Universidade de Aveiro Departamento de Biologia
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DIANA SALVADOR DE SÁ DIAS

CARACTERIZAÇÃO MOLECULAR DE ISOLADOS CLÍNICOS RESISTENTES A ANTIBIÓTICOS

MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT CLINICAL ISOLATES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Cláudia Sofia Soares de Oliveira, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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"*In every job that must be done, there is an element of fun*." -- Mary Poppins (*Mary Poppins*)

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palavras-chave

Resistência a antibióticos; Isolados clínicos; Enterobacteriaceae; β-lactâmicos; cefalosporinas de 3ª geração; β-lactamases; ESBLs; AmpC; tipagem molecular; elementos genéticos móveis; integrões; plasmídeos; replicon typing.

resumo

A prevalência de isolados bacterianos, da família *Enterobacteriaceae,* resistentes a cefalosporinas de largo espectro, produtores de β-lactamases de largo espectro (ESBLs) e/ou AmpCs tem vindo a aumentar. Tem sido também reportada a coocorrência de genes que codificam para ESBLs e genes que conferem resistência a outras classes de antibióticos. Esta situação tem como consequências a limitação de opções terapêuticas e o insucesso da terapia, traduzindo-se em taxas de mortalidade e morbilidade mais elevadas.

Este trabalho focou-se na caracterização molecular de 46 isolados clínicos resistentes a antibióticos, pertencentes à família *Enterobacteriaceae.* Os isolados foram previamente obtidos no Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E., e foram selecionados para análise com base na suspeita de produção de ESBLs e/ou AmpCs, de acordo com o sistema automático Vitek2®. Os isolados foram caraterizados em termos de diversidade genotípica, presença de genes de resistência e elementos genéticos móveis. Foram observados determinantes genéticos responsáveis pela resistência a β-lactâmicos (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M}$ e *bla*$_{CMY}$), fluoroquinolonas (*qnrA, qnrB* e *aac(6')-ib-cr)*, sulfonamidas (*sul*1 e *sul*2) e aminoglicosídeos (*aac(6')-ib).* O gene *bla*$_{TEM}$ foi o mais prevalente (97,8%), enquanto o gene *bla*$_{SHV}$ foi o menos prevalente (6,5%) entre os isolados. A variante *bla*$_{CMY}$ do gene *bla*$_{AmpC}$ foi a mais frequente em contexto adquirido (n=10 isolados). A variante *bla*$_{CTX-M-15}$ foi observada na maioria (94,4%) dos isolados *bla*$_{CTX-M}^{+}$ e surge associada à sequência de inserção IS*Ecp1*. Foram identificados genes codificantes para o fenótipo de resistência a aminoglicosídeos (*aadA1, aadA2, aadA5* e *aadB*), trimetoprim (*dfrA1*, *dfrA12, dfrA15* e *dfrA17*), cloranfenicol (*catB3)* e estreptotricina (*sat2*) nas regiões variáveis dos integrões de classes 1 e 2, presentes em 34 e 1 isolados, respetivamente. Além dos integrões, foram encontrados outros elementos genéticos móveis como sequências de inserção, nomeadamente, *IS*Ecp1 e plasmídeos conjugativos pertencentes aos grupos IncF, IncK, IncB/O e IncI1. Foram realizados ensaios de conjugação para 8 isolados *bla*$_{CTX-M-15}^{+}$ e 7 isolados *bla*$_{CMY}^{+}$, originando 2 e 3 transconjugantes, respetivamente.

Com este trabalho foi possível concluir que a resistência às cefalosporinas de 3ª geração neste hospital está predominantemente associada a mecanismos de resistência adquirida. Os resultados referentes à tipagem molecular, recorrendo a BOX-, ERIC- e rep-PCR, sugerem que a disseminação de produtores de ESBLs e/ou AmpCs neste hospital será policlonal, dada a variabilidade intra-espécies observada. Não se identificaram clones prevalentes em diferentes serviços ou associados à comunidade. De facto, a disseminação dos determinantes de resistência parece estar relacionada com transferência de elementos genéticos móveis, nomeadamente dos replicões IncB/O, K, I1 e Frep. A presença de integrões nestes isolados resulta com frequência em fenótipos de multirresistência. Serão necessários mais estudos para caracterizar os elementos genéticos móveis com maior detalhe. O estudo aqui apresentado representa uma contribuição importante para a definição de estratégias de controlo da disseminação de bactérias resistentes a cefalosporinas de largo espectro no Hospital Infante D. Pedro.

keywords

Antibiotic resistance; clinical isolates; Enterobacteriaceae; β-lactams; 3rd generation cephalosporins; β-lactamases; ESBLs; AmpC; molecular typing; mobile genetic elements; integrons; plasmids; replicon typing .

abstract

The prevalence of *Enterobacteriaceae* isolates displaying resistance to broad-spectrum cephalosporins, harboring extended-spectrum β-lactamases (ESBLs) and/or AmpC enzymes is increasing. Moreover, the co-occurrence of ESBLs and genes conferring resistance to other antibiotic classes is frequently reported. This situation leads to a limitation of therapeutic options and unsuccessful therapy and consequently to higher rates or mortality and morbidity.

This work focused on the molecular characterization of 46 antibiotic resistant clinical isolates belonging to *Enterobacteriaceae* family. These isolates were previously obtained from *Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E.,* and were selected for analysis based on the suspicion of ESBL and/or AmpC production provided by the Vitek2® Automatic System. The isolates were characterized in terms of genetic diversity, resistance genes and mobile genetic elements. Genetic determinants conferring resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{CMY}), fluoroquinolones (*qnrA, qnrB* and *aac(6')-ib-cr)*, sulfonamides (*sul*1 and *sul*2) and aminoglycosides (*aac(6')-ib)* were observed. bla_{TEM} was the most prevalent gene (97.8%) while bla_{SHV} gene was the least prevalent (6.5%) among isolates. bla_{CMY} variant of the bla_{AmpC} genes was the most frequent in an acquired context (n=10 isolates). $bla_{CTX-M-15}$ variant was found in the majority of the isolates that tested positive for the bla_{CTX-M} gene (94.4%) and is frequently associated with the insertion sequence *IS*Ecp1. Resistance genes coding resistance phenotype to aminoglycosides (*aadA1, aadA2, aadA5* and *aadB*), trimetoprim (*dfrA1, dfrA12, dfrA15* and *dfrA17*), chloramphenicol (*catB3*), and streptothricin (*sat2*) were also identified in the variable regions of class 1 and 2 integrons, found in 34 and 1 isolates, respectively. Aside from integrons, other mobile genetic elements like insertions sequences, *IS*Ecp1 and conjugative plasmids belonging to groups IncF, IncK, IncB/O and IncI1, were also identified. Mating assays were performed for 8 $bla_{CTX-M-15}^{+}$ isolates and 7 bla_{CMY}^{+} isolates, originating 2 e 3 transconjugants, respectively.

It was possible to conclude that resistance to 3rd generation cephalosporins in this hospital is predominantly associated with acquired mechanisms. Molecular typing results, obtained by BOX-, ERIC- and rep-PCR, suggest a non-clonal dissemination of the ESBL and/or AmpC-producing isolates, based on the high intra-species diversity observed. Clones prevalent in the hospital or prevalent in the community were not identified. In fact, the dissemination of the resistance determinants seems to be related to the transference of mobile genetic elements, namely IncB/O, K, I1 and Frep replicons. Also, the presence of integrons often results in a multidrug phenotype. Further studies need to be conducted to identify these elements with more detail. This study represents an important contribution to the development of effective strategies aiming to reduce the dissemination of broad-spectrum cephalosporins resistant bacteria in *Hospital Infante D. Pedro.*

INDEX

I. INTRODUCTION

1. Antibiotics

An antibiotic can be defined as a substance that has the capacity to kill (bactericides) or selectively inhibit the growth of bacteria (bacteriostatic) by specific interactions with cell targets. These compounds can either have a synthetic or a natural origin and can be used to treat or to prevent bacterial infections (Davies & Davies, 2010).

In 1928, Alexander Fleming accidently discovered penicillin, a natural compound with antimicrobial properties. While examining his culture-plates of *Staphylococcus*, Fleming noticed that around the colonies of the contaminating mold, *Staphylococcus* colonies did not grow. There were made subcultures of this mold, later identified as *Penicillium notatum*, with the aim to isolate the antimicrobial substance (Fleming, 1980). Penicillin, along with other antimicrobials discovered in the Antimicrobial Era, that is the large-scale production and introduction of antibiotics in clinical facilities, mark the future of antimicrobials discovery (Aminov, 2010).

Penicillin had the capacity to kill or affect the growth of the majority of common pathogenic bacteria when discovered. A few years later, in 1945, after mass production and distribution, penicillin was used as treatment for infections of sick and wounded soldiers in the U.S. and Allies' military forces. Thereafter, penicillin became a widely used antibiotic for infectious diseases that were previously untreatable (Aminov, 2016).

After the discovery of penicillin, numerous antibiotics were discovered and developed. Today, there are several classes of antibiotics which differ in their targets but they all act on central processes/structures distinctive of bacterial cells. As a consequence, their negative effects in eukaryotic cells are minimized (Neu, 1992).

The discovery of these compounds and their application in medical practice to treat and prevent diseases had a great health impact on human morbidity and mortality rates thus saving numerous lives. Besides their application in human medicine, antibiotics can also be used in veterinary medicine and agricultural practices.

1.1 Classes of antibiotics and their mechanism of action

As mentioned before, since the discovery of penicillin the number of antibiotics available increased, which required a system to classify them. The most practical system is the one where antibiotics are classified based on their mechanism of action (Neu, 1992). These mechanisms include: inhibition of protein synthesis, inhibition of synthesis or metabolism of nucleic acids, inhibition of the synthesis or damage of the bacterial cell wall or modification of energy metabolism of the prokaryotic cell.

Table I.1 is a summary of the classification of the main classes of antibiotics according to their mechanism of action.

Table I.1. Classification of antibiotic classes according to their action mechanism.

Mechanism of Action	Antibiotic Class
Inhibition of protein synthesis	Aminoglycosides Phenicols Macolides-Lincosamides-Streptogramins B (MLS) Tetracyclines
Interaction with the synthesis of nucleic acids (DNA and RNA)	(Fluoro)quinolones
Inhibition of the synthesis/damage of the bacterial cell wall	β-lactams Glycopeptides
Modification of the energy metabolism of the bacterial cell	Sulfonamides Trimethoprim

1.1.1. β-Lactams

Due to their versatility, low toxicity, and broad spectrum of action as well as their high safety, efficacy, and availability, β-lactams play a major role in the treatment of bacterial infections (Bush, 2010; Bush & Jacoby, 2010).

β-lactams act on the bacteria cell wall, by inhibiting its synthesis when they bind to penicillin-binding proteins (PBPs) thus preventing terminal transpeptidation in the prokaryotic cell wall. Consequently, since the bacteria cell wall is weaker, occurs cytolysis or death due to osmotic pressure (Van Hoek et al., 2011).

Identification of 6-aminopenicillanic acid as the core of penicillin allowed a starting point for the development of derivatives. Thus, this class of antibiotics now includes several sub-groups, namely: penicillins and derivatives, cephalosporins, carbapenems, monobactams and β-lactamase inhibitors. Aside from β-lactamase inhibitors, all of these sub-classes contain a β-lactamic ring in their structure which is characteristic of this family of antibiotics (Van Hoek et al., 2011).

The sub-group of **penicillins and derivatives** is composed by natural and semisynthetic penicillins, such as derivatives that are active against Gram-negative bacteria like ampicillin and amoxicillin (Aminov, 2016).

Cephalosporins can be grouped in 1st, 2nd, 3rd and 4th generation cephalosporins according to their spectrum of activity and time of introduction as therapeutic agents (Donowitz & Mandell, 1988). 3rd and 4th generation cephalosporins have a broader antibiotic activity spectrum compared to 1st and 2nd generations (early-cephalosporins). 3rd generation cephalosporins have an improved Gram-negative and variable Gram-positive activity and 4th generation cephalosporins have a good broad spectrum of activity against Gram-negatives as well as Gram-positives. Ceftazidime and cefotaxime are examples of 3rd generation cephalosporins and cefepime is an example of 4rd generation (Van Hoek et al., 2011).

Carbapenems, like imipenem and meropenem, are considered as broad spectrum β-lactams due to their broad spectrum of activity (Van Hoek et al., 2011).

Sub-group **β-lactamase inhibitors** have minor antimicrobial activity and are used in combination with other β-lactams to overcome resistant bacteria that express β-lactamases. Tazobactam, sulbactam and clavulanic acid are clinical irreversible β-lactamase inhibitors since they will cause the destruction of the enzymatic activity (Van Hoek et al., 2011).

1.1.2. Other classes of antibiotics

Aside from β-lactam antibiotics, there are other classes of antibiotics that can be used to treat Gram-negative infections, for example, aminoglycosides, chloramphenicol, (fluoro)quinolones, sulfonamides, trimethoprim and tetracyclines.

Aminoglycosides, like streptomycin (discovered in the early 1940s), neomycin and kanamycin, isolated from *Streptomyces* species and gentamicin, isolated from *Micromonospora purpurea*, have a broad spectrum activity (Schatz & Waksman, 1944). They inhibit protein synthesis and/or alter the integrity of the cell membranes and can act in combination with other antibiotics (Vakulenko & Mobashery, 2003).

As aminoglycosides, **chloramphenicol** (1947) and its synthetic derivatives act as inhibitors of protein synthesis. They bind to a peptidyltransferase of a subunit of the 70S ribosome and prevent peptide chain elongation (Schwarz et al., 2004).

Fluoroquinolones (1962) are the second generation of quinolones and the result of an addition of a fluoride atom to the structure of a quinolone molecule. Ciprofloxacin is an example of these molecules. This alteration improved their biological activity so further alterations were made, resulting in the third generation fluoroquinolones (e.g. levofloxacin) which have activity against both Gram-negative and Gram-positive bacteria (Van Hoek et al., 2011; Wolfson & Hooper, 1989).

Sulfonamides are the oldest synthetic therapeutics (1932) and they act on the folic acid pathway. Due to its structural analogy to *p*-aminobenzoic acid, sulfonamides competitively inhibit the enzyme dihydropteroate synthase (DHPS), inactivating the thymine production which affects the cell growth. Nowadays, sulfamethoxazole is the most commonly used sulfonamide and is used in combination with **trimethoprim**. Like sulfonamides, trimethoprim is a class of completely synthetic drugs and they act on the metabolism of the cell as well, in this case, trimethoprim inhibits the enzyme dihydrofolate reductase (DHFR) that is part of the folic acid pathway like DHPS (Roberts, 2002).

Tetracyclines are second after penicillin in world consumption because of their broad spectrum of activity, relative safety and low cost. They inhibit the growth of the bacteria by interacting with the ribosomes then blocking the protein synthesis. One example of compounds included in this class is chlortetracycline (Roberts, 2002).

2. Antibiotic Resistance

In 1940, before the introduction of penicillin in medical practices, two members of the penicillin discovery team observed that some *Escherichia coli* bacteria had the ability to destroy penicillin through the production of an enzyme, named penicillinase, thus becoming resistant to penicillin (Abraham & Chain, 1940). However, the scenario was more or less optimist. It was assumed that the frequency of mutations that led to antibiotic resistance was negligible and the exchange of genetic material between bacteria was not expected (Davies & Davies, 2010).

Alexander Fleming was one of the firsts to alert to this resistance to penicillin. In 1945, in his Nobel Lecture, Fleming stated that penicillin would be easily available to anyone and this, associated with an inadequate treatment, either due to under-dosages or smaller treatment periods than the recommended ones, would result in resistant bacteria (Fleming, 1945). The scenario presented for penicillin and the emergence of resistance, is also seen in other antibiotics. Upon the introduction of an antibiotic, within a few years, resistance mechanisms emerge, compromising the efficiency of the antibiotic (Van Hoek et al., 2011).

Besides self-medication and misprescription of antibiotics, there are numerous factors influencing the increasing emergence of antibiotic resistance and most of them are anthropogenic, namely: (i) the use of antibiotics as growth promotors in animals, (ii) the prophylactic use in humans, animals and aquaculture and (iii) agriculture usage as pest control. All of these activities release antibiotics into the environment providing a constant selection and pressure for resistant populations which result in environmental reservoirs of resistance (Davies & Davies, 2010).

Mutation and selection, together with mechanisms of gene transfer among bacteria, provide a rapid adaptation of these bacteria to the introduction of an antibiotic in their environment.

2.1 Intrinsic and acquired resistance

Antibiotic resistance can either be intrinsic or acquired. Intrinsic resistance refers to an innately resistance conferred by genes in the bacteria genomes that could lead to a resistance phenotype. In this case, bacteria are innately resistant to one (or more) classes of antibiotics (Davies & Davies, 2010).

Acquired resistance emerges when a bacteria that was initially susceptible to one antibacterial agent starts to display a resistance phenotype that allows its proliferation in the presence of that agent. This resistance can arise via new mutations or by acquisition of genetic information encoding resistance from other bacteria. The first event occurs spontaneously in the chromosome and can either cause resistance through several mechanisms or can just reduce the susceptibility of the bacteria to an antibiotic allowing its initial survival until the bacteria acquires other mutations or genes that confer antibiotic resistance. The second event is termed horizontal gene transfer (HGT) and occurs through genetic exchange mechanisms. This event plays the major role in the evolution and transmission of resistance to antibiotics among bacteria (Magiorakos et al., 2012; Tenover, 2006).

2.1.1 Mechanisms of horizontal gene transference

There are three main processes that promote horizontal HGT: transformation, transduction and conjugation. Transformation occurs when a competent bacteria is capable of uptake, integrate and express fragments of DNA present in the extracellular environment. In transduction, bacteriophages can transfer genes between bacteria. Bacterial DNA, along with phage DNA, is packaged into the phage head and then can be integrated and expressed in the recipient bacterial cell. Conjugation events require cell to cell contact since DNA transference from the donor cell to the recipient cell occurs via cell surface pili (Von Wintersdorff et al., 2016).

Transformation occurs without an intermediary platform. On the other hand, transduction and conjugation require a vehicle for gene transference. This first involves bacteriophages and the second requires exchange of genetic elements like plasmids, transposons, integrons and/or insertion sequences.

2.1.1.1 Plasmids

Plasmids are circular or linear extra chromosomal elements that replicate autonomously in a host cell and can be found in almost all bacterial species. In their structure, plasmids contain genes essential for replication and may contain accessory genes that confer useful phenotypes to their host, like virulence or antibiotic resistance genes (Carattoli, 2009). Although plasmids can contribute for genetic diversity, they do not necessarily increase the host fitness because their presence carries a fitness burden for the host.

The simplest of these structures contain an origin of replication and associated genes. Mobilizable plasmids, apart from genes associated with replication, also contain an origin of transfer (*oriT*) and conjugative plasmids contain an origin of replication (*oriR*), an *oriT* and genes encoding functions allowing them to transfer to another host (Van Hoek et al., 2011).

Since some plasmid types cannot coexist in the same bacterial cell, the system of plasmid classification relies on incompatibility (Inc) groups. Plasmids that do not propagate on the same cell line belong to the same Inc group while plasmids that belong to different Inc groups can coexist on the same cell and can both be transferred to the daughter cells. This classification system also allows the evaluation of the transferability of plasmids carrying resistance determinants among bacteria. In *Enterobacteriaceae* the major plasmid families found are HI2, HI1, I1-Ɣ, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K and B/O (Carattoli, 2009).

2.1.1.2 Transposons, integrons and insertion sequences

Integrons are genetic platforms that acquire gene cassettes in their environment by recombination, and convert them into functional genes by ensuring their correct expression, thus increasing genetic diversity in bacteria (Mazel, 2006). From the structural point of view, integrons are composed of three key elements: a gene encoding an integrase (*intI*), a recombination site (*attI*) and a promoter (P_c) that directs transcription of the captured genes. These elements allow the capture of exogenous genes, like antibiotic resistance genes (Mazel, 2006). Figure I.1 illustrates the structure of an integron and the mechanism of exogenous capture of gene cassettes.

There are three major integron classes which are frequently found in clinical environments, classes I, II and III, which differ in the sequence of the integrase gene. Integrons are not considered mobile DNA elements for themselves since they do not include any genes encoding for mobility. However, they can be integrated into mobile elements, like plasmids or transposons, contributing to the dissemination of antibiotic resistance determinants among bacteria (Boucher et al., 2007).

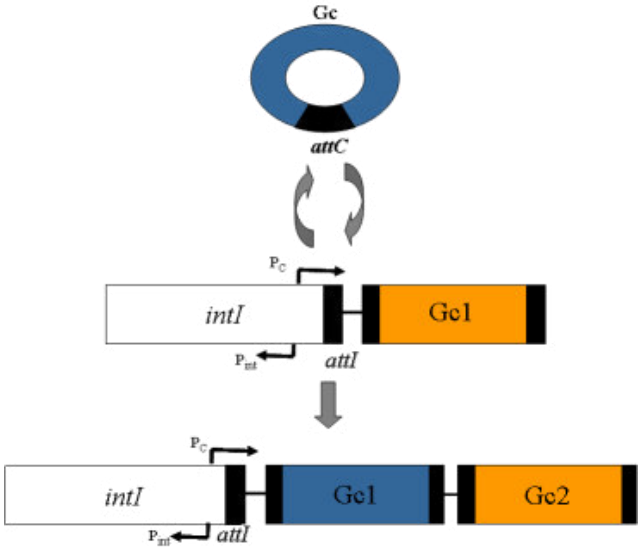

Figure I.1 - Integration of gene cassettes into an integron. Retrieved from http://integrall.bio.ua.pt/?intro

Insertion sequences (IS) are the simplest of mobile genetic elements. They consist in a segment of DNA with the ability of insert at multiple sites in a target DNA (Boucher et al., 2007). Insertion sequences, like IS*Ecp1*, have been implicated in the mobilization of antibiotic resistance genes onto plasmids since they facilitate this transference (Jacoby, 2009). Among IS, there is one particular type called Insertion Sequences Common Region (IS*CR*) which are elements close related to the IS*91* family and are transposed by a mechanisms called rolling-circle transposition. While most IS need two copies to flank the mobilized gene, IS*CR*s, can transpose adjacent DNA sequences mediated by a single copy of the element (Toleman et al., 2006a).

The majority of these elements are closely associated with antibiotic resistance genes, including resistance to β-lactams, aminoglycosides, chloramphenicol, trimethoprim and quinolones, and are also found beyond the 3' Conserved Region (3' CS) of class 1 integrons. This suggests that IS*CR* elements may play a role in the rapid transmission of the bacteria multi-drug resistance (Toleman et al., 2006a).

Insertion sequences containing genes that are not involved in its translocation are called **transposons**. Transposons are mobile DNA elements which have the ability to relocate within the genome due to the presence of a transposase. In some cases, can also be transferred to a recipient cell, like plasmids, and then they are termed conjugative transposons. This type of transposons have characteristic features of plasmids but do not contain an *oriR* and for that reason need to be integrated into DNA in order to be maintained (Van Hoek et al., 2011).

Given these dissemination mechanisms, is easy to acknowledge that bacterial cells have a large potential to adapt to environments with the presence of antibiotics. They do so by acquiring antibiotic resistance genes either from other bacteria or directly from the extracellular environment. The presence of antimicrobial agents in the bacteria environment, forces the development of new mechanisms of resistance and this, associated with the capacity of rapid adaptation to selective pressures that bacteria possess, makes the dissemination of resistance to antibiotics an emergent problem since bacteria will continue to adapt (Tenover, 2006).

2.2 Bacterial resistance mechanisms

Bacteria may manifest resistance to antimicrobial agents through a variety of mechanisms, including: (i) modification of the target, (ii) permeability alterations in the bacterial cell wall thus restricting the access of the antibiotic to its target, (iii) efflux of the antimicrobial agent from the bacteria, (iv) enzymatic modifications or degradation of the antibiotic, (v) overproduction of the target enzyme and (vi) alternative pathways to overcome the inhibition of the pathways inhibited by the antimicrobial agent (Van Hoek et al., 2011). Bacterial biofilms can also be considered a resistance mechanism to antimicrobial agents. Moreover, in these structures, HGT events and mutation frequency are significantly increased thus contributing to multidrug resistance phenotypes (Driffield et al., 2008; Molin & Tolker-Nielsen, 2003).

2.2.1. β-lactams

There are several mechanisms that confer resistance to β-lactams. The most common and important among them is the production of enzymes named β-lactamases. Aside from that mechanism, when bacteria have altered penicillin-binding proteins (PBPs), β-lactams cannot bind to the PBPs, and as consequence, the antibiotic does not disrupt the cell wall with the same efficiency, leading to reduced sensibility of the bacteria to β-lactams (Dallenne et al, 2010; Van Hoek et al., 2011).

2.2.1.1 β-lactamases

β-lactamases are enzymes that have the capacity to hydrolyze the β-lactamic ring characteristic of this class of antibiotics. Consequently, the antibiotic loses the ability to kill the bacteria (Bush, 2010).

These enzymes are encoded by *bla* genes and, due to their continuous mutations, there is, currently, a wide variety of β-lactamases, which led to classification systems. One system divides them according to amino acid sequences – molecular classes, where Ambler classes A, C and D comprise β-lactamases with serine at their active site and β-lactamases that belong to Ambler class B are metallo-enzymes, requiring zinc as a metal cofactor (Ambler, 1980). Apart from molecular classes, functional groups divide β-lactamases according to their inactivating properties, based on the hydrolysis and inhibition profiles of the enzymes. Serine β-lactamases belong to groups 1, 2 and 4 while members of group 3 are metallo-β-lactamases (Bush et al., 1995).

2.2.1.1.1 AmpC and extended-spectrum β-lactamases (ESBLs)

AmpC β-lactamases, also known as cephalosporinases, belong to molecular class C and functional group 1. They confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins, cephamycins and monobactams, and generally, are not inhibited by clavulanic acid, tazobactam or sulbactam (Jacoby, 2009).

The production of AmpC β-lactamases can be chromosomally or plasmid mediated. AmpC are inducible enzymes found in chromosomes of most *Enterobacteriaceae* (Bush, 2010). They can be expressed at low and high levels, the latter due to mutations in the repressor gene *ampD* or in the regulator AmpR. High levels of

AmpC production confer resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone. This can be problematic since a bacteria isolate that was initially susceptible to these β-lactam antibiotics may become resistant during therapy (Jacoby, 2009).

Plasmid-mediated AmpC β-lactamases have arisen through HGT of chromosomal AmpCs by transmissible plasmids among different species. For that reason AmpC β-lactamases have been found on bacterial species that previously lacked the chromosomal bla_{AmpC} gene or expressed AmpC at low levels (Jacoby, 2009).

There are several types of plasmid mediated AmpC β-lactamases which are irregularly named, like: CMY, FOX, MOX and LAT, named according to the resistance produced to cephamycins, cefoxitin, moxalactam and latamoxef, respectively; ACT (AmpC type) and ACC (Ambler class C), named according with the type of β-lactamase and according with the site of discovery, such as Miriam Hospital Providence, R.I. (MIR-1) or Dhahran Hospital in Arabian Saudi (DHA), or the name of the patient from whom the microorganism was isolated such as Bilal (BIL-1) (Beceiro & Bou, 2004; Philippon et al., 2002). Figure I.2 represents a phylogenetic tree obtained in this work with the amino acid sequences of the representative variants of the AmpC cephalosporinases types described so far in order to represent the known diversity of these enzymes.

Regarding the phylogeny of AmpC β-lactamases, it is worth to emphasize that CMY sequences grouped in two clusters that share the origin of the enzymes. The CMY-1 group, represented by the varieties CMY-1, -8, -9, -10 and -11, is related to chromosome-encoded AmpC β-lactamases in *Aeromonas* spp., while the CMY-2 group (represented only by a part of the currently known varieties) is related to AmpC enzymes of *Citrobacter freundii*. CMY-2 is the most frequent plasmid-mediated AmpC β-lactamase worldwide (Jacoby, 2009) and is also the most recent common ancestor of all plasmid-borne AmpC of *C. freundii* (Barlow & Hall, 2002). LAT enzymes have a similar origin, comparing to CMY-2. Of the four original LAT enzymes, it was discovered, by sequence analysis, that LAT-2 was identical to CMY-2, LAT-3 was identical to CMY-6 and LAT-4 was actually identical to LAT-1, which is the only LAT enzyme remaining unique (Barlow & Hall, 2002; Jacoby, 2009).

Also, BIL-1 is not represented since its sequence is identical to CMY-2. For the same reason, LAT-2 is not represented either (Barlow & Hall, 2002). That said, CMY-2 could be listed as CMY-2-BIL-1-LAT-2, the common ancestor of plasmid cephalosporinases.

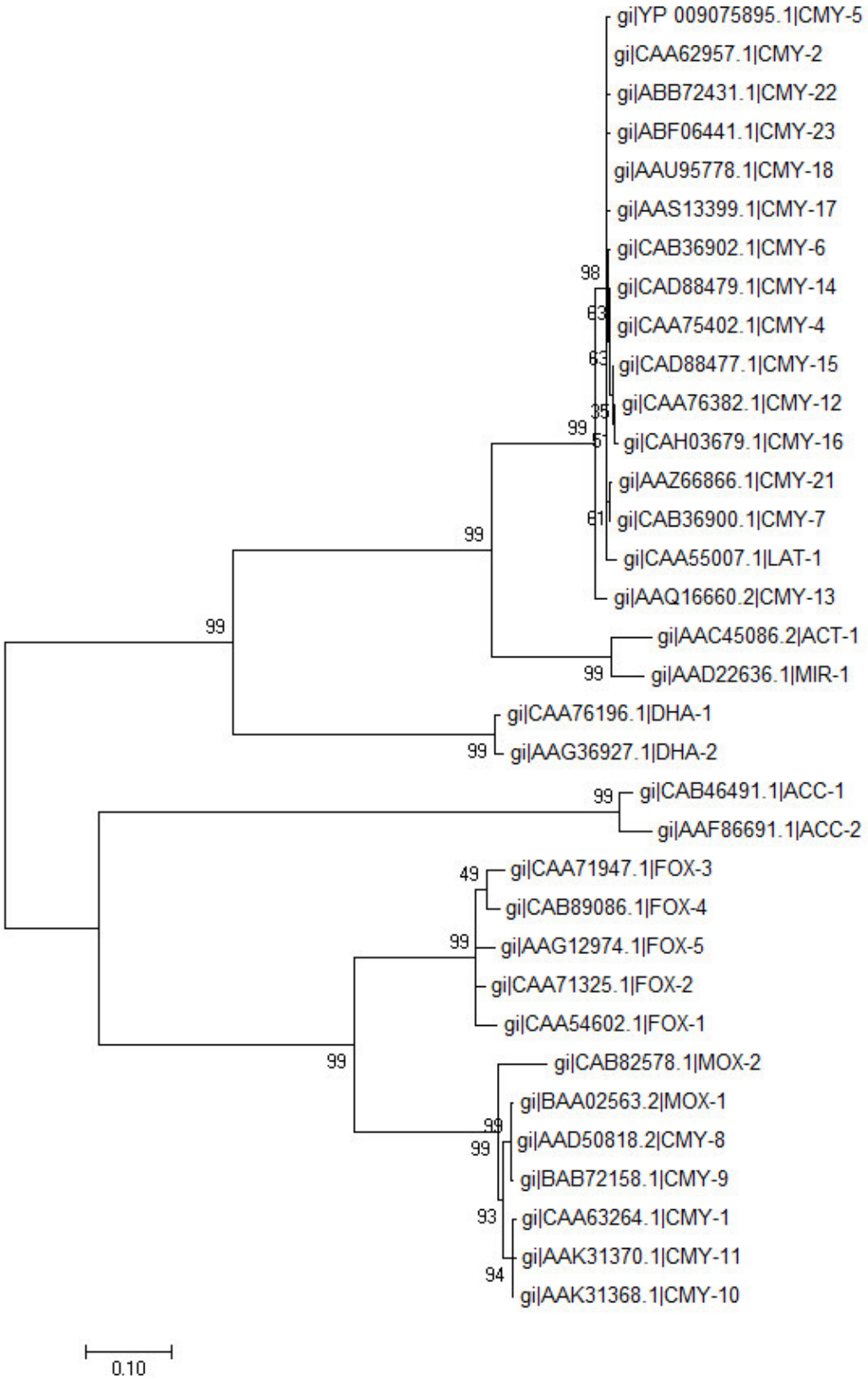

Figure I.2 - Molecular Phylogenetic analysis by Maximum Likelihood method using MEGA7. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The designation used in the tree includes the amino acid sequence accession number for each enzyme. The tree includes only sequences representatives of each family among all the variants described so far.

ESBLs belong to functional group 2be and molecular class A. These enzymes are capable of hydrolyzing early- and expanded-spectrum cephalosporins and monobactams, but not cephamycins and carbapenems. Additionally, ESBLs are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) (Bush, 2010). This property and the susceptibility to cephamycines distinguishes ESBLs from AmpCs.

TEM, SHV and CTX-M are the three main families of ESBLs described. The genes encoding these ESBLs are generally found in plasmids that confer resistance to multiple antibiotic classes and that are easily transferable among bacteria (Bush, 2010).

Initially, all ESBLs were identified as variants of the TEM-1 and SHV-1 β-lactamases, since they arise via point mutations from these two narrow-spectrum β-lactamases. TEM-1 and SHV-1 penicillinases (group 2b/class A) include the SHV-1 enzyme found in *Klebsiella pneumoniae* and the TEM-1 and -2 β-lactamases found in *Neisseria gonorroheae* and *Haemophilus influenza*, encoded by bla_{SHV}, $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-2}}$, respectively (Bush, 2010). These enzymes hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalothin are inhibited by clavulanic acid, sulbactam and tazobactam (Bush & Jacoby, 2010). This group of enzymes were the most common plasmid β-lactamases identified in the 1970s and early 1980 (Bush & Jacoby, 2010) and were prevalent among *Enterobacteriaceae* before the introduction of the broad-spectrum cephalosporins (Bush, 2010). It is thought that the use of 3rd generation cephalosporins acted as a selective pressure thus being responsible for the arise of these variants displaying resistance to 3rd generation cephalosporins (Paterson & Bonomo, 2005). Despite these variants of TEM-1 and SHV-1 penicillinases, in the late 1980s CTX-M family – encoded by $bla_{\text{CTX-M}}$ genes, arose and within a decade, became the predominant ESBL family, outnumbering most of the TEM- and SHV-derived ESBLs (Seiffert et al., 2013).

CTX-M family preferentially hydrolyzes cefotaxime and some CTX-M-type ESBL actually have hydrolyzing activity against ceftazidime thus conferring resistance (Paterson & Bonomo, 2005). In addition to the unique feature of the rapid hydrolysis of cefotaxime, CTX-M enzymes, are also distinctive in the fact that they are better inhibited by tazobactam than by sulbactam and clavulanic acid (Shaikh et al., 2015).

It appears that CTX-M β-lactamases have a different origin than TEM and SHV ESBLs. These latter ESBLs are originated by amino acid substitutions of their parent enzymes but CTX-M family is thought to be originated by HGT mechanisms since they

are closely related to chromosomal β-lactamases of *Kluyvera* spp.. As an example, CTX-M-8 shares 99% amino acid identity with chromosomal β-lactamase KLUG-1 of *Kluyvera* spp. and compared to TEM and SHV β-lactamases, CTX-M-type, only have 40% or less identity (Paterson & Bonomo, 2005).

The number of CTX-M has been rapidly expanding and so is their geographic dissemination. Among all variants, CTX-M-15 is the most frequent and one of the most spread CTX-M β-lactamases in the community and in clinical settings (Cantón & Coque, 2006).

2.2.2 Other classes of antibiotics

2.2.2.1 Aminoglycosides

Among all of the resistance mechanisms against aminoglycosides, the main one is the inactivation of the antibiotic by aminoglycoside-modifying enzymes. These enzymes are classified, according to the type of modification, in the following major classes: ACC (acetyltranferases), ANT (nucleotidyltransferases/ adenyltransferases) and APH (phosphotransferases) (Vakulenko & Mobashery, 2003).

2.2.2.2 Chloramphenicol

Resistance to chloramphenicol emerges mostly by enzymatic inactivation by acetylation through chloramphenicol acetyltransferases (CATs) which are coded by *catA* and *catB* genes. Besides this mechanism, inactivation by phosphotransferases, mutations of the target site, permeability barriers and efflux systems – coded by *floR* and *cmlA* genes, were also reported (Schwarz et al, 2004).

2.2.2.3 Fluoroquinolones

Resistance to fluoroquinolones can be chromosome-encoded and plasmid-mediated. Chromosomal resistance results, mainly, from mutations in the target enzymes of fluoroquinolones, DNA gyrase and DNA topoisomerase IV. These mutations occur at specific "quinolone resistance determining regions" in the genes *gyrA*, *gyrB, parC* and *parE*, that are located in the DNA-binding surface, thus reducing the affinity of the quinolone biding. Apart from this system, chromosome-encoded resistance can also result by decreasing the intracellular concentration of the antibiotic either by overexpression of efflux pumps or by diminished expression of outer membrane porins (Jacoby, 2005).

Plasmid-mediated resistance can emerge from three different genes families, *qnr, aac(6')-Ib-cr* and *qepA*. The first one encodes a protein, called Qnr protein, capable of protecting DNA gyrase from quinolones. Currently, there are five families of *qnr* genes: *qnrA*, *qnrB*, *qnrC, qnrD, qnrS* and *qnrVC*. The second gene, *aac(6')-Ib-cr*, is a variant of *aac(6')-Ib,* which confers resistance to aminoglycosides, and has the ability of reducing ciprofloxacin activity. The last mechanism, coded by *qepA* genes, is an efflux pump (Robicsek et al, 2006; Strahilevitz et al, 2009).

2.2.2.4 Sulfonamides

Sulfonamides are synthetic antibiotics so it was not expected that enzymes capable of degrade or modify sulfonamides would emerge. Nevertheless, low level chromosomal resistance occurs by mutations in the *folP* gene that encodes DHPS (Sköld, 2001). Plasmid-mediated resistance can also occur by *sul1, sul2* and *sul3* genes, coding variants of DHPS (Perreten & Boerlin, 2003; Radstrom & Swedberg, 1988; Swedberg & Skold, 1983).

2.2.2.5 Trimethoprim

Trimethoprim as well sulfonamides, is a synthetic antibiotic. Low level resistance can occur via mutations of the *folA* gene which encodes DHFR. High level resistance occurs through acquisition of DHFRs variants, coded by *dfrA* and *dfrB genes* (Sköld, 2001).

2.2.2.6 Tetracyclines

There are three main mechanisms conferring resistance to tetracyclines: efflux-pumps, ribosomal protection proteins (PBPs) and enzymatic inactivation – this latter mechanism has only been identified in Gram-negative bacteria so far. These mechanisms are coded by *tet* (tetracycline resistance) and *otr* (oxytetracycline resistance) genes (Chopra & Roberts, 2001).

3. Antibiotic resistance in clinical environment

Clinical infections can be hospital- or community-acquired and can be caused by a variety of organisms, including viral, fungal and bacterial pathogens.

Hospital-acquired infections, also known as nosocomial infections, are acquired in hospitals or healthcare service units and are unrelated to the original illness that led the patient to the healthcare facility. These infections appear, at least, 2 days after the patient admission or within 30 days after the patient discharge (Horan et al., 2008). By opposition, community-acquired infections are contracted outside the healthcare setting and are present in the moment of admission.

Nosocomial infections represent a real concern since they interfere with the appropriate treatment of the patient and according to *Direção-Geral da Saúde* (Direção-Geral da Saúde, 2014), about 1/3 of these bacterial infections are inevitable. These infections can be transferred by direct or indirect contact either among patients, healthcare workers, visitors, contaminated objects or environmental sources (e.g. hospital food). There are patient and environmental related risk factors for the development of these infections, namely: age more than 70 years, major trauma, acute renal failure, prior use of antibiotics, mechanical ventilation, drugs that affect the immune system (e.g. chemotherapy), indwelling (urethral or suprapubic) catheters and prolonged hospital stay (more than 3 days) (Siegel et al, 2007).

Additionally, nursing homes may serve as a portal for the entry of these organisms into hospital settings. Antibiotic use is high in this type of facilities and the residents require frequent contact with health care providers. These arguments, associated with the fact that hand washing rates of nursing home personnel is low, makes nursing homes a focus of infectious organisms (Paterson & Bonomo, 2005).

Hospital- and community-acquired infections can be monoclonal or polyclonal. Monoclonal infections are caused by organisms clonally related and are the most common type of epidemiology in hospital onset. This outbreak implies a transmission among patients, for example, through carriage on the hands of health care personnel, thus requiring the introduction of a strategy that controls this transmission. Polyclonal infections are more prevalent in the community but can also occur in hospital settings. This type of infection is probably due to selective pressure that is imposed by antibiotic use (Paterson & Bonomo, 2005).

Antibiotic resistant pathogens are increasingly associated with nosocomial infections. Moreover, longer hospital stay promotes the selection of resistant microorganisms. As a consequence, the growing number of resistant pathogens places a burden on healthcare systems by augmenting the mortality and morbidity rates and the diagnostic uncertainties, leading to an increase in treatment costs (Khan & Khan, 2016).

Recently, the term "ESKAPE pathogens" has been used to describe a group of nosocomial pathogens that cause the majority of hospital infections and that have the ability of "escape" the effective therapy. This group encompasses both Gram negative and Gram positive species, namely: *Enterococcus faecium*, *Straphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Rice, 2008). Some authors, besides Louis B. Rice, consider that "K" encompasses both *Klebsiella* and *Escherichia coli* ESBL-producing species (Boucher et al., 2009; Peterson, 2009) while others suggested an alteration to the last "E" to "*Enterobacteriaceae family"* since isolates of *E. coli*, *K. pneumonia*, *Enterobacter* species and other members of this family are a threat that needs more attention (Khan & Khan, 2016).

3.1. Antibiotic resistance in clinical isolates of *Enterobacteriaceae*

The *Enterobacteriaceae* family, comprises several genera of Gram-negative bacteria including human pathogens, such as, *Citrobacter*, *Enterobacter, Escherichia*, K*lebsiella, Morganella, Providencia, Salmonella, Serratia* and *Proteus.* These microorganisms are considered as opportunistic pathogens as they normally cause infection in patients whose defenses are compromised (Brenner & Farmer, 2015).

Enterobacteriaceae bacteria often harbor virulence factors and are responsible for a wide variety of hospital- and community-acquired infections, namely, urinary-tract infections (UTI), bloodstream infections, healthcare-associated pneumonias and several intra-abdominal infections. In particular, *E. coli* is a frequent cause of UTIs and surgical site infections, *Klebsiella* spp. and *Enterobacter* spp. cause pneumonias and, along with other members of this family, have been associated in bloodstream and intra-abdominal infections, like cholangitis and peritonitis (Paterson, 2006). Although UTIs are the most common infections, either community- or hospital acquired – being the latter highly

associated with catheterization, lower respiratory tract and bloodstream infections are the most lethal (Horan et al., 2008).

The primary choice for the treatment of serious infections caused by *Enterobacteriaceae* bacteria are 3rd generation cephalosporins. However, the production of ESBL and AmpC β-lactamases by these organisms leads to resistance against these antibiotics and complicates therapy, remaining an important reason for its failure (Peleg & Hooper, 2010). Organisms like *Enterobacter spp*. and *Morganella morganii* have intrinsic resistance to 3rd generation cephalosporins due to the overproduction of AmpC β-lactamases. The typical scenario when these antibiotics are administered is characterized by initial response followed by recurrence of the infection since the use of 3rd generation cephalosporins results in selection of AmpC-overproducing mutants (Paterson, 2006).

K. pneumoniae and *E. coli* are considered the major ESBL-producing organisms isolated worldwide. In Portugal, *K. pneumoniae* resistance to 3rd generations cephalosporins has been increasing (Figure I.3) and places this country in the second group with higher resistance rates in Europe (Direção-Geral da Saúde, 2016). This scenario is also valid for *E. coli* isolates (Figure I.4), although the resistance rates are lower for these isolates than for *K. pneumoniae* isolates.

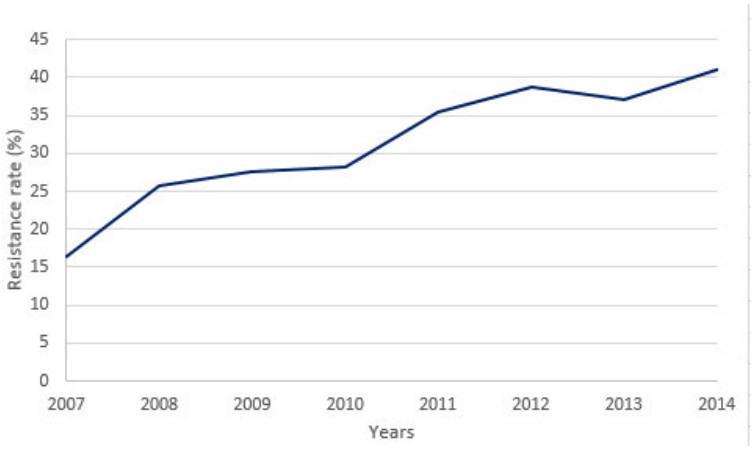

Figure I.3 - K. pneumoniae resistance to 3rd generation cephalosporins in invasive isolates, in Portugal (2007-2014) (Direção-Geral da Saúde, 2016)

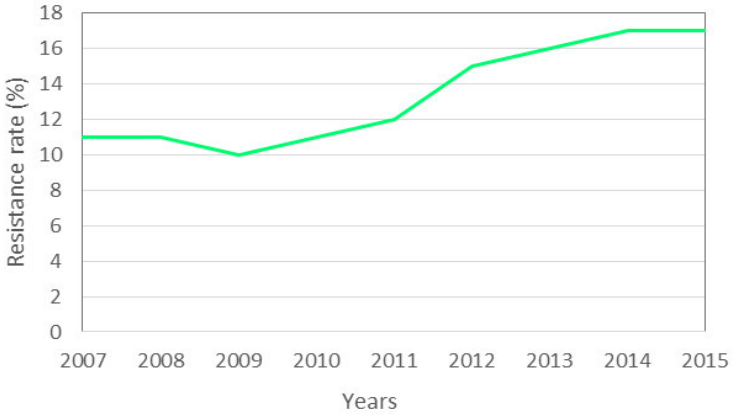

Figure I.4 – Resistance rates to 3rd generation cephalosporins in E. coli isolates found in Portugal (2007-2015). Adapted from: ResistanceMap, available at https://resistancemap.cddep.org/AntibioticResistance.php

Aside from *K. pneumoniae* and *E. coli*, ESBL have also been identified in other members of the *Enterobacteriaceae* family. Regularly, these ESBL-producing organisms are multiresistant. This is due to the presence of ESBL encoding genes and genes coding for resistance to other antibiotic classes on the same plasmid. Typically, ESBL encoding genes are associated to genes that encode resistance to aminoglycosides, sulfonamides and fluoroquinolones (Paterson, 2006; Pitout & Laupland, 2008; Tacão et al, 2014).

This multiresistance has serious consequences for the selection of adequate empirical therapy. Empirical therapy is the initial therapy when the infection was already diagnosed but the results of bacterial cultures identification and antimicrobial susceptibility tests are yet unknown. The presence of a multiresistant phenotype limits the therapeutic choices for infections caused by ESBL-producing bacteria from *Enterobacteriaceae* family and it is known that delays in the administration of appropriate antibiotic therapy are associated with elevated mortality rates (Pitout & Laupland, 2008).

There are many concerns about the adequate therapy when multiresistant *Enterobacteriaceae* organisms cause infections because of the relative ease by which bacteria can develop antibiotic resistance mechanisms. As a result, some of the treatment options rely on carbapenems, including meropenem, imipenem, ertapenem and doripenem, as the first choice for serious infections caused by ESBL-producing bacteria (Peleg & Hooper, 2010). The potential selection of carbapenem-resistant variants sometimes leads to the choice of older and previously discarded antimicrobials such as colistin, which are associated with high toxicity, instead of carbapenems (Boucher et al., 2009). These multiresistant *Enterobacteriaceae* bacteria pose such a serious threat to public health that World Health Organization (WHO) considers them as a "critical group"

for which research and development of antibiotics are needed (Tacconelli & Magrini, 2017).

The misuse and overuse of antibiotics along with the genetic plasticity of bacteria contributed to an increase in antibiotic resistance while the process of discovery and development of new antibiotics has suffered a reduction. This reduction is due to a number of factors, among them are the challenge of screening for new compounds, the time required to approve an antibiotic and the concern about new resistance emergency (Peleg & Hooper, 2010). Taken together, the rapid bacteria adaptation, the absence of new drugs and relative ease by which bacterial infections can be acquired generate a worrying public health concern regarding antibiotic resistance in bacteria.

4. Aims of this work

The *Enterobacteriaceae* family includes a wide diversity of relevant pathogens namely, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii* and *Morganella morganii*. The increasing antibiotic resistance among these bacteria is a global concern, particularly in clinical settings. 3rd generation cephalosporins are a group of antibiotics widely used to treat infections caused by Gram-negative bacteria and are considered critically relevant in human medicine.

A total of 46 isolates were obtained from different human biological samples from several wards in the *Hospital Infante D. Pedro*, *Centro Hospitalar do Baixo Vouga*, *E.P.E.*, Portugal, between January and December of 2014. These isolates were obtained in the scope of a previous work and were selected for analysis based on the suspicion of β-lactamase production provided by the Vitek2® Automatic System. Vitek2® was also used to determine the Minimum Inhibitory Concentration (MICs) to a range of antibiotics for each isolate and to identify the isolate pathogen.

The main goal of this work is to characterize the genetic basis of the resistance phenotypes as well as the dissemination potential of the genes present in these isolates, in this particular clinical setting, in order to contribute to the development of effective strategies to reduce resistance prevalence.

Specific goals are:

- To establish the genetic relatedness between isolates;
- To detect and characterize antibiotic resistance (AR) genes;
- To determine the genetic context of the identified genes;
- To detect and characterize mobile genetic elements such as integrons and plasmids.

II. MATERIAL AND METHODS

1. Clinical isolates

The sampling process, bacteria isolation and identification were performed in the scope of a MSc thesis (Roxo, 2015). All clinical isolates characterized in this work were obtained in *Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E.*, from January to December of 2014.

The 46 isolates selected for this work were obtained from human biological samples from different wards (Table II.1) and were selected based on the suspicion of AmpC production given by Vitek2® Automated System. This was confirmed by a phenotypic test with cefoxitin discs. The co-production of an AmpC and an ESBL was also analyzed. Strains identified by Vitek2® as ESBL producers were confirmed using phenotypic test E-test with a cephalosporin and cephalosporin associated with clavulanic acid.

Vitek2® automated system was also used to determine the MICs to a range of antibiotics for each isolate and to identify the isolate. The results obtained from Vitek2® concerning bacteria identification and resistance phenotype are presented in Table II.1. Results regarding antibiotic susceptibility testing are presented in supplemental material as Table S.1.

Bacterial cells selected for this work (n=46) were cryopreserved at in glycerol at -80ºC.

Table II.1. Origin of the isolates used in this work: provenance of the patient and type of sample from which the isolate was obtained. Bacteria identification and resistance phenotype given by Vitek2® Automated System.

Isolate	Species	Resistance Phenotype	Provenance		Sample
			Inpatients	*Outpatients*	
ER1	*Escherichia coli*	AMC, AMP[a]		X	Urine
ER2	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Urine (Foley catheter)
ER3	*Escherichia coli*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER4	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER5	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Urine
ER6	*Escherichia coli*	AMC, AMP, CIP, LVX, SXT		X	Urine
ER7	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER8	*Escherichia coli*	AMC, AMP, CAZ, CTX, LVX, SXT	X		Urine
ER9	*Enterobacter cloacae*	AMC, AMP		X	Urine (bladder puncture)
ER10	*Enterobacter aerogenes*	AMC, AMP	X		Urine
ER11	*Escherichia coli*	AMC, AMP, CAZ, CTX	X		Pus
ER12	*Serratia marcescens*	Unknown	X		Sputum
ER13	*Citrobacter freundii*	AMC, AMP,CAZ, CTX, CIP, LVX, SXT		X	Urine
ER14	*Morganella morganii*	AMC, AMP, CIP, LVX		X	Pus
ER15	*Escherichia coli*	AMC, AMP, CAZ, CTX	X		Pus
ER16	*Enterobacter aerogenes*	AMC, AMP, CAZ, CTX	X		Urine
ER17	*Enterobacter cloacae*	AMC, AMP	X		Urine (Foley catheter)
ER18	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Pus
ER19	*Escherichia coli*	AMC, AMP, CIP, LVX, SXT	Unknown		Urine
ER20	*Morganella morganii*	Unknown	X		Sputum
ER21	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Urine (Foley catheter)
ER22	*Morganella morganii*	Unknown	X		Sputum
ER23	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Catheter tip
ER24	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER25	*Enterobacter aerogenes*	Unknown	X		Sputum
ER26	*Escherichia coli*	AMC, AMP, CAZ, CTX	X		Pus
ER27	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER28	*Citrobacter freundii*	AMC, AMP, TZP,CAZ, CTX, CIP, LVX, SXT		X	Urine
ER29	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX	X		Pus
ER30	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	X		Urine (Foley catheter)
ER31	*Escherichia coli*	AMC, AMP, CAZ, CTX		X	Urine
ER32	*Morganella morganii*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	X		Pus
ER33	*Escherichia coli*	AMC, AMP, TZP, CAZ, CTX		X	Urine
ER34	*Morganella morganii*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT		X	Urine
ER35	*Escherichia coli*	AMC, AMP, CAZ, CTX	X		Urine
ER36	*Escherichia coli*	AMC, AMP, CAZ, CTX, LVX	X		Urine
ER37	*Enterobacter cloacae*	AMC, AMP, CAZ, CTX		X	Urine
ER38	*Morganella morganii*	AMC, AMP, CAZ, CTX, CIP, LVX		X	Urine (Foley catheter)
ER39	*Escherichia coli*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	X		Urine
ER40	*Escherichia coli*	AMC, AMP, TZP, CIP, LVX		X	Urine (Foley catheter)
ER41	*Escherichia coli*	AMC, AMP, TZP, CIP, LVX		X	Urine
ER42	*Enterobacter aerogenes*	AMC, AMP, TZP,CAZ, CTX	X		Pus
ER43	*Escherichia coli*	AMC, AMP, TZP, CAZ, CTX	X		Pus
ER44	*Enterobacter aerogenes*	AMC, AMP, TZP,CAZ, CTX	X		Pus
ER45	*Enterobacter aerogenes*	AMC, AMP, TZP, CAZ, CTX	X		Pus
ER46	*Providencia stuartii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT		X	Urine

[a]Antibiotic abbreviations: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CTX, cefotaxime; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

2. Genomic DNA extraction

Genomic DNA was extracted from bacterial cells using Silica Bead DNA Gel Extraction Kit #K0513 (ThermoFisher Scientific, USA). The protocol used is described below.

The success of the extraction experiment was confirmed by electrophoresis. A volume of 4µL of DNA was loaded on a 0.8% (w/v) Seakem® LE Agarose (Lonza, USA) gel 1x TAE (5Prime, Deutschland), in a ratio of 3:1 to 6x Loading Dye (MBI Fermentas, Lithuania).

Preparation of genomic DNA from bacterial cells

A. Cell-Lysis

- Lysis protocol:

1. Prepare pellets from fresh cells in Eppendorf tubes, keep frozen if necessary;
2. Resuspend (thawed) cell pellet in 100 µL of Buffer B1;
3. Prepare mastermix of B1 with enzymes: per 1 mL add 2 µL of RNase A, 20 µL of Lysozym and 45 µL of Protease;
4. Add 100 µL mastermix to each cell suspension;
5. Vortex vigorously, incubate for at least 30 minutes at 37ºC;
6. Add 70 µL of Buffer B2;
7. Vortex vigorously, incubate for at least 30 minutes at 55ºC.

At this point, the lysates can be used directly for DNA preparation or can be stored frozen.

- DNA Preparation protocol:

8. Add 500 µL of Binding Solution to the 270 µL of lysate, vortex;
9. Resuspend the Silica Suspension by vortexing for 1 minutes and add 5 µL;
10. Incubate 5 minutes at 55ºC, invert from time to time;
11. Centrifuge 5 seconds, remove supernatant;
12. Suspend the pellet in 500 µL ice cold Wash Buffer;
13. Centrifuge 5 seconds, remove supernatant;
14. Repeat steps 11 and 12;
15. Centrifuge the pellet again and remove all traces of the supernatant;

16. Air-dry the pellet at room temperature (~10 minutes);
17. Resuspend the pellet in 50 µL of TE;
18. Incubate 5 min at 55ºC;
19. Centrifuge 2 minutes;
20. Remove the supernatant = genomic DNA.

Repeat the last centrifugation in case of transferring any silica particles to the DNA extract because this may inhibit subsequent enzymatic reactions, like restriction analysis or PCR.

3. Polymerase Chain Reaction (PCR) amplification

PCR was performed with extracted DNA or cell suspension from all 46 clinical isolates. PCR reactions were performed in a volume of 25 µL in MyCycler Thermal Cycler (BioRad, USA) and all primers used were ordered from StabVida, Portugal or from Sigma, USA. Table II.2 represents the general reaction mixture, used for most of the amplification experiments. In each experiment negative and positive controls were used. The first one consisted in a substitution of the template DNA by the same volume of dH_2O, in the reaction mixture, and the latter contained a DNA or cell suspension positive for the searched gene.

Table II.2. General PCR reaction mixture.

Sample reaction mixture	Volume (µL)
NZYtech 2x Green Master Mix (2,5mM $MgCl_2$; 200µM dNTPs; 0.2U/µL DNA Polymerase)	6.25
Primer Forward (10 µM)	0.75
Primer Reverse (10 µM)	0.75
DNA (50-100ng/µL)/Cell suspension (0.5 McFarland)	1.00
dH_2O	16.75

Table II.3. PCR programs used for DNA amplification.

	PCR Programs							
Temperature (ºC)	A	B	C	D	E	F	G	**Number of cycles**
94	5’	5’	10’	5’	5’	5’	5’	(x1)
94	30’’	30’’	40’’	30’’	30’’	30’’	1’	
X	30’’	30’’	40’’	30’’	30’’	30’’	30’’	(x 30)
72	30’’	1’	1’	90’’	3’	2’	1’	
72	7’	7’	7’	5’	10’	10’	5’	(x1)

X - Annealing temperature for each primer set; A to G – different PCR programs used in this study.

a. Molecular typing of resistant isolates

Molecular typing analysis was performed to evaluate the diversity among the isolates. Three different PCR typing methods were used: BOX element polymerase chain reaction (BOX-PCR), Enterobacterial repetitive intergenic consensus (ERIC)-PCR and Repetitive element sequence-based PCR (REP-PCR). The combination of these thee PCR methods is known as rep-PCR. All primers used in this assay are presented in Table II.4. The PCR program used was Program H and it is presented in Table II.5. Annealing temperatures were 53ºC for BOX-PCR, 52ºC for ERIC-PCR and 40ºC for REP-PCR. The reaction mixture used for ERIC- and REP-PCR is the one described in Table II.2. For BOX-PCR, since it requires only one primer, a volume of 2µL of this primer is added to 6.25µL of NZYtech 2x Green Master Mix and a volume of 16.25µL of dH_2O is added to make a final volume of 25µL.

PCR products were loaded in agarose gels for electrophoresis and the resulting band patterns were normalized and analyzed with software GelCompar II 6.1 (Applied Maths). Similarity matrices were calculated using the Dice coefficient and cluster analysis of similarity matrices was performed using the unweighted-pair group method using arithmetic average (UPGMA).

Table II.4. Primers used for molecular typing assay.

Target	Primer Sequences (5'-3')	Primers Reference
BOX element	BOX A1R: CTACGGCAAGGCGACGCTGACG	(Versalovic et al., 1994)
ERIC element	ERIC 1: AAGTAAGTGACTGGGGTGAGC ERIC 2: ATGTAAGCTCCTGGGGATTCAC	(Versalovic et al., 1994)
Rep element	REP 1R: IIIICGICGICATCIGGC REP 2F: NCGICTTATCIGGCCTAC	(Versalovic et al., 1991)

Table II.5. PCR program used for isolates molecular typing.

	PCR Program timing	
Temperature (ºC)	H	Number of cycles
95	7'	(x1)
95	1'	(x 30)
X	1'	
65	8'	
65	16'	(x1)

X= 53ºC for BOX-PCR; X=52ºC for ERIC-PCR and X=40ºC for REP-PCR.

b. Screening for antibiotic resistance genes

AR genes were screened by PCR according to the phenotype given by Vitek® Automated System**.**

The reaction mixture used for amplification of the AR genes is the same as in Table II.2 and the PCR programs that were used are summarized in Table II.3 as A, B and C. All reaction conditions for each gene targeted are described in Table II.6.

Table II.6. PCR conditions for AR genes amplifications. Programs used are described in table II.3.

Target	Program	Annealing Temperature (ºC)	Primer Sequence (5'-3')	Primers Reference	Amplicon size (bp)	Control Strains	Control Strains Reference
bla$_{CTX-M}$	B	55	CTX-M F Lu: SCVATGTGCAGYACCAGTAA CTX-M R Lu: GCTGCCGGTYTTATCVCC	(Lu et al., 2010)	652	*E. coli* C65	(Tacão et al., 2012)
*qnr*A	A	53	qnrA_F: TTCTCACGCCAGGATTTG qnrA_R: CCATCCAGATCGGCAAA	(Guillard et al., 2011)	521	*Shewanella algae Sh2*	(Tacão et al., unpublished
*qnr*B	A	53	qnrB_F: GGMATHGAAATTCGCCCACTG qnrB_F: TTYGCBGYYCGCCAGTCG	(Cattoir et al., 2007) (Guillard et al., 2011)	261	*K. pneumoniae* Kp1	(Alves et al., 2014)
*qnr*S	A	54	qnrS_F: GCAAGTTCATTGAACAGGGT qnrS_R: TCTAAACCGTCGAGTTCGGCG	(Cattoir et al., 2007)	428	*K. oxytoca* Ko25	(Alves et al., 2014)
aac(6')-ib	B	54	Aac-6'-ib_F: TTGCGATGCTCTATGAGTGGCTA Aac-6'-ib_R: CTCGAATGCCTGGCGTGTTT	(Park et al., 2006)	482	-	-
sul1	A	50	Sul1_F: CTG AACGATATCCAAGGATTYCC Sul1_R: AAAAATCCCACGGRTC	(Heuer & Smalla, 2007)	239	*Aeromonas media*	(Alves et al., 2014)
sul2	A	60	Sul2_F: GCGCTCAAGGCAGATGGCAT Sul2_R: GCGTTTGATACCGGCACCCG	(Henriques et al., 2006)	293	*E. coli* A237	(Alves et al., 2014)
bla$_{TEM}$	C	60	MultiTEM_For: CATTTCCGTGTCGCCCTTATTC MultiTEM_Rev: CGTTCATCCATAGTTGCCTGAC	(Dallenne et al., 2010)	800	*E. coli* A237	(Alves et al., 2014)
bla$_{SHV}$	C	60	MultiSHV_For: AGCCGCTTGAGCAAATTAAAC MultiSHV_Rev: ATCCCGCAGATAAATCACCAC	(Dallenne et al., 2010)	713	*E. coli* A237	(Alves et al., 2014)

c. Detection of *bla*$_{\text{AmpC-Like}}$ genes

Detection of plasmid-mediated AmpC-Like β-lactamases coding genes was performed by multiplex PCR, according to (Dallenne et al., 2010). The PCR program used is listed in Table II.3 as PCR Program C.

Primers and respective concentrations are summarized in Table II.7. The reaction mixture differed from the one in Table II.2, since the search of *bla*$_{\text{AmpC-Like}}$ genes is carried out by a multiplex PCR, and it is presented in Table II.8.

Table II.7. Primers and respective concentrations for *bla*$_{\text{AmpC-Like}}$ genes.

Target	Primer Sequences	Amplicon Size (bp)	[Primers] (pmol/ µL)
bla$_{\text{ACC}}$	ACC_F:CACCTCCAGCGACTTGTTAC ACC_R:GTTAGCCAGCATCACGATCC	346	0.2
bla$_{\text{FOX}}$	FOX_F:CTACAGTGCGGGTGGTTT FOX_R:CTATTTGCGGCCAGGTGA	162	0.5
bla$_{\text{MOX}}$	MOX_F:GCAACAACGACAATCCATCCT MOX_R:GGGATAGGCGTAACTCTCCCAA	895	0.2
bla$_{\text{DHA}}$	DHA_F: TGATGGCACAGCAGGATATTC DHA_R: GCTTTGACTCTTTCGGTATTCG	997	0.5
bla$_{\text{CIT}}$	CIT_F: CGAAGAGGCAATGACCAGAC CIT_R: ACGGACAGGGTTAGGATAGY	538	0.2
bla$_{\text{EBC}}$	EBC_F: CGGTAAAGCCGATGTTGCG EBC_R: AGCCTAACCCCTGATACA	683	0.2

Table II.8. Reaction mixture for *bla*$_{\text{AmpC-Like}}$ genes Multiplex PCR.

Sample reaction mixture	Volume (µL)
Mix NzyTech (2.5mM $MgCl_2$; 200µM dNTPs; 0.2U/µL DNA Polymerase)	6.25
Primer F MOX (10µM)	0.50
Primer R MOX (10µM)	0.50
Primer F CIT (10µM)	0.50
Primer R CIT (10µM)	0.50
Primer F FOX (10µM)	1.25
Primer R FOX (10µM)	1.25
Primer F ACC (10µM)	0.50
Primer R ACC (10µM)	0.50
Primer F DHA (10µM)	1.25
Primer R DHA (10µM)	1.25
Primer F EBC (10µM)	0.50
Primer R EBC (10µM)	0.50
DNA (50-100ng/µL)	1.00
dH_2O	8.75

d. Determination of genomic context of antibiotic resistance genes

Genomic context of the detected AR genes was investigated by analyzing the regions surrounding these genes. This was performed by PCR and subsequent sequencing of the resulting amplicons. Isolates carrying *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes were characterized. *bla*$_{CTX-M}$-positive isolates were characterized by inspecting for the presence of the IS*Ecp1* insertion sequence upstream of the gene, and downstream by the presence of *orf477*. *bla*$_{CMY}$-positive isolates were tested for IS*Ecp1*, located upstream of the gene. The reaction mixture used for the determination of the genomic context is the one listed in Table II.2 and Table II.9 describes PCR conditions and primers used for characterization of these regions.

Table II.9. PCR conditions and primers used to characterize AR genes surrounding regions.

Target	Program	Annealing Temperature (ºC)	Primer Sequence (5'-3')	Primer Reference
IS*Ecp1*	D	52	ISEcp1_U1_F: AAAAATGATTGAAAGGTGGT CTX_R Lu: GCTGCCGGTYTTATCVCC	(Saladin et al., 2002) (Lu et al., 2010)
	D	52	ISEcp1_U1_F: AAAAATGATTGAAAGGTGGT CIT_R: ACGGACAGGGTTAGGATAGY	(Saladin et al., 2002) (Dallenne et al., 2010)
IS*26*	D	52	IS26U: AGCGGTAAATCGTGGAGTGA CTX_R Lu: GCTGCCGGTYTTATCVCC	(Saladin et al., 2002) (Lu et al., 2010)
orf477	D	52	CTX_F Lu: SCVATGTGCAGYACCAGTAA Orf477_R: ACTTCAAAAATTATGCCACC	(Lu et al., 2010) (Eckert et al., 2006)

e. Determination of integrase genes and integron gene arrays

The presence of integrase genes was evaluated by searching the genes *intI1* and *intI2*, which code for class 1 and 2 integrases, respectively. The integron content was also characterized for the isolates that harbored the *intI1* and/or *intI2* genes. This was done by PCR and subsequent sequencing reaction of the resulting amplicons. The reaction mixture used is present in Table II.2. Table II.10 describes PCR conditions and primers used to characterize integrase genes and the integrons' arrays.

Table II.10. PCR conditions and primers used to characterize integrase genes and surrounding regions.

Target	Program	Annealing Temperature (ºC)	Primer Sequence (5'-3')	Amplicon size (bp)	Primers Reference
intI1 gene	A	55	IntI1_F: CCTCCCGCACGATGATC IntI_R: TCCACGCATCGTCAGGC	280	(Kraft et al., 1986)
intI2 gene	A	50	IntI2_F: TTATTGCTGGGATTAGGC IntI2_R: ACGGCTACCCTCTGTTATC	233	(Goldstein et al., 2001)
Content of class 1 integrons	E	55	5'CS: GGCATCCAAGCAGCAAG 3'CS: AAGCAGACTTGACCTGA	Variable	(Levesque et al., 1995)
	F	52	IntI1_F: CCTCCCGCACGATGATC 3'CS: AAGCAGACTACTTGACCTGA		(Kraft et al., 1986) (Levesque et al., 1995)
Content of class 2 integrons	E	55	Hep F: CGGGATCCCGGACGGCATGCACGATTTGTA Hep R: GATGCCATCGCAAGTACGAG	Variable	(White et al., 2001)

4. DNA electrophoresis and visualization

PCR products were separated by electrophoresis at 80V for 1h20 for confirming DNA extraction, detection of AR genes and genomic context, and 2h45 for molecular typing. PCR products were loaded on a Seakem® LE Agarose (Lonza, USA) gel 1x TAE (5Prime, Deutschland), of 0.8% (w/v) for DNA extraction and 1.5% (w/v) for the remaining experiments. In each gel was loaded the molecular weight marker GeneRuller™ DNA Ladder Mix (MBI Fermentas, Lithuania).

For visualization, the gels were stained with a solution of 0.5 µg/mL ethidium bromide (Sigma, USA) for 10-15 minutes and washed in dH_2O for 15-20 minutes. Gel images were obtained using Molecular Imager® GelDoc™ XR+ System (Bio-Rad, USA) under UV light.

5. PCR products sequencing

Products resulting from the PCR experiments were used as template in the sequencing reaction carried out by GATC Company (Germany).

The final sequences were visualized and edited with FinchTV program (Geopiza, USA). Similarities were searched using BLAST software at the National Biotechnology Information (NCBI) website against sequence databases.

6. Conjugation experiments

Conjugation assays were performed for strains containing *bla*$_{\text{CTX-M}}$ genes and for strains containing *bla*$_{\text{AmpC}}$ genes. For *bla*$_{\text{CTX-M}}$ genes, from 18 isolates containing these genes, only 8 were used as donors based on their resistance phenotype to cefotaxime. These donors were previously identified as *M. morganii* (n=6) and *C. freundii* (n=2) carrying *bla*$_{\text{CTX-M-15}}$ variant. For *bla*$_{\text{AmpC}}$ genes, the resistance phenotype to cefotaxime also reduced the number of donors, in this case, from 11 to 7 isolates, from which: *E. coli* (n=6) and *E. aerogenes* (n=1), carrying *bla*$_{\text{CMY}}$ genes. The rifampicin resistant *E. coli* CV601 was used as recipient strain. Conjugation protocol is described below.

Resulting transconjugants were selected in Plate Count Agar (PCA) plates supplemented with rifampicin (150 mg/mL) and cefotaxime (4 mg/mL). To confirm the identity of the transconjugant and the acquisition of the plasmid, BOX-PCR and detection of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ genes was performed as described above. Upon the confirmation of the transconjugant, genomic characterization was performed in order to identify the genes previously found in the donor isolate.

Conjugation Protocol

Day 1: Overnight grow of donors and recipient strains in 6 mL of LB (Merck, Germany) broth.
Adjust the optical density (OD) at 600nm in a way that it falls between 0.6-0.8.
Place donors and recipient strains in selective plates for mutants' rate evaluation.

Day 2: Mix 900 µL of donor cells and 900 µL of recipient cells culture (1:1 ratio).
Centrifuge at 10.000 rpm for 5 minutes at room temperature.
Discard liquid and add 1 mL of fresh LB medium.
Incubate overnight, without shaking, at 37ºC.

Day 3: Centrifuge at 10.000 rpm for 5 minutes at room temperature.
Resuspend cells in 1 mL of NaCl 0.9%.
Inoculate directly (10^0) in plates with antibiotics or dilute:
10^{-1}: dilute 100 µL of 10^0 in 900 µL of NaCl 0.9%;
10^{-2}: dilute 100 µL of 10^{-1} in 900 µL of NaCl 0.9%.
Spread 100 µL of each in plates with antibiotics.

Day 4: Incubate overnight, at 37ºC or 28ºC.

7. Plasmid DNA extraction

Plasmid DNA, from donor cells that gave a positive result in the conjugation experiment and respective transconjugant, was extracted with E.Z.N.A.® Plasmid Mini Kit II (Omega, bio-tek, USA). The protocol used was Spin Protocol and it is described below.

An alteration was performed on step 22 of the protocol. Instead of using 80-100µL of Elution Buffer, plasmid DNA was eluted in 40µL of Elution Buffer.

E.Z.N.A.® Plasmid Mini Kit II – Spin Protocol

1. Isolate a single colony from freshly streaked selective plate, and inoculate a culture of 10-15mL (50µg/mL) LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hours at 37ºC with vigorous shaking (~300rpm). Use culture tube or a flask with a volume at least 4 times the volume of the culture. It is strongly recommended that an end A negative strain of *E. coli* be used for routine plasmid isolation.
2. Centrifugation at 5,000 x *g* for 10 minutes at room temperature.
3. Decant or aspirate the medium and discard.
4. Add 500µL Solution I/RNase A. Vortex or pipet up and down to mix it thoroughly. Complete resupension of cell pellet it vital for obtaining good yields.
5. Transfer suspension into new 2mL microcentrifuge tube.
6. Add 500µL of Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minutes incubation may be necessary.
7. Add 700µL Solution III. Immediately invert several times until a flocculent white precipitate forms.
8. Centrifuge at maximum speed (≥13,000 x *g*) for 10 minutes at room temperature. A compact white pellet will form. Promptly proceed to the next step.
9. Insert a HiBind® DNA Mini Column into a 2mL Collection Tube.

   Optional Protocol for Column Equilibration:

   1. Add 100µL 3M NaOH to the HiBind® DNA Mini Column.
   2. Centrifuge at maximum speed for 30-60 seconds.
   3. Discard the filtrate and reuse the collection tube.

10. Transfer 700µL cleared lysate from step 8 CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
11. Centrifuge at maximum speed for 1 minute.
12. Discard the filtrate and reuse the collection tube.
13. Repeat steps 10-12 until all cleared lysate has been transferred to the HiBind® DNA Mini Column.
14. Add 500µL HBC Buffer.
15. Centrifuge at maximum speed for 1 minute.
16. Discard the filtrate and reuse collection tube.
17. Add 700µL DNA Wash Buffer.

18. Centrifuge at maximum speed for 1 minute.
19. Discard the filtrate and reuse collection tube.

    Optional: Repeat steps 17-19 for a second DNA Wash Buffer wash step.

20. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
21. Transfer the HiBind® DNA Mini Column to a clean 1,5mL microcentrifuge tube.
22. Add 40µL Elution Buffer directly to the center of the column membrane.
23. Let sit at room temperature for 1 minute.
24. Centrifuge at maximum speed for 1 minute.
25. Store DNA at -20ºC.

8. Replicon typing

Detection of IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, Frep subgroups), IncHI1, IncHI2, IncI1, IncK, IncX, IncL/M, IncN, IncP, IncT, IncW and IncY replicons was performed by simplex PCR for Frep subgroup and multiplex PCR for the remaining groups, according to the PCR-based inc/rep typing method developed by Alessandra Carattoli et al. (Carattoli et al., 2005). The PCR program used for this amplification is listed in Table II.3 as Program G and the annealing temperatures were 60ºC and 52ºC for the multiplex and simplex PCR, respectively. Reaction conditions and primers are summarized in Table II.11 and Table II.12, respectively. Positive controls used in replicon typing assay are cloned amplicons and were gently given by Alessandra Carattoli (Carattoli et al., 2005).

Table II.11. Reaction mixture for *bla*$_{\text{AmpC-Like}}$ genes Multiplex PCR.

	Volume (µL)			
Sample Reaction Mixture	**Multiplex 1**	**Multiplex 2**	**Multiplex 3**	**Simplex 1**
Mix NzyTech (2.5mM $MgCl_2$; 200µM dNTPs; 0.2U/µL DNA Polymerase)	6.25	6.25	6.25	6.25
Primer F A/C (10µM)	1.00	-	-	-
Primer R A/C (10µM)	1.00	-	-	-
Primer F FIC (10µM)	1.00	-	-	-
Primer R FIC (10µM)	1.00	-	-	-
Primer F P (10µM)	1.00	-	-	-
Primer R P (10µM)	1.00	-	-	-
Primer F T (10µM)	1.00	-	-	-
Primer R T (10µM)	1.00	-	-	-
Primer R B/O (10µM)	1.00	-	-	-
Primer F K/B (10µM)	1.00	1.00	-	-
Primer R K (10µM)	-	1.00	-	-
Primer F W (10µM)	-	1.00	-	-
Primer R W (10µM)	-	1.00	-	-
Primer F FIIA (10µM)	-	1.00	-	-
Primer R FIIA (10µM)	-	1.00	-	-
Primer F FIA (10µM)	-	1.00	-	-
Primer R FIA (10µM)	-	1.00	-	-
Primer F FIB (10µM)	-	1.00	-	-
Primer R FIB (10µM)	-	1.00	-	-
Primer F Y (10µM)	-	1.00	-	-
Primer R Y (10µM)	-	1.00	-	-
Primer F I1 (10µM)	-	-	1.00	-
Primer R I1 (10µM)	-	-	1.00	-
Primer F X (10µM)	-	-	1.00	-
Primer R X (10µM)	-	-	1.00	-
Primer F HI1 (10µM)	-	-	1.00	-
Primer R HI1 (10µM)	-	-	1.00	-
Primer F HI2 (10µM)	-	-	1.00	-
Primer R HI2 (10µM)	-	-	1.00	-
Primer F N (10µM)	-	-	1.00	-
Primer R N (10µM)	-	-	1.00	-
Primer F L/M (10µM)	-	-	1.00	-
Primer R L/M (10µM)	-	-	1.00	-
Primer F Frep (10µM)	-	-	-	1.00
Primer R Frep (10µM)	-	-	-	1.00
H_20	13.75	5.75	5.75	15.75
Cell suspension (0.5 McFarland)	1.00	1.00	1.00	1.00

Table II.12. .Reaction conditions and primers used for the detection of plasmid Inc groups.

Target	Primer Sequences (5'-3') (Carattoli et al., 2005)	Amplicon size (bp)	PCR Group
A/C	A/C F: GAGAACCAAAGACAAAGACCTGGA A/C R: ACGACAAACCTGAATTGCCTCCTT	465	Multiplex 1
FIC	FIC F: GTGAACTGGCAGATGAGGAAGG FIC R: TTCTCCTCGTCGCCAAACTAGAT	262	
P	P F: CTATGGCCCTGCAAACGCGCCAGAAA P R: TCACGCGCCAGGGCGCAGCC	534	
T	T F: TTGGCCTGTTTGTGCCTAAACCAT T R: CGTTGATTACACTTAGCTTTGGAC	750	
B/O	B/O R: TCTGCGTTCCGCCAAGTTCGA	159	
K/B	K/B F: GCGGTCCGGAAAGCCAGAAAAC K R: TCTTTCACGAGCCCGCCAAA	160	Multiplex 2
W	W F: CCTAAGAACAACAAAGCCCCCG W R: GGTGCGCGGCATAGAACCGT	242	
FIIA	FIIs F: CTGTCGTAAGCTGATGGC FIIs R: CTCTGCCACAAACTTCAGC	270	
FIA	FIA F: CCATGCTGGTTCTAGAGAAGGTG FIA R: GTATATCCTTACTGGCTTCCGCAG	462	
FIB	FIB F: GGAGTTCTGACACACGATTTTCTG FIB R: CTCCCGTCGCTTCAGGGCATT	702	
Y	Y F: AATTCAAACAACACTGTGCAGCCTG Y R: GCGAGAATGGACGATTACAAAACTTT	765	
I1	I1 F: CGAAAGCCGGACGGCAGAA I1 R: TCGTCGTTCCGCCAAGTTCGT	139	Multiplex 3
X	X F: AACCTTAGAGGCTATTTAAGTTGCTGAT X R: TGAGAGTCAATTTTTATCTCATGTTTTAGC	376	
HI1	HI1 F: GGAGCGATGGATTACTTCAGTAC HI1 R: TGCCGTTTCACCTCGTGAGTA	471	
HI2	HI2 F: TTTCTCCTGAGTCACCTGTTAACAC HI2 R: GGCTCACTACCGTTGTCATCCT	644	
N	N F: GTCTAACGAGCTTACCGAAG N R: GTTTCAACTCTGCCAAGTTC	559	
L/M	L/M F: GGATGAAAACTATCAGCATCTGAAG L/M R: CTGCAGGGGCGATTCTTTAGG	785	
Frep	Frep F: TGATCGTTTAAGGAATTTTG Frep R: GAAGATCAGTCACACCATCC	270	Simplex 1

III. RESULTS

1. Molecular typing

The 46 isolates, obtained from different human biological samples and wards in the *Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E.,* were obtained in the scope of a previous work and were selected for analysis based on the suspicion of β-lactamase production provided by the Vitek2® Automatic System. After the species identification, the 46 clinical isolates, identified as: *Citrobacter freundii* (n=2), *Escherichia coli* (n=16), *Enterobacter aerogenes* (n=6), *Enterobacter cloacae* (n=3), *Morganella morganii* (n=17), *Providencia stuartii* (n=1), *Serratia marcescens* (n=1), were typed to assess the genetic relatedness between them.

Molecular typing was done by BOX-, ERIC- and REP-PCR (rep-PCR). Figure III.1 represents the electrophoresis result of the ERIC-PCR. Upon the combined analysis of the fingerprints using the software GelCompar II ®, the resulting dendrogram (Figure III.2) was evaluated to assess de genetic diversity of the collection. Isolates displaying fingerprints with more than 95% similarity were considered clonal.

In general, the isolates clustered according to their phylogenetic affiliation, in accordance to the species identification given by Vitek2® automated system. *M. morganii* isolates grouped in one cluster, exhibiting less than 50% similarity with other clusters. *E. coli* and *E. aerogenes* isolates also grouped in a distinct cluster (less than 40% similarity with other clusters). However, for isolates ER17 (*E. cloacae*), ER46 (*P. stuartii*) and ER12 (*S. marcescens*), their grouping was not evident, appearing in different clusters. There is a high diversity between isolates among the different clusters, composed by the species present in the collection. A total of 45 different patterns were detected, since isolates ER42 and ER44 were considered to derive from the same strain based on the 95% similarity cutoff. Despite this consideration, both isolates were included in all tests performed in this study.

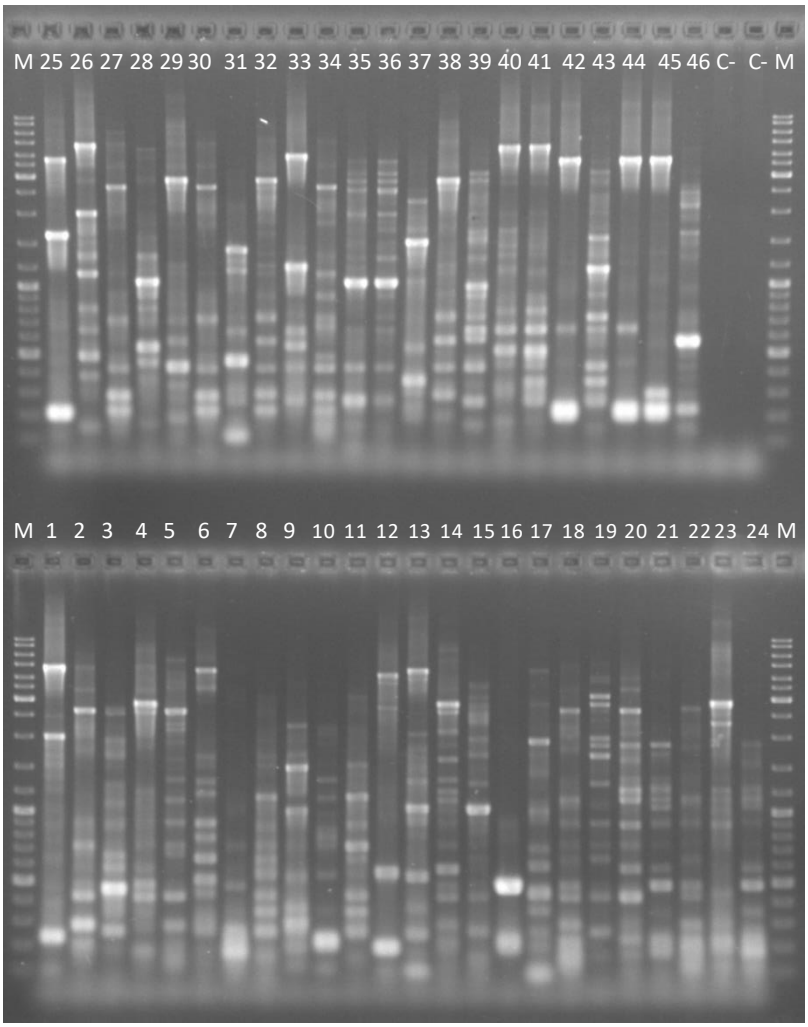

Figure III.1 – DNA fingerprtins obtained with ERIC-PCR method. M – DNA molecular marker; 1-46 – Clinical isolates; C⁻ – negative control.

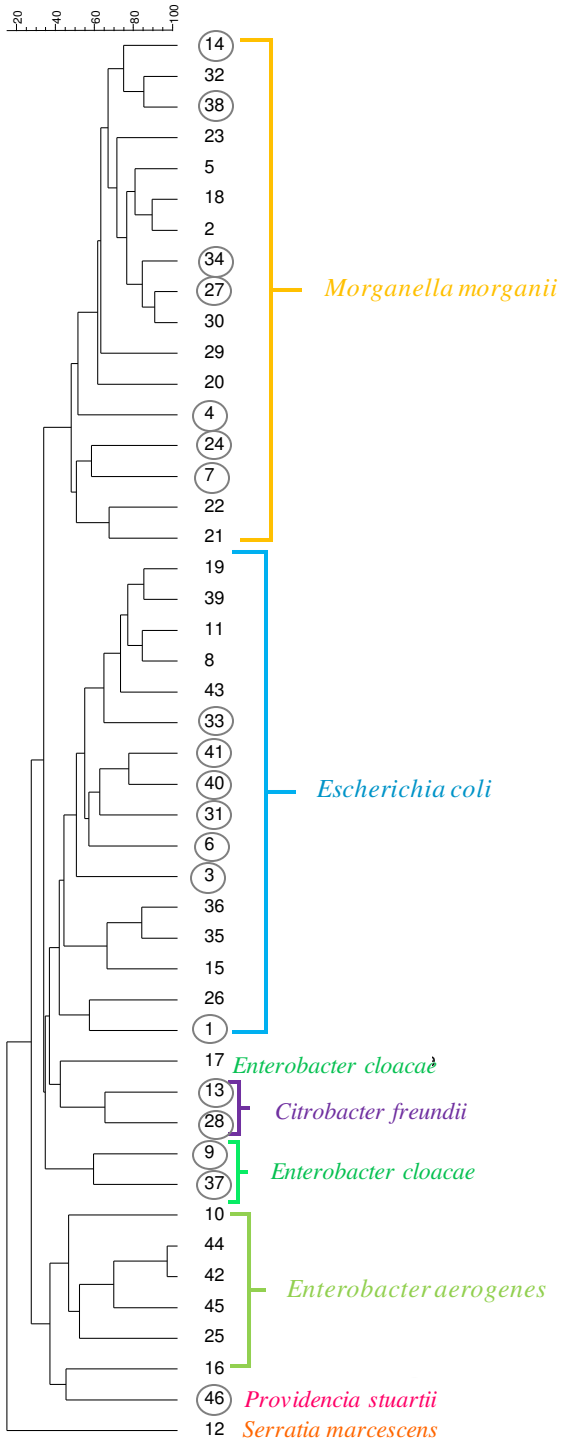

Figure III.2 – Dendrogram showing genetic relatedness of all 46 isolates determined by analysis of rep-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster methods. Circled identifications correspond to the isolates with origin in the community.

2. Antibiotic resistance genes

The presence of clinically relevant AR genes was confirmed by PCR amplification using primers whose gene targets are conserved regions. The choice of these genes relied on the resistance phenotype given by Vitek2® for a certain range of antibiotics, belonging to the following families: penicillins, 2nd and 3rd generation cephalosporins, carbapenems, fluoroquinolones, sulfamethoxazole/trimethoprim, aminoglycosides and nitrofurantoin. Based on this information, PCR analysis was performed in order to detect the presence of genes that are known to confer resistance phenotype to these antibiotics. Table III.1 summarizes genes tested for each antibiotic family.

Table III.1. List of tested genes according to the resistance phenotype given by Vitek2® Automated System.

Antibiotic families	Tested Genes
Penicillins (AMP, AMC, TZP) **3rd generation cephalosporins** (CAZ, CTX)	bla_{SHV} bla_{TEM}
3rd generation cephalosporins (CAZ, CTX)	bla_{CTX-M} bla_{AmpC}
Fluoroquinolones (CIP, LVX)	*qnr*A *qnr*B *qnr*S *aac(6')-ib-cr*
Sulfamethoxazole/ trimethoprim (SXT)	*sul1* *sul2*

Results obtained by PCR screening of the AR genes referred above, along with the resistance phenotypes given by Vitek2®, are presented in Table III.2.

Table III.2. Characterization of clinical *Enterobacteriaceae* isolates: antibiotic resistance phenotypes and genotypes, presence of integrases and integron content.

Isolate	Species	Resistance Phenotype	β-lactamases genes				Fluoroquinolones R genes			*aac(6')-ib* gene	Sulfonamides R genes		Integrase	Gene cassettes array[b]
			bla$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{AmpC}$	*qnrA*	*qnrB*	*qnrS*		*sul1*	*sul2*		
ER1	*E. coli*	AMC, AMP	+	-	-	-	-	-	-	+	-	-	1	ND[c]
ER2	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER3	*E. coli*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	+	-	-	-	-	-	-	+	-	-	1	*dfrA15*
ER4	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	-	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER5	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER6	*E. coli*	AMC, AMP, CIP, LVX, SXT	+	-	+	-	-	-	-	+	+	-	1	*dfrA17, aadA5*
ER7	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	-	*DHA*	-	-	-	+	+	+	1	*hyp prot, dfrA12, aadA2*
ER8	*E. coli*	AMC, AMP, CAZ, CTX, LVX, SXT	+	-	-	*CMY*	-	-	-	+	+	+	1	ND
ER9	*E. cloacae*	AMC, AMP	+	-	-	*CMY*	-	-	-	+	-	-	1	ND
ER10	*E. aerogenes*	AMC, AMP	+	-	-	-	-	-	-	+	-	-	1	ND
ER11	*E. coli*	AMC, AMP, CAZ, CTX	+	-	-	*CMY*	-	-	-	+	-	-	1	ND
ER12	*S. marcescens*	Unknown	+	-	-	-	-	-	-	+	-	-	1	ND
ER13	*C. freundii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*CMY*	-	+	-	*aacA4cr*[d]	+	+	1	ND
ER14	*M. morganii*	AMC, AMP, CIP, LVX	+	-	-	*DHA*	-	-	-	+	+	-	1	*aadB*
ER15	*E. coli*	AMC, AMP, CAZ, CTX	+	-	-	-	-	-	-	+	+	-	1	ND
ER16	*E. aerogenes*	AMC, AMP, CAZ, CTX	+	-	-	-	-	-	-	+	-	-	1	ND
ER17	*E. cloacae*	AMC, AMP	+	-	-	*EBC*	-	-	-	+	-	-	1	ND
ER18	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER19	*E. coli*	AMC, AMP, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	-	-	1	ND
ER20	*M. morganii*	Unknown	+	-	+	*DHA*	-	-	-	+	-	-	1	*dfrA15*
ER21	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	+	1	ND
ER22	*M. morganii*	Unknown	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER23	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*aadB, catB3*
ER24	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	+	1	*dfrA12, hyp prot, aadA2*
ER25	*E. aerogenes*	Unknown	+	+	-	-	-	-	-	+	-	-	-	
ER26	*E. coli*	AMC, AMP, CAZ, CTX	+	-	-	*CMY*	-	-	-	+	-	-	-	
ER27	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER28	*C. freundii*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	+	+	+	-	+	-	-	+	+	+	1, 2	*ND; dfrA1, sat2, aadA1*
ER29	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX	+	+	+	*DHA*	-	-	-	+	-	-	-	
ER30	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER31	*E. coli*	AMC, AMP, CAZ, CTX	+	-	-	*CMY*	-	-	-	-	-	-	-	
ER32	*M. morganii*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	+	-	-	*DHA*	-	-	-	+	+	-	1	*aadB*
ER33	*E. coli*	AMC, AMP, TZP, CAZ, CTX	+	-	-	*CMY*	-	-	-	+	-	+	1	ND
ER34	*M. morganii*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	+	-	+	*DHA*	-	-	-	+	+	-	1	*dfrA15*
ER35	*E. coli*	AMC, AMP, CAZ, CTX	+	-	+	-	-	-	-	+	-	-	1	*dfrA15*

(continued on the next page)

Table III.2. (continued)

Isolate	Species	Resistance Phenotype[a]	β-lactamases genes				Fluoroquinolones R genes			*aac(6')-ib* gene	Sulfonamides R genes		Integrase	Gene cassettes array
			bla_{TEM}	bla_{SHV}	bla_{CTX-M}	bla_{AmpC}	*qnrA*	*qnrB*	*qnrS*		*sul1*	*sul2*		
ER36	*E. coli*	AMC, AMP, CAZ, CTX, LVX	+	-	+	-	-	-	-	+	-	-	-	
ER37	*E. cloacae*	AMC, AMP, CAZ, CTX	+	-	-	-	-	-	-	+	-	-	-	
ER38	*M. morganii*	AMC, AMP, CAZ, CTX, CIP, LVX	+	-	-	*DHA*	-	-	-	+	+	-	1	*aadB*
ER39	*E. coli*	AMC, AMP, TZP, CAZ, CTX, CIP, LVX, SXT	+	-	-	*CMY*	-	-	-	+	-	+	1	ND
ER40	*E. coli*	AMC, AMP, TZP, CIP, LVX	+	-	-	-	-	-	-	+	-	+	-	
ER41	*E. coli*	AMC, AMP, TZP, CIP, LVX	+	-	-	*CMY*	-	-	-	+	-	-	-	
ER42	*E. aerogenes*	AMC, AMP, TZP,CAZ, CTX	+	-	-	-	-	-	-	-	-	-	-	
ER43	*E. coli*	AMC, AMP, TZP, CAZ, CTX	+	-	-	*CMY*	-	-	-	+	-	-	-	
ER44	*E. aerogenes*	AMC, AMP, TZP,CAZ, CTX	+	-	-	*CMY*	-	-	-	+	-	+	-	
ER45	*E. aerogenes*	AMC, AMP, TZP, CAZ, CTX	-	-	-	-	-	-	-	+	-	-	-	
ER46	*P. stuartii*	AMC, AMP, CAZ, CTX, CIP, LVX, SXT	+	-	-	-	-	-	-	+	+	+	1	ND

[a]Antibiotic abbreviations: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CTX, cefotaxime; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

[b] Gene cassettes identified by nucleotide sequencing and comparison against the sequences available in GenBank database using BLAST software.

[c] ND, not detected; + and -, indicate gene presence and absence, respectively.

[d] *aacA4cr* or *aac(6')-ib-cr* –gene coding the aminoglycoside acetyltransferase variant, aac(6')-ib-cr , which confers resistance to fluoroquinolones.

Isolates displaying resistance to penicillins and 3rd generation cephalosporins were tested for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{AmpC}$ presence. The gene *bla*$_{TEM}$ was the most frequently detected, present in 45 isolates. *bla*$_{SHV}$ gene was only present in 3 isolates identified as *E. aerogenes, M. morganii* and *C. freundii*. The presence of *bla*$_{CTX-M}$ gene was detected in 18 isolates from which: *M. morganii* (n=12), *E. coli* (n=4) and *C. freundii* (n=2). The strategy used to search for the presence of *bla*$_{AmpC}$ genes allowed the distinction among the AmpC β-lactamases types. *bla*$_{AmpC}$ genes were detected in a total of 30 isolates from which: *bla*$_{DHA}$ (n=18) in isolates of *E. coli* (n=1) and isolates of *M. morganii* (n=17), *bla*$_{EBC}$ (n=1) in 1 isolate of *E. aerogenes, bla*$_{CMY}$ (n=11) in isolates of *E. coli* (n=8), *E. aerogenes* (n=1), *E. cloacae* (n=1), *C. freundii* (n=1).

All isolates were tested for genes that confer resistance or at least reduce the susceptibility to fluoroquinolones, namely: *qnrA, qnrB* and *qnrS*. *qnrA* gene was found only in one *C. freundii* as well as *qnrB*, although in distinct isolates. Gene *qnrS* was absent in all 46 *Enterobacteriaceae* isolates. aac(6')-ib-cr is a variant of an aminoglycoside acetyltransferase, coded by *aac(6')-ib-cr* gene, that has the ability to modify certain quinolones. *aac(6')-ib* gene was present in 44 isolates belonging to all 7 identified species. Only isolate ER13 was sequenced and it was confirmed that it harbors *aac(6')-ib-cr* gene variant. For the remaining isolates, the variant was not confirmed.

sul1 and *sul2* genes were present in 21 and 11 isolates, respectively. *sul1* was present in *M. morganii* (n=15), *E. coli* (n=3), *C. freundii* (n=2) and *P. stuartii* (n=1). *sul2* was present in less isolates than *sul1*, from which: *M. morganii* (n=3), *E. coli* (n=4), *C. freundii* (n=2), *E. aerogenes* (n=1) and *P. stuartii* (n=1). A total of 9 isolates harbor both *sul1* and *sul2* genes.

2.1. Genomic context and variant of the *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes

Isolates carrying *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes were characterized in order to evaluate their genomic context. Sequencing analysis of the PCR amplicons provided the information about the gene variant.

The presence of the IS*Ecp1* insertion sequence upstream of the *bla*$_{CTX-M}$ gene and the presence of *orf477* downstream was searched in *bla*$_{CTX-M}$-positive isolates. From 18 *bla*$_{CTX-M}$-positive isolates, 17 were positive for the presence of IS*Ecp1* upstream of the

gene and *orf477* downstream. Using BLAST software, the gene variant was confirmed to be *bla*$_{\text{CTX-M-15}}$ in all 17 isolates. Figure III.3 represents the genomic context of bla$_{\text{CTX-M-15}}$.

The isolate ER6 is the only one for which the upstream region and the gene variant are unknown, only the downstream region is known – *orf477* is present. For this isolate, amplification between IS*Ecp1* and the *bla*$_{\text{CTX-M}}$ gene gave a negative PCR result. Another possible context was tested, namely, the presence of IS*26* insertion sequence. Although the PCR amplification resulted in a band with a size similar to the positive control, the sequencing analysis was not successful and did not allow the characterization of this PCR amplicon.

bla$_{\text{CMY}}$-positive isolates were tested for IS*Ecp1*, located upstream of the gene. From 11 *bla*$_{\text{CMY}}^{+}$ isolates, 6 *E. coli* isolates gave a positive PCR result for the presence of IS*Ecp1*. Figure III.4 represents the genomic context of these 6 *E. coli* isolates. The genomic context of the other 5 isolates, belonging to species *E. coli* (n=2), *C. freundii* (n=1), *E. cloacae* (n=1) and *E. aerogenes* (n=1), remains unknown. With the same strategy used for *bla*$_{\text{CTX-M}}^{+}$ isolates, the gene variant was not known since the *bla*$_{\text{CMY}}$ gene was not completely sequenced. Although, the sequence retrieved by the sequencing analysis was large enough to identify the variant as part of the CMY-2 group.

To address this problem, a multiple sequence alignment was performed in ClustalOmega (available at: http://www.ebi.ac.uk/Tools/msa/clustalo/) with all amino acid sequences of β-lactamases belonging to CMY-2 group available in CARD database (available at: https://card.mcmaster.ca/) as well as the sequences from isolates that tested positive for b*la*$_{\text{CMY}}$ with the IS*Ecp1* located upstream the gene (sequences used are presented in supplemental material). Results (Figure III.5) indicate that the CMY variant can either be CMY-2 or CMY-22.

Figure III.3 – Genomic context of bla$_{\text{CTX-M-15}}$ *positive isolates.* IS*Ecp1, partial sequence; bla*$_{\text{CTX-M-15}}$*, β-lactamase CTX-M.15 complete coding sequence; orf477, partial sequence.*

Figure III.4 – Genomic context of bla$_{\text{CMY}}$ *positive isolates.* IS*Ecp1, partial sequence; bla*$_{\text{CMY}}$*, β-lactamase CMY-2 or CMY-22 partial sequence.*

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gi|BAC76072.1|CFE-1        MMKKSICCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYY
gi|CMY-40                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-38                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-12                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-15                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CAA55007.1|LAT-1        MMKKSLCSALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-53                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-20                  -MKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-45                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-36                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|AHW47897.1|CMY-111      MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|AGC54799.1|CMY-95       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-99                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-59                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITP LMQEQAIPGMAVAVIYQGKPYY
gi|CMY-42                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-31                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-30                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-29                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-27                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
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gi|CMY-23                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
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gi|CMY-6                   MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-5                   MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
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gi|CMY-33                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-22                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-18                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-2                   MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER8                        MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER11                       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER26                       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER31                       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER33                       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
ER39                       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-49                  MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYY
gi|AGZ20169.1|CMY-108      MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY
gi|CMY-13                  MMKKSLCCALLLTASFSTFASAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYY
gi|CMY-26                  MMKKSLCCALLLTASFSTFASAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKTYY
                             ****:*.************:******************************:***** **

gi|BAC76072.1|CFE-1        FTWGKADIANNRPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTQYWPELTGKQ
gi|CMY-40                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-15                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CAA55007.1|LAT-1        FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDCIARGEIKLSDPVTKYWPELTGKK
gi|CMY-53                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-36                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDCIARGEIKLSDPVTKYWPELTGKQ
gi|AHW47897.1|CMY-111      FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|AGC54799.1|CMY-95       FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-23                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-16                  FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-5                   FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKFSDPVTKYWPELTGKQ
gi|CMY-4                   FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|AGC54798.1|CMY-94       FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|CMY-44                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-33                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-22                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-18                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-2                 FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
ER8                      FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
ER11                     FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
ER26                     FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
ER31                     FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
ER33                     FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYW PELTGKQ
ER39                     FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-49                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|AGZ20169.1|CMY-108    FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKL SDPVTKYWPELTGKQ
gi|CMY-13                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
gi|CMY-26                FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ
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gi|BAC76072.1|CFE-1      WQGISLLHLATYTAGGLPLQVPDDVTDKAALLRFYQNWQPQWAPGAKRLYANSSIGLFGA
gi|CMY-40                WQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNWQPQWTPGAKRLYANSSIGLFGT
gi|CMY-38                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-12                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYSNSSIGLFGA
gi|CMY-15                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CAA55007.1|LAT-1      WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-53                WQGICLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-20                WQGISLLHLATYTAGGLPLQIPDDVTDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-45                WQGIRLLHLATYTAGGLPLKIPDDVRDKAALLHFYQNWQPQWTPGAKRLYSNSSIGLFGA
gi|CMY-36                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|AHW47897.1|CMY-111    WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|AGC54799.1|CMY-95     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-99                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYSNSSIGLFGA
gi|CMY-59                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-42                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-31                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNW QPQWTPGAKRLYANSSIGLFGA
gi|CMY-30                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-29                WQGIRLLHLATYTAGGLPLQFPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGE
gi|CMY-27                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-25                WQGIRLLHLATYTAGGLPLQIPDDVTDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-24                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-23                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-21                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGE
gi|CMY-16                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYSNSSIGLFGA
gi|CMY-14                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-7                 WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGE
gi|CMY-6                 WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-5                 WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-4                 WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|AGC54798.1|CMY-94     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-44                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-33                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-22                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-18                WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-2                 WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER8                      WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER11                     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER26                     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER31                     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER33                     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
ER39                     WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|CMY-49                WQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNWQPQWTPGAKRLYANSSIGLFGA
gi|AGZ20169.1|CMY-108    WQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNWQPQWAPGAKRLYANSSIGLFGA
gi|CMY-13                WQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNWQPQWAPGAKRLYANSSIGLFGA
gi|CMY-26                WQDISLLHLATYTAGGLPLQIPDDVTDKTALLHFYQNWQPQWAPGAKRLYANSSIGLFGA
                         **.* **************:.**** **:***:*********:*******:********

gi|BAC76072.1|CFE-1      LAVKPSGMSYEEAMTTRVLQPLKLAHTWITVPQSEQKDYALGYREGKPVHVSPGQLDAEA
gi|CMY-40                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-38                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLNAEA
gi|CMY-12                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|CMY-15                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|CAA55007.1|LAT-1      LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGRLDAEA
gi|CMY-53                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-20                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-45                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|CMY-36                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPENEQKDYAWGYREGKPVHVSPGQLDAEA
gi|AHW47897.1|CMY-111    LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDVEA
gi|AGC54799.1|CMY-95     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHASPGQLDAEA
gi|CMY-99                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
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gi|CMY-59                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHASPGQLDAEA
gi|CMY-42                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHSSPGQLDAEA
gi|CMY-31                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGRLDAEA
gi|CMY-30                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHGSPGQLDAEA
gi|CMY-29                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-27                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYACGYREGKPVHVSPGQLDAEA
gi|CMY-25                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-24                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVYVSPGQLDAEA
gi|CMY-23                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAGA
gi|CMY-21                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGKLDAEA
gi|CMY-16                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|CMY-14                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|CMY-7                 LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-6                 LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYALGYREGKPVHVSPGQLDAEA
gi|CMY-5                 LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-4                 LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSPGQLDAEA
gi|AGC54798.1|CMY-94     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-44                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-33                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-22                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-18                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-2                 LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
ER8                      LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
ER11                     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
ER26                     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
ER31                     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
ER33                     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP GQLDAEA
ER39                     LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-49                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVTPGQLDAEA
gi|AGZ20169.1|CMY-108    LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYRE GKPVHVSPGQLDAEA
gi|CMY-13                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA
gi|CMY-26                LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYREGKPVHVSPGQLDAEA
                         *************** ****************:.****** ********: :**:*:. *

gi|BAC76072.1|CFE-1      YGVKSSVVDMTRWVQANMDASQVQEKTLQQGIKLAQSRYWRIGDMYQGLGWEMLNWPVKA
gi|CMY-40                YGVKSSVIDMAHWVQANMDASHVQEKTLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-38                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-12                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-15                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CAA55007.1|LAT-1      YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-53                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-20                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-45                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-36                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|AHW47897.1|CMY-111    YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|AGC54799.1|CMY-95     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-99                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-59                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSR YWRIGDMYQGLGWEMLNWPLKA
gi|CMY-42                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-31                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-30                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-29                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-27                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-25                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-24                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-23                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-21                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-16                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-14                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-7                 YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-6                 YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-5                 YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-4                 YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|AGC54798.1|CMY-94     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-44                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEM LNWPLKA
gi|CMY-33                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-22                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-18                YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-2                 YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
ER8                      YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
ER11                     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIA------------------------
ER26                     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
ER31                     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
ER33                     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
ER39                     YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-49                YGVKSNVTDMARWIQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|AGZ20169.1|CMY-108    YGVKSSVIDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
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gi|CMY-13                YGVKSNVTDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
gi|CMY-26                YGVKSNVTDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA
                         *****.* **:::*:*.*****:**********

gi|BAC76072.1|CFE-1      DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-40                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGYTGGFGSYVAFVPEKNLGIVM
gi|CMY-38                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-12                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-15                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CAA55007.1|LAT-1      DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-53                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-20                DSIINGS--DNKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-45                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTVGFGSYVAFVPEKNLGIVM
gi|CMY-36                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|AHW47897.1|CMY-111    DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|AGC54799.1|CMY-95     DSIINGS--DSKVASAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-99                DSIINGS--DSKVALAALPAVEVNPPATAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-59                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-42                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-31                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-30                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-29                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-27                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-25                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-24                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-23                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-21                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-16                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-14                DSIINGS--DSKVALAALPAVEINPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-7                 DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-6                 DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-5                 DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-4                 DSIINGSD--SKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|AGC54798.1|CMY-94     DSIINGSD--SKVASAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-44                DSIINGSDSK------VAPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-33                DSIINGSDS----KVAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-22                DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-18                DSIINGNGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-2                 DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
ER8                      DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI--
ER11                     ----------------------------------------------------------
ER26                     DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI--
ER31                     DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI--
ER33                     DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKN-----
ER39                     DSIING--SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKN-----
gi|CMY-49                DSIINGS--DSKVALAALHTVEVNPPAPAVKASWVHKTGSTGGFGSYVAFIPEKNLGIVM
gi|AGZ20169.1|CMY-108    DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-13                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM
gi|CMY-26                DSIINGS--DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVM


gi|BAC76072.1|CFE-1      LANKSYPNPARVEAAWRILEKLQ
gi|CMY-40                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-38                LANKNYPNPVRVEAAWRILEKLQ
gi|CMY-12                LANKNYPNPVRVEAAWRILEKLQ
gi|CMY-15                LANKNYPNPVRVEAAWRILEKLQ
gi|CAA55007.1|LAT-1      LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-53                LANKSYPNPARVEAAWRILEKLQ
gi|CMY-20                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-45                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-36                LANKSYPNPVRVEAAWRILEKLQ
gi|AHW47897.1|CMY-111    LANKSYPNPVRVEAAWRILEKLQ
gi|AGC54799.1|CMY-95     LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-99                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-59                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-42                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-31                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-30                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-29                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-27                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-25                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-24                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-23                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-21                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-16                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-14                LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-7                 LANKSYPNPVRVEAAWRILEKLQ
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gi|CMY-6                    LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-5                    LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-4                    LANKSYPNPVRVEAAWRILEKLQ
gi|AGC54798.1|CMY-94        LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-44                   LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-33                   LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-22                   LANKSYPNPVRVEAYWRILEKLQ
gi|CMY-18                   LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-2                    LANKSYPNPVRVEAAWRILEKLQ
ER8                         ------------------------
ER11                        ------------------------
ER26                        ------------------------
ER31                        ------------------------
ER33                        ------------------------
ER39                        ------------------------
gi|CMY-49                   LANKSYPNPVRVEAAWRILEKLQ
gi|AGZ20169.1|CMY-108       LANKSYPNPVRVEAAWHILEKLQ
gi|CMY-13                   LANKSYPNPVRVEAAWRILEKLQ
gi|CMY-26                   LANKSYPNPVRVEAAWRILEKLQ
```

Figure III.5 – Results regarding multiple sequence alignment performed in ClustalOmega with all amino acid sequences of β-lactamases belonging to CMY-2 group available in CARD database. CMY-2 and CMY-22 sequences are highlighted.

2.2. Integrase genes and integron gene arrays

The presence of integrons was detected by PCR amplification of the integrase genes. Results are presented in Table III.2. The integrase gene *intI1* was detected in 34 isolates, identified as *C. freundii* (n=2), *E. coli* (n=10), *E. aerogenes* (n=2), *E. cloacae* (n=2), *M. morganii* (n=16), *P. stuartii* (n=1), *S. marcescens* (n=1). Gene *intI2* was only detected in 1 *C. freundii* isolate. This isolate harbors both *intI1* and *intI2* genes.

A total of 35 class 1 and 2 integrons were analyzed for the presence of inserted gene cassettes in the variable region with the primers and PCR conditions presented in Table II.10. In 15 isolates harboring class 1 integrons, only the presence of the integrase gene was determined since the PCR results for the presence of inserted DNA were negative. For the remaining integrons, 7 different arrays were detected (Table III.2). The majority (n=11) contained *dfrA15* gene cassettes, which confers resistance to trimethoprim. Other gene cassettes were detected, among them: *aadA1, aadA2, aadA5* and *aadB* which confer resistance to aminoglycosides, *dfrA1, dfrA12* and *dfrA17* (resistance to trimethoprim), *catB3*, known to confer resistance to chloramphenicol, and *sat2*, conferring resistance to streptothricin.

3. Conjugation experiments

Conjugation assays were performed for strains containing $bla_{\text{CTX-M}}$ genes and for strains containing bla_{AmpC} genes. For $bla_{\text{CTX-M}}$ genes, 2 out of 8 donors, belonging to species *C. freundii,* generated transconjugants resistant to cefotaxime. For bla_{AmpC} genes, 3 out of 7 isolates identified as *E. coli* generated transconjugants. To confirm the identity of the transconjugant and the acquisition of the plasmid, BOX-PCR and detection of $bla_{\text{CTX-M}}$ and bla_{CMY} genes was performed as described in Chapter II.

For the resultant transconjugants, genomic characterization was performed in order to identify the genes previously found in the donor isolate.

Tables III.3 and III.4 summarize the genomic characterization of the AR genes present in the transconjugants based on the resistance genotype of the donors, for $bla_{\text{CTX-M}}$ and bla_{CMY} genes, respectively.

For conjugation experiments regarding isolates *bla*$_{CTX-M-15}$$^{+}$, *bla*$_{TEM}$ was present on ER13 and ER28 but it was only present in Tj13. *bla*$_{SHV}$ was present in ER28 and was transferred to the respective transconjugant. Class 1 integrase was detected in all donors and transconjugants; class 2 integrase was not detected in the transconjugant Tj28. Resistance to sulfonamides genes *sul1* and *sul2* were present in both donors but *sul2* was only detected in Tj13 while *sul1* gene was only present in Tj28. About fluoroquinolones resistance determinants, ER13 harbored *aac(6')-ib-cr* gene and *qnrB* while ER28 harbored *qnrA*. In Tj13 only one gene conferring resistance to fluoroquinolones is present, *qnrB*. In Tj28, *qnrA* is not present. In transconjugant Tj28, *aac(6')-ib* gene is present. Isolate ER13 also harbored *bla*$_{CMY}$ gene, which was not detected in Tj13.

Table III.3. Resistance genotypes of *bla*$_{CTX-M-15}$ donors and transconjugants.

Donor	**Species**	***bla* gene**	**Transconjugant**	**Resistance Genotype**	
				Donor	**Transconjugant**
ER5	*M. morganii*	*bla*$_{CTX-M-15}$	**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib*, *sul1*, *intI1*	**-**
ER13	*C. freundii*		**Tj13**	*bla*$_{TEM}$, *bla*$_{CMY}$, *qnrB, aac(6')-ib-cr , sul1, sul2, intI1*	*bla*$_{TEM}$. *qnrB, sul2, intI1*
ER18	*M. morganii*		**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib*, *sul1*, *intI1*	-
ER22	*M. morganii*		**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib*, *sul1*, *intI1*	-
ER23	*M. morganii*		**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib*, *sul1*, *intI1*	-
ER27	*M. morganii*		**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib*, *sul1*, *intI1*	-
ER28	*C. freundii*		**Tj28**	*bla*$_{TEM}$, *bla*$_{SHV}$ *qnrA,aac(6')-ib, sul1, sul2, intI1, intI2*	*bla*$_{SHV}$, *aac(6')-ib, sul1, intI1*
ER30	*M. morganii*		**-**	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib, sul1*, *intI1*	**-**

For *bla*$_{CMY}$$^{+}$ isolates, *bla*$_{TEM,}$ class 1 integrase gene, aminoglycoside resistance gene *aac(6')-ib* and genes conferring resistance to sulfonamides were detected. *bla*$_{TEM}$ was present in all 3 donors and respective transconjugants. *aac(6')-ib* gene was present in all donors. *intI1* and *sul2* genes were both present in ER8 and ER33, this two isolates' genotypes differed in the presence of *sul1* gene – isolate ER8, was the only isolate harboring both *sul1* and *sul2*.

Aside from *bla*$_{TEM}$ and *aac(6')-ib* gene, ER8 donor possessed *sul1, sul2* and *intI1* genes, while in Tj8 only *sul2* and *intI1* genes were detected. ER26 also harbored resistance gene *aac(6')-ib* and this gene was not detected in Tj26. Only *bla*$_{TEM}$ was detected in Tj26. *aac(6')-ib* gene, *sul2* and *intI1* genes were present in ER33. From these 3 genes, only *sul2* was detected in Tj33. When present in the donor, neither *sul1* or *aac(6')-ib* genes were detected in the transconjugant.

Table III.4. Resistance genotype of *bla*$_{CMY}$ donors and transconjugants.

Donor	**Species**	*bla* gene	**Transconjugant**	**Resistance Genotype**	
				Donor	**Transconjugant**
ER8	*E. coli*	*bla*$_{CMY}$	**Tj8**	*bla*$_{TEM,}$ *aac(6')-ib, sul1, sul2, intI1*	*bla*$_{TEM}$, *sul2, intI1*
ER11	*E. coli*		**-**	*bla*$_{TEM}$, *aac(6')-ib, intI1*	-
ER26	*E. coli*		**Tj26**	*bla*$_{TEM}$, *aac(6')-ib*	*bla*$_{TEM}$
ER31	*E. coli*		**-**	*bla*$_{TEM}$	-
ER33	*E. coli*		**Tj33**	*bla*$_{TEM}$, *aac(6')-ib, sul2, intI1*	*bla*$_{TEM}$, *sul2*
ER39	*E. coli*		**-**	*bla*$_{TEM}$, *aac(6')-ib, sul2, intI1*	-
ER44	*E. aerogenes*		**-**	*bla*$_{TEM}$, *aac(6')-ib, sul2*	-

Plasmid DNA was extracted from donors and transconjugants to assess the plasmid DNA profiles of these isolates (Figure III.6).

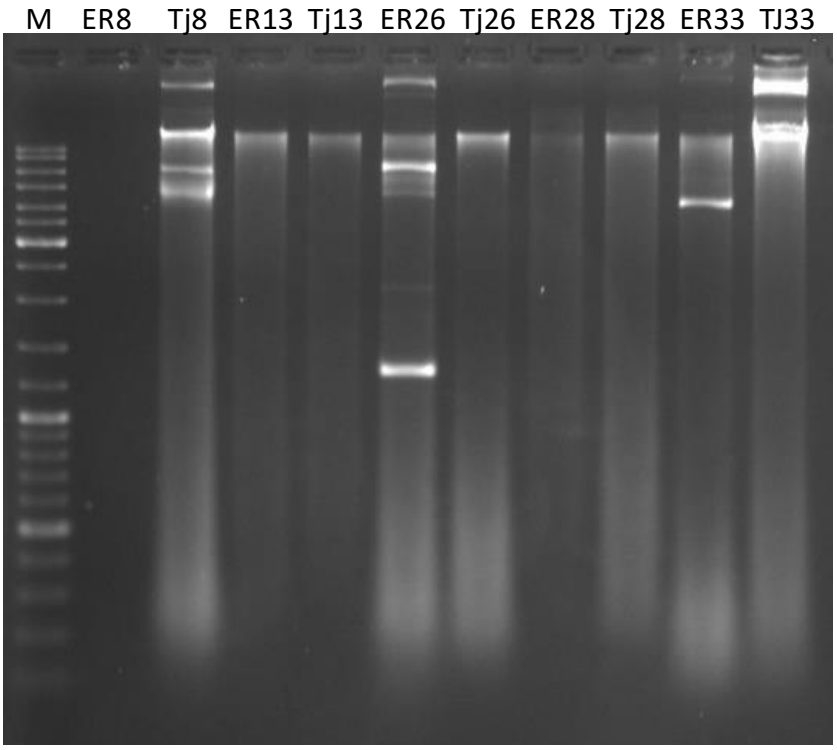

Figure III.6 – Results of Plasmid DNA Extraction. M – molecular marker; ER – isolates used as donors; Tj – transconjugants

4. Replicon typing

Detection of IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, Frep subgroups), IncHI1, IncHI2, IncI1, IncK, IncX, IncL/M, IncN, IncP, IncT, IncW and IncY replicons was performed by PCR. Results are summarized in Table III.5.

The inc/rep PCR-based typing method was applied to all 46 isolates and transconjugants that resulted from the mating assay (n=5). Thus, cell suspensions were used for transconjugant and donor cells as well as for the remaining isolates.

In a total of 46 isolates and 5 transconjugants, 13 isolates and 3 transconjugants gave a positive result for at least one Inc group. All 16 strains belong to *E. coli* species.

IncF, IncK, IncI1 and IncB/O specific sequences were detected among the isolates. IncF Frep subgroup was the most frequently identified, it was detected in 14 isolates. Among these 14 isolates, 10 isolates harbored Frep subgroup and one or more replicon belonging to subgroup FIB, groups IncB/O, IncI1 and IncK. The remaining isolates harbored only Frep plasmids. FIB subgroup is present in 5 isolates, always associated with other replicon sequences. FIB subgroup is the only plasmid group that is present in one donor and absent in the respective transconjugant.

IncK group was detected in ER8, ER26 and the respective transconjugants and always appears associated with either Frep subgroup or associated with both Frep and IncI1 replicons. IncI1 group appears in a total of 4 isolates and in 2 of them it is not associated with any other replicon sequences. In the other 2 groups, IncI1 appears associated with Frep subgroup and IncK and associated with IncF subgroups Frep and FIB.

IncB/O is present only in ER33 and respective transconjugant.

Analysis of the presence of certain AR genes and the replicon typing, does not suggest a direct correlation between the presence of one gene and the presence of an Inc group, or vice-versa.

Table III.5. Results of the inc/rep PCR-based typing method. Only strains that gave a positive result in at least one PCR experiment were included in this table.

Strain	Multiplex 1					Multiplex 2						Multiplex 3						Simplex	Resistance Genotype
	B/O	F/C	A/C	P	T	K	W	FIIA	FIA	FIB	Y	I1	X	HI1	N	HI2	L/M	Frep	
ER6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *aac(6')-ib, sul1, intI1*
ER8	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib, sul1, sul2, intI1*
Tj8	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *sul2, intI1*
ER11	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib, intI1*
ER15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *aac(6')-ib, sul1, intI1*
ER19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{DHA}$, *aac(6')-ib, intI1*
ER26	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib*
Tj26	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$
ER31	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$
ER33	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib, sul2, intI1*
Tj33	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *sul2*
ER35	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *aac(6')-ib, intI1*
ER36	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *aac(6')-ib*
ER40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*bla*$_{TEM}$, *aac(6')-ib, sul2*
ER41	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib*
ER43	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	*bla*$_{TEM}$, *bla*$_{CMY}$, *aac(6')-ib*

IV. DISCUSSION

This study aimed to contribute for the development of effective strategies to reduce resistance prevalence in clinical settings by characterizing the genetic basis of the resistance phenotypes associated with infectious bacteria in *Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E.*, as well as the mobility potential of the identified genes. Since antibiotic resistance is a worldwide problem with serious consequences in the clinical setting, there is a need to define strategies that can be applied in this environment to contribute for the future mitigation of antibiotic resistance dissemination. Controlling antibiotic resistance in healthcare systems might increase successful antimicrobial therapy rates and, as consequence, reduce mortality and morbidity rates as well as the treatment costs as the hospital stay would decrease.

To define effective control strategies it is imperative to characterize the bacteria that occur in the study environment. Know not only the provenance of the isolates or identify the species but also the genetic relatedness among them by performing a typing analysis. Molecular typing allows the establishment of epidemiologic relationships among isolates giving evidence for clonal or horizontal dissemination. While clonal isolates share the same DNA fingerprint, polyclonal infections are characterized by unrelated isolates which have different DNA profiles. If isolates with the same DNA profile are collected from different patients, it implies a transmission among patients or a common contamination source and requires a strategy aiming this spread among patients or wards. Unrelated isolates are commonly found in a community-acquired scenario. However, they can be found in a nosocomial scenario and this is probably due to the selective pressure that is imposed by the antibiotics in this settings (Paterson & Bonomo, 2005).

The isolates used in this work were collected from inpatients, which represented the nosocomial infections, and outpatients (patients attending the emergency room) which represented the community-acquired infections since the community environment is also a reservoir of antibiotic resistance (Roxo, 2015).

Molecular typing experiments allowed to assess the genetic diversity between the 46 isolates characterized in this study and the combined use of three PCR typing methods (ERIC-, BOX and REP-PCR) allowed a better discrimination of the isolates.

Analyzing the dendrogram, it was possible to observe a good distinction between species, with a few exceptions, which increases the confidence in Vitek2®'s results. Within the species clusters there was a high intra-species variability, suggesting that the dissemination of ESBL-producers and *bla*$_{AmpC}$-carriers was not clonally related. Furthermore, species clustering does not correlate with the isolates origin (clinic or community). This variability is probably due to the diverse origins of the isolates, both coming from the community and the hospital environment.

The presence of clinically relevant resistance genes was searched by PCR. These genes encoded resistance to β-lactams, fluoroquinolones and sulfonamides.

Regarding β-lactams, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{AmpC}$ genes were searched in the context of explaining a resistance phenotype to penicillins and broad-spectrum cephalosporins. Resistance to broad-spectrum cephalosporins in *Enterobacteriaceae* can be due to: i) production of ESBLs, ii) overexpression of chromosomal *bla*$_{AmpC}$ genes, and iii) plasmid-encoded *bla*$_{AmpC}$ genes (Conen et al., 2015; Pfeifer et al., 2010).

bla$_{CTX-M}$ gene was found in 18 of 46 isolates (39.1% prevalence). 17 isolates displayed a resistance phenotype against broad-spectrum cephalosporins and harbored the CTX-M-15 variant. Previous works demonstrated that this variant is frequently found in clinical environments and associated with *Enterobacteriaceae* isolates (Conen et al., 2015; Dedeic-Ljubovic et al., 2009; Eckert et al., 2004). *bla*$_{CTX-M-15}$ variant is also frequently associated with insertion sequence IS*Ecp1* located upstream the gene as well as *orf477* downstream. It is important to evaluate the genomic context to assess the possible mobilization of the *bla* genes. It was possible to observe that the array IS*Ecp1*-*bla*$_{CTX-M-15}$-*orf477* is a success structure in this clinical setting, as seen in previous studies (Eckert et al., 2006; Salladin et al., 2002).

Insertion sequences, namely IS*Ecp1*, serve as vehicle for the dissemination of these genes (Eckert et al., 2006). Aside from IS*Ecp1*, other insertion sequences have been detected surrounding this *bla* gene, namely, IS*26* (Cullik et al., 2010). This insertion sequence was screened in isolate ER6 since this was the only isolate harboring a *bla*$_{CTX-M}$ gene for which IS*Ecp1* was not detected upstream of the gene. Sequence analysis of the PCR amplicon was not successful so the variant and the upstream genomic context remained unknown. ER6 is also the only isolate that was susceptible to broad-spectrum cephalosporins. This is an uncommon situation since CTX-M enzymes have high hydrolytic activity against cefotaxime, hence the name CTX-M – active on **C**efo**T**a**X**ime,

isolated on Munich (Bush & Fisher, 2011). Based on the fact that the PCR amplicon for IS*26* gave a positive band with similar size to the positive control, one hypothetical explanation for the lack of expression of this CTX-M could be that the IS*26* is interrupting the coding sequence of the *bla*$_{\text{CTX-M}}$ gene. However, to best of our knowledge, there are no reports regarding the interruptions of *bla*$_{\text{CTX-M}}$ genes by IS*26* that support this hypothesis. There is one study describing the interruption of a coding sequence, encoding a porin *OmpK36*, by IS*26* (Mena et al., 2006).

Variants *bla*$_{\text{CMY}}$, *bla*$_{\text{DHA}}$ and *bla*$_{\text{EBC}}$ of *bla*$_{\text{AmpC}}$ genes were found, collectively, in 65.2% of the isolates. According to previous studies, the variant *bla*$_{\text{CMY}}$ descended from a *bla*$_{\text{AmpC}}$ located in the chromosome of *C. freundii*, the variant *bla*$_{\text{DHA}}$ derived from *M. morganii bla*$_{\text{cAmpC}}$ gene and *bla*$_{\text{EBC}}$ (coding ACT-1 and MIR-1 β-lactamases) is descended from a *bla*$_{\text{cAmpC}}$ gene found in *E. cloacae* (Barlow & Hall, 2002). Based on this information, *bla*$_{\text{DHA}}$ genes found in *M. morganii* isolates were considered to be intrinsic, and the same is valid for *bla*$_{\text{CMY}}$ found in *C. freundii* and *bla*$_{\text{EBC}}$ found in *E. cloacae*. In opposite, *bla*$_{\text{DHA}}$ genes found in *E. coli* and *bla*$_{\text{CMY}}$ genes found in *E. coli*, *E. aerogenes* and *E. cloacae*, were considered to be acquired. In accordance to other studies, in this work cAmpCs were more prevalent than pAmpC (Conen et al., 2015).

Chromosomal AmpC β-lactamases can either be over-produced through mutations in the promotor region which derepresses the AmpC production or induced by the exposition to β-lactams (Conen et al., 2015). *Enterobacteriaceae* isolates harboring cAmpC can initially be susceptible to cefotaxime, which is the case of isolates ER14 and ER17, but, in a clinical situation, the administration of 3$^{\text{rd}}$ generation cephalosporins will result in selection of mutants and consequently recurrence of the infection (Paterson, 2006). In a context of resistance dissemination, pAmpC are more relevant than cAmpC since the identification of a species that harbors these β-lactamases is enough to indicate their presence and avoid the administration of broad-spectrum cephalosporins (Thomson, 2010). Also, pAmpC enzymes are usually associated with penicillinases or other cephalosporinases (Jacoby, 2009) and that was showed in this study, when present, a pAmpC was always associated with another β-lactamase, either TEM, SHV or CTX-M.

The variant of the *bla*$_{\text{CMY}}$ present in an acquired scenario was determined by sequence analysis but it was only possible to identify the variant as part of the CMY-2 group. Within this group, the variant present in the 11 isolates can either be CMY-2 or CMY-22. Considering the fact that CMY-2 is the most prevalent and with the widest geographic distribution (Philippon et al., 2002) the genomic context was inspected for

insertion sequence IS*Ecp1* since it is associated with the mobilization of acquired AmpCs (Verdet et al., 2009). For 6 isolates, IS*Ecp1* was present suggesting a role in the mobilization of this gene. IS*5* has also been reported associated with *bla*$_{CMY-2}$ (Verdet et al., 2009) gene but was not present in none of the isolates. One possible solution for accessing the CMY variant is to amplify the *bla*$_{CMY}$ gene using a reverse primer designed to ligate to the *blc* gene which is frequently located downstream the *bla*$_{CMY-2}$ variant (Ahmed & Shimamoto, 2008; Mata & Navarro, 2012).

Some isolates displayed resistance to broad-spectrum cephalosporins but *bla*$_{CTX-M}$ and *bla*$_{AmpC}$ (either chromosomal or plasmidic) genes were absent. Therefore, *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were also screened since TEM- and SHV-type ESBLs could explain these phenotypes. *bla*$_{TEM}$ was the most prevalent gene in the collection (97.8%), detected in all isolates except isolate ER45, and *bla*$_{SHV}$ was the least frequent (6.5%), present in isolates ER25, ER28 e ER29. Despite the presence of these genes, the variant was not assessed, therefore, it was not possible to distinguish between ESBLs and TEM-1 and SHV-1 families. While *bla*$_{SHV-1}$, *bla*$_{TEM-1}$ and *bla*$_{TEM-2}$ genes encode resistance to penicillins and early cephalosporins, TEM- and SHV-type ESBL also confer resistance to broad-spectrum cephalosporins. In some cases, the presence of *bla*$_{SHV}$ or *bla*$_{TEM}$ suggests the presence of TEM-1 and SHV-1 penicillinases, since the isolate only displays resistance to penicillins, while in others, suggests the presence of an ESBL type based on the broad-spectrum cephalosporins resistance phenotype (Bush, 2010; Bush & Jacoby, 2010). In this case, the knowledge of the gene variant would be determinant to distinguish the β-lactamase present and make a comparison between the resistance phenotype and the genotype. Both TEM- and SHV-type ESBL and TEM-1 and SHV-1 families have been detected in *Enterobacteriaceae* (Pfeifer et al., 2010).

It is worth to highlight that ER45 does not harbor any *bla* gene tested and displays resistance to 3rd generation cephalosporins. One possible explanation for this situation, is that isolate ER45 harbors other ESBL types like VEB or PER, which are less common but were found in *Enterobacteriaceae*, and could explain the resistance phenotype to broad-spectrum cephalosporins and the susceptibility to carbapenems (Paterson & Bonomo, 2005; Weldhagen et al, 2003).

In a clinical scenario, the distinction between pAmpC producers and ESBL producers is imperative, since organisms producing high levels of pAmpCs can be misclassified as ESBL producers. Another problematic situation occurs when the presence of a pAmpC masks the effect of the ESBL and the isolate is considered ESBL-

negative (Munier et al., 2010; Robberts et al., 2009). This results in inappropriate therapy. Until the moment, there is no time-effective method available that allows a reliable identification (Conen et al., 2015).

Plasmid mediated resistance to fluoroquinolones can be due to three mechanisms. The first mechanism is mediated by *qnr* genes, which code for proteins that protect DNA gyrase and topoisomerase IV from quinolone action. The second, involves a variant of the aminoglycoside acetyltransferase, *aac(6')-ib-cr*, that has the ability to modify and reduce the activity of quinolones. Finally, the last mechanism involves efflux of the antibiotic coded by pumps' genes *qepA*. These mechanisms only provide low-level resistance but facilitate the emergence of high-level resistance in the presence of therapeutic levels of fluoroquinolones (Jacoby et al., 2014; Robicsek et al., 2006). In this context, variants *qnrA*, *qnrB* and *qnrS* of *qnr* genes and *aac(6')-ib-cr* were searched.

The *qnrA* and *qnrB* genes were found only in *C. freundii* isolates. According to other studies, *qnrB* is the most frequent variant found in clinical *Enterobacteriaceae* (Jacoby et al., 2011) and *Citrobacter* spp. is considered as the origin of *qnrB* allele (Ribeiro et al., 2015). *qnrA* is also found in *C. freundii* isolated from clinical *Enterobacteriaceae* (Cambau et al., 2006).

The screening of *aac(6')-ib-cr* gene was performed with the same primers used for *aac(6')-ib*. Without sequencing, it is not possible to distinguish between these two variants. Only one amplicon was sequenced, corresponding to isolate ER13 and it was confirmed to be the *aac(6')-ib-cr* variant. This isolate harbors two genetic determinants conferring low-level resistance to fluoroquinolones and displays resistance to CIP and LVX. *aac(6')-ib* gene is only absent in two isolates, ER31 and ER42, both of them are susceptible to LVX and CIP and are also susceptible to the aminoglycosides tested, namely amikacin, gentamicin and tobramycin. For the remaining isolates, among the ones that tested positive for the presence of *aac(6')-ib*, there are isolates displaying resistance to fluoroquinolones and aminoglycosides and isolates that are susceptible to both classes. Moreover, there are isolates that harbor the gene and are susceptible to aminoglycosides and resistant to fluoroquinolones. Sequencing the amplicon would allow to assign the gene variant to a certain antibiotic class and understand which phenotype it confers.

Sulfamethoxazole is the most commonly used sulfonamide and is used in combination with trimethoprim forming sulfamethoxazole/trimethoprim (or cotrimoxazol). Since some isolates displayed resistance to cotrimoxazol, *sul1* and *sul2* were screened in all isolates. Both genes have been reported in clinical isolates of

Enterobacteriaceae members, some of which were ESBL-producing isolates (Antunes et al., 2005; Frank et al., 2007; Singha et al., 2015). Isolates ER3 and ER19 displayed resistance to cotrimoxazol, however, neither *sul1* nor *sul2* genes were present. In these cases, the presence of *sul3* gene might explain the phenotype (Perreten & Boerlin, 2003). This variant has been found in *Enterobacteriaceae* clinical isolates (Grape et al., 2003). However, resistance can arise from other mechanisms that were not studied in this work.

ESBL-producing organisms frequently display co-resistance to other antibiotic classes, namely, aminoglycosides, tetracyclines, sulfonamides and fluoroquinolones. This was observed in this study and it is probably due to horizontal gene transfer due to dissemination agents (Cantón & Coque, 2006).

This leads to the other main goal of this work that was to detect the potential mobility of the resistance genes screened. In this sense, detection and characterization of mobile genetic elements, like insertion sequences, integrons and plasmids, was performed.

Detection of insertions sequences was performed when the genomic context of $bla_{\text{CTX-M}}$ and bla_{CMY} was evaluated and only IS*Ecp1* was found. In *Enterobacteriaceae* bacteria, the presence of $bla_{\text{CTX-M}}$ genes associated to this insertion sequence were suggested to be embedded in transposon elements, thus enhancing the occurrence of transposable events (Poirel et al., 2003). Besides IS*Ecp1*, other insertions sequences, namely IS*26* and IS*CR*, have been associated with *bla* genes (Cantón & Coque, 2006). The IS*CR* element has been associated to $bla_{\text{CTX-M}}$ and bla_{CMY} as well as fluoroquinolones' resistance gene *qnrA*. Moreover, IS*CR1* was reported in a complex structure involving class 1 integrons (Toleman et. al, 2006b).

A class 1 integron is composed of a variable region flanked by two conserved segments, 5'CS and 3'CS. On the 5' terminal, there are three key elements: an integrase gene (*intI1*), a recombination site (*attI*) and a promoter (P_c). The 3'CS region englobes the *qacEΔ1* gene, coding resistance to quaternary ammonium compounds and the *sul1* gene, which confers resistance to sulfonamides (Domingues et al., 2012). Integrons, by themselves, are not considered mobile elements because they lack of self-mobility functions, however they are able to integrate gene cassettes so they have a roll in multidrug phenotype (Boucher et al., 2007). In this sense, class 1 and class 2 integrase genes were searched in all isolates. When present, the variable region was also

characterized, since it can harbor antibiotic resistance genes. Class 1 integrons were highly prevalent among the isolates (73.9%) while only one class 2 integron was found in the collection (2.2%). Comparing to a recent study in *Enterobacteriaceae* clinical isolates, the number of class 1 integrons was higher while the number of class 2 integrons was similar (Malek et al., 2015).

The gene cassettes found consisted in 7 different arrays. The most frequent array contained *dfrA15* gene cassette, which confers resistance to trimethoprim. Other gene cassettes were detected, among them: *aadA1, aadA2*, *aadA5* and *aadB* (resistance to aminoglycosides), *dfrA1*, *dfrA12* and *dfrA17* (resistance to trimethoprim), *catB3* (chloramphenicol) and *sat2* (streptothricin). In some isolates, the presence of these gene cassettes explained a multidrug resistance phenotype. These arrays were previously reported according to the information available at the INTEGRALL Database (available at: http://integrall.bio.ua.pt/).

Based on the fact that *sul1* gene is a part of the structure of the integron, it was expected that all the isolates harboring *intI1* gene, also harbored *sul1* gene. But, that was not the case. In 13 isolates, the *intI1* gene was present and *sul1* was absent. There are reports of integrons lacking the 3'CS region. One of the possible explanations for this, is the substitution of the *sul1* gene for the *sul*3 gene in the 3'CS region. If these isolates tested positive for the presence of *sul3*, the variable region could be tested using a forward primer for the *intI1* gene and a reverse primer for the *sul3* region (Grape et al., 2003; Toleman et al., 2006b).

bla$_{\text{CTX-M}}$ genes have also been associated to epidemic plasmids. These plasmids frequently harbor other antibiotic resistance genes besides *bla*$_{\text{CTX-M}}$ genes, located in the same plasmid, conferring resistance to various antibiotic classes (Cantón & Coque, 2006). Since these plasmids can be transferred among isolates, their mobility was evaluated by performing conjugation experiments. To attribute the identified plasmid to certain Inc groups, after the mating assays, replicon typing was performed.

The mating assays were performed for *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ genes. For *bla*$_{\text{CTX-M}}$ genes only 2 donor strains among the 8 *bla*$_{\text{CTX-M}}^{+}$ isolates were able to pass the phenotype to the recipient cells. And for *bla*$_{\text{CMY}}^{+}$, the same was observed for 3 out of 7 donors. The number of transconjugants obtained is similar to the number obtained in other studies using *E. coli* J53 Azide^{R} as recipient cell (Tacão et al., 2014). This recipient was also tested, but the donors were able to grow on culture media supplemented with azide maximum concentration tested for the growth of recipient cell, so it was not possible to

use another recipient strain. The recipient strain used was *E. coli* CV601 RIF^R and it is not frequently described in conjugation experiments. Although, in previous studies, using this recipient strain and *Enterobacteriaceae* donors, the conjugation success was higher than the one obtained in this study (Moura et al., 2014; Smalla et al., 2000).

The identification of plasmid replicon types is important in the plasmid epidemiological surveillance because some Inc groups are typically associated with the carriage of certain resistance genes. In *Enterobacteriaceae* the major plasmid families present are: IncA/C, IncB/O, IncF (FIA, FIB, FIC, FIIA, Frep subgroups), IncHI1, IncHI2, IncI1, IncK, IncX, IncL/M, IncN, IncP, IncT, IncW and IncY (Carattoli, 2009). All these families were screened but only IncF, IncK, IncI1 and IncB/O replicons were found among the isolates and transconjugants for bla_{CMY} gene.

Plasmid DNA extraction results revealed in gel a multiband pattern for the bla_{CMY}^+ isolates, namely, Tj8, ER26, ER33 and Tj33 (Figure III.6). These isolates were also positive for more than one Inc group. These results suggest the presence of multiple plasmids belonging to several Inc groups. However, it is not possible to affirm this considering the fact that some plasmids can harbor more than one type of replicon in order to have a broader host range. The presence of multireplicon plasmids cannot be discarded in this situation (Boyd et al., 1996; Osborn et al., 2000).

$bla_{CTX\text{-}M\text{-}15}$ is associated with plasmids belonging to the IncF group, more frequently in subgroups FIIA, FIA and FIB, in isolates originated from humans (Carattoli, 2009). This was observed in the 2 $bla_{CTX\text{-}M\text{-}15}^+$ isolates that were positive for at least one replicon, both of them harbored FIB replicons, as well as Frep subgroup sequences. Moreover, the structure IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$ is found in the IncF group in hospital isolates and it is frequently associated with $bla_{TEM\text{-}1}$ (Boyd et al., 2004). In this study, isolates $bla_{CTX\text{-}M}^+$ that harbored IncF plasmids, were also positive for bla_{TEM} genes, however, the gene variant was not confirmed to be $bla_{TEM\text{-}1}$. IncF plasmids originated from human samples are also associated with bla_{CMY} and bla_{DHA} as well as aminoglycosides and fluoroquinolones resistance genes (Carattoli, 2009), which is in accordance with the results found in this study. IncI1 group appears associated with bla_{TEM} and bla_{CMY} genes (Carattoli, 2009; Hordijk et al., 2013a) which is also in accordance with the results obtained.

Plasmids belonging to IncK group are highly related to the ones belonging to group B/O (Praszkier et al., 1991). Both of these replicons have been reported in *Enterobacteriaceae*, although, these replicon types are typically associated with $bla_{CTX\text{-}}$

M-14 and *bla*CTX-M-9 gene variants and this was not verified in this study (Carattoli, 2009; Lyimo et al., 2016). Recently, IncK plasmids were found associated with *bla*CMY-2 in animal (Hordijk et al., 2013b) and human samples (Hansen et al., 2016; Seiffert et al., 2017). As well as IncB/O plasmids (Seiffert et al., 2017).

The isolates studied in this work were characterized in terms of genetic relatedness, genes conferring resistance phenotype to several antibiotic classes and potential mobility of the resistance genes found. Except isolate ER45, all isolates harbor at least one ESBL or an AmpC β-lactamase. As said before, ESBL-producing organisms frequently harbor resistance to other antibiotic classes and this is probably due to the co-occurrence of genetic determinants in the same mobile genetic elements. Results regarding mating experiments and the characterization of the variable regions of the class 1 and 2 integrons supported this hypothesis. Our results confirm that mobile genetic elements play a major role in the dissemination of resistance determinants in the analyzed hospital, contributing to the spread of successful resistance arrays. Future strategies to control the dissemination of resistance should be designed taking this information into account.

V. CONCLUSION

Enterobacteriaceae isolates displaying resistance to broad-spectrum cephalosporins or harboring ESBLs and/or AmpC enzymes are relevant in the clinical setting. This family of antibiotics is the main choice to treat infections caused by Gram-negative bacteria and is considered critically relevant in human medicine. Moreover, the presence of ESBLs is frequently associated with genes conferring resistance to other antibiotic classes which diminishes the therapeutic options.

The fact that multidrug resistant isolates are becoming more prevalent in the clinical settings, shows the importance of evaluating the provenance of the isolates found in the hospital, since the clinical setting is a "hotspot" of different resistance determinants. This, associated with the ease by which these resistance determinants can spread among bacteria, thanks to horizontal gene transfer mechanisms, makes it imperative to define strategies that reduce this dissemination among clinical wards.

This study provided a general sight of the resistance genotypes found in isolates recovered from patients that were treated in *Hospital Infante D. Pedro, Centro Hospitalar do Baixo Vouga, E.P.E.*, during a one year period, which complements the phenotypic study done before.

It was possible to conclude that resistance to 3rd generation cephalosporins in this hospital is predominantly associated to acquired mechanisms. Moreover, the dissemination of these resistance determinants seems to be related with the transference of mobile genetic elements rather than with clonal spread.

However, it was not possible to point a certain resistance genotype to one specific dissemination vehicle. The results obtained in this study suggest that the resistance phenotype present in this particular clinical setting results from the horizontal transference of more than one mobile genetic element. Further studies need to be conducted to characterize these elements with more detail.

In summary, some final considerations concerning this study include:

- High intra-species diversity suggests a non-clonal spreading among wards;
- ESBL-encoding *bla*$_{\text{CTX-M-15}}$ was frequently detected, in accordance with previous studies;
- *bla*$_{\text{CTX-M-15}}$ context includes insertion sequences typically associated with this gene;
- Acquired AmpC-encoding genes were also frequent, particularly among *E. coli* isolates;
- The majority of *bla*$_{\text{CMY}}$ gene in an acquired scenario is associated with an insertion sequence frequently found linked to this gene;
- *qnr* genes were only found in *Citrobacter freundii* isolates, in accordance with previous studies;
- Class 1 integrons were highly prevalent, in some cases explaining a multi-drug phenotype;
- Plasmids belonging to IncF, IncI1 and IncB/O groups were found in these clinical isolates harboring *bla* genes, which is in accordance with previous studies.

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VII. SUPPLEMENTAL MATERIAL

Presented below are the amino acid sequences of AmpC β-lactamases belonging to CMY-2 group available in CARD database. These sequences along with the sequences from ER isolates were used to perform the multiple sequence alignment with ClustalOmega.

>gi | CMY-2 [*Klebsiella pneumoniae*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-4 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-5 [*Klebsiella oxytoca*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKFSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-6 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYALGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-7 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGELAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-12 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYSNSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKNYPNPVRVEAAWRILEKLQ

>gi|CMY-13 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFASAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYYFTWGKADIANNHPVTQQTL
FELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNW
QPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPG
QLDAEAYGVKSNVTDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-14 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEINPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-15 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKNYPNPVRVEAAWRILEKLQ

>gi|CMY-16 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYSNSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-18 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGN
GSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-20 [*Escherichia coli*]

MKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQTLF
ELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKAALLHFYQNWQ
PQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQ
LDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGSDN
KVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-21 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGELAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GKLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-22 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAYWRILEKLQ

>gi|CMY-23 [*Salmonella enterica* subsp. *enterica serovar Senftenberg*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAGAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-24 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVYVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-25 [*Klebsiella pneumoniae*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVTDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-26 [*Klebsiella oxytoca*]

MMKKSLCCALLLTASFSTFASAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKTYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQDISLLHLATYTAGGLPLQIPDDVTDKTALLHFYQN
WQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSNVTDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIING
SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-27 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYACGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-29 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQFPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGELAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-30 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHGS
PGQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIING
SDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-31 [*Klebsiella pneumoniae*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GRLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-33 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-36 [*Klebsiella pneumoniae*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDCIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPENEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-38 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLNAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKNYPNPVRVEAAWRILEKLQ

>gi|CMY-40 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQN
WQPQWTPGAKRLYANSSIGLFGTLAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMAHWVQANMDASHVQEKTLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGYTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-42 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-44 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVAPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-45 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLKIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYSNSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTVGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-49 [*Citrobacter freundii*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYYFTWGKADIANNHPVTQQTL
FELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQNW
QPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVTPG
QLDAEAYGVKSNVTDMARWIQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGSD
SKVALAALHTVEVNPPAPAVKASWVHKTGSTGGFGSYVAFIPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-53 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGICLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPARVEAAWRILEKLQ

>gi|CMY-59 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHASP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CMY-99 [*Proteus mirabilis*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYSNSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYARGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPATAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|AGC54798.1|CMY-94 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVASAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|AGC54799.1|CMY-95 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHASP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVASAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|AGZ20169.1|CMY-108 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKAALLRFYQN
WQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQSEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQVNMDASRVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWHILEKLQ

>gi|AHW47897.1|CMY-111 [*Serratia marcescens*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDVEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|CAA55007.1|LAT-1 [*Klebsiella pneumoniae*]

MMKKSLCSALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDCIARGEIKLSDPVTKYWPELTGKKWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GRLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPVRVEAAWRILEKLQ

>gi|BAC76072.1|CFE-1 [*Escherichia coli*]

MMKKSICCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAIIYQGKPYYFTWGKADIANNRPVTQQTL
FELGSVSKTFNGVLGGDAIARGEIKLSDPVTQYWPELTGKQWQGISLLHLATYTAGGLPLQVPDDVTDKAALLRFYQN
WQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTTRVLQPLKLAHTWITVPQSEQKDYALGYREGKPVHVSPG
QLDAEAYGVKSSVVDMTRWVQANMDASQVQEKTLQQGIKLAQSRYWRIGDMYQGLGWEMLNWPVKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLANKSYPNPARVEAAWRILEKLQ

>ER8 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI

>ER11 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIA

>ER26 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI

>ER31 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGI

>ER33 [*Escherichia coli*]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKN

>ER39 [Escherichia coli]

MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYYFTWGKADIANNHPVTQQT
LFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQWQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQN
WQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSP
GQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKADSIINGS
DSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKN

Table S.1 summarizes all the information regarding antibiotic susceptibility tests given by Vitek2® automated system.

Table S.1. Results regarding antibiotic susceptibility testing given by Vitek2® Automated System.

Isolate	Species	MICs of each antibiotic																	
		AMK	AMC	AMP	CEF	CTX	CAZ	CXMa	CMXs	CIP	SXT	ERT	GEN	IPM	LVX	MEM	NIT	TZP	TOB
ER1	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	I (≤4)	S (≤1)
ER2	*M. morganii*	I (8)	R (≥32)	R (≥32)	R (≥64)	R (≥64)	R (2)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (0.5)	-	I (≤4)	R (≥16)
ER3	*E. coli*	R	R (≥32)	R	R	R	R	-	-	R	R	S	R	S	R	S	R	R	R
ER4	*M. morganii*	S (≤2)	R (≥32)	R (≥32)	-	R (8)	R (4)	R (≥64)	R (≥64)	R (2)	R (≥320)	S (≤0.5)	R (≥16)	-	R (2)	S (≤0.25)	R (128)	I (≤4)	R (2)
ER5	*M. morganii*	R (8)	R (≥32)	R (≥32)	-	R (≥64)	R (8)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (≤0.25)	R (128)	I (8)	R (≥16)
ER6	*E. coli*	S (≤2)	R (16)	R (≥32)	-	S (≤1)	S (≤1)	R (≥64)	R (≥64)	R (≥4)	R (≥32)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (≤16)	S (8)	S (≤1)
ER7	*M. morganii*	I (8)	R (≥32)	R (≥32)	-	R (8)	R (8)	R (≥64)	R (≥64)	R (2)	R (≥320)	S (≤0.5)	R (≥16)	-	R (2)	S (0.5)	R (128)	I (≤4)	R (8)
ER8	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (4)	R (4)	R (16)	R (16)	R (≥4)	R (≥320)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (64)	I (≤4)	S (≤1)
ER9	*E. cloacae*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S (≤1)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	I (64)	S (≤4)	S (≤1)
ER10	*E. aerogenes*	S (≤2)	R (≥32)	R (8)	-	S (≤1)	S (≤1)	S (4)	S (4)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	I (64)	S (≤4)	S (≤1)
ER11	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (4)	R (16)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	I (8)	S (≤1)
ER12	*S. marcescens*	Unknown																	
ER13	*C. freundii*	S (≤2)	R (8)	R (≥32)	-	R (≥64)	R (8)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (≤16)	S (≤4)	S (≤1)
ER14	*M. morganii*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S (≤1)	R (≥64)	R (≥64)	R (≥4)	S (≤20)	S (≤0.5)	R (8)	-	R (≥8)	S (≤0.25)	R (128)	S (≤4)	R (8)
ER15	*E. coli*	S (4)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	I (8)	S (≤1)
ER16	*E. aerogenes*	S (≤2)	R (≥32)	R (≥32)	-	R (16)	R (≥64)	R (≥64)	R (≥64)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	R (128)	R (≥128)	S (≤1)
ER17	*E. cloacae*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S (≤1)	R (4)	S (4)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (32)	S (≤4)	S (≤1)
ER18	*M. morganii*	I (16)	R (≥32)	R (≥32)	-	R (≥64)	R (4)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (≤0.25)	R (128)	I (≤4)	R (≥16)
ER19	*E. coli*	S (≤2)	R (16)	R (≥32)	-	S (≤1)	S (≤1)	R (16)	R (16)	R (≥4)	R (≥320)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	R (256)	S (8)	S (≤1)
ER20	*M. morganii*	Unknown																	
ER21	*M. morganii*	S (4)	R (≥32)	R (≥32)	-	R (32)	R (8)	R (≥64)	R (≥64)	R (2)	R (≥320)	S (≤0.5)	R (≥16)	-	R (2)	S (0.5)	R (128)	I (≤4)	R (8)
ER22	*M. morganii*	Unknown																	
ER23	*M. morganii*	S (4)	R (≥32)	R (≥32)	-	R (32)	R (≥64)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (8)	-	R (≥8)	S (≤0.25)	R (128)	I (≤4)	R (8)
ER24	*M. morganii*	I (8)	R (≥32)	R (≥32)	-	R (4)	R (8)	R (≥64)	R (≥64)	R (2)	R (≥320)	S (≤0.5)	R (≥16)	-	R (2)	S (0.5)	R (128)	I (8)	R (8)
ER25	*E. aerogenes*	Unknown																	
ER26	*E. coli*	-	R (≥32)	R (≥32)	-	R (2)	R (4)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	I (≤4)	S (≤1)
ER27	*M. morganii*	I (16)	R (≥32)	R (≥32)	-	R (≥64)	R (2)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (≤0.25)	R (128)	I (≤4)	R (≥16)
ER28	*C. freundii*	I (16)	R (≥32)	R (≥32)	-	R (8)	R (≥64)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	S	R (≥8)	S (≤0.25)	R (256)	R (32)	R (≥16)
ER29	*M. morganii*	S (4)	R (≥32)	R (≥32)	-	R (16)	R (16)	R (≥64)	R (≥64)	R (2)	S (≤20)	S (≤0.5)	S (≤1)	-	R (4)	S (0.5)	R (256)	I (≤4)	S (≤1)
ER30	*M. morganii*	I (16)	R (≥32)	R (≥32)	-	R (≥64)	R (2)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (≤0.25)	R (128)	I (≤4)	R (≥16)
ER31	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (0.5)	S (≤0.25)	S (≤16)	I (≤4)	S (≤1)
ER32	*M. morganii*	S (4)	R (≥32)	R (≥32)	-	R (≥64)	R (≥64)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (8)	-	R (≥8)	S (0.5)	R (128)	R (≥128)	R (8)
ER33	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (8)	R (16)	R (32)	R (32)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	R (64)	S (≤1)
ER34	*M. morganii*	I (16)	R (≥32)	R (≥32)	-	R (≥64)	R (4)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	R (≥16)	-	R (≥8)	S (≤0.25)	R (128)	I (≤4)	R (≥16)
ER35	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (16)	R (16)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (1)	S (≤0.25)	S (≤16)	I (8)	S (≤1)
ER36	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (32)	R (32)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (1)	S (≤0.25)	S (≤16)	I (8)	S (≤1)
ER37	*E. cloacae*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (≥64)	R (≥64)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (0.5)	S (≤0.25)	R (128)	I (8)	S (≤1)
ER38	*M. morganii*	S (≤2)	R (≥32)	R (≥32)	-	R (4)	R (4)	R (≥64)	-	R (≥4)	S (≤20)	S (≤0.5)	R (8)	-	R (≥8)	S (≤0.25)	S (≤2)	I (≤4)	R (8)
ER39	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (8)	R (16)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (≤16)	R (64)	S (≤1)
ER40	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S (≤1)	R (16)	R (16)	R (≥4)	S (≤20)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (≤16)	R (64)	S (≤1)
ER41	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	S (≤1)	S (≤1)	R (16)	R (16)	R (≥4)	S (≤20)	S (≤0.5)	S (≤1)	S	R (≥8)	S (≤0.25)	S (≤16)	R (≥128)	S (≤1)
ER42	*E. aerogenes*	S (≤2)	R (≥32)	R (≥32)	-	R (8)	R (≥64)	R (≥64)	R (≥64)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	I (64)	R (≥128)	S (≤1)

ER43	*E. coli*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (4)	R (32)	R (32)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	S (≤16)	R (≥128)	S (≤1)
ER44	*E. aerogenes*	S (≤2)	R (≥32)	R (≥32)	-	R (8)	R (16)	R (≥64)	R (≥64)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	R (128)	R (≥128)	S (≤1)
ER45	*E. aerogenes*	S (≤2)	R (≥32)	R (≥32)	-	R (16)	R (16)	R (≥64)	R (≥64)	S (≤0.25)	S (≤20)	S (≤0.5)	S (≤1)	S	S (≤0.12)	S (≤0.25)	I (64)	R (≥128)	S (≤1)
ER46	*P. stuartii*	S (≤2)	R (≥32)	R (≥32)	-	R (2)	R (16)	R (≥64)	R (≥64)	R (≥4)	R (≥320)	I (1)	R (8)	-	R (≥8)	S (1)	R (256)	I (≤4)	R (8)

Antibiotic abbreviations: AMK – amikacin; AMC - amoxicillin-clavulanic acid; AMP – ampicillin; CEF – cephalothin; CTX – cefotaxime; CAZ – ceftazidime; CXMa – cefuroxime axetil; CXMs –cefuroxime sodium; CIP – ciprofloxacin; SXT – trimethoprim-sulfamethoxazole; ERT – ertapenem; GEN – gentamicin; IPM – imipenem; LVX – levofloxacin; MEM – meropenem; NIT – nitrofurantoin; TZP - piperacillin/tazobactam; TOB – tobramycin