprofloxacin (96%), levofloxacin (96%), trimethoprim-sulfamethoxazole (96%), and gentamicin (88%). They were susceptible to meropenem and ertapenem. KPC enzymes has already been found in Lisbon, Portugal (Machado et al., 2010). Isolates presented the usual *bla*<sub>SHV</sub> gene, and 68% produced CTX-M-15 and TEM-1b beta-lactamases. The plasmid replicon Inc FIIA was the unique type identified, detected in 32% of the isolates. In Portugal, previous studies reported the presence of *bla*<sub>CTX-M-15</sub> in *K. pneumoniae* (Conceição et al., 2005; Mendonça et al., 2009), but to our knowledge, this is the first study made in renal transplant patients. IncFII, IncA/C, IncL/M, IncL1 plasmids are frequently found in Enterobacteria and associated with ESBL dissemination (Carattoli, 2011). However, we could not associate FIIA replicon type with CTX-M-15 gene. This suggests the presence of other plasmids or a chromosomal location, and that some lineages of *K. pneumoniae* are evolving to maintain specific antimicrobial determinants. Recently, CTX-M-15 gene was found integrated in *K. pneumoniae* chromosome (Coelho et al., 2010).

So far, the extent of knowledge of the virulence traits content in *K. pneumoniae* compared to uropathogenic *E. coli* is limited. The biological basis of the clone
lineages for divergent clinical behavior is unclear. However, it likely involves great
differences in disease-relevant traits, i.e., specific virulence factors. PAI IV536 was
found in 64% of the isolates. PAI III536 and PAI IIcft073 were detected simultaneously
in 36% of the isolates. PAI I536, PAI II536, PAI IJ96, PAI IIJ96 and PAI ICFT073 were not
detected. PAIs are well described in *E. coli* uropathogenic strains (Bronowski et al.,
2008; Sabaté et al., 2006). However, the presence of these clusters virulence genes
are not so well known in *Klebsiella* spp., including in isolates associated with urinary
infections. To our knowledge, with the exception of PAI IV536, (Koczura & Kaznowski,
2003), the presence of PAIs in *K. pneumoniae* strains has not been investigated.

The virulence encoding-genes *fimH, mrkD, uge, wabG* and *ureA* were detected
in all the isolates, while *kfu* was detected in 64% of the isolates. *alls, rmpA, and
cf29A* genes and the capsular genes K1, K2, K5, K20, K54 and K57 were not
detected.

The analysis of PFGE data revealed the identification of four clusters at >80%
degree of genetic similarity (Figure 8.1).

![Dendrogram](image)

**Figure 8.1** – Dendrogram based on the band-based coefficient of similarity of PFGE profiles. Coefficient value >80% were considered to belong to the same clone.

Five isolates did not group at this cut-off. Clusters I and II were related at a
72% degree of similarity, while clusters III and IV grouped together at 76%. Table 8.1
shows the antimicrobial profiles of the main clusters with the β-lactamase resistance
determinants and the virulence genes identified.

The isolates seemed to have distinct origins. Indeed, some patients could be
colonized before admission to the hospital, since they were submitted to
hemodialysis elsewhere, or they were transferred from regional hospitals. Thus, the
screen for colonization and the knowledge of clinical history on the admission to the
tertiary care hospital may be helpful to prevent and manage further infections.
Moreover, the emergence of multidrug resistant strains in renal transplant recipients
fuels the discussion on whether antibiotherapy should be implemented before the
transplant. Molecular characterization of bacterial populations can give insight on the
sources and modes of transmission of microorganisms which is useful to improve or
design infection control strategies.
Table 8.1 – Antimicrobial resistance and virulence determinants of the main genetic

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Resistance profilea</th>
<th>Replicon typing</th>
<th>β-lactamases genes</th>
<th>Virulence markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>AMP, CTX, CAZ, CIP, LEV, GEN, SXT</td>
<td>NDb</td>
<td>bla\textsubscript{CTX-M-15}, bla\textsubscript{TEM-1b}, bla\textsubscript{SHV-type}</td>
<td>PAI IV\textsubscript{536}, fimH, mrkD, kfu, uge, wabG, ureA</td>
</tr>
<tr>
<td>II</td>
<td>AMP, CTX, CAZ, CIP, LEV, GEN, SXT</td>
<td>ND</td>
<td>bla\textsubscript{CTX-M-15}, bla\textsubscript{TEM-1b}, bla\textsubscript{SHV-type}</td>
<td>PAI IV\textsubscript{536}, fimH, mrkD, kfu, uge, wabG, ureA</td>
</tr>
<tr>
<td>III</td>
<td>AMP, CTX, CAZ, CIP, LEV, GEN, SXT</td>
<td>FIIA</td>
<td>bla\textsubscript{SHV-type}</td>
<td>PAI III\textsubscript{536}, PAI II\textsubscript{CFT073}, fimH, mrkD, uge, wabG, ureA</td>
</tr>
<tr>
<td>IV</td>
<td>AMP, CTX, CAZ, CIP, LEV, GEN, SXT</td>
<td>FIIA</td>
<td>bla\textsubscript{SHV-type}</td>
<td>PAI III\textsubscript{536}, PAI II\textsubscript{CFT073}, fimH, mrkD, uge, wabG, ureA</td>
</tr>
</tbody>
</table>

aAMP - Ampicillin; CTX - Cefotaxime; CAZ - Cefazidime; LEV - Levofloxacin; GEN - Gentamicin; SXT - Trimethprim-sulfamethoxazole
bND - Not Detected
The capsular genes K1, K2, K5, K20, K54 and K57, virulence genes allS, rmpA, and cf29A and PAIs PAI \textsubscript{l036}, PAI \textsubscript{l036}, PAI \textsubscript{l036}, PAI \textsubscript{l036}, PAI \textsubscript{l036}, PAI \textsubscript{l036}, PAI \textsubscript{l036}, and PAI \textsubscript{l036} were not detected.
Chapter 9

General Discussion and Concluding Remarks
1. General Discussion

Along with the worldwide increase of resistance and the growing importance of bacterial virulence, questions related to the association between resistance and virulence started to arise: Are resistant strains also more virulent? Is there a trade-off between resistance and virulence? This study aimed to understand the relation between virulence and resistance in *E. coli* and *Klebsiella* spp. as important human pathogens. Diverse origins of strains were considered, including clinical isolates from Portugal and from a country with a different use of antibiotics (Nigeria), and wastewater treatment plants isolates, and the variety of their virulence and resistance determinants was characterized.

The phylogenetic group of *E. coli* is used in the characterization of isolates, as an indicator of the pathogenic potential of bacteria. The genetic characterization of clinical *E. coli* strains, allowed the finding of a new profile of genes, which was not previously reported or even considered in the method of Clermont et al. (Clermont et al., 2000). Chapter 2 describes this new profile and characterizes the virulence and resistance characteristics of the strain. After the publication of this unusual yjaA/TspE4.C2 profile, Skjot-Rasmussen et al. also reported the identification of this profile in four isolates: two from human samples (urine and blood), one from pig and one from broiler chicken meat (Skjot-Rasmussen, Jakobsen, Olsen, Frimodt-Moller, & Hammerum, 2013). The identification of this unusual PCR profile partially contributed to the development of new strategies to identify the phylogroup of the strains. Doumith et al. developed an updated version of the original multiplex PCR based on this and other reports, and facing the observation of the existence of polymorphic regions in the annealing sites of the primers previously described by Clermont et al. (Doumith, Day, Hope, Wain, & Woodford, 2012). Furthermore, Clermont et al. published a revision of the original method, introducing an extended quadruplex method which identified eight *E. coli* phylogroups: A, B1, B2, C, D, E, F belonging to *E. coli* sensu stricto, and the eighth which was *Escherichia* cryptic clade I (Clermont, Christenson, Denamur, & Gordon, 2013).

Aiming to understand the relation between virulence and resistance in *E. coli*, a collection of UPEC isolates was characterized for phylogroup, virulence genes, PAIs, antimicrobial resistance profile and resistance determinants (Chapter 3). Conjugation assays and fitness studies were performed to understand if the carriage
of PAIs would influence the fitness cost of the cell by a resistance plasmid acquisition.

Pathogenicity islands PAI I\textsubscript{536}, PAI II\textsubscript{536}, PAI III\textsubscript{536} and PAI II\textsubscript{J96}, were found to be associated to phylogroup B2. The exclusive detection of these PAIs among B2 strains may be related to the fact that generally higher concentrations of virulence factors have been reported among the phylogroup B2 (Picard, Garcia et al. 1999).

Two main theories may explain the higher concentrations of virulence factors in this phylogroup: the occurrence of a particular compatibility between virulence genes and genetic background of phylogroup B2; or as result of the interaction between chance and timing (Johnson and Kuskowski 2000). The first theory defends that a specific genetic background may be required for the acquisition of virulence genes and that phylogroup B2 may have genetic characteristics that are suitable for the acquisition and or maintenance of these traits, leading to the emergence of a virulent clone (Picard, Garcia et al. 1999, Johnson and Kuskowski 2000). Or in addition, the occurrence of adaptive mutations among the B2 phylogroup may help to compensate the acquisition of virulence genes contributing to their maintenance in the lineage, although the bacteria initially may not have been receptive to these exogenous genes (Johnson and Kuskowski 2000).

Another theory claims that there is no particular affinity between phylogroups B2 and virulence genes, and that these genes may have been acquired randomly by a B2 ancestor, and were further vertically inherited by the majority of the members during clonal expansion. The acquisition of these virulence traits may also be so recent that the evolutionary time has not yet been sufficient for these traits to diffuse to the remaining groups (Johnson and Kuskowski 2000).

Additionally, among strains carrying these non-frequent PAIs, PAI I\textsubscript{536}, PAI II\textsubscript{536}, PAI III\textsubscript{536} and PAI II\textsubscript{J96}, a trade-off between virulence and resistance was observed. The carriage of these islands was generally found to be associated with susceptibility to antibiotics. In fact, this trade-off seems to be verified for the majority of the virulence genes, as higher prevalence of these determinants are verified among susceptible isolates compared to the resistant ones. This trade-off does not seem to be associated with a depletion of B2 phylogroup, as in this study, this group was generally more prevalent among resistant isolates when compared to the susceptible counterparts. Exceptions were observed for resistance to ampicillin, ciprofloxacin and sulfamethoxazole-trimethoprim, however a shift towards non-B2
phylogroups was only observed for sulfamethoxazole-trimethoprim. Considering resistance to ampicillin and ciprofloxacin, though the susceptible isolates presented higher prevalence’s of B2 group compared to the resistant ones, B2 was still the most prevalent phylogroup among resistant strains. This observation is supported by the study performed by Johnson and colleagues, in which they report that non-B2 strains didn’t have higher capacity to acquire resistance (Johnson et al., 2005a). These authors also suggest that the inverted relation between resistance and virulence is related to the importation of resistant strains from an external selection reservoir, such as food animals, carrying a susceptible source population exhibiting the same low virulence and non-group-B2 profile similar to resistant human clinical isolates.

On the other hand, a positive relation was observed for the most frequent PAIs (PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073}) and resistance to amoxicillin-clavulanic acid, cephalothin, cefotaxime, ceftaizidime and gentamicin. This association between these islands and resistance associated with plasmidic determinants may be indicative that these PAIs may be using resistance plasmids to enhance their dissemination.

Although an inverted relation between the number of the plasmids identified and the number of PAIs carried by the isolates seemed to occur, the experiments demonstrated that this relation was not fully verified. Conjugation assays were used to transfer a resistance plasmid carrying a \textit{bla}_{CTX-M-15}. Fitness studies indicated that the carriage of a higher number of PAIs didn’t implicate a direct rise in the fitness cost of the acquisition of the resistance plasmid, at least in strains harboring until three PAIs. Nonetheless, conjugation was not successful in strains harboring more than three PAIs, including the less commonly detected (Chapter 3). It is important to notice that the best fitness value was obtained for the transconjugant which harbored the plasmid carrying \textit{bla}_{CTX-M-15} and the combination of three PAIs (PAI IV\textsubscript{536}, PAI I\textsubscript{CFT073} and PAI II\textsubscript{CFT073}). This supports the previous observation that this combination of PAIs and \textit{bla}_{CTX-M-15} were more prevalent among the ST131 isolates (Chapter 4), and therefore is another indicator that this can constitute an optimal combination of virulence and resistance features.

A wide variety of plasmid types were found to be carried by the UPEC isolates. IncF and IncK were the most common. \textit{aac (6')-lb-cr} was frequently found among the isolates and, in addition, \textit{bla}_{CTX-M-15}, \textit{qnrA} and \textit{qnrS} resistance determinants were also detected.
The genetic relation between CTX-M-15 producers was investigated, strains were characterized and the sequence type (ST) was determined (Chapter 4). With the exception of one isolate belonging to ST10, the remaining isolates were assigned to ST131.

Regardless of the genetic variability, the majority of ST131 strains were found to present a profile composed by PAI IV$_{536}$, PAI I$_{CFT073}$ and PAI II$_{CFT073}$, IncF plasmid, bla$_{CTX-M-15}$ and aac(6')-lb-cr genes. This prevalent virulence and resistance profile may constitute an optimal combination of factors which allow *E. coli* ST131 to maintain both features, becoming concomitantly virulent and extremely resistant.

In fact, despite of the apparent trade-off between virulence and resistance, particularly concerning quinolones, *E. coli* ST131 is an example of a clonal group that contradicts this tendency, as it combines resistance and virulence genes, and this feature may be contributing to its successful dissemination worldwide (Johnson et al., 2010a). Contrarily to other ExPEC isolates from phylogroup B2, ST131 isolates are commonly producers of ESBLs, including CTX-M-15, and the great majority are also resistant to fluoroquinolones (Nicolas-Chanoine et al., 2014). In addition, ST131 *E. coli* isolates are considered to be truly pathogenic, not only because of the diversity of infections they cause either in community or hospital settings, but also because of the wide number of virulence-associated genes they carry.

The wide distribution of clone ST131 and its success as a human pathogen may in part be explained by the co-selection of resistance genes including bla$_{CTX-M}$ and virulence genes. IncF group, widely detected among ST131 clone has been associated with the dissemination of both virulence and resistance genes (Beceiro, Tomás, & Bou, 2013). During this study, an IncF plasmid which carried bla$_{CTX-M-15}$, aac(6')-lb-cr and PAI IV$_{536}$ was identified in a ST131 isolate from a urine sample. Antibiotic selective pressure and acquisition of plasmid that co-carry virulence factors and resistance determinants seem to be the key for the equilibrium of this important human pathogen, which is, nowadays, worldwide disseminated.

A human *E. coli* isolate ST131 was also identified from a high vaginal swab from Nigeria. This strain, similarly to the ST131 isolates detected among the Portuguese collection studied, was multidrug resistant and also presented bla$_{CTX-M-15}$ and aac(6')-lb-cr genes, and an IncFIB replicon (which in this case was shown to be conjugative). Resembling the Portuguese ESBL producers, the Nigerian ESBL producers carried mainly IncF plasmids and in some cases also carried IncK
plasmids. In addition, they carried IncHI plasmids not detected among the Portuguese ESBL producers. The PMQR genes detected were also similar except that \(qnrB\) was only detected among the Nigerian isolates. Once again it was shown that although there are regional differences among the isolates, clone ST131, IncF plasmids and \(\text{bla}_{\text{CTX-M-15}}\) are widely and successfully disseminated worldwide.

The dissemination of resistance and virulence determinants by horizontal gene transfer may contribute to the emergence of more virulent and resistant bacteria. This concern is not only valid for the clinical setting but also to the environment. WWTPs are a focus of concentration of sewage from several origins, and therefore, a place where bacteria from different sources and with different characteristics become in contact with each other. Considering that water may be a good matrix for horizontal gene transfer, if the treatments performed in the WWTPs are unable to remove potentially pathogenic and/or resistant bacteria from water, they will be disseminated into the environment constituting a threat, not only to public health but also to the natural ecosystems.

\(E.\ coli\) from municipal and hospital WWTPs were characterized to understand if bacteria carrying resistant and virulence traits are being disseminated into the environment (Chapter 6).

Likewise in the clinical studies of both Portuguese and Nigerian ESBL producers, ST131 clone was also detected in the effluent of a hospital WWTP. This isolate was multidrug resistant and carried the prevalent combination of resistance determinants and PAIs previously referred. In addition, it also presented \(\text{bla}_{\text{TEM}}, qnrS, iutA, sfa/foc\) and \(\text{papAH}\) genes. Other isolates containing several resistance and virulence determinants were also detected. Nonetheless, this isolate from ST131 clone was the one which presented simultaneously more virulence and resistance determinants and, thus, was the one constituting a higher risk for public and environmental health.

Despite of the high diversity of bacterial sources that converge into the WWTPs, when compared to the Portuguese clinical isolates, these \(E.\ coli\) generally present fewer variety of plasmid types, resistance determinants and virulence factors. Nonetheless, the observed presence of strains carrying potentially disseminative elements at the effluent of the WWTPs, those of them persisting along the treatment station for several days, constitutes a real threat. Dissemination of these virulence and resistance determinants between bacteria may lead to the appearance of more
virulent and/or resistant bacteria including among natural ecosystems. Therefore, it is important to re-think the treatments used in WWTPs and increase the efficiency on the removal of potentially pathogenic bacteria from wastewaters. Perhaps a higher control would be needed for particular species including \textit{E. coli}, and, thus, the number of allowed colonies from this species in microbiological control should be reduced.

\textit{E. coli} is known to be an important pathogen among \textit{Enterobacteriaceae} family. Nonetheless, \textit{K. pneumoniae} and at a lesser extent \textit{K. oxytoca} are also important human pathogens, particularly in nosocomial infections. Their clinical importance is increasing, not only because of the enhancement of resistance among these species, but also to the fact that they are being more frequently identified as causative agents of several types of infections. However, studies on these species are generally focused in resistance, and investigations on virulence are scarce. To understand the relation between virulence and resistance in these species the characteristics of two distinct populations were studied.

The relation between virulence and resistance was evaluated for \textit{K. pneumoniae} and \textit{K. oxytoca} from several clinical samples (Chapter 7).

The presence of PAI II\textsubscript{CFT073}, frequently found among the \textit{E. coli} isolates, and also detected in the \textit{K. pneumoniae} strains from the renal transplant patients (Chapter 8), was firstly identified among \textit{K. oxytoca} in this study. The presence of this island in this species confirms the dissemination of uropathogenic associated PAIs within the genus \textit{Klebsiella}.

A statistically significant relation between PAI IV\textsubscript{536} and resistance to gentamicin and sulfamethoxazole- trimethoprim in \textit{K. pneumoniae} was observed. Furthermore, PAI II\textsubscript{CFT073} was significantly associated with resistance to cephalothin, cefotaxime, ceftazidime, ciprofloxacin, and gentamicin in \textit{K. oxytoca}. The co-carriage of pathogenicity islands in resistance plasmids may be one possible explanation for these observations, which could constitute one of the research line emerging from this work.

Among the clinical syndromes caused by \textit{K. pneumoniae}, pyogenic liver abscess is a serious condition which can lead to extra-hepatic complications. This explains why many of the virulence studies focus on \textit{K. pneumoniae} from this source. Several virulence determinants have been associated with this condition including \textit{allS}, involved in the allantoin metabolism (Chou et al., 2004). In this study, this gene
was prevalently detected among strains from urinary infections. This may indicate that this virulence factor generally involved in PLA may also be an important urovirulence determinant. In fact, allantoin is found in human urine as a product of uric acid metabolism, and therefore the possibility of using this substance as an energy source may also constitute an advantage for bacterial growth among urine. To really understand the pathogenic potential and the possible role of the different virulence factors among *Klebsiella* spp. further studies should expand the range of clinical samples studied.

Contrarily to the observed in *E. coli*, where a trade-off between virulence and resistance seems to occur, has in our strains the majority of the genes were more prevalent among susceptible isolates, this is generally not verified in *Klebsiella* spp. In fact, in *K. pneumonia*, higher prevalence of virulence factors was detected among isolates resistant to ciprofloxacin and gentamicin and at a lesser extent to cefotaxime and sulfamethoxazole/trimethoprim. Furthermore, higher prevalence of virulence genes was observed in resistance to sulfamethoxazole-trimethoprim in *K. oxytoca*. Nonetheless, for this species, in the remaining antibiotics, the prevalence was more balanced, as about half of the virulence genes were more prevalent among resistant strains, while the others were more prevalent on susceptible strains. These results suggest that the interplay between resistance and virulence are largely influenced by the genus and species, the virulence factor, as well as the resistance phenotype.

Finally, a set of *K. pneumoniae* isolates from renal transplant patients, all of them ESBL producers, were characterized. The genetic relatedness was also assessed to understand if an outbreak was occurring (Chapter 8). Uropathogenic associated PAIs are commonly found among *E. coli* strains (Bronowski et al., 2008; Sabaté et al., 2006). Nonetheless, these elements are generally not investigated in *K. pneumoniae*. For the first time, the islands PAI II\textsubscript{CFT073} and PAI III\textsubscript{536} were reported among *K. pneumoniae*. Previously, only PAI IV\textsubscript{536} had been identified in this species (Koczura & Kaznowski, 2003). The identification of these mobile genetic elements in isolates from different genus, indicates that PAIs may be disseminating among *Enterobacteriaceae* members. The presence of PAI III\textsubscript{536}, one of the most unstable PAIs in *E. coli* (Middendorf et al., 2004), among *K. pneumoniae* strains may be suggestive that they may provide a favourable genetic background for the acquisition or maintenance of this island. In fact, while in our *E. coli* clinical strains this PAI was only detected in isolates susceptible to ciprofloxacin, in this case the carriers of this
island were resistant to ciprofloxacin. This indicates that resistance to ciprofloxacin in *K. pneumoniae* is not incompatible with the presence of PAI III\textsubscript{536}. The relation between resistance and the presence of certain virulence determinants seems therefore to be species-related.

Likewise among *E. coli*, in *K. pneumoniae* ESBL producers from renal transplant patients, *bla\textsubscript{CTX-M-15}* was also prevalent. Nonetheless, while in *E. coli* strains this determinant was generally associated with IncF plasmids, in *K. pneumoniae* producing CTX-M-15, none of the replicon types circulating among *Enterobacteriaceae* were detected. Therefore, either the gene encoding these enzymes was integrated in the chromosome of *K. pneumoniae* or other plasmid types, not identified by the Caratolli et al. scheme, are circulating among this species.

In addition to the characterization of *K. pneumoniae* strains, the genetic study of these isolates allowed to understand that the isolates had different origins. This excluded the possibility of an outbreak caused by a single internal clone disseminating among the Renal Transplant Unit. This information was very important to the hospital, and highlighted the importance of the screening for colonization of patients before admission in these units to prevent further infections, and dissemination of resistant strains. As these patients are generally immunocompromised hosts, the dissemination of potentially pathogenic and resistant bacteria in these units may even be fatal.

## 2. Concluding Remarks

Overall, this PhD dissertation provides new insights in the knowledge of the relation between virulence and resistance among *E. coli* and *Klebsiella* spp. This relation seems to be complex and dependent on several factors including genus and species, individual virulence factor and resistance phenotype. While in some virulence factors there seems to be a trade-off between virulence and resistance, particularly in *E. coli*, others seem to be positively related to resistance.

Therefore, it is important not only to conduct other studies in other virulence factors but also consider other emerging resistance phenotypes, including resistance to carbapenems that, nowadays, start to emerge in our country. Furthermore, genetic relatedness studies should also be performed between strains from human and
animal origin. This would allow understanding if the trade-off observed for some virulence factors and resistance determinants is really verified, or if it is related to the importation of less virulent but more resistant strains from animal origin.


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