Universidade de Aveiro Departamento de Biologia
2020

Nuno Valente Pinto

Carbapenemase-producing bacteria in urban aquatic environments

Bactérias produtoras de carbapenemases em ambientes aquáticos urbanos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Marta Tacão, investigadora do Departamento de Biologia da Universidade de Aveiro e da Doutora Isabel Henriques, Professora Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

A ciência será sempre uma busca e jamais uma descoberta.
É uma viagem, nunca uma chegada.
Karl Popper

o júri

presidente

Professora Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar c/ Agregação, Universidade de Aveiro

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agradecimentos

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palavras-chave resistência a antibióticos; ambientes aquáticos urbanos; carbapenemases; elementos genéticos móveis.

resumo

A resistência a antibióticos é um problema sério e crescente a nível global. Se nada for feito, prevê-se um futuro onde as infeções que atualmente são facilmente tratáveis poderão matar milhões de pessoas anualmente. Os carbapenemos são um grupo de antibióticos de último recurso, considerados antibióticos chave no tratamento de infeções graves. A disseminação de genes que codificam enzimas capazes de hidrolisar e inutilizar estes antibióticos, através de elementos genéticos móveis (EGM), constitui uma ameaça séria à Saúde Pública. As bactérias resistentes a antibióticos (BRA) e genes de resistência a antibióticos (GRA) são frequentemente associados ao âmbito hospitalar e pouco se sabe acerca da sua presença em pequenos lagos urbanos, situados em áeas recreativas, onde humanos e animais poderão ser facilmente expostos. Este estudo teve como objetivo avaliar a prevalência e caracterizar bactérias produtoras de carbapenemases em sistemas aquáticos urbanos.

A prevalência de bactérias resistentes a cefotaxima e de bactérias resistentes a imipenemo foi avaliada em cinco pequenos lagos urbanos e num canal estuarino durante 6 meses. Durante esse tempo, um lago urbano mostrou consistentemente a maior taxa tanto de bactérias resistentes a imipenemo como de bactérias resistentes a cefotaxima. As bactérias resistentes a imipenemo foram detetadas apenas em 2 lagos urbanos e num canal estuarino.

Estabeleceu-se uma coleção de 30 isolados resistentes a imipenemo, que foram posteriormente identificados e submetidos a tipagem molecular através de BOX-PCR. Estes isolados foram caracterizados com base em suscetibilidade a antibióticos, presença de genes que codificam carbapenemases, conteúdo plasmídico e ensaios de conjugação. Foram selecionados 3 isolados para sequenciação do genoma e os seus resistomas, mobilomas e genes associados a fatores de virulência foram avaliados.

Os isolados afiliaram com *Klebsiella* (n = 1), *Raoultella* (n = 11), *Enterobacter* (n = 3), *Citrobacter* (n = 8) e *Aeromonas* (n = 7). A maioria deles eram resistentes a todos os antibióticos β-lactâmicos testados, 76,6 % eram organismos multirresistentes e nenhum dos antibióticos testados foi eficaz contra todos os isolados. A presença de genes que codificam carbapenemases foi detetada em 27 isolados: bla_{KPC} (n=20; 8 *Citrobacter*, 11 *Raoultella* e 1 *Enterobacter*); bla_{GES-5} (n=13; 6 *Aeromonas*, 5 *Raoultella*, 1 *Enterobacter* e 1 *Klebsiella*) e bla_{VIM-1} (n=1; *Citrobacter*). Observou-se também a presença de dois genes que codificam carbapenemases em simultâneo (bla_{KPC} e bla_{GES-5}, n=6; bla_{KPC} e bla_{VIM-1}, n=1). Foram detetados integrões de classes 1 e 3 em 80 % e 36,7 % dos isolados, respetivamente. A presença do gene bla_{GES-5} esteve sempre associada a integrões de classe 3. A análise genómica mostrou que o gene bla_{KPC-3} se encontrava associado a transposões e plasmídeos. GRA adicionais também foram detetados (ex: *catB3*, *aacA4-cr*, *qnrS1*, *sul1*, *dfrA14*, *tet(A)* and *macA*). Alguns dos integrões detetados possuíam vários GRA. Apesar de não ter sido possível obter transconjugantes através de ensaios de conjugação, confirmou-se a presença de plasmídeos conjugativos através da

análise genómica. Foram detetados vários fatores de virulência nos genomas sequenciados e os organismos foram previstos como patogénicos para humanos. Com este estudo conclui-se que alguns dos ambientes aquáticos estudados possuem cargas microbianas acima do desejável e que as BRA detetadas possuíam genes de resistência a carbapenemos e a outras classes de antibióticos, habitualmente associados a EGM envolvidos em surtos graves e que podem contribuir para a disseminação de GRA entre diferentes bactérias e ambientes. São necessários estudos posteriores para elucidar a fonte destes GRA e tentar prevenir a sua disseminação, com o objetivo de abrandar a resistência a antibióticos, especialmente a resistência a antibióticos de último recurso.

keywords

antibiotic resistance; urban aquatic environments; carbapenems resistance; mobile genetic elements.

abstract

Antibiotic resistance is an increasingly serious problem globally. If nothing is done, is predictable a future where infections that are currently easily treatable would kill millions of people each year. Carbapenems are a group of last-resort antibiotics, considered key drugs in the treatment of severe infections. The spread of genes encoding carbapenem-hydrolyzing enzymes through mobile genetic elements is a serious threat to Public Health. Antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARG) are often associated with clinical settings and little is known about their presence in urban aquatic systems located in recreational areas, where humans and animals may be easily exposed. This study aimed to assess the prevalence and characterize carbapenemase-producing bacteria in urban aquatic systems.

The prevalence of imipenem and cefotaxime-resistant bacteria was monitored in five urban ponds and in an urban estuarine channel over six months. Over this time, one urban pond showed consistently the highest rate of both imipenem- and cefotaxime-resistant bacteria. Imipenem-resistant bacteria were detected only in 2 urban ponds and in the estuarine channel site.

A collection of 30 imipenem-resistant isolates was established, which were subsequently identified and submitted to molecular typing by BOX-PCR. These isolates were characterized regarding the antibiotic susceptibility, carbapenemase-encoding genes, plasmid content and mating assays. Three isolates were selected for whole genome sequencing and their resistome, mobilome and virulence related genetic determinants were evaluated.

Isolates affiliated to *Klebsiella* (n = 1), *Raoultella* (n = 11), *Enterobacter* (n = 3), *Citrobacter* (n = 8) and *Aeromonas* (n = 7). Most of them were resistant to all β-lactam antibiotics tested, 76.6 % were multidrug-resistant organisms and none of the tested antibiotics was effective against all isolates. The presence of carbapenemase-encoding genes was detected in 27 isolates: bla_{KPC} (n=20; 8 *Citrobacter*, 11 *Raoultella* and 1 *Enterobacter*); bla_{GES-5} (n=13; 6 *Aeromonas*, 5 *Raoultella*, 1 *Enterobacter* and 1 *Klebsiella*) and bla_{VIM-1} (n=1; *Citrobacter*). The simultaneous presence of two carbapenemase genes was observed (bla_{KPC} and bla_{GES-5}, n=6; bla_{KPC} and bla_{VIM-1}, n=1). Class 1 and 3 integrons were detected in 80 % and 36.7 % of the isolates, respectively. The presence of bla_{GES-5} was always associated with class 3 integrons. The whole genome analysis showed that bla_{KPC-3} was found associated to transposons and plasmids. Additional ARG were also detected (e.g *catB3*, *aacA4-cr*, *qnrS1*, *sul1*, *dfrA14*, *tet(A)* and *macA*). Some of the integrons detected harboured multiple ARG. Although it was not possible to obtain transconjugants through mating assays, the presence of conjugative plasmids was confirmed by WGS analysis. The presence of several virulence determinants was detected in sequenced genomes and these organisms were predicted as human pathogens.

With this study, we concluded that some of the studied aquatic environments possess microbial loads above the desirable and that the detected ARB harbored both genetic determinants of resistance to

carbapenems and to other classes of antibiotics, often associated with mobile genetic elements already linked to serious outbreaks, that may contribute to their spread among bacteria and between environments. Further studies are needed to elucidate the source of these ARG to try preventing their spread, in order to slow-down the problem of antibiotic resistance, especially the last-resort antibiotic resistance.

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Abbreviations

ºC	Degree Celsius
AMR	Atimicrobial resistance
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance genes
ATU	Area of technical uncertainty
CFU	Colony forming unit
CPE	Carbapenemase-producing *Enterobacteriaceae*
DDD	Defined daily dose
dDDH	Digital DNA-DNA hibridization
DNA	Deoxyribonucleic acid
EARS	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum β-lactamase
EURGen-Net	European Antimicrobial Resistance Genes Surveillance Network
EuSCAPE	European Survey on Carbapenemase-Producing *Enterobacteriaceae*
µg	Microgram
GES	Guiana extended-spectrum β-lactamase
GC	Gene cassette
HGT	Horizontal gene transfer
KPC	*Klebsiella pneumoiae* carbapenemase
Km	Kilometer
L	Liter
µm	Micrometer
mL	Mililiter
mm	Milimeter
MDR	Multidrug resistant
MGE	Mobile genetic element
min	Minute
NDM	New Delhi metallo- β-lactamase
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
rpm	Rotation per minute

RNA	Ribonucleic acid
VIM	Verona Integron-Borne Metallo-β-Lactamase
WGS	Whole genome sequencing
WHO	World Health Organization
WWTP	Wastewater treatment plant

I. Introduction

1. Antibiotics: The life-saving weapons

Earth is inhabited by approximately 5 X 10^{30} bacteria. Through evolutionary processes, some microorganisms have become capable of synthesizing antagonistic compounds of bacteria, known as antibiotics (Finley et al., 2013). Although mankind has used microorganisms for many centuries, to try to control microbial infections (Sengupta et al., 2013), it was Selman Waksman who in 1941 used the term "antibiotic" for the first time (Clardy et al., 2009). In 1947, Waksman published a paper, describing an antibiotic as "(…) *a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree* (…)" (Waksman, 1947). Currently, this designation has been changing, since in addition to totally natural antibiotics, produced by environmental fungi and bacteria, there are those that are synthetically modified and those that are entirely synthetic (Aslam et al., 2018). Nowadays, an antibiotic can be defined as "*an organic chemical of natural or synthetic origin that inhibits or kills pathogenic bacteria at low concentrations and possesses selective toxicity, i.e., maximal toxicity for a pathogen and minimal toxicity for the* host (Bentley & Bennett, 2003). In 1909, Paul Ehrlich discovered a chemical compound derived from arsenic, the salvarsan, which proved to be effective in syphilis treatment and was the first modern antimicrobial agent. However, salvarsan did not fit into the definition made by Waksman, once it was not a natural compound produced by microorganisms (Gould, 2016; Waksman, 1947). From the 30s to the 60s of the last century, humanity experienced a period called "golden era of antibiotic discovery" (Nathan & Cars, 2014). During this period many new antibiotics were discovered (Buchy et al., 2019), including most of the naturally occurring antibiotics used today (Brown & Wright, 2016).

Penicillin was the first natural discovered antibiotic, by Alexander Fleming, in 1928, from a fungus of the genus *Penicillium* (Peterson & Kaur, 2018) and it was implemented in the clinical practice in 1943 (Palumbi, 2001). This compound proved to be less toxic with a higher activity spectrum than salvarsan and replaced it (Swain, 2018). These advances, made during the golden age of antibiotic discovery, were one of the main advances in medical field (van Hoek et al., 2011). Antibiotics, in addition to saving patients' lives, also play a crucial role in the development of medicine and surgery. This compounds allow to prevent or treat infections that can occur in patients who are

receiving chemotherapy treatments, patients with chronic diseases, patients who have undergone surgery, organ transplants, among others (Ventola, 2015), acting as both therapeutic and prophylactic agents, thus reducing morbidity and mortality, and contributing to the increase of life expectancy (van Hoek et al., 2011).

There are several antibiotic classes, which possess different modes of action. Some examples of cellular targets are cell membrane (lipopeptides), cell wall biosynthesis (β-lactams), DNA gyrase (fluoroquinolones), DNA synthesis (sulfonamides), fatty acid synthesis (chlorophenol), protein synthesis, by 30S ribosomal subunit binding (tetracyclines) or by 50S ribosomal subunit binding (macrolides, amphenicols) and RNA synthesis (rifamycins) (O'Rourke et al., 2020) (figure 1).

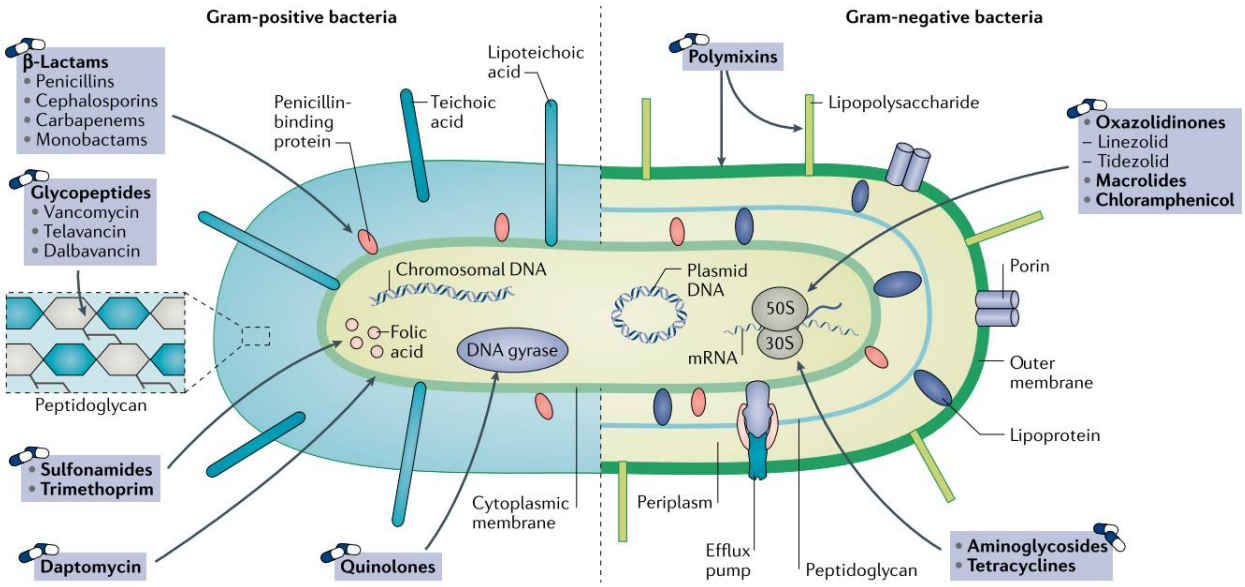

Figure 1. Examples of antibiotics and their mode of action in Gram-positive and Gram-negative bacteria (Wang et al., 2019)

Regarding the spectrum of action, antibiotics that are effective only against a limited set of bacteria are called narrow-spectrum antibiotics, with the advantage of preventing the formation of resistance by bacteria that are not the target of treatment. On the other hand, antibiotics that target a wide variety of bacteria are called broad-spectrum antibiotics, with the advantage of allowing physicians to treat an infection empirically, with a greater likelihood of success. However, there is a greater risk of antibiotic resistance development by bacteria that are not the target of treatment (Maxson & Mitchell, 2016; Sarpong & Miller, 2014).

There are some new antibiotics in clinical trials, however, it may take 10 years until their commercialization (Renwick & Mossialos, 2018). An example of time-consuming antibiotic development and commercialization is the platensimycin, a promising drug which act as inhibitor of FabF proteins, discovered in 2006 (Wang et al., 2006), that is still in clinical trials (Deng et al., 2019).

2. β-lactams: The scientific path taken to cope with the emergence of resistance

β-lactam antibiotics are a class of antibiotics that includes several molecules, classified according to their chemical structure (Nordmann et al., 2012). Penicillin G (benzylpenicillin), the original molecule extracted from *Penicillium rubens* by Alex Fleming in 1928, was the first β-lactam used in the clinical practice. Its clinical trials started in 1942 (Houbraken et al., 2011; Lobanovska & Pilla, 2017; Raynor, 1997). Since its discovery, many other natural and semi-synthetic β-lactams have been described (Essack, 2001). These compounds are among the most effective agents in the treatment of bacterial infections (Öztürk et al., 2015). β-lactams molecules have a β-lactam ring that can be fused to other rings, creating variable structures. Additionally, the substitutions on branches linked to the β-lactam ring influence antibacterial activity, stability against β-lactamases and toxicity to the patient (Essack, 2001). Based on the structure of the β-lactam molecule it is possible to group these molecules in subgroups: penicillins, cephalosporins, carbapenems and monobactams (Bush & Bradford, 2019; Tahlan & Jensen, 2013) (figure 2).

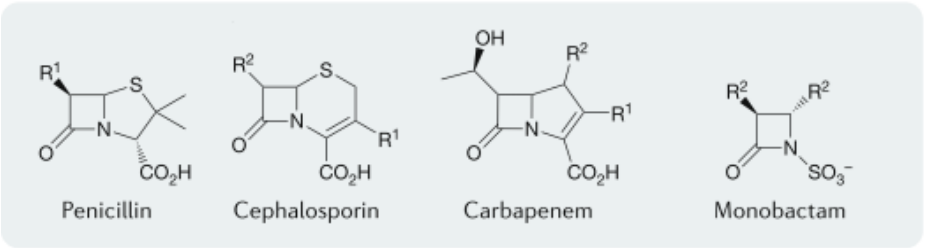

Figure 2. **Members of β-lactam class (Bush & Bradford, 2019).**

In addition to all these molecules sharing a β-lactam ring, they also have the same mechanism of action, the penicillin binding proteins (PBP) inhibition (Bush & Bradford, 2019). During the late 1960s, the emergence of β-lactamases threatened the effectiveness of penicillins (Papp-Wallace et al., 2011). Due to this, research and development of new and more stable β-lactam compounds, and also of β-lactamase inhibitors began (Bush &

Bradford, 2016). In 1979, Kahan et al., discovered thienamycin, a metabolite produced by *Streptomyces cattleya*. This new β-lactam molecule was the first representative of the carbapenems family. It had a greater spectrum of action than the natural antibiotics known to that date, acting against Gram-positive, Gram-negative bacteria including β-lactamase-producing bacteria resistant to penicillins and cephalosporins (Kahan et al., 1979). In addition to its broad-spectrum activity, it also acted as a β-lactamase inhibitor (Papp-Wallace et al., 2011). Despite its antibacterial activity, it was chemically unstable. In 1979 it was chemically stabilized by the addition of an N-formimidoyl group, which resulted in a compound named MK0787 (Leanza et al., 1979), currently known as imipenem (Bush & Bradford, 2016). In 1985, imipenem became the first carbapenem available to treat complex microbial infections (Papp-Wallace et al., 2011). Since the prevalence of resistance to first-line antibiotic such as 3rd generation cephalosporins is increasing, physicians are forced to administer more effective drugs (Gashe et al., 2018; Lin et al., 2019; Marinho et al., 2016; Meyer et al., 2010; Shelton et al., 2016), among them, carbapenems, which are considered last-resort antibiotics. Their use is reserved, in several countries, such as Portugal, for infected patients that are seriously ill, when there is a suspicion that the infection is caused by a multi-resistant bacteria or when no other antibiotic is effective (Papp-Wallace et al., 2011; Sekyere, 2016; Tacão et al., 2015). Although carbapenems are β-lactams, such as penicillins and cephalosporins, they differ in their chemical structure (Hawkey & Livermore, 2012) and in their greater range of action when compared to penicillins, cephalosporins and β-lactam/β-lactamase inhibitor combinations (Papp-Wallace et al., 2011). In addition to imipenem, there are other carbapenems, such as meropenem, doripenem, ertapenem, panipenem and biapenem (Codjoe & Donkor, 2018). This group of antibiotics is highly effective to treat bacterial infections and has low toxicity (Kattan et al., 2008). In Portugal, although carbapenems consumption has decreased in recent years, they still represent 20.7 % of the non-penicillin β-lactam antibiotics used in clinical settings during 2018 (0.081 defined daily dose (DDD) per 1000 inhabitants and per day) (ECDC, 2019) (figure 3).

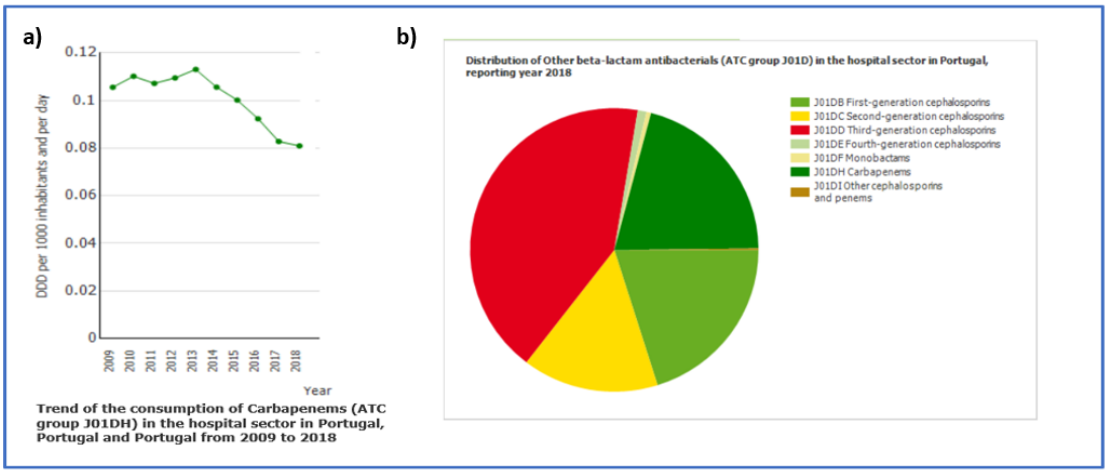

Figure 3. a) Consumption of carbapenems in clinical settings from 2009 to 2018 in Portugal. b) Consumption of non-penicillin β lactam antibiotics during 2018 in Portugal (https://www.ecdc.europa.eu/en/antimicrobial-consumption/database/country-overview).

2.1. How do they act? A molecular perspective

Most bacteria have an essential structural element, the cell wall, consisting of a peptidoglycan matrix, which is formed from glucan chains cross-linked by peptides (Cho et al., 2014; Lobanovska & Pilla, 2017). Cell wall is essential to maintain cell function, ensure its shape, integrity and prevent macromolecules from migrating into the cell (Lobanovska & Pilla, 2017; Raynor, 1997). Cross-linking is mediated by transpeptidases, usually called PBP, which use an active serine site to carry out their catalytic cycle (Wilke et al., 2005). The number of different types of PBP per bacterial species is variable, frequently ranging from 3 to 8, differing in affinity with which they bind to penicillin or other β-lactam antibiotics (Bush & Bradford, 2016; Nordmann et al., 2012). β-lactams act as analogs of PBP' substrates, since they resemble the structure of D-alanyl-D-alanine, binding irreversibly to Ser403 residue of PBP active site (Nordmann et al., 2012; Oliva et al., 2003) and inhibiting its catalytic activity (Raynor, 1997). Under normal conditions, peptidoglycan precursors signal for bacterial cell wall reorganization, activating cell wall autolytic hydrolases. Since transpeptidases inhibition cause precursors accumulation, digestion of existing peptidoglycan by autolytic hydrolases takes place without new peptidoglycan production (Nordmann et al., 2012). Through these autocatalytic mechanisms, leading to osmotic pressure and cell lysis, cell death occurs (Lobanovska & Pilla, 2017; Raynor, 1997) (figure 4).

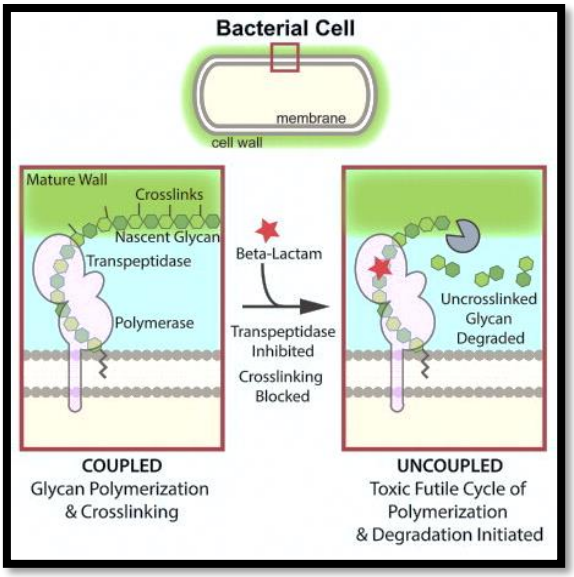

Figure 4. Mechanism of action of β-lactams (Adapted from Cho et al., 2014).

3. Antibiotic resistance: one step forward, two steps backward

Since they were discovered and introduced in the clinical practice in the 1930s, antibiotics have been highly effective against bacterial infections. Nowadays, we face the problem of antibiotic resistance increasing, since bacteria carrying antibiotic resistance genes (ARG) are resistant to most commonly prescribed treatments, giving rise to more prolonged illnesses and a higher death risk (Biyela et al., 2004; Y. Yang, Liu, et al., 2017). In a broad sense, antibiotic resistance can be defined as any reduction in susceptibility of a bacterial strain when compared to the susceptible wildtype strain (Kraemer et al., 2019).

Antimicrobial resistance (AMR) is a natural and ancestral phenomenon that precedes human use of antibiotics. However, infections caused by bacteria resistant to these compounds and their rapid spread have been described after their human use to treat diseases (Agga et al., 2015; Goethem et al., 2018). Antibiotic resistance seriously compromises Public Health, causing thousands of deaths per year (Z. Zhou et al., 2018). Data collected from European Antimicrobial Resistance Surveillance Network (EARS-Net), from January to December 2015, indicate that in Europe and in countries belonging to the European economic area, 671,689 people were infected with antibiotic-resistant bacteria (ARB), of which 33,110 resulted in death (Cassini et al., 2019). Although there are some continents and countries where it is possible to quantify the AMR effect, there are also other regions where epidemiological data are scarce, making it difficult to measure the real impact of AMR globally (Marston et al., 2016). The emergence of infections caused by ARB and the lack of new antibiotics development threatens humanity to return to the pre-antibiotic era (Lamba et al., 2017). In a world without

effective antibiotics, 11 million people a year would lose their lives due to bacterial infections and there would be a reduction in the global economy of 0.1-3.1% by 2050 (O'Neill, 2014). Unfortunately, health care changes in the last 50 years, such as overuse and misuse of antibiotics have created new niches for some microorganisms to emerge as important human pathogens (Mathers et al., 2019; Ventola, 2015). The continuous increase of AMR in clinical isolates has been attributed to selective pressure exerted by anthropogenic action (Agga et al., 2015). In the environment, bacteria can be exposed during long periods of time to low concentrations of antibiotics, that are present due to release of pollutants derived from anthropogenic activities (Wistrand-Yuen et al., 2018). Excessive use of antibiotics, biocides, heavy metals and other chemicals has led to their accumulation in the environment, providing an opportunity for exposure of microbial populations to these compounds, which may select for antibiotic resistance (Almakki et al., 2019; Biyela et al., 2004; Haberecht et al., 2019). In polluted environments, ARG can be co-selected with resistance genes to other pollutants, such as heavy metal resistance genes, since they can be located on the same mobile genetic element (MGE) (Mills & Lee, 2019).

Bacterial resistance can be classified into intrinsic resistance, adaptive resistance and acquired resistance (Hughes & Andersson, 2017). In intrinsic resistance, also known as innate resistance, bacterial ability to resist to an antibiotic is due to intrinsic functional or structural properties (Aslam et al., 2018). It is a trait shared within the species level, it is independent of previous exposure to an antibiotic and is not related to horizontal gene transfer. Examples of this resistance are the natural activity of efflux pumps (Reygaert, 2018) or the slow uptake of the drug due to possession of a semi-permeable external membrane (Fernández & Hancock, 2012; Hughes & Andersson, 2017). Secondly, the adaptive resistance, is induced when a bacteria is exposed to antibiotics (Reygaert, 2018), causing a temporary increase in bacteria's ability to survive to its action and even to other antibiotics to which it was not exposed. Unlike other types of resistance, this resistance is not transferred vertically (Fernández & Hancock, 2012; Hughes & Andersson, 2017). Some examples are epigenetic modifications which can lead to efflux pump overexpression, increasing antibiotic resistance levels (Du et al., 2018). Thirdly, acquired resistance arises through mutations in chromosomal DNA or through horizontal gene transfer (HGT). Acquired resistance can also happen through a combination of these two mechanisms (Aslam et al., 2018; Giedraitienė, 2011). Resistance acquired through HGT is one of the main pathways for bacterial evolution and plays a central role in antibiotic

resistance spread among clinically relevant bacteria (Mathers et al., 2019; Munita & Arias, 2016). Acquired resistance genes are usually "packaged" within mobile DNA, that is, a segment of DNA that is capable of translocating from one region of the genome to another or even between genomes (van Hoek et al., 2011). Main mechanisms of HGT are transduction, transformation and conjugation (figure 5) (Munita & Arias, 2016). The first mechanism is mediated by bacteriophages, in which bacterial DNA and phages DNA are packed together in their heads and subsequently injected into recipient bacteria. The second mechanism is based on the acquisition of naked DNA from the extracellular environment by both Gram-positive and Gram-negative competent bacteria (van Hoek et al., 2011). In this process the genes are incorporated into the genome of the recipient cell by homologous recombination or transposition. During this process, gene DNA sequences can suffer nucleotide alterations (Giedraitienė, 2011). The DNA molecule acquired by transformation may be able to replicate autonomously (e.g. plasmids) and therefore it doesn't need to be incorporated into the host DNA (van Hoek et al., 2011). Conjugation, the third mechanism, consists of HGT through cell-cell contact (Munita & Arias, 2016) and is mediated by conjugative transposons and conjugative plasmids that have all the genetic information necessary for their transfer between cells. Mobilizable elements also can be transferred through this mechanism. In this case, they use conjugation machinery of conjugative elements present in the cell to transfer themselves to another host (van Hoek et al., 2011). Through HGT mediated by conjugative plasmids, an ARG can also be transferred from a chromosome to another chromosome (Manson et al., 2010). Conjugation is very efficient with regard to HGT, for example, in the hospital setting, emergence of resistant bacteria is often associated with this mechanism, and it is believed to occur at high rates in the gastrointestinal tract of individuals who are under antibiotic treatment (Munita & Arias, 2016). Acquisition of resistance through mechanisms above described can be temporary or permanent (Reygaert W., 2018).

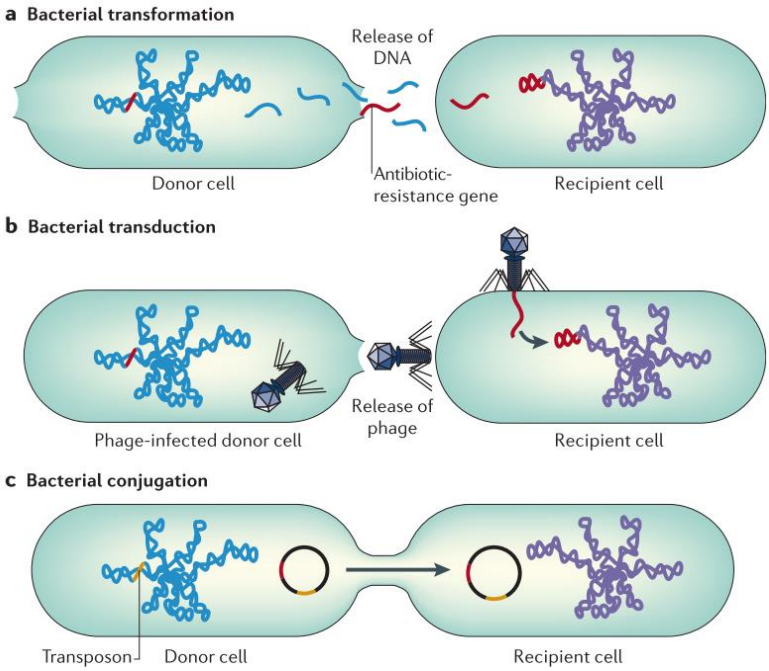

Figure 5. HGT mechanisms: Transformation, transduction and conjugation (Furuya & Lowy, 2006).

Integrons are genetic platforms, firstly described in 1987 (Stokes & Hall, 1989), responsible for the capture, rearrangement and expression of gene cassettes (GC), thus providing a rapid adaptive capacity to bacteria (Ghaly et al., 2019). The GC, which are free in the circular form, and linearized when are integrated into integrons (Recchia & Hall, 1995), possess a mobilizable open reading frame and an *attC* recombination site (Hall & Collis, 1995). Once the majority of GC don't harbor their own promoter, they depend on the integron promoter to be expressed (Jové et al., 2010).

Integrons have a typical structure: a gene that encodes an integrase (*intI*), a promoter (Pc) and a recombination site (*attI*) (Guérin et al., 2011). Integrase is both responsible for inserting the GC on integron, through recombination of *attC* and *attI* sites and responsible for GC excision, through recombination between *attC* sites (Stalder et al., 2012) (figure 6). The integrase is responsible for the insertion of GC in the correct orientation, ensuring that it can be expressed by the integron promoter (Nivina et al., 2016). In addition to Pc promoter, some class 1 integrons also harbour a second promoter, around 90 bp downstream away from Pc (Xiao et al., 2019).

Although integrons are not mobile by themselves, they can be associated with MGE, such as transposons and plasmids, thus contributing to the spread of ARG among bacteria (Stalder et al., 2012).

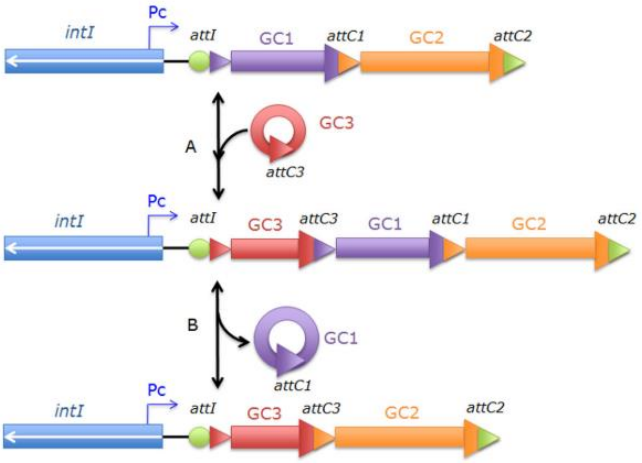

Figure 6. General structure of integrons. A) GC3 integration, through integrase mediated recombination between *attI* and *attC3* sites. B) GC1 excision, through integrase mediated recombination between *attC1* and *attC3* sites (Stalder et al., 2012).

When the bacterial cell is under stress conditions, integrase expression, reorganization of GC and capture of new determinants of resistance can be increased (Baharoglu et al., 2010; Guerin et al., 2009). Moreover, it has been suggested that the expression of integrase is regulated by SOS response. In the absence of stress condition, the repressor protein LexA binds to P_{intL} and avoids integrase transcription. On the other hand, when a bacterium is exposed to a stress condition, such as an antibiotic, the repressor protein is cleaved and the transcription of the integrase gene occurs normally, allowing the integration, excision and reorganization of GC. This expression regulation makes these structures inexpensive for the cell when are not needed (Guerin et al., 2009; Lacotte et al., 2017; Tansirichaiya et al., 2019).

3.1. Molecular mechanisms of antibiotic resistance

Antibiotic resistance can be acquired through chromosomal mutations or HGT of resistance genes, with several molecular mechanisms involved in this resistance (Rolain, 2013). The four main mechanisms that confer resistance to antibiotics are: a) decrease in drug uptake, due to changes in permeability of bacterial cell wall; b) modification of drug target due to post-translational modifications or due to genetic mutations in gene that encodes the target (Reygaert W., 2018; Aslam et al., 2018), c) drug inactivation by enzymatic modifications or hydrolysis (degradation of the antibiotic); d) drug efflux (Reygaert W., 2018; Aslam et al., 2018) (figure 7). Since interaction between antibiotic and its target is highly specific, small changes in target can influence antibiotic binding (Giedraitienė et al, 2011); Other mechanisms include the acquisition of alternative

metabolic pathways and overproduction of target enzyme (van Hoek et al., 2011). Acquisition of alternative metabolic pathways can be ensured for example by a production of an antibiotic resistant replacer enzyme, which performs the same function as the native enzyme. In this situation, antibiotic blocks native enzyme activity, however, due to the action of the replacer enzyme that is resistant to antibiotic action, cell activity is not affected (Giedraitienė, 2011).

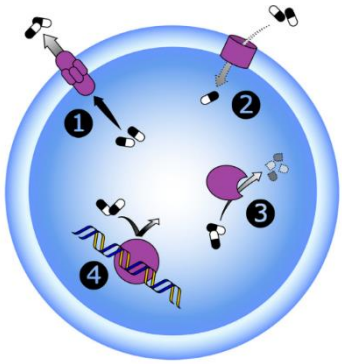

Figure 7. Main mechanisms of antibiotic resistance: 1) drug efflux; 2) decrease in drug uptake; 3) drug inactivation; 4) modifications in drug target (Laws et al., 2019)

3.1.1. β-lactamases: The biggest threat to β-lactams efficiency

Among Gram-negative organisms, the main resistance mechanism to β-lactams is the production of degrading enzymes, known as β-lactamases (Philippon et al., 2002). β-lactamases are a heterogeneous group of enzymes, with more than 1800 variants described so far, and are classified either according to their protein sequence homology, by Ambler classification, or according to their phenotypic profile, by the Bush-Jacoby-Medeiros classification (Brandt et al., 2017). In Ambler classification, β-lactamases are divided into four classes, from A to D, in which, enzymes that belong to groups A, C and D are serine β-lactamases and those belonging to group B are metallo-β-lactamases (Paterson & Bonomo, 2005). β-lactamases of Ambler classes A, C and D catalyze hydrolysis through attack using a catalytic serine, whereas in class B enzymes, which have one or two zinc ions in their active site, the attack is promoted by a hydroxide ion (Palzkill, 2018). On the other hand, in Bush-Jacoby-Medeiros classification, enzymes are grouped according to their substrate and inhibitor profiles. This classification encloses four main groups and several organizational subgroups (Paterson & Bonomo, 2005). The first β-lactamase identified, an AmpC in *Escherichia coli*, was described before the widespread use of β-lactams (Abraham & Chain, 1940; Jacoby, 2009), and β-lactamases were found in remote Alaska soil, demonstrating that they can be present in

environmental isolates even without anthropogenic pressure (Allen et al., 2009). However, extensive use of antibiotics led to their emergence and dissemination (Tooke et al., 2019), since exposed strains are induced continuously to produce β-lactamases, which in turn also acquire mutations and modify their action spectrum (N Samaha-Kfoury & Araj, 2003) (figure 8).

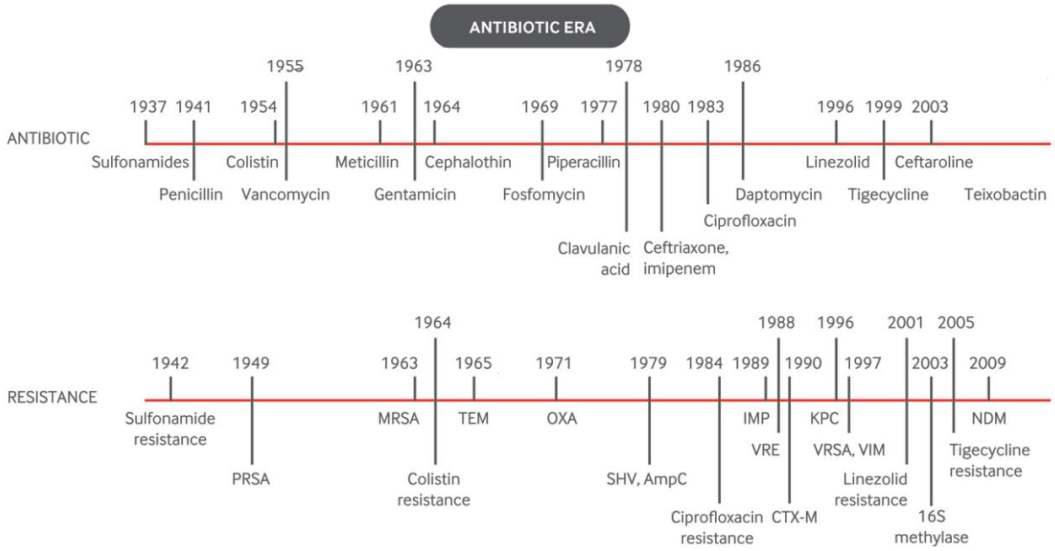

Figure 8. Timeline showing clinical implementation of antibiotics and the first report of resistance to those antibiotics. PRSA- penicillin-resistant *Staphylococcus aureus*. MRSA – Methicillin-resistant *Staphylococcus aureus* (Adapted from Iredell et al., 2015).

The first plasmid encoded β-lactamase, TEM-1, was described in 1965 (Datta & Kontomichalou, 1965), having spread worldwide among different bacterial species (Grover et al., 2013). When penicillins lost their capacity to treat most bacterial infections, more potent penicillins and cephalosporins were developed (Bush, 2010). During 1980s, introduction of 3rd generation cephalosporins was a main step in the treatment of infections caused by β-lactamase producing bacteria (Paterson & Bonomo, 2005). Shortly after introduction of these cephalosporins, in 1983, a plasmid-mediated β-lactamase, capable of hydrolyzing extended-spectrum cephalosporins was described (Knothe et al., 1983). These enzymes, called extended-spectrum β-lactamases (ESBLs), evolved from β-lactamases TEM-1, TEM-2 and SHV-1, through point mutations (N Samaha-Kfoury & Araj, 2003), possessing the ability to inactivate penicillins, 1st, 2nd and 3rd generation cephalosporins, aztreonam and most of enzymes in this group may also confer resistance to 4th generation cephalosporins. However, they do not have the ability to hydrolyze cephamycins or carbapenems and are inhibited by β-lactamase inhibitors, such as clavulanic acid (Ye et al., 2017). Another β-lactamases group is the AmpC group. These

enzymes are not inhibited by classic β-lactamase inhibitors, they confer resistance to cephamycins but do not efficiently hydrolyze cefepime (Rodriguez-Baño et al., 2018). Both ESBL and AmpC can be found in plasmids and are able to spread quickly (Barlow & Hall, 2002; Rodriguez-Baño et al., 2018). After extended-spectrum cephalosporins introduction in clinical practice, ESBLs proliferated, making treatment with these β-lactams more difficult (Bush, 2010).

Carbapenems are the drugs of choice in treatment of infections caused by ESBLs and AmpC-producing *Enterobacteriaceae* (Rodriguez-Baño et al., 2018). During the late 20th century, it seemed that almost all resistance mediated by β-lactamases could be overcome using carbapenems (Bush, 2010). Although first reports of carbapenemases were published in the 1980s (Codjoe & Donkor, 2018), until the early 1990s they were associated to certain species and were chromosome-encoded enzymes (Queenan & Bush, 2007). First report of plasmid encoded carbapenemase, IMP-1, an enzyme conferring imipenem resistance, occurred in 1990 (Watanabe et al., 1991). Since then, other carbapenemases have been isolated and characterized, encoded in MGE, such as KPC-1 in 1996 (Yigit et al., 2001) and GES-2 in 2000 (L. Poirel et al., 2001). According to Ambler classification, carbapenemases belong mainly to classes A, B and D. Although Kim et al., 2006 described a class C enzyme, CMY-10, able to hydrolyze imipenem, enzymes of this class are not considered true carbapenemases since their carbapenem hydrolysis activity is low or nonexistent (Bonomo et al., 2018; Kim et al., 2006; Nordmann & Poirel, 2019). Some examples of carbapenemases are described in figure 9.

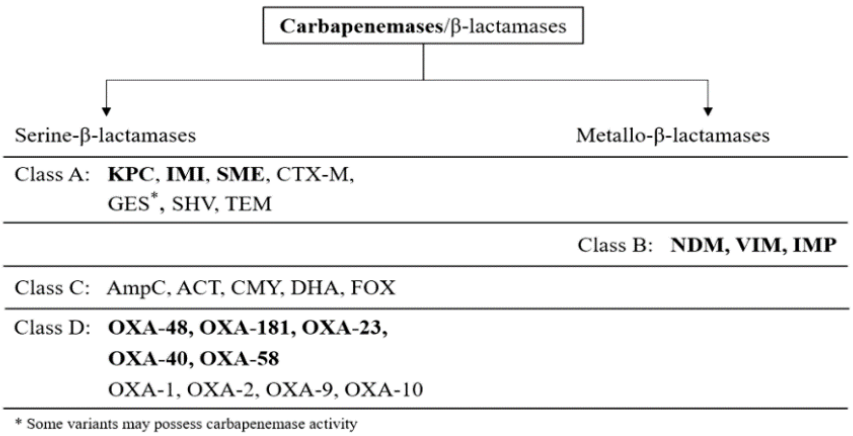

Figure 9. Classification of carbapenemases and β-lactamases according Ambler classification (Nordmann & Poirel, 2019).

Due to widespread and consecutive dissemination of carbapenemases, leading to carbapenem resistance, treatment options available to overcome infections caused by Gram-negative bacteria are quite limited, and in some cases, bacteria become resistant to all available antibiotics, since the MGE that carry carbapenemase genes can also possess additional resistance genes to other antibiotics (Hrabák et al., 2014). A strain is considered multidrug resistant (MDR) when it is not susceptible to one or more antimicrobials in three or more antibiotic classes (Koulenti et al., 2019). To make matters worse, MGE that harbor ARG may also have genes that encode virulence factors (Harbarth et al., 2015). In addition to this coexistence of resistance genes to various antibiotics, for example, coexistence of the KPC enzyme with other carbapenemases has been globally described (Giakkoupi et al., 2009; S. N. Richter et al., 2012; Rojas et al., 2013). Due to their significant activity as carbapenemases, the ability to hydrolyze not only carbapenems but also broad-spectrum cephalosporins and because they are globally found, KPC, VIM, NDM and IMP are considered the most relevant carbapenemases found in *Enterobacteriaceae* family (Poirel et al., 2012) (table 11).

Table 1. Main carbapenemases found in *Enterobacteriaceae* and its hydrolysis activity against different β-lactam antibiotics. (-) represents not detectable hydrolysis, (+) represents detected hydrolysis and (++) a stronger hydrolysis detected (Nordmann et al., 2012).

Ambler class	Name of the enzyme	Plasmid/ chromosome	Hydrolysis spectrum						Inhibitor
			Penicillins	First generation cephalosporins	Second generation cephalosporins	Third generation cephalosporins	Aztreonam	Carbapenems	
A	SME-1 to -3	Chromosome	++	++	–	+	+	+	Clavulanate, tazobactam, sulbactam, NXL-104
	NMC-A	Chromosome	++	++	–	+	–	++	
	IMI-2	Plasmid	++	++	–	+	–	++	
	GES-4, -5, -6	Plasmid	++	++	+	+	–	+	
	KPC-2 to -12	Plasmid	++	++	–	++	+	++	Clavulanate, tazobactam, boronic acid, sulbactam
B	IMP-1 to -33	Plasmid	++	++	++	++	–	++	EDTA
	VIM-1 to -33	Plasmid	++	++	++	++	–	++	
	NDM-1 to -6	Plasmid	++	++	++	++	–	+	
	KHM-1	Plasmid	++	++	++	++	–	++	
D	OXA-48	Plasmid	++	++	+/–	+/–	–	+	NaCl
	OXA-181	Plasmid	++	++	+/–	+/–	–	+	

Although infections caused by ESBL-producing and carbapenemase-producing bacteria were commonly associated with healthcare settings, in recent years, community-acquired infections caused by these bacteria have increased significantly (Tanner et al., 2019). For example, a study conducted by Tang *et al*., reported that almost 33 % of human

infections with carbapenem-resistant *Enterobacteriaceae* were community-acquired (H. J. Tang et al., 2016).

Over the past few years there has been an attempt to assess the incidence of carbapenem-producing *Enterobacteriaceae* (CPE) in European countries. According to the first epidemiological studies under the European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE) project (Albiger et al., 2015; Glasner et al., 2013; Grundmann et al., 2010) and the European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net) program (Brolund et al., 2019), countries are clustered in different stages (0, 1, 2, 2b, 3, 4 and 5) (figure 10), depending on CPE incidence, where 0 represents " no case reported" and 5 represents "endemic situation (most hospitals in a country are repeatedly seeing cases admitted from autochthonous sources)". Portugal, which in 2010 and 2013 was in phase 1 (sporadic occurrence (epidemiologically unrelated single cases)), reached to phase 2b (sporadic hospital outbreaks (unrelated hospital outbreaks with epidemiologically unrelated introduction or different strains, no autochthonous inter-institutional transmission reported)) in 2014-15 and it was in phase 3 (regional spread (more than one epidemiologically related hospital outbreak confined to hospitals that are part of the same region or health district, indicating regional autochthonous inter-institutional transmission)) in 2018. Thus, it shows a gradual increase of CPE dissemination. In 2018, in addition to Portugal, the remaining 36 European countries also reported cases of CPE (Brolund et al., 2019). In 2019, David *et al.*, analyzed 1717 *Klebsiella pneumoniae* genomes, from strains studied under EuSCAPE program and observed that the predominant carbapenems resistance mechanism in Portugal was the production of KPC (David et al., 2019).

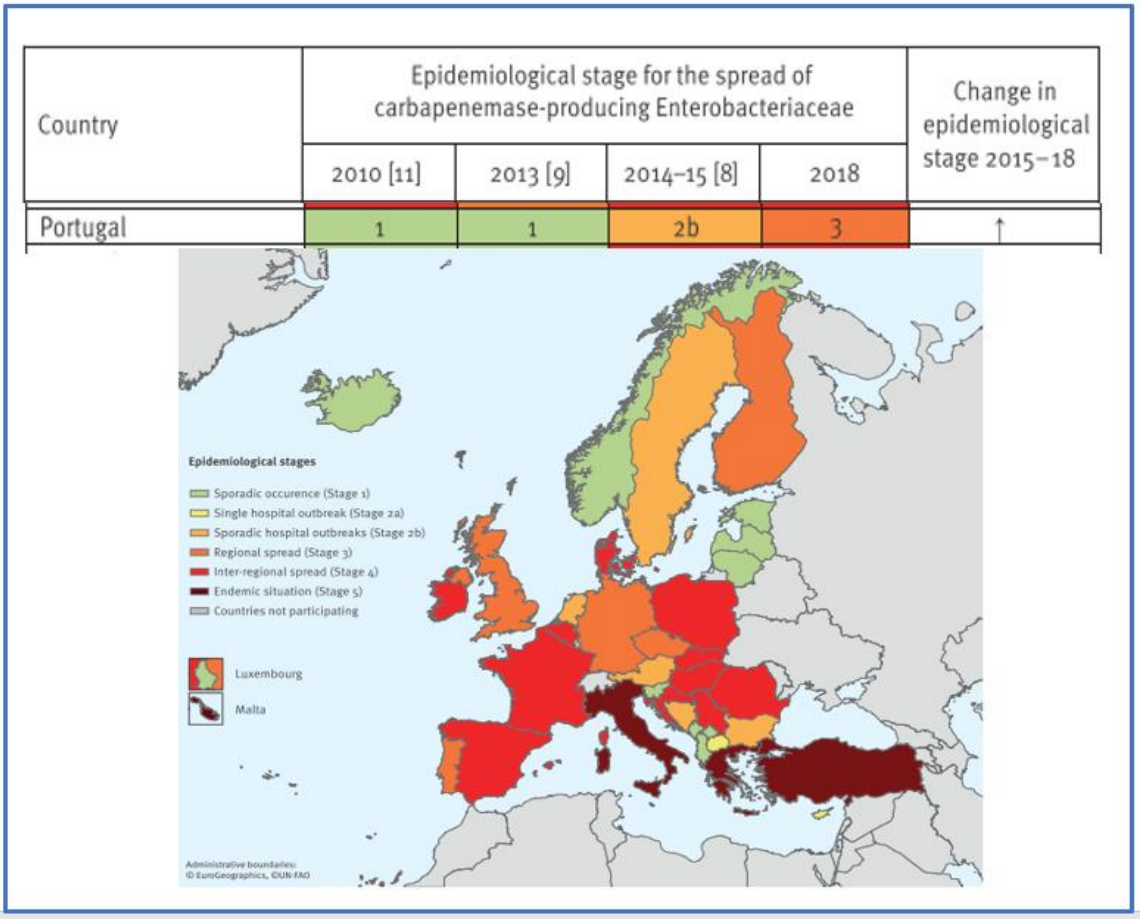

Figure 10. Epidemiological stages regarding the spread of carbapenemase-producing *Enterobacteriaceae* from 2010 to 2018 in different countries in Europe in 2018 (Brolund et al., 2019).

In addition to carbapenemases associated to MGE, there are microorganisms which harbour intrinsic carbapenemase coding genes, such as $bla_{\text{OXA-51-Like}}$ in *Acinetobacter baumannii*, bla_{L1} in *Stenotrophomonas maltophilia* and bla_{cphA} in *Aeromonas* spp., this knowledge is important to guide clinicians to choose an effective antibiotic (Lee et al., 2012; Rosso et al., 2019; Z. Yang et al., 2014).

4. MDR organisms, a Public Health concern: Focusing on *Enterobacteriaceae* and *Aeromonas*

In 2017, the World Health Organization (WHO) published a guideline indicating the most important ARB globally, for which there is an urgent need for new treatments, containing a list of priorities to guide research, discovery and development of new antibiotics (WHO, 2017). Among these microorganisms, are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., coined as ESKAPE (Rice, 2008). These organisms are associated to a growing increase of antibiotic resistance, are increasingly associated with

diseases in humans and the infections caused by them take longer to be treated, being associated to a higher mortality rate (Pogue et al., 2015). In 2018, the European Centre for Disease Prevention and Control (ECDC) published an antibiotic resistance report, with data collected from 2015 to 2018 in European Union countries (ECDC, 2019). Microorganisms isolated and characterized are part of those for which WHO in 2017 has established that there is an urgent need of antibiotics research and development. Among them, there were MDR organisms. The sets of antibiotics to which the microorganisms were resistant, giving an MDR phenotype and the proportion they represent are described in table 2.

Table 2. Microorganisms isolated from 2015 to 2018 in European countries, the sets of antibiotics to which they are resistant giving an MDR phenotype and the proportion they represent (ECDC, 2019).

Organism	MDR resistance phenotype	number of isolates	proportion of resistant bacteria (%)
Escherichia coli	Aminopenicillins + fluoroquinolones + aminoglycosides	2814	2.3
	Aminopenicillins + third-generation cephalosporins + fluoroquinolones + aminoglycosides	5904	4.9
Klebsiella pneumoniae	Third-generation cephalosporins + fluoroquinolones + aminoglycosides	4978	13.7
	Third-generation cephalosporins + fluoroquinolones + aminoglycosides + carbapenems	1799	5.0
Pseudomonas aeruginosa	Fluoroquinolones + aminoglycosides + carbapenems	169	0.9
	[Piperacillin ± tazobactam] + fluoroquinolones + aminoglycosides + carbapenems	235	1.3
	Fluoroquinolones + ceftazidime + aminoglycosides + carbapenems	139	0.8
Acinetobacter spp.	Fluoroquinolones + aminoglycosides + carbapenems	2848	45.7
Staphylococcus aureus	MRSA + fluoroquinolones + rifampicin	254	0.5

Gram-negative bacteria can become MDR, since MGE can carry multiple ARG, such as carbapenemases and resistance determinants to other antibiotic classes, conferring resistance to several or all available antibiotics (Hrabák et al., 2014). Although MDR bacteria are often associated with nosocomial infections, there are some cases in which these bacteria have been quite prevalent causes of community-acquired infections, leading to an increase in exposed population at risk and the subsequent increase in the

number of infections caused by these bacteria (van Duin & Paterson, 2016; Villafuerte et al., 2020).

Organisms belonging to *Enterobacteriaceae* family (such as the genera *Klebsiella*, *Raoultella*, *Citrobacter*, *Enterobacter*, etc.) are Gram-negative organisms, ubiquitous in nature. Some of them can be MDR associated with severe infections in humans and animals (Djenadi et al., 2018; Duin & Doi, 2017; Potter et al., 2016). In WHO report, the *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., and *Providencia* spp, *Morganella* spp microorganisms, members of *Enterobacteriaceae* family resistant to carbapenems and 3rd generation cephalosporins, were classified as "priority number 1", a critical group, for which discovery and development of new antibiotics are urgent. This classification was based on several criteria, such as mortality rate, if they are treatable or not, widespread presence of resistance, preventability in community and healthcare settings, trends of resistance based on 10-years data, healthcare and community burden and transmissibility (WHO, 2017). Although the CPE are now considered "top 1", this resistance phenotype was not observed when clinically use of carbapenems started. At that time, almost all *Enterobacteriaceae* were susceptible to them, however this is no longer true (Rida et al., 2018). One of the MGE responsible for MDR phenotypes in *Enterobacteriaceae* are the plasmids. For example, the plasmids incF, conjugative plasmids found in members of this family can possess resistance determinants to several antibiotics, such as carbapenems and other β-lactams, aminoglycosides, sulphonamides, quinolones and tetracycline (Rozwandowicz et al., 2018).

Other clinically relevant microorganisms are the members of *Aeromonas* genus, Gram-negative bacteria, which include clinical relevant species (Figueras et al., 2000). They inhabit aquatic environments, being also habitually found in meat, vegetables and seafood (Ugarte-Torres et al., 2018). Members of *Aeromonas* genus are considered as emerging pathogens and may cause disease both in cold-blooded animals, such as fish, and in immunocompromised individuals (Fernández-Bravo & Figueras, 2020; Janda & Abbott, 2010). In human they can cause diseases such as wound infections, septicemia, gastroenteritis (Fernández-Bravo & Figueras, 2020) Within this genus, have been described MDR strains, isolated from both aquatic environments and clinical settings (patients) (Odeyemi & Ahmad, 2015; Zhou et al., 2019). MDR plasmids have been also described in these organisms, making them resistant to several classes of antibiotics. Some studies demonstrated through conjugation assays that some of these MDR plasmids

are able to conjugate and to be transferred to *Escherichia coli*, a member of the *Enterobacteriaceae* family, demonstrating that the spread of ARG through MGE is not limited to the exchange between members of the same family (Del Castillo et al., 2013; Hedges et al., 1985; Rhodes et al., 2000; Sørum et al., 2003).

5. Environment: Both final destination and origin of resistance genes

In the natural environment, bacteria have to overcome several challenges, including competition and predation. Antibiotic producing bacteria use them to deal with these challenges (Nguyen et al., 2019). In addition to genes responsible for antibiotic biosynthesis, bacteria tend to harbour ARG in the same operon, encoding a defense mechanism against their own antibiotics (D'Costa et al., 2006). On the other hand, to survive, antibiotic susceptible bacteria inhabiting the same ecological niche also tend to develop resistance mechanisms (Galán et al., 2013). Thus, the origin of ARG can be found in both antibiotic-producing and non-producing bacteria (Cantón, 2009). Once antibiotics are often found in insufficient concentrations to act as toxic agents against other bacteria, it is thought that they are able to perform other functions, such as signalling molecules for the biofilm formation (Fajardo et al., 2009). Likewise, the product of some ARG seems to play physiological functions. An example is the AmpC enzyme, which confers resistance to some β-lactams but is also involved in peptidoglycan biosynthesis (Galán et al., 2013). The resistome can be defined as "(…) *the collection of all the antibiotic resistance genes, including those usually associated with pathogenic bacteria isolated in the clinics, non-pathogenic antibiotic producing bacteria and all other resistance genes*", including "*cryptic resistance genes (which are not necessarily expressed)*" (Wright, 2007). The environmental resistome comprises ARG from different environments, such as built environments, animals, atmosphere and aquatic environments, which may interact with microorganisms present in humans (Surette & Wright, 2017) (figure 11).

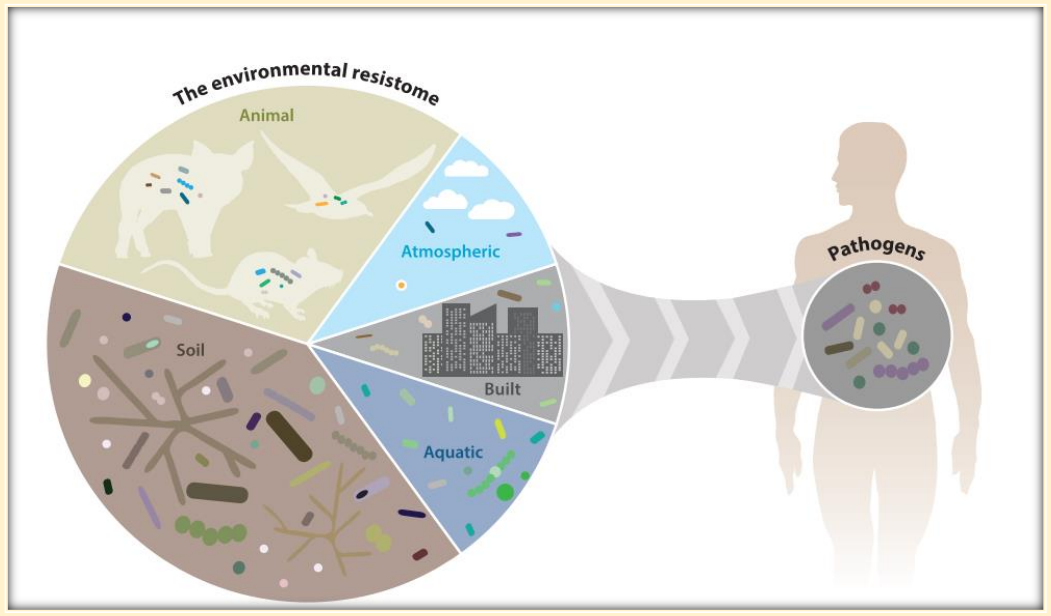

Figure 11. The environmental resistome and its connection to human pathogens (Surette & Wright, 2017).

The environmental resistome is ancestral, quite diverse and can mobilized (Surette & Wright, 2017). Environmental bacteria are thought to be natural reservoirs of ARG and probably are the origin of ARG found in clinical environments (Surette & Wright, 2017; Wright, 2007). Although almost 50 years ago Benveniste & Davies noticed that aminoglycosides inactivating enzymes present in *Actinomyces*, producers of aminoglycosides, are similar to those found in clinically relevant bacteria (Benveniste & Davies, 1973), the role of natural environment in antibiotic resistance was ignored for many years (Surette & Wright, 2017). In recent years, genes orthologous to those present in antibiotic-producing organisms have been found in MGE present in clinically relevant bacteria (D'Costa et al., 2006). Some examples of the ARG found in clinical settings that evolved from environmental bacteria genes are: *bla*$_{\text{CTX-M}}$, from *Kluyvera ascorbate* (Humeniuk et al., 2002), *bla*$_{\text{OXA-48}}$ from *Shewanella* spp. (Oxacillinase et al., 2004; Tacão et al., 2013; Tacão et al., 2017), *bla*$_{\text{OXA-181}}$ from *Shewanella xiamenensis* (Potron et al., 2011), *qnr*-like genes from *Vibrionaceae* (Poirel et al., 2005a), *qnrA* from *Shewanella algae* (Poirel et al., 2005b), *mecA* from *Staphylococcus sciuri* (Wu & Tomasz, 2001) and *bla*$_{\text{KPC}}$ from *Chromobacterium* spp. (Gudeta et al., 2016; Teixeira et al., 2020). Other ARG, with unknown origin, have been also found both in clinical settings and aquatic environments, suggesting that there may be an exchange of ARG between the environment and the clinic (Pereira et al., 2013; Tacão et al., 2014; Tacão et al., 2012).The genetic context of ARG contribute to their dissemination (Lupo et al., 2012). The genetic fraction responsible for the exchange of ARG is called mobilome (Almakki et al., 2019).

ARG found in the environment can be integrated in integrons and successively mobilized to human pathogens through plasmids and transposons (Kristiansson et al., 2011). Gillings *et al*. suggested that class 1 integrons may emerge from natural environments and that upon reaching the clinical environment, integrated in transposons, they spread to human bacteria, both commensal and pathogenic. The acquisition of ARG by pathogenic bacteria conferred an adaptive advantage against administered antibiotics (Gillings et al., 2008). Although class 3 integrons are less abundant in the clinical environment, they may play an important role in the environment (Stalder et al., 2012). The HGT is more influenced by environmental factors than by microbial phylogeny or geographic distribution. For example, HGT between human associated bacteria (microbiome) is 25 times higher than in non-human associated bacteria (Smillie et al., 2011).

5.1. Contamination of aquatic environments

ARG can be found in low-impacted environments, such as in a study by Fonseca *et al* that found metallo-β-lactamases homolog genes in water samples, collected from different pristine oceans. It is thought that these genes do not have significant clinical relevance, since they are distant homologous of clinical relevant ARG, are located on chromosome, and are present in pristine regions, not representing a threat of ARG dissemination (Fonseca et al., 2018). On the other hand, in environments with high anthropogenic pressure, such as contaminated aquatic environments, ARG may disseminate and have a high impact in human heath (Amarasiri et al., 2019; Czekalski et al., 2015; Yang et al., 2017). These ARG can be co-selected even in absence of selective pressure exerted by antibiotics (Zhang et al., 2020). Although ARG had their origin in natural environments, is the selective pressure exerted by anthropogenic activities the driven-force that accelerates the spread of these ARG (Chen et al., 2013).

Since the end of the last century, antibiotics have been considered emerging pollutants (Yang, et al., 2017) and ARG have been considered environmental contaminants since 2006 (Pruden 2006). Among the causes that contribute to the increase in the prevalence of ARG in aquatic environments, whether by selection or promotion of ARG HGT, is the overuse of antibiotics in veterinary medicine, livestock and aquacultures, where antibiotics are used as prophylactic measure, growth promoting agents and therapeutic agents; use of manure as fertilizer in agriculture, which may contain non-metabolized antibiotics; overpopulation; wildlife spread; poor sanitation and

sewerage infrastructures (Aslam et al., 2018; Vaz-Moreira et al., 2020; Wang et al., 2018). The wide use of antibiotics causes an increase in its discharges into the environment, often in active form, contributing to the spread of ARG and ARB (Gbylik-Sikorska et al., 2014; Silva et al., 2018; Marta Tacão et al., 2015)(figure 12).

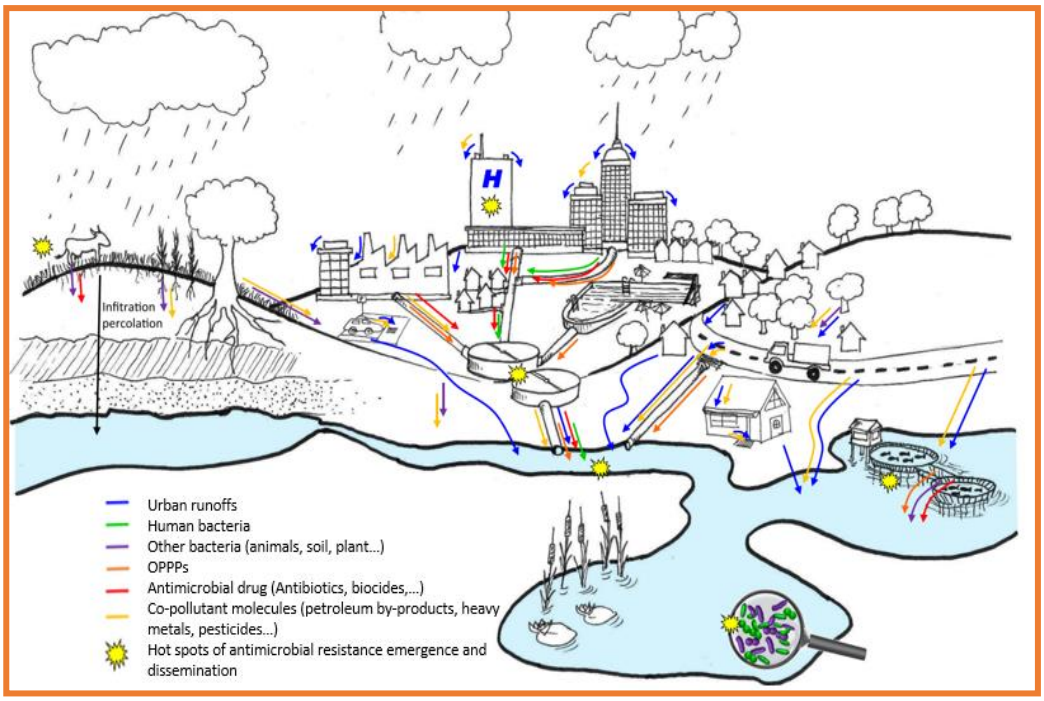

Figure 12. Route of urban runoffs from the origins to aquatic environments (Adapted from Almakki et al., 2019).

Larsson *et al*. observed that effluents from a pharmaceutical industry contained a ciprofloxacin antibiotic concentration of 31,000 µg/L, values 1000 times higher than those needed to cause the death of some bacteria (Larsson et al., 2007). Urbanization can affect surface waters quality and change the composition of aquatic microbial communities. One mechanism by which human activity directly affects surface water is through wastewater treatment process, in which human waste is collected, treated and treated wastewater is eventually released into surface waters (Lambirth et al., 2018), such as rivers, seas, oceans and lakes (Pazda et al., 2019). Wastewater treatment plants (WWTP) may receive a mixture of effluents from hospitals, communities, farms, industries and agricultural sectors, which can be contaminated with ARG and ARB, therefore they are considered the largest source of ARB and resistance genes to aquatic environment (Amarasiri et al., 2019; Devarajan et al., 2015). When wastewaters are treated in WWTP, the total microbial load is significantly reduced. Unfortunately, the

fraction that ARB represent is not proportionally reduced, and in some cases may even increase (Alexander et al., 2015; Czekalski et al., 2012).

Upon reaching aquatic environments, antibiotics and other compounds with anthropogenic origin can be retained, accumulating and persisting over the time, for this reason, these environments are considered main receptors of these compounds of anthropogenic origin (Tacão et al., 2015), acting as both ARB and ARG reservoirs (Surette & Wright, 2017). The pollution of aquatic environments also contributes to the increase of integrons, that spread to clinical environments (Lupo et al., 2012). Aquatic environments are known as ideal hotspots for ARG acquisition and dissemination (Amarasiri et al., 2019). They are inhabited by a high diversity of native bacteria that act as a large reservoir of ARG, and by allochthonous bacteria from several origins, including potential pathogens that are already resistant to antibiotics. Consequently, aquatic environments become a local of HGT and subsequent emergence of ARB (Almakki et al., 2019). Some carbapenem resistance genes and their genetic contexts found in aquatic environments are described in table 3.

Table 3. Carbapenem-resistance genes and their genetic contexts found in different bacterial species isolated from aquatic environments.

Organisms	Source	Country	Gene	Genetic context	Reference
C. freundii	River	Portugal	*bla*$_{GES-5}$	Plasmid-borne class 3 integron	(Teixeira et al., 2020)
K. pneumoniae	River	Portugal	*bla* $_{KPC-3}$	Plasmid-borne transposon	
E. roggenkampii	River	Portugal	*bla*$_{NDM-1}$	Plasmid-borne transposon	
K. pneumoniae	wastewater	Portugal	*bla*$_{GES-5}$	not detected	(Manageiro et al., 2014)
K. pneumoniae	River	Philippines	*bla* $_{KPC-2}$	Plasmid	(Suzuki et al., 2020)
K. pneumoniae	wastewater	Austria	*bla*$_{OXA-48}$; *bla*$_{KPC-2}$	not screened	(Galler et al., 2014)
K. pneumoniae	wastewater	Germany	*bla*$_{NDM}$, *bla*$_{OXA-48}$	not screened	(Müller et al., 2018)
Enterobacter sp.	Coastal water	Brazil	*bla*$_{GES-5}$	not screened	(Montezzi et al., 2015)
Aeromonas sp.	Coastal water	Brazil	*bla*$_{KPC-2}$	not screened	
K. pneumoniae	River	Switzerland	*bla*$_{VIM}$	not screened	(Zurfluh et al., 2013)

In recent years, much attention has been given to rivers as reservoirs of ARG (Yang, et al., 2017). Since these aquatic systems receive sewage from urban effluents, they become the main source of ARB and ARG (Goñi-urriza et al., 2000). However, lakes have a longer retention time for pollutants, thus having a greater capacity to store and accumulate more ARG than rivers. Urban lakes have been seriously contaminated due to rapid urbanization. Due to its great role in ecological and economic area, such as ecological habitats, water sources, recreational activities and urban landscape, water quality is of great importance since there is a high probability of direct contact of this water with human beings (Yang, et al., 2017). A pond can be defined as "*(…) small (1 m2 to about 5 ha), man-made or natural shallow waterbodies which permanently or temporarily hold water*" Although word "lakes" are often used to describe both lakes and ponds, there is evidence that ponds are functionally different from lakes (Céréghino et al., 2008). It is important to establish and understand the role of this environment in the transmission of ARB to humans (Huijbers et al., 2015). Without inclusion of all pathways for spread of these bacteria in the environment, plans to try to contain their spread may not achieve the desired goals, thus compromising the effectiveness of existing and future antibiotics (Singer et al., 2016). Unfortunately, the role of the environment as a source of ARG and ARB dissemination and transmission is understudied (Mills & Lee, 2019).

II. Scope and aims of this thesis

Antibiotic resistance is one of the biggest threats to public health. With the increase of antibiotic resistance levels, a future without effective antibiotics is a possible scenario, where nowadays easy-to-treat infections would kill millions of people each year. The β-lactams are the most widely used class of antibiotics in a clinical setting, with several antibiotics currently used, ranging from penicillin to last-resort antibiotics, carbapenems. The use of carbapenems is frequently reserved to the clinic and administered only when no other antibiotics work. These antibiotics represent the ultimate strategy to overcome bacterial defenses and save human lives. For this reason, resistance to them is extremely worrying. Many of the studies carried out focus on the hospital environment, where carbapenem-producing bacteria are usually detected. However, natural environments, suggested as the source and a reservoir of ARG, may play an important role in the spread of antibiotic resistance. Little is known about antibiotic

resistance in urban ponds, aquatic environments in recreational areas where humans and animals can easily have direct contact with ARB.

With the constant increase in population growth and urbanization, it is expected that in the coming years, these urban environments will be more prevalent and that there will be more people in contact with them. The evaluation of the risk that they represent both in ARG dissemination and in community-acquired infections is fundamental to ensure effective Public Health measures.

Based on this knowledge, this work aimed to:

- To determine the prevalence of *Enterobacteriaceae* resistant to first-line, 3rd generation cephalosporins, and to last resort antibiotics, carbapenems, in urban aquatic systems;
- To characterize the phenotypes and genotypes of carbapenem-resistant bacteria isolated from urban aquatic systems;
- To perform whole-genome sequence analysis of clinically relevant carbapenemase-producing *Enterobacteriaceae* isolated from urban aquatic systems.

Hence, specific aims were:

- To determine the prevalence of cefotaxime- and imipenem- resistant *Enterobacteriaceae* along a 6-month period in 5 urban ponds and 1 estuarine channel;
- To establish a collection of carbapenem-resistant isolates obtained from urban aquatic systems;
- To determine the phylogenetic affiliation, clonality, antibiotic susceptibility profiles and plasmid content of a set of carbapenem-resistant isolates;
- To detect the presence of carbapenemase-enconding genes and integrases;
- To evaluate carbapenemase-encoding genes transferability through mating assays;
- To select isolates for whole genome sequence (WGS) analysis;
- To analyze the genetic context of carbapenemase-encoding genes and other antibiotic resistance determinants;
- To estimate the virulence and pathogenicity profiles based on WGS analysis.

III. Material and Methods

1. Sampling and enumeration of resistant bacteria

Water samples were recovered from surface water using sterile glass bottles and kept in ice during transportation. Water samples were recovered from 5 urban aquatic ponds in the city of Aveiro and from an urban estuarine channel downstream of the urban pond 1 in Dos Santos Mártires channel ((figure 13), from September, 2019 to February, 2020, comprising 6 campaigns. In January, no samples were collected in the Ria de Aveiro.

Figure 13. Location and designation of the sampling sites.

Location, designation and coordinates of the sampling sites are described in table 4.

Table 4. Location, designation and coordinates of the sampling sites. For ponds, areas and perimeters were estimated using Google Maps.

Location	Designation	Coordinates	Area (m^2)	perimeter (m)	aquatic environment
Pond 1	P1	40°38'18.9"N 8°39'21.9"W	810	185	pond
Pond 2	P2	40°38'08.3"N 8°39'13.6"W	6775	548	pond
Pond 3	P3	40°38'04.2"N 8°39'05.5"W	1126	263	pond
Pond 4	P4	40°37'57.4"N 8°39'40.3"W	4120	350	pond
Pond 5	P5	40°38'17.9"N 8°38'23.7"W	2526	379	pond
Dos Santos Mártires channel	E1	40°38'20.1"N 8°39’26.0"W	-	-	estuary

Water samples were filtered using a 0.45 µm mixed cellulose ester membrane (GN-6 Metricel®) and then membranes were placed in 50 mm Petri dishes containing m-Faecal Coliform agar (m-FC agar) (VWR), with or without antibiotics. From each location 0.1 mL, 1 mL and 100 mL of water were filtered in triplicate and placed in m-FC plates, m-FC supplemented with cefotaxime (4 µg/mL) and m-FC supplemented with imipenem (8 µg/mL), respectively. Plates were incubated at 37 °C for 18 hours and the typical coliform colonies (blue colonies) were counted. Lastly, colonies resistant to imipenem were selected and transferred to a new Petri plate dish containing m-FC agar supplemented with imipenem (8 µg/mL) and incubated at 37 ºC for 18 hours. This process was repeated until isolated colonies were obtained. Following purification, individual colonies were stored in 15 % glycerol at -80ºC.

2. Molecular typing

Imipenem resistant strains were typed with BOX-PCR. One colony was suspended in 20 µL of sterile distilled water and the suspension was used as DNA template. Primer used is described in table A1 (see appendix). A 25 µL master mix was prepared containing 6.25 µL of 5000 U NZYTaq 2x Green Master Mix (NzyTech, Portugal), 15.75 µL of sterile distilled water and 2 µL of primer BOXA1R (initial concentration of 10 µM) (Versalovic et al. 1994). After that, 1 µL of cell suspension was added to each polymerase chain reaction (PCR) tube. Negative and positive controls were added. After a short spin, PCR tubes were placed in a thermocycler. PCR program details are presented in table A2, see appendix. Amplicons were loaded onto a 1.5 % agarose gel and ran in an electrophoresis tank at 60 volts for 2 hours. Obtained results were analyzed using Gel Compar II software - version 6.1. Similarity matrix was constructed based on Pearson correlation coefficient and the dendrogram was built by the UPGMA method (Applied Maths, Belgium).

3. Phylogenetic affiliation

The 16S rRNA gene sequence analysis was used to determine phylogenetic affiliation of all isolates. For that, one colony was suspended in 20 µL of sterile distilled water and used as DNA template. Then, a 25 µL master mix was prepared containing 6.25 µL of 5000 U NZYTaq 2x Green Master Mix (NzyTech, Portugal), 16.25 µL of sterile distilled water and 0.75 µL of primer forward (27_F) and reverse (1492_R) (initial

concentration of 10 µM) (table A1, see appendix) and 1 µL of cell suspension. Negative and positive controls were added. PCR conditions are presented in table A2. Amplicons were loaded on an agarose gel (1.5 %) and run in an electrophoresis tank at 90 Volts for 1 h. 16S rRNA gene fragments were sequenced and analyzed against the GenBank database at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) using BLAST software (Altschul et al., 1990) and against Ezbio Cloud database (Yoon et al., 2017). 16S rRNA gene amplicons were purified and sequenced as described below (section 5). Sequences were aligned using Clustal X v2.0 program (Larkin et al., 2007). 16S rRNA gene based phylogenetic tree was constructed using Mega-X program V10.1.7 (Kumar et al., 2018), with maximum likelihood statistical method, 1000 bootstrap replications and K2+G+I or K2 model.

When necessary, Bruker MALDI Biotyper IVD equipment was used to confirm the results obtained from 16S rRNA gene analysis. This analysis was conducted at the Hospital Infante Dom Pedro, Portugal.

4. Antibiotic susceptibility testing

Antibiotic susceptibility patterns were determined by the Kirby-Bauer Disk Diffusion Susceptibility Test (Hudzicki, 2016) against 16 antibiotics from 5 classes (table 5). For that, isolates were inoculated in LA medium supplemented with imipenem (concentration of 4 µg/mL**)** and incubated overnight at 37 ºC. One colony from each strain was transferred to microtubes containing 500 µL of NaCl solution (0.9 %). Microtubes were vortexed and optical density was adjusted to 0.5 McFarland. A sterile swab was dipped and bacterial inoculum was spread over the plate of Muller Hinton Agar and antibiotics placed using a dispenser (Oxoid). Inoculated plates were incubated for 18 h at 37 ºC. After that, inhibition halos were measured and analyzed according EUCAST Clinical Breakpoints, 2020. Quality control was performed using *Escherichia coli* ATCC 25922.

Table 5. Antibiotic discs used in this study.

Antibiotic	Amount (µg)	Brand
Piperacillin (PRL)	30	Oxoid
Piperacillin/Tazobactam (TZP)	36	Oxoid
Cefepime (FEP)	30	Oxoid
Cefotaxime (CTX)	5	Oxoid
Ceftazidime (CAZ)	10	Oxoid
Imipenem (IPM)	10	Oxoid
Ertapenem (ETP)	10	Oxoid
Meropenem (MEM)	10	Oxoid
Aztreonam (ATM)	30	Oxoid
Tetracycline (TE)	30	Oxoid
Tigecycline (TGC)	15	Oxoid
Gentamicin (CN)	10	Oxoid
Amikacin (AK)	30	Oxoid
Ciprofloxacin (CIP)	5	Oxoid
Sulphamethoxazole/Trimethoprim (SXT)	25	Oxoid
Chloramphenicol (C)	30	Oxoid

5. Screening of antibiotic resistance genes and integrons

In order to detect resistance genes (*bla*$_{IMP,}$ *bla*$_{VIM,}$ *bla*$_{KPC,}$ *bla*$_{GES,}$ *bla*$_{NDM,}$ *bla*$_{OXA-48,}$ *bla*$_{cphA,}$ *bla*$_{CTX-M}$ and *mcr1)*, integrons and their variable regions (*intI1*, *intI2*, *intI3*, *intI1* 5'CS-3'CS) the same procedure of PCR amplification and detection, as described previously (2) was performed using primers and programs described in tables A1 and A2 (see appendix).

6. PCR products purification and sequencing

PCR products were purified using NZYGelpure kit (Nzytech, Portugal), through an adaption to manufacturer's protocol. Briefly, 20 µL of PCR products and 100 µL of binding buffer were added to 1.5 mL microtubes and mixed. In new 2 mL microtubes, spin columns were added, mixture was transferred into the spin columns and they were centrifuged for 1 minute at 12.000 rpm. The flow-throughs were discarded, 600 µL of washing buffer was added into spin columns and they were centrifuged for 1 minute at 12.000 rpm. The flow-throughs were discarded, spin columns were placed into columns and were centrifuged at 12.000 rpm for 1 minute. Spin columns were transferred to 2 mL microtubes, 25 µL of sterile distilled water was added to each tube, and then they were

centrifuged at 12.000 rpm for 1 minute. Spin columns were discarded and microtubes containing eluted DNA were stored at -20 ºC until analysis. After purification, Sanger sequencing (Eurofins Genomics, Germany) was performed.

7. Characterization of plasmid content

Replicon typing, analysis of the extracted plasmid DNA profile and screening of plasmids pBK30661 and pBK30683 by PCR were performed to determine and characterize plasmids content.

7.1. Replicon typing

Replicon typing to detect replicons belonging to 18 incompatibility groups (table 6) was performed as described by Carattoli *et al*., 2005, following the procedure described previously (2). Primers and programs used are described in table A3 and A4 (3 multiplex- and 1 simplex-PCR). Negative and positive controls were added.

Table 6. Panels (multiplex-PCR), simplex-PCR and target genes.

	Panel 1	Panel 2	Panel 3	simplex-PCR
Replicon	IncB/O	IncK/B	IncI1	Frep
	IncFIC	IncW	IncX	
	IncA/C	IncFIIA	IncHI1	
	IncP	IncFIA	IncN	
	IncT	IncFIB	IncHI2	
		IncY	IncL/M	

7.2. Plasmid DNA extraction

Bacterial strains were inoculated in tubes containing 3-5 mL of LB broth supplemented with imipenem (4 µg/mL) and incubated for 14 h at optimal growth temperature at 180 rpm. Plasmid DNA was extracted using Nzytech NZYMiniprep (NZYTech, Portugal) or Zyppy Plasmid Miniprep Kit (Zymo Research, U.S.A) following manufacturer's protocol.

7.3. Screening of pBK30661 and pBK30683 plasmids

Screening of pBK30661 and pBK30683 plasmids (table 7) was performed using procedure described previously (2). Primers and programs used are described in tables A5 and A6 (see appendix). This assay was performed as described by (Liang Chen et al., 2014).

Table 7. Panels, primers and target genes for screening of pBK30661 and pBK30683 plasmids.

Panel	Amplification	Primer	target	Plasmid
I	1	IA-1f	IncFIA *repA*	pBK30661 and pBK30683
		IA-1r		
	2	IA-2f	Second IncFII *repA*	pBK30683
		IA-2r		
II	3	IA-3f	Tn4401 upstream junction between *chrB* gene and *ISKpn6*	pBK30661 and pBK30683
		IA-3r		
	4	IA-4f	Tn4401 downstream junction between Tn4401 *tnpR* gene and neighboring Tn3 *tnpA* gene	pBK30661 and pBK30683
		IA-4r		
III	5;6	IA-5f		
		IA-56r	Region between putative cytoplasmic protein gene and adenine-specific methyltransferase gene (*met1*)	pBK30683
		IA-6f	Region between hypothetical protein gene and adenine specific methyltransferase gene (*met1*)	pBK30661 and pBK30683
IV	7;8	4401v-r (3781L)	Tn4401d isoform	pBK30661 and pBK30683
		4401v-r1		
		4401v-f (3098U)		

8. Liquid conjugative mating assay

Donor strains were inoculated in LA media supplemented with imipenem (4 µg/mL). Receptor strain, *Escherichia coli* J53, was inoculated in LA media supplemented with azide (200 µg/mL**).** Strains were incubated overnight at 37 ºC at 180 rpm. After that, donor strains were inoculated in tubes containing 5 mL of LB broth supplemented with imipenem (4 µg/mL) and receptor strain was inoculated in a tube containing 5 mL of TSB broth supplemented with azide (200 µg/mL). Strains were incubated overnight in an incubation shaker at 37 ºC at 180 rpm. With a spectrophotometer, optical densities were measured at 600 nm and adjusted to 0.6-0.8 Abs using TSB medium. After that, 900 µL

of each donor strain and receptor strain were transferred to 2 mL microtubes and centrifuged at 10.000 rpm for 5 min, supernatant was discarded and 1 mL of TSB was added. Tubes were incubated overnight at 37 ºC without agitation. To perform quality control, 100 µL of donor strain were inoculated through spread plate method in LA medium supplemented with azide (200 µg/mL) and 100 µL of receptor strain were inoculated in LA medium supplemented with imipenem (4 µg/mL). Plates were incubated overnight at 37 ºC. Microtubes containing donor recipient strains were centrifuged at 10.000 rpm for 5 min, supernatant was discarded, and the pellet was resuspended in 1 mL of 0.9 % NaCl. 100 µL of the bacterial suspensions were inoculated through spread plate method on LA media supplemented with imipenem (4 µg/mL) and azide (200 µg/mL). Plates were incubated overnight at 37 ºC. After that, selected grown colonies were transferred to LA media supplemented with imipenem (4 µg/mL) and azide (200 µg/mL) and incubated overnight at 37 ºC. Putative transconjugants were confirmed by BOX-PCR and by screening of carbapenemase-enconding genes as described previously, in section 2.

9. Whole genome sequencing and analysis

DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol. Paired-end libraries were created using Illumina HiSeq 2500 platform. The raw reads quality was verified using FastQC software and submitted to the trimming process in order to exclude those that contained a phred quality score below 20 using Trimmomatic v.0.36 (parameters used: illuminaclip ON, slidingwindow 4:15, leading, trailing 3, crop OFF, minlen 36). The genomes were assembled using SPAdes version 3.14.0 program. Draft genomes were annotated using RAST – Rapid Annotation using Subsystem Technology (Aziz et al., 2008). The rRNA were predicted using RNAmmer 1.2 Server ((Lagesen et al., 2007) and tRNA were predicted using tRNAscan-SE 2.0 (Chan & Lowe, 2019). Sequence typing was attributed through Multi Locus Sequence Typing analysis using MLST v2.0 (Larsen et al., 2012) and PubMLST v1 (Jolley et al., 2018). ANIb (based on Blast+) and ANIm (based on Mummer) were calculated by using JSpeciesWS tool (M. Richter et al., 2015) and dDDH was calculated by using Genome-to-Genome Distance Calculator v2.1 (Auch et al., 2010). Phylogenetic trees based on whole genome analysis were obtained using Tree builder, a tool integrated on Type (Strain) Genome Server (TYGS) platform which uses FastMe 2.1.4 software to perform the analysis (Meier-Kolthoff & Göker, 2019). Pan-

genome comparisons were performed using PGAdb-builder V1 (Yen Yi Liu et al., 2016). Resistance genes were screened using Resfinder v3.2 (Zankari et al., 2012), CARD (McArthur et al., 2013) and compared against NCBI database using BLAST (Altschul et al., 1990). Genetic context of carbapenemases were designed using SimpleSynteny v1 tool (Veltri et al., 2016). *In silico* screening of plasmid replicons was performed using PlasmidFinder v2.1 (Carattoli et al., 2014). Virulence factors were identified using VFanalyzer from VFDB - Virulence Factor Database (Lihong Chen et al., 2005) and probability of pathogenicity was estimated using PathogenFinder 1.1 (Cosentino et al., 2013).

IV. Results

1. Prevalence of cefotaxime- and imipenem-resistant bacteria in urban aquatic systems

The results obtained from the counting of bacteria colony-forming units (CFU) and the proportion of cefotaxime- and imipenem-resistant bacteria with typical coliform morphology on m-FC agar are described in figures 14 and 15. In February 2019 and March 2019 (in the scope of a previous study), samples were collected only from Pond 1 and Pond 2. In the present study, in September 2019 no samples were collected in Dos Santos Mártires channel, Ria de Aveiro.

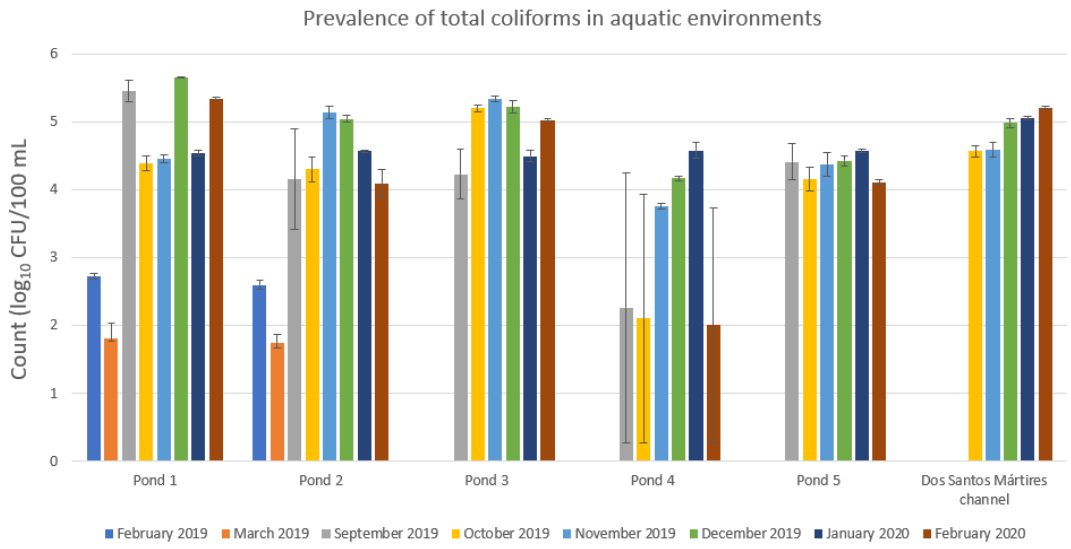

Figure 14. Average counting of typical coliform in CFU/100 mL (log_{10}) with standard deviation, collected in February 2019, March 2019 and from September 2019 to February 2020, from 5 ponds and an estuarine channel.

The total CFU on m-FC agar in studied aquatic environments (5 ponds and 1 urban estuarine channel) varied from 5.7-log in Pond 1 (February 2019) to 1.7-log in Pond 2 (March 2019). In general, the Pond 1 showed the highest values (4,3-log on average) and the Pond 4 the lowest (3.4-log on average). Although a temporal variation on CFU counts was observed, no particular trend was evident from our results and variation depended on the pond.

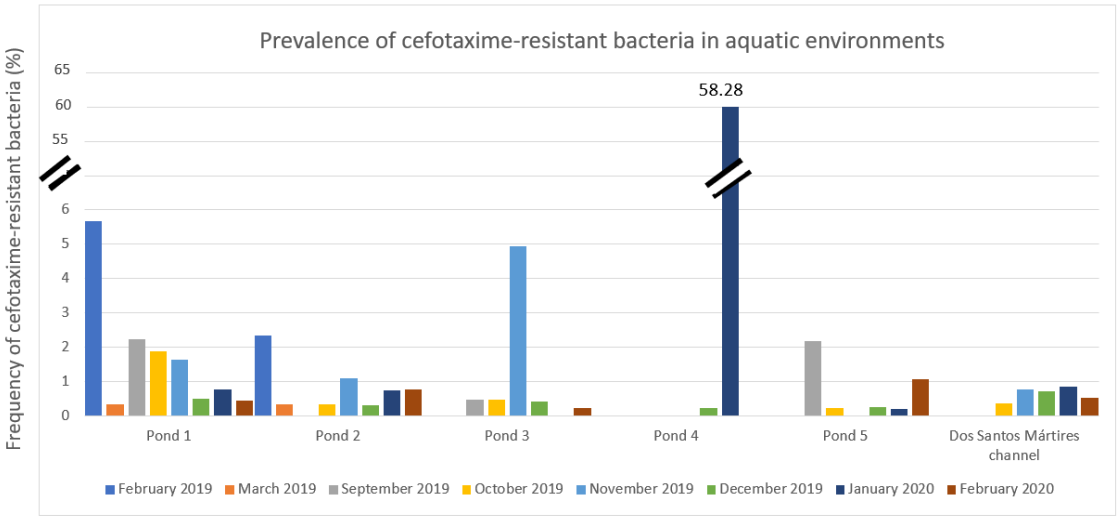

Figure 15. Percentage of cefotaxime-resistant bacteria in the sampled ponds and estuarine channel, in February 2019, March 2019 and September 2019 to February 2020.

Temporal fluctuations in the number of cefotaxime-resistant bacteria were observed, depending on the sampled environment.

The percentage of cefotaxime-resistant bacteria ranged from 0 % for instance in Pond 4 in September 2019, October 2019 and November 2019 to 58.276 % in January 2020, also in Pond 4. In a general way, the highest values of cefotaxime-resistant bacteria were detected at Pond 1 (1.676 % on average) and the lowest amounts were detected at Pond 4 (0.05 % on average), excluding January 2020. With the exception of Pond 4 in January 2020 where 58.276 % of cefotaxime-resistant bacteria were detected, none of the other sites during the sampled months (February 2019, March 2019 and September 2019 to February 2020) exceeded 6.000 % of cefotaxime-resistant bacteria.

Imipenem-resistant bacteria were detected only in three sites: Pond 1 Pond 3 and Dos Santos Mártires channel.

The percentage of imipenem-resistant bacteria in Pond 1 was 0.97 % in February 2019, 0.01 % in March 2019, 0.16 % in September 2019, 0.01 % in October 2019 and 0.06 % in November 2019. In December 2019, January 2020 and February 2020 the percentage was lower than 0.00 %. In Pond 3 imipenem-resistant bacteria were detected only in November 2019, however it represents less than 0.00 % of total CFU. In Dos Santos Mártires Channel imipenem-resistant bacteria were detected in all months sampled, however, excluding November 2019 when 0.06 % of imipenem-resistant bacteria were detected, the remaining percentages were lower than 0.00 %.

When detected, the proportion of imipenem-resistant bacteria varied from 0.002 % (6 CFU/100 mL), for instance in Pond 1 (February 2020), up to 0.967 % (6 CFU/100 mL) in Pond 1 (February 2019). Broadly, Pond 1 was the place where higher counts of imipenem-resistant bacteria were obtained, ranging from 0.002 to 0.967 %.

2. Molecular typing and phylogenetic affiliation of imipenem-resistant isolates

Based on the colony morphology in m-FC agar supplemented with imipenem, a total of 24 isolates were selected for further analysis. Additionally, 6 *Citrobacter* strains isolated from Pond 1 in February 2019 in a previous study were included (table 8). Imipenem-resistant bacteria found in Pond 2 in February and March 2019, in the scope of a previous study, were not included since 16S rRNA gene sequencing analysis demonstrated that the isolates collected from those samples belong to *Shewanella* genus, which harbors intrinsic mechanisms of resistance to imipenem (Potron et al., 2011; Tacão et al., 2018). Hence the collection here analysed comprised 30 isolates collected from September to February 2019 in Pond 1 (P1; n= 26 isolates), Pond 3 (P3; n=2) and Dos Santos Mártires channel (E1; n=2). Phylogenetic identification depicted from 16S rRNA gene analysis is shown in table 1, along with the closest relatives. From all 30 isolates included in this study, 8 affiliated with *Citrobacter* (26.7 %), 11 affiliated with *Raoultella* (36.7 %), 7 affiliated with *Aeromonas* (23.3 %), 3 affiliated with *Enterobacter* (10.0 %) and 1 affiliated with *Klebsiella* (3.3 %). *Enterobacteriaceae* family represents 76.7 % (n=23) of all isolates included in this study.

Table 8. 16S rRNA gene-based phylogenetic affiliation of bacterial isolates used in this study. Sampling dates and sites are indicated along with similarity with the type strain based on EzBiocloud database, and closest relatives based on NCBI database search. BOX-profiles for each isolate are indicated with the same letter indicating a similar BOX-profile.

Isolates	Sampling site	Sampling date	Closest type strain in EzBiocloud (% similarity)	Genbank closest strain relatives (% identity; Acc. number)	16S rRNA gene fragment size (bp)	Box Profile
F1	P1	February 2019	*C. freundii (99.81)*	*C. freundii* (100; CP049015.1)	1071	A
			C. braakii (99.53)	*C. freundii* (100; LR699006.1)		
F2	P1	February 2019	*C. freundii (99.79)*	*C. freundii* (100; CP049015.1)	967	A
			C. braakii (99.59)	*Citrobacter sp.* (100; CP047606.1)		
F3	P1	February 2019	*C. freundii (99.81)*	*C. freundii* (100; CP049015.1*)*	1071	A
			C. braakii (99.53)	*C. freundii* (100; LR699006.1)		
F4	P1	February 2019	*C. freundii (99.91)*	*C. freundii* (100; CP049015.1)	1101	A
			C. braakii (99.64)	*C. freundii* (100; MK471377.1)		
F5	P1	February 2019	*C. freundii (99.80)*	*C. freundii* (100; CP049015.1)	1022	B
			C. braakii (99.51)	*C. freundii* (100; LR699006.1)		
F6	P1	February 2019	*C. freundii (99.73)*	*Citrobacter sp.* (100; CP047606.1)	1098	A
			C. braakii (99.45)	*C. freundii* (100; CP042534.1)		
S1	P1	September 2019	*R. ornithinolytica (99.82)*	*R. planticola* (99.91; LR134195.1)	1091	E
			R. planticola (99.54)	*Raoultella sp.* (99.91; CP030874.1)		
S2	P1	September 2019	*A. caviae (99.73)*	*A. hydrophila* (99.91; MN865804.1)	1095	J
			A. enteropelogenes (99.63)	*Aeromonas sp.* (99.91; MK165122.1)		
S3	P1	September 2019	*R. ornithinolytica (99.72)*	*R. planticola* (100.00; LR134195.1)	1080	E
			R. planticola (99.63)	*Raoultella sp.* (100.00; CP030874.1)		
S4	P1	September 2019	*R. ornithinolytica (99.82)*	*R. electrica* (99.82; CP041247.1)	1113	E
			R. planticola (99.64)	*R. planticola* (99.82; LR134195.1)		
S5	P1	September 2019	*R. ornithinolytica (99.82)*	*R. electrica* (99.82; CP041247.1)	1128	E
			R. planticola (99.65)	*R. planticola* (99.82; LR134195.1)		
O1	P1	October 2019	*R. ornithinolytica (99.81)*	*R. electrica* (99.91; CP041247.1)	1065	E
			R. planticola (99.72)	*R. ornithinolytica* (99.91; CP033683.1)		

O2	P1	October 2019	*A. caviae (99.70)*	*A. hydrophila* (99.90; MN865804.1)	1004	J
			A. enteropelogenes (99.60)	*Aeromonas sp.* (99.90; MK165122.1)		
O3	P1	October 2019	*A. caviae (99.91)*	*A. caviae* (100; MG737563.1)	1093	K
			A. enteropelogenes (99.82)	*A. caviae* (100; MG737562.1)		
O4	P1	October 2019	*A. caviae (99.90)*	*A. caviae* (99.90; MN737498.1)	1028	L
			A. enteropelogenes (99.81)	*A. caviae* (99.90; CP039832.1)		
O5	P1	October 2019	*R. planticola (99.79)*	*R. electrica* (100; CP041247.1)	969	E
			R. ornithinolytica (99.69)	*R. planticola* (100; LR134195.1)		
O6	P1	October 2019	*A. caviae (99.73)*	*A. hydrophila* (99.91; MN865804.1)	1095	K
			A. enteropelogenes (99.63)	*Aeromonas sp.* (99.91; MK165122.1)		
O7	P1	October 2019	*A. veronii (99.62)*	*A. veronii* (99.81; MT345040.1)	1057	C
			A. ichthiosmia (99.62)	*Aeromonas sp.* (99.81; MT026965.1)		
N1	P1	November 2020	*R. ornithinolytica (100)*	*Raoultella sp.* (100.00; MN428658.1)	959	F
			R. electrica (99.90)	*R. ornithinolytica* (100.00; MF428820.1)		
N4	P1	November 2020	*A. caviae (99.91)*	*Aeromonas sp.* (100.00; MK165122.1)	1088	J
			A. enteropelogenes (99.82)	*A. caviae* (100.00; CP024198.1)		
N5	P1	November 2020	*C. portucalensis (100)*	*C. portucalensis* (100.00; AP022378.1)	1100	D
			C. werkmanii (99.91)	*C. portucalensis* (100.00; CP046348.1)		
N6	P1	November 2020	*C. freundii (99.63)*	*Citrobacter sp.* (99.91; CP047606.1)	1080	A
			C. braakii (99.35)	*C. freundii* (99.91; CP042534.1)		
N8	P1	November 2020	*R. ornithinolytica (100)*	*Raoultella sp.* (99.91; MK789736.1)	1103	F
			R. electrica (99.46)	*Raoultella sp.* (99.91; MN540107.1)		
N9	P1	November 2020	*R. ornithinolytica (100)*	*R. ornithinolytica* (100.00; CP049752.1)	994	F
			R. electrica (99.90)	*Raoultella sp.* (100.00; MK789736.1)		
N10	P1	November 2020	*Leclercia adecarboxylata (99,81)*	*Enterobacter sp.* (100.00; MH084944.1)	1031	H
			E. ludwigii (99.71)	*E. cancerogenus* (100.00; CP025225.1)		
N11	P3	November 2020	*E. cloacae subsp. cloacae (99.82)*	*Enterobacter sp.* (100.00; KF984470.1)	1098	I

			E. cloacae subsp. dissolvens (99.82)	*E. cloacae* (99.91; MT138639.1)		
N12	E1	November 2020	*R. ornithinolytica (100)*	*R. ornithinolytica* (100.00; CP049752.1)	1026	**F**
			R. electrica (99.42)	*Raoultella sp.* (99.90; MK600538.1)		
N13	E1	November 2020	*R. ornithinolytica (100)*	*Raoultella sp.* (100.00; MK789736.1)	1087	**F**
			R. electrica (99.45)	*Raoultella sp.* (100.00; MN540107.1)		
N14	P1	November 2020	*Klebsiella michiganensis (99.70)*	*K. michiganensis* (99.91; CP024643.1)	1066	**G**
			K. oxytoca (99.53)	*K. pasteurii* (99.91; MN104669.3)		
N15	P3	November 2020	*E. cloacae subsp. dissolvens (99.91)*	*E. cloacae* (100.00; CP020089.1)	1130	**I**
			E. cloacae subs. cloacae (99.73)	*E. cloacae* (99.91; MT138639.1)		

Further analyses were performed by building phylogenetic trees based on 16S rRNA partial gene sequences to determine the evolutionary relationships among isolates affiliated with the *Aeromonas* genus (figure 16) and with the *Enterobacteriaceae* family (figure 17).

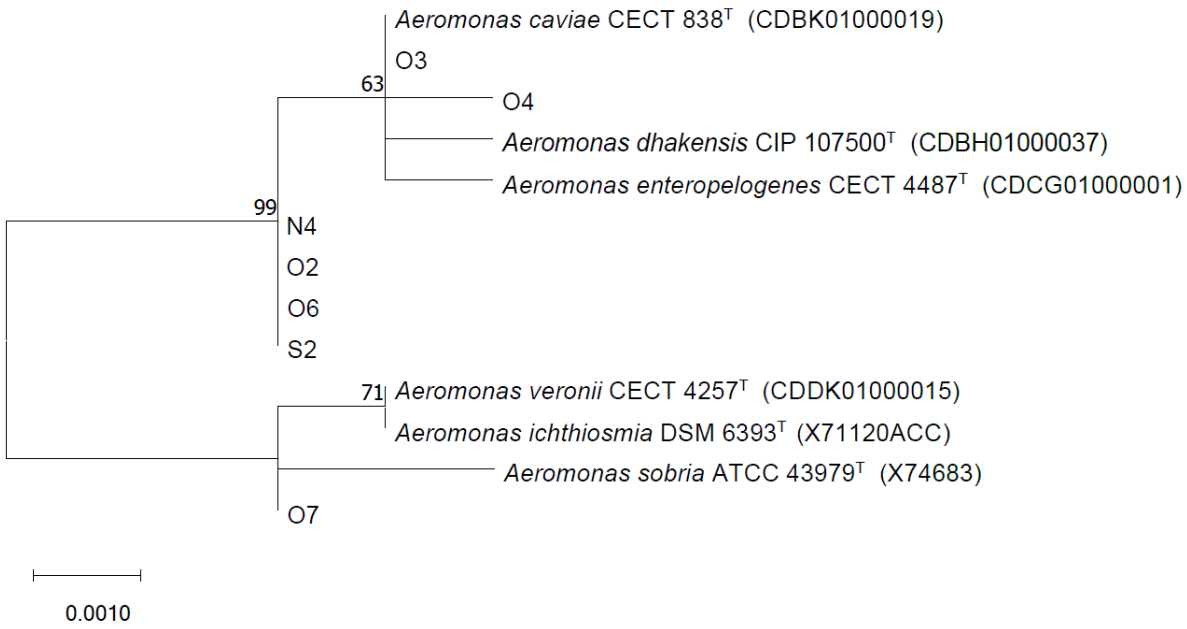

Figure 16. 16S rRNA gene-based phylogenetic relationships between *Aeromonas* isolates obtained during this study and the closest related type strains using maximum likelihood method and a bootstrap analysis with 1000 replications. Bootstrap confidence is shown in %.

From the analysis of the phylogenetic tree, isolate O7 is part of a cluster which includes the type strains of species *A. veronii*, *A. ichthiosmia* and *A. sobria*. Isolates N4, O2, O6 and S2 are closely related and are part of the same cluster as isolates O3 and O4, along with the type strains of species *A. enteropelogenes*, *A. dhakensis* and *A. caviae*. This is a well-supported cluster with a 99 % bootstrap.

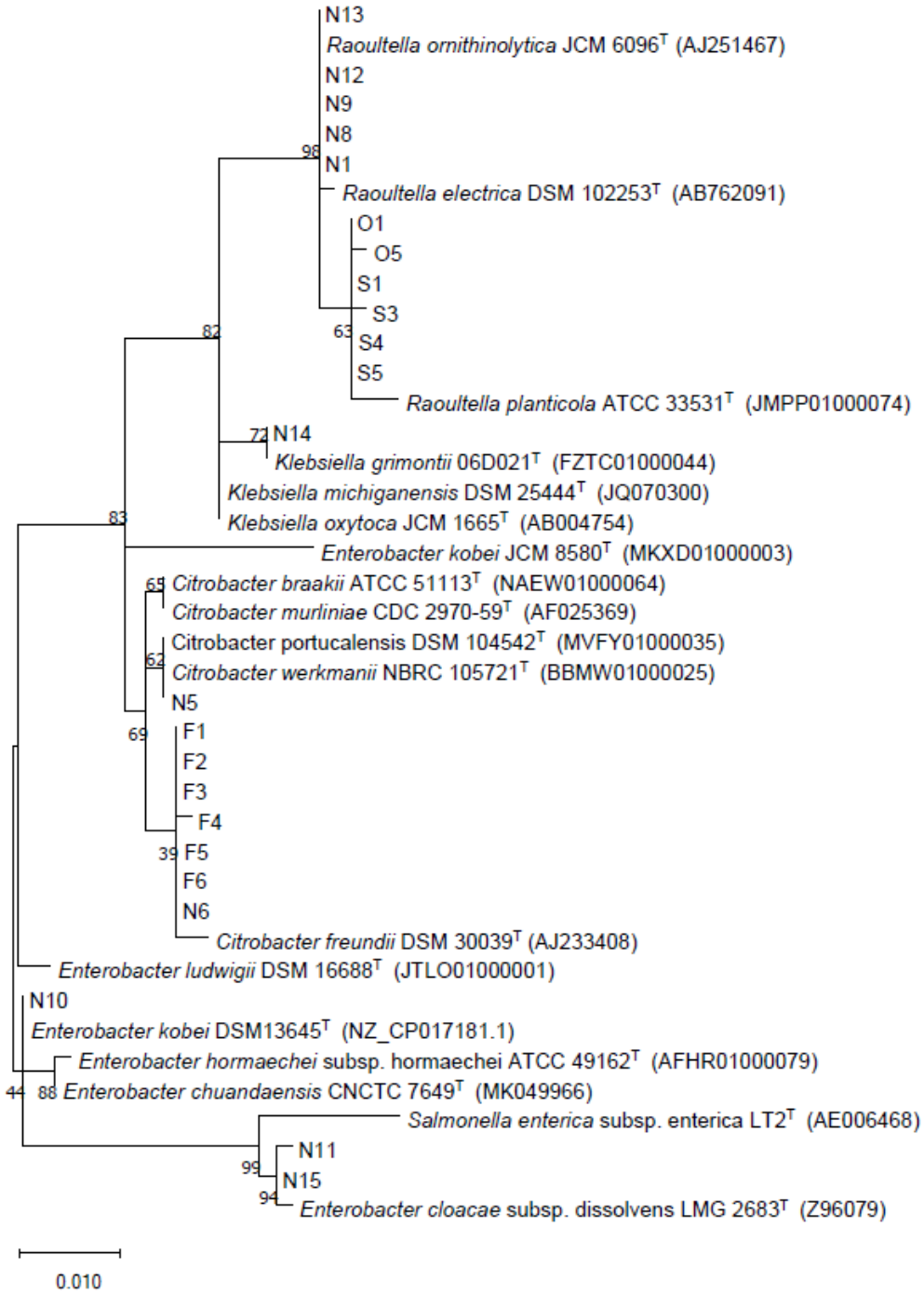

Figure 17. 16S rRNA gene-based phylogenetic relationships between *Enterobacteriaceae* isolates obtained in this study and the closest type strains. The tree was generated using maximum likelihood method with a bootstrap analysis with 1000 replications. Bootstrap confidence is shown in %.

According to the phylogenetic analysis, isolates S1, S3, S4, S5, O1, O5, N1, N8, N9, N12 and N13, collected in September, October and November 2019, belong to the genus *Raoultella* forming a well-supported cluster (98 %) with the type strains of the species included in this genus.

Isolates S1, S3, S4, S5, O1 and O5 and *R. planticola* type strain are clustered together. N14 isolate is gathered with *Klebsiella grimontii* type strain, with a bootstrap of 72 %. The phylogenetic analysis confirmed the affiliation of isolates F1, F2, F3, F4, F5, F6, N6 and N5 with the *Citrobacter* genus in a cluster supported by 69% bootstrap. Among *Enterobacter* isolates several clusters were formed within the tree. N11 and N15 isolates are clustered together with *Enterobacter cloacae* subsp. dissolvens type strain, supported by a bootstrap of 94 %, and N10 is in a different *Enterobacter* cluster.
MALDI-TOF analysis confirmed that all *Raoultella* isolates affiliated to *Raoultella ornithinolytica*.

Clonal relationships among imipenem-resistant isolates (n=30) were assessed by BOX-PCR. Araújo *et al*. used a similarity cutoff of 85 % to associate two box profiles to the same strain (Araújo et al., 2014). In our study we adjusted this value to 88 %. Clustering analysis of BOX-PCR patterns obtained from GelCompar II is shown in figure 18.

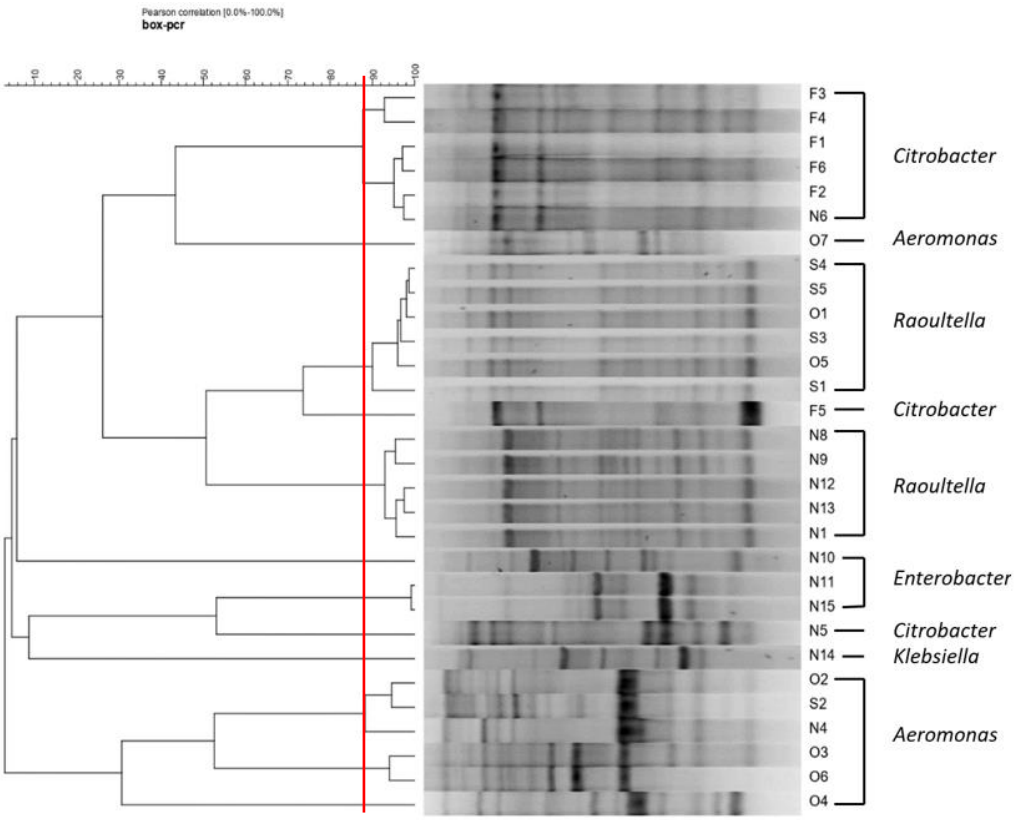

Figure 18. Clustering analysis of BOX-PCR patterns of all isolates used in this study, obtained from GelCompar II using Person correlation coefficient and UPGMA clustering method. The similarity cutoff value considered in this study is shown by a red line.

Using a cutoff of 88 %, where isolates sharing ≥ 88 % of similarity were considered the same strain and < than 88 % were considered distinct strains, twelve distinct BOX profiles were observed, with 6 of them represented by a single isolate (*Aeromonas* sp. O4, *Aeromonas* sp. O7, *Citrobacter* sp. F5, *Citrobacter* sp. N5, *Enterobacter* sp. N10 and *Klebsiella* sp. N14).

Among *Citrobacter* genus 3 distinct BOX profiles were detected. The F3, F4, F1, F6, F2 and N6 bacterial isolates, collected in February 2019 and November 2019 shared at least 88 % of BOX profile similarity and as previously said, isolates F5 and N5 represented single BOX profiles.

Among *Aeromonas* genus 4 distinct BOX profiles were detected. The O2, S2 and N4 isolates shared a BOX profile similarity of at least 88 %. Within this cluster, isolates O2 and S2, collected in October 2019 and September 2019, shared a BOX profile similarity of 96 %. The O6 and O3 isolates, collected both in October 2019, shared a similarity of 96 % in BOX profiles, belonging to the same cluster. O4 and O7 isolates, as previously mentioned, have unique BOX profiles.

Among *Enterobacter* isolates, N11 and N15, collected in Pond 3, shared a similarity of 98 % and were considered identical. *Enterobacter* N10, collected in Pond 1, only shares 10 % of BOX-profile similarity with N11 and N15 isolates.

Within *Raoultella* genus two distinct BOX clusters were observed. The isolates S4, S5, O1, S3, O5 and S1, collected in September and October 2019, exhibit 88 % similarity and belong to the same BOX cluster. Isolates N1, N8, N9, N12, N13 collected in Pond 1 (N1, N8 and N9) and Dos Santos Mártires channel (N12, N13), in November 2019, shared a similarity of 94 %. These two *Raoultella* clusters shared only 50 % similarity between them.

3. Antibiotic susceptibility profiles

Antibiotic susceptibility profiles are described on table 9. Isolates included in the categories ATU (area of technical uncertainty) and R (resistant) were considered as resistant in the interpretation of antibiotic susceptibility test results.

Table 9. Replicon typing, integrases and carbapenemase genes detected by PCR and antibiotic susceptibility profiles.

Isolate	Replicons	Integrases genes	Carbapenemase genes	Phenotypic resistance profile
Citrobacter sp. F1	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, TGC, AK, SXT
Citrobacter sp. F2	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, CIP
Citrobacter sp. F3	IncN	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, CIP, C
Citrobacter sp. F4	IncL/M	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, TGC, CIP, SXT, C
Citrobacter sp. F5	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, AK, CIP, SXT, C
Citrobacter sp. F6	IncN, IncL/M	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, CIP, SXT
Citrobacter sp. N6	IncN	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, CIP
Citrobacter sp. N5	IncN	*intI1*	bla_{KPC}, bla_{VIM-1}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, TE, CN, CIP, SXT
R. ornithinolytica S1	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica S3	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica S4	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica S5	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica O1	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica O5	nd	*intI1*	bla_{KPC}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
R. ornithinolytica N1	nd	*intI1, intI3*	bla_{KPC}, bla_{GES-5}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, SXT
R. ornithinolytica N8	nd	*intI1, intI3*	bla_{KPC}, bla_{GES-5}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, AK, SXT
R. ornithinolytica N9	nd	*intI1, intI3*	bla_{KPC}, bla_{GES-5}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, SXT
R. ornithinolytica N12	nd	*intI1, intI3*	bla_{KPC}, bla_{GES-5}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, AK, SXT
R. ornithinolytica N13	nd	*intI1, intI3*	bla_{KPC}, bla_{GES-5}	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, SXT

Klebsiella sp. N14	nd	*intI3*	*bla*$_{GES-5}$	PRL, TZP, CTX, CAZ, IPM, ETP, MEM, CN, CIP
Enterobacter sp. N10	IncL/M	*intI1, intI3*	*bla*$_{KPC}$, *bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CIP
Enterobacter sp. N11	nd	nd	nd	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM
Enterobacter sp. N15	nd	nd	nd	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM
Aeromonas sp. S2	nd	*intI1, intI3*	*bla*$_{GES-5}$	PRL, FEP, CTX, CAZ, IPM, ETP, MEM, CIP
Aeromonas sp. O2	nd	*intI1, intI3*	*bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, CIP
Aeromonas sp. N4	nd	*intI1, intI3*	*bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
Aeromonas sp. O3	nd	nd	*bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
Aeromonas sp. O4	nd	*intI1, intI3*	*bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, CIP
Aeromonas sp. O6	nd	nd	*bla*$_{GES-5}$	PRL, TZP, FEP, CTX, CAZ, IPM, ETP, MEM, ATM, CN, CIP, SXT
Aeromonas sp. O7	nd	nd	nd	IPM, ETP, MEM, CIP

Piperacillin (PRL), Piperacillin-tazobactam (TZP), Cefepime (FEP), Cefotaxime (CTX), Ceftazidime (CAZ), Imipenem (IPM), ertapenem (ETP), meropenem (MEM), aztreonam (ATM), tetracycline (TE), tigecycline (TGC), Gentamicin (CN), Amikacin (AK), ciprofloxacin (CIP), Trimethoprim-sulfamethoxazole (SXT) and Chloramphenicol (C). nd: not detected.

Since isolates were selected in culture media supplemented with imipenem, as expected all isolates were resistant to this antibiotic. Moreover, they were also resistant to other tested carbapenems (ertapenem and meropenem) (figure A1). Additionally, most isolates were resistant to all β-lactams, with exception of isolates *Klebsiella* sp. N14 (susceptible to cefepime and aztreonam), *Aeromonas* sp. S2 (susceptible to piperacillin-tazobactam and aztreonam), *Aeromonas* sp. O2 and O4 (susceptible to aztreonam) and *Aeromonas* sp. O7 (susceptible to piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime and aztreonam). Among tested antibiotics, none was effective against all isolates. With the exception of *Enterobacter* isolates N10, N11, and N15, and *Aeromonas* isolates S2, O2, O4 and O7, all the remaining isolates (76.7 % of the total) were multidrug resistant (MDR), since they were resistant to at least one antibiotic from at least 3 different antibiotic classes (Rolain et al., 2016). Of these, all members of *Raoultella* (n=12), *Citrobacter* (n=8) and *Klebsiella* (n=1) genera were MDR.

All *Raoultella* isolates showed resistance or ATU phenotypes to all β-lactams tested and were susceptible to tested tetracyclines (tetracycline and tigecycline) and chloramphenicol. A total of 81.8 % of the *Raoultella* isolates were resistant to gentamicin

and remaining 18.2 % exhibited an ATU phenotype to this antibiotic. A fraction of the isolates (18.2 %) exhibited a resistance phenotype to amikacin and the remaining 81.2 % were susceptible. Regarding ciprofloxacin, 54.5 % of the isolates were resistant and the remaining 45.5 % were susceptible.

Klebsiella isolate exhibited a resistance phenotype to all β-lactams tested, apart from cefepime and aztreonam, for which it was susceptible. It also exhibited resistance to ciprofloxacin, ATU phenotype to gentamicin and was susceptible to the tested tetracyclines, amikacin and trimethoprim-sulfamethoxazole.

All *Enterobacter* isolates exhibited resistance or ATU phenotypes to all β-lactams tested and were susceptible to the remaining tested antibiotics, except N10 isolate which was resistant to ciprofloxacin.

All *Aeromonas* isolates were resistant to tested carbapenems, exhibiting different susceptibility profiles to the remaining β-lactams. However, most isolates, except O7 isolate, were resistant to most of the tested β-lactams. Regarding other classes of tested antibiotics, 100 % of the isolates were susceptible to tested tetracyclines, amikacin and chloramphenicol. A total of 42.9 % of the isolates were resistant to gentamicin and trimethoprim-sulfamethoxazole. Regarding ciprofloxacin, 85.7 % of the isolates were resistant and O7 isolate exhibited an ATU phenotype.

Within *Citrobacter* genus, all isolates (n=8) were resistant to all tested β-lactams and tetracycline. Some isolates were also resistant to ciprofloxacin (n=7, 87.5 %), amikacin (n=2, 25.0 %) and tigecycline (n=2, 25.0 %). Additionally, *Citrobacter* sp. N5 exhibited a resistance phenotype to at least one antibiotic of all classes of antibiotics tested (all β-lactams, tetracycline/tetracyclines, gentamicin/aminoglycosides, ciprofloxacin/fluoroquinolones, and trimethoprim-sulfamethoxazole/miscellaneous agents).

4. Occurrence and diversity of ARG

The ARG detected by PCR are described in table 3. From all tested genes encoding carbapenemases (*bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{GES}$, *bla*$_{OXA-48-like}$ and *bla*$_{VIM}$), three genes, *bla*$_{KPC}$ (n=20 isolates; 66.6 %), *bla*$_{GES}$ (n=13; 43.3 %) and *bla*$_{VIM}$ (n=1; 3.3 %), were detected. In three isolates, *Enterobacter* sp. N11, *Enterobacter* sp. N15 and *Aeromonas* sp. O7, ARG weren't detected. The *bla*$_{KPC}$ was detected in *Raoultella* (n=11; 100 % of *Raoutella* isolates), *Citrobacter* (n= 8; 100 %) and *Enterobacter* (n= 1; 33.3 %). The *bla*$_{GES}$ was found in *Raoultella* (n=5; 45.5 % of *Raoutella* isolates), *Enterobacter* (n=

1; 33.3 %), *Aeromonas* (n= 6; 85.7 %) and *Klebsiella* (n= 1; 100 %). The *bla*$_{VIM}$ was found only in *Citrobacter* sp. N5. In 7 isolates (representing 23.3 % of the isolates in this collection), the presence of two carbapenemase-encoding genes was detected.

Within *Raoultella* genus, isolates S1, S3, S4, S5, O1 and O5, collected in September and October 2009 in Pond 1, harboured *bla*$_{KPC.}$ The N1, N8, N9, N12, N13 isolates collected in November 2019, harboured both *bla*$_{KPC}$ and *bla*$_{GES.}$

Within *Enterobacter* genus, N10 strain harboured both *bla*$_{KPC}$ and *bla*$_{GES.}$ Within *Citrobacter* genus, N5 isolate, in addition to *bla*$_{VIM}$ also harboured *bla*$_{KPC.}$ The remaining isolates of this genus harboured only *bla*$_{KPC.}$

As previously mentioned, 6 *Aeromonas* isolates harboured *bla*$_{GES}$, so, this carbapenemase gene was present in 85.7 % isolates of this genus. The primers used to detect the *cphA* gene, encoding an intrinsic carbapenemase present in some *Aeromonas* species, did not amplified any fragment.

The amplicons of *bla*$_{GES,}$ amplified by PCR were sequenced using Sanger sequencing and the variant detected in *bla*$_{GES}$ positive isolates was *bla*$_{GES-5,}$ which encodes a carbapenemase.

The presence of *bla*$_{CTX-M}$, a gene encoding ESBL responsible for conferring resistance to 3rd generation cephalosporins and *mcr-1* gene, responsible for conferring resistance to colistin, a last-resort antibiotic, were not detected in isolates of this collection.

5. Integron screening and characterization

The results obtained from the screening of genes encoding integrases by PCR are described in table 3.

Genes encoding integrases of class 1 were detected in 24 of 30 isolates (80.0 %), namely in *Citrobacter* (n=8 isolates), *Raoultella* (n=11), *Enterobacter* (n=1) and *Aeromonas* (n=6). Genes encoding integrases of Class 3 were detected in 11 isolates, *Raoultella* (n=5), *Enterobacter* (n=1), *Aeromonas* (n=6) and *Klebsiella* (n=1), representing 36.7 % of all isolates. The presence of class 2 integrons was not detected.
All isolates belonging to the genera *Citrobacter* (n=8) and *Raoutella* (n=11) harbour the *intI1* gene. Additionally, isolates N10 (*Enterobacter*), S2, O2, O4 and N4 (*Aeromonas*) also carried an *intI1* gene.
Excluding isolate O7, all isolates belonging to the genus *Aeromonas* possess class 3 integrons. Further PCR analysis confirmed that *Aeromonas* S2, O2, O4 N4, *Raoultella*

N1, N8, N9, N12 and N13, *Klebsiella* sp. N14 and *Enterobacter* sp. N10 possessed a *bla*$_{GES-5}$ gene cassette inserted into a class 3 integron.

The genetic context of the *bla*$_{VIM-1}$ gene in *Citrobacter* sp. N5 was also determined by PCR and sequence analysis, demonstrating that the *bla*$_{VIM-1}$ gene was inserted in a class 1 integron with the following gene cassette array: 1-5'CS- *bla*$_{VIM-1}$-*aacA4*-(**...**)-*aadA1*-*catB2*-3'CS-*qAcEΔ1*-*sul1*. This integron also includes gene cassettes encoding resistance to aminoglycosides (*aacA4; aadA1*), chloramphenicol (*catB2*), sulfonamides (*sul1*) and quaternary ammonium detergents (*qAcEΔ1*).

According to results obtained from screening of class 1 integrons variable regions (5'CS-3'CS), *Citrobacter* sp. F4 and F6, *Aeromonas* spp. O4 and N4 and *Enterobacter* sp. N10 isolates seem to harbour empty integrons. However, these results should be confirmed by Sanger sequencing.

6. Plasmid content and mating assays

Plasmid DNA extraction was successful for the 30 strains as shown in figure 19, originating 15 distinct plasmid profiles (A to O).

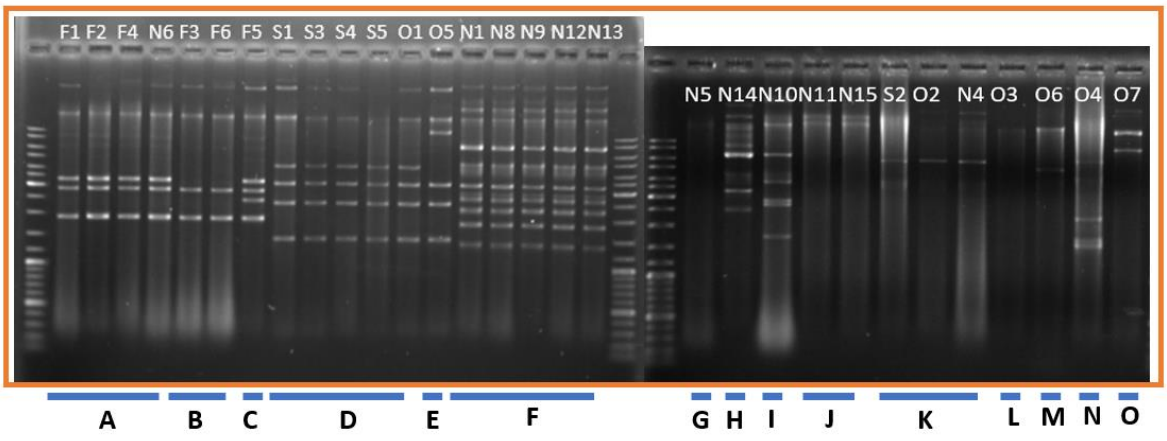

Figure 19. Plasmid DNA profiles of all isolates.

Of these plasmid profiles, 4 were from isolates that belong to *Citrobacter* (A, B, C and F), 3 to *Raoultella* (D, E and F) 1 to *Klebsiella* (H), 2 to *Enterobacter* (I and J) and 5 to *Aeromonas* (K, L , M, N and O). Isolates *Citrobacter* sp. F5, O5, *Citrobacter* sp. N5, *Klebsiella* sp. N14, *Enterobacter* sp. N10 and *Aeromona*s spp. O3, O6, O4 and O7 (30 %) possessed unique plasmid profiles.

The *Citrobacter* F1, F2, F3, F4, F6, collected in February and *Citrobacter* N6 collected in November, which grouped in the same BOX-PCR cluster, harboured 2 different plasmid profiles (A and B).

Within the genus *Raoultella*, isolates S1, S3, S4, S5, collected in September, and O1 and O5, collected in October, grouped in the same BOX-PCR cluster, and originated 2 distinct plasmid profiles (D and E). *Raoultella* isolates N1, N8, N9, N12 and N13, collected in November in Pond 1 and Dos Santos Mártires channel, grouped in the same BOX-PCR cluster and shared the same plasmid profile.

Among *Enterobacter* isolates, two distinct plasmid profiles were identified (I and J). N11 and N15 isolates, which shared the same BOX-profile, originated identical plasmid profiles.

Among *Aeromonas* isolates, 5 distinct plasmid profiles were observed. O2, S2 and N4 isolates, collected in October, September and November shared the same plasmid profile (K). Isolates O3 and O6, which shared the same BOX-profile, originated two distinct plasmid profiles (L and M).

Replicon typing was performed for all isolates and results obtained are shown in table 2. With the primers and conditions tested, only two replicons were identified: IncL/M was observed in isolates *Enterobacter* sp. N10 and *Citrobacter* sp. F4 and F6; and the IncN replicon was detected in *Citrobacter* sp. F3, F6, N5 and N6. Thus, for isolate *Citrobacter* sp. F6 both incL/M and incN were detected.

In order to understand if pBK30661 and pBK30683 plasmids could be involved in the spread of bla_{KPC} among isolates of this collection, we chose 12 bla_{KPC} – positive isolates (based on the detected ARG, BOX-profiles and plasmid profiles) and proceeded to the detection of these two plasmids by multiplex-PCR. The results obtained are shown in figure 20.

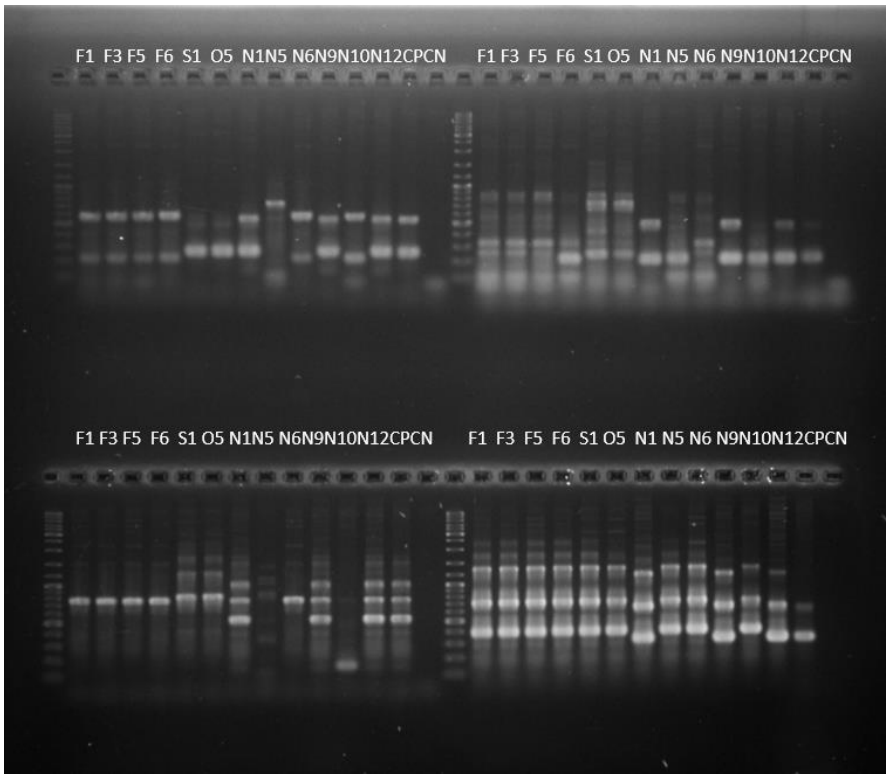

Figure 20. Screening of pBK30661 and pBK30683 plasmids by PCR in all isolates from this study.

According to results obtained, N1, N9 and N12 isolates harbor a pBK30683-like plasmid, since they demonstrated a positive result for all 8 PCR performed, corresponding to 8 conserved regions of this plasmid.

Mating assays were conducted for all 30 isolates but under the tested conditions, no transconjugants were obtained.

7. Whole genome sequencing (WGS) analysis

Based on the results obtained with phylogenetic affiliation using 16S rRNA gene, resistance phenothypes, carbapenemase- and integrase- encoding genes detected and plasmid profiles, three isolates were selected for WGS and analysis: *Citrobacter freundii*. F6, *Raoultella ornithinolytica* N9 and *Enterobacter* sp. N10. The results obtained are described in this section.

7.1. General genomic features

The general features of the *Citrobacter* sp. F6, *R. ornithinolytica* N9 and *Enterobacter* sp. N10 draft genomes are described in table 10 and figure A2.

Table 10. General features of the *Citrobacter* sp. F6, *Raoultella ornithinolytica* N9 and *Enterobacter* sp. N10 draft genomes.

	Citrobacter sp. *F6*	*R. ornithinolytica N9*	*Enterobacter sp. N10*
Size	5,100,708 bp	6,212,323 bp	5,363,533 bp
GC Content (%)	51.7	55.2	54.4
N50	88228	110464	65421
L50	20	17	29
Number of Contigs	124	176	221
Number of Subsystems	578	589	591
Number of Coding Sequences	4989	6010	5129
Predicted tRNA	80	84	80
Predicted rRNA	2	2	1
Sequenced reads	6,124,550	7,038,940	7,008,910
Sequenced bases	1,837,365,000	2,111,682,000	2,102,673,000
Coverage	180^{X}	170^{X}	115^{X}

Citrobacter sp. F6 draft genome consisted of 5,100,708 bp, with a G+C content of 51.7 %, organized in 124 contigs, with 4111 predicted CDS. Most of genes are related to metabolism and cellular functions essential to cell survival. However, 126 genes related to virulence, disease and defense and 114 genes related to phages, prophages, transposable elements and plasmids were detected. The sum of these two subsystems represents a total of 5.8 % of all genes present in the cell (figure A10).

R. ornithinolytica N9 draft genome consisted of 6,212,323 bp, with a GC content of 55.2 %, organized in 176 contigs, with 4569 predicted CDS. Most of genes annotated in *R. ornithinolytica* N9 are associated to metabolism and cellular functions, however, 147 genes related to virulence, disease and defense and 107 genes related to phages, prophages, transposable elements and plasmids were detected. These two subsystems represent 5.6 % of all genes present in the cell (figure A10).

Enterobacter sp. N10 draft genome consisted of 5,363,533 bp, with a GC content of 54.4 %, organized in 221 contigs, with 4024 predicted CDS. Most of genes annotated in *Enterobacter* sp. N10 are associated to metabolism and cellular functions, however, 155 genes related to virulence, disease and defense and 73 genes related to phages,

prophages, transposable elements and plasmids were detected. It represents a total of 5.7 % of all genes present in the cell (figure A10).

7.2. *In silico* phylogenetic analysis

The similarity between the genome of the type strain *C. freundii* ATCC 8090^T and the genome of *Citrobacter* sp. F6 was evaluated to confirm taxonomic affiliation. For that, the average nucleotide identity based on two algorithms, ANIb, and ANIm, and the digital DNA-DNA hybridization (dDDH), and G+C content divergence were calculated. The ANIb and ANIm values were 98.32 % and 98.98 %, respectively, thus above the established threshold of 95-96% (Richter & Rosselló-Móra, 2009) for species delineation. Likewise, the predicted dDDH value of 90.50 % obtained, between *Citrobacter* sp. F6 and *C. freundii* ATCC 8090^T is above the threshold of 70 % (Chun et al., 2018) for species definition based on DDH values. Finally, G+C content differed in 0.01 % between these two genomes, thus in less than 1 % as suggested previously to occur within species (Meier-Kolthoff et al., 2014). In order to further confirm these results the draft genome of *Citrobacter* sp. F6 was analysed together with 13 *Citrobacter* genomes available (including 9 type strains) and submitted to the Type (Strain) Genome Server (TYGS) platform.

As expected, TYGS analysis confirmed that strain F6 affiliates to *Citrobacter freundii* (figure 21). Overall results based on WGS analysis (ANI, dDDH and TYGS analysis) support that strain F6 affiliates with *C. freundii*.

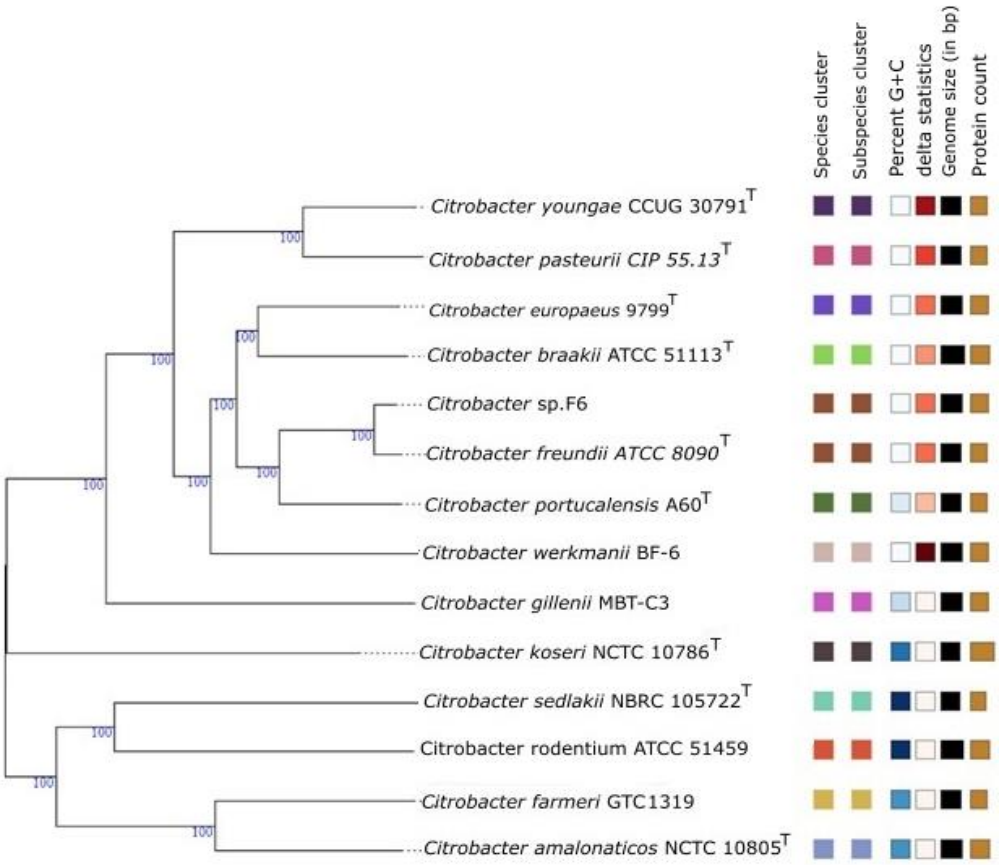

Figure 21. Phylogenetic tree of *Citrobacter* spp. based on whole genome analysis obtained from TYGS. Species and subspecies cluster colors represent different clusters. Percent of G+C varies from white (64.0) to dark blue (69.3). δ statistic values varies from white (0.1) to brown (0.2). Genome size (bp) varies from (4.2 Mb) to (8.0 Mb), dark rectangle size represents genome size. Protein count varies from (3211) to (7675), golden rectangle size represents number of proteins. Accession numbers: NCTC10805, NZ_UFVN01000003.1; ATCC 51113, NZ_NAEW00000000.1; 97/99, NZ_FLYB01000036.1; GTC 1319, NZ_BBMX00000000.1; ATCC, 8090T, NZ_JMTA00000000.1; MBT-C3 NZ_QVEK01000037.1; NCTC10786, NZ_UAVY01000004.1; P080C CL, QFVP01000003.1; CIP 55.13, NZ_CDHL01000056.1; A60, MVFY01000001.1; ATCC 51459, NZ_JXUN00000000.1; NBRC 105722, NZ_BBNB01000030.1, BF-6, NZ_CP019987.1; CCUG 30791, NZ_RPOI00000000.1.

MLST analysis based on *arcA*, *aspC*, *clpX*, *dnaG*, *fadD*, *lysP* and *mdh* loci showed that *Citrobacter freundii* F6 affiliated to ST 270 (table 11).

Table 11. Allelic analysis and sequence type of *C. freundii* F6.

Sequence type: 270						
Locus	Identity	Coverage	Alignment Length	Allele Length	Gaps	Allele
arcA	100	100	435	435	0	arcA_5
aspC	100	100	513	513	0	aspC_10
clpX	100	100	567	567	0	clpX_12
dnaG	100	100	444	444	0	dnaG_11
fadD	100	100	483	483	0	fadD_7
lysP	100	100	477	477	0	lysP_11
mdh	100	100	549	549	0	mdh_6

***Raoultella ornithinolytica* N9**

To confirm the phylogenetic affiliation of N9 strain to *R. ornithinolyitica* as suggested by previous results based on MALDI-TOF analysis, the values of ANIb, ANIm, dDDH and the difference in G+C were calculated against the type strain *R. ornithinolytica* NBRC 105727^{T}.

The ANIb and ANIm values of 99.16 % and 99.53 %, respectively, the dDDH value of 94.90 % and difference in % G+C of 0.47 suggest an affiliation to *R. ornithinolytica.*

Additionally, the phylogenetic affiliation based on Multi locus sequence analysis (MLSA) (figure 22) and whole genome analysis using TYGS (figure 23) were also performed. Since not all type strains of *Raoultella* species are available, whenever necessary a representative of each *Raoultella* species was used for the analysis. Hence, we selected a total of 4 *Raoultella* that together with *R. ornithinolytica* N9 were used for MLSA analysis based on *atpD, gyrB, infB* and *rpoB* concatenated housekeeping genes sequences and WG-based analysis in TYGS platform.

Both MLSA and WG based- analysis showed that strain N9 affiliates with *R. ornithinolytica*.

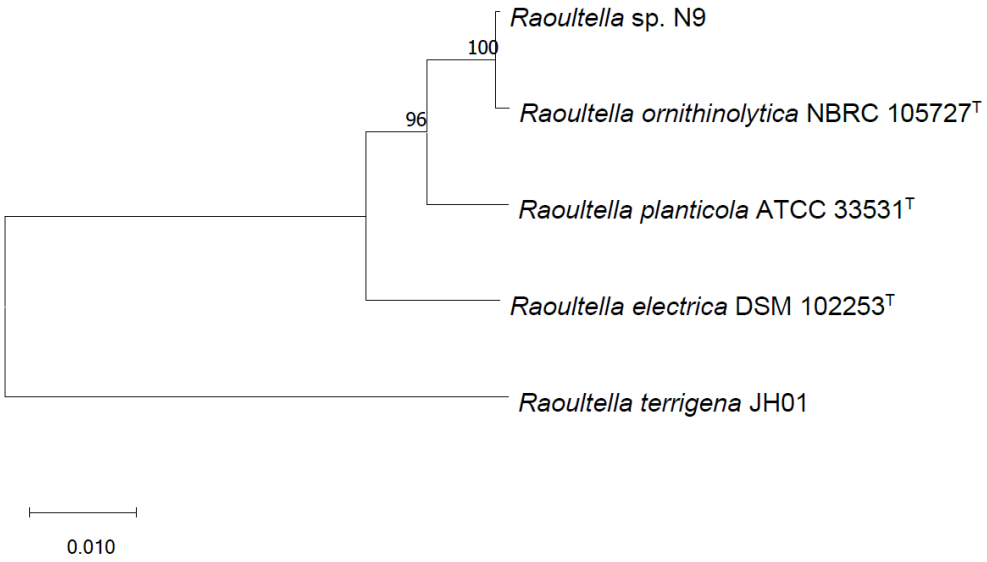

Figure 22. Phylogenetic tree of MLSA using a bootstrap analysis with 1000 replications based on *atpD, gyrB, infB* and *rpoB* concatenated housekeeping genes of *R. ornithinolytica* N9 and other representatives of different type strains of genus *Raoultella*. Genbank accession numbers: *Raoultella planticola* ATCC 33531T (JMPP01000014); *Raoultella ornithinolytica* NBRC 105727T (BCYR00000000.1) ; *Raoultella electrica* DSM 102253T (CP041247); *Raoultella terrigena* JH01 (CP050508.1).

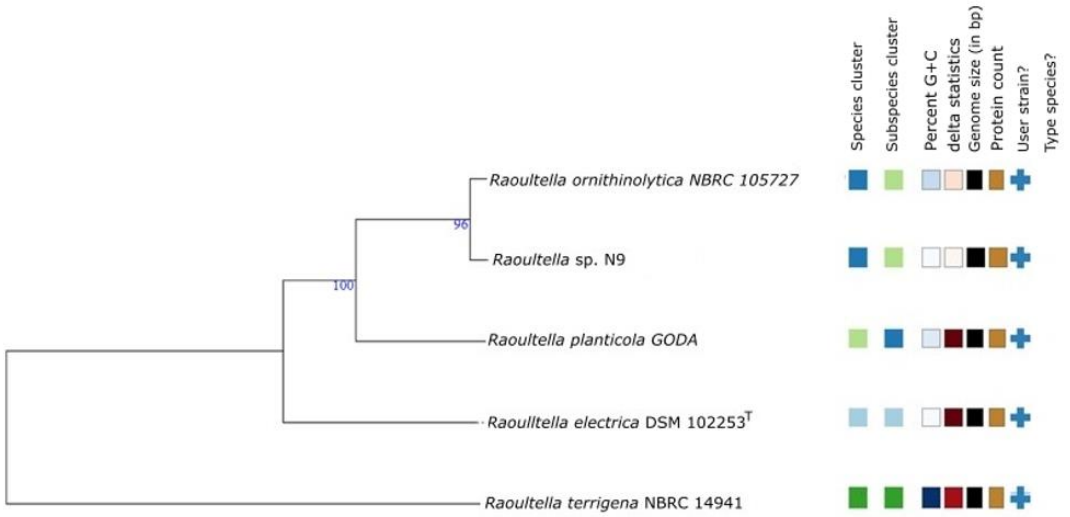

Figure 23. Phylogenetic tree based on whole genome analysis of the *Raoultella* sp. obtained from TYGS. Species and subspecies cluster colors represent different clusters. Percent of G+C varies from white (64.0) to dark blue (69.3). δ statistic values varies from white (0.1) to brown (0.2). Genome size (bp) varies from (4.2 Mb) to (8.0 Mb), dark rectangle size represents genome size. Protein count varies from (3211) to (7675), golden rectangle size represents number of proteins. Genbank accession numbers: NBRC 105727T, BCYR00000000.1; NBRC 14941, BJNO00000000.1; GODA, NZ_CP019899.1; DSM 102253T, GCA_006711645.1.

Based on TYGS phylogenetic analysis, strain N9 belongs to *R. ornithynolitica* species.

According to all phylogenetic analysis results, strain N9 belongs to *R. ornithynolitica* species, supporting results obtained from MALDI-TOF.

In order to correctly affiliate *Enterobacter* sp. N10 strain, the ANIb, ANIm, dDDH, difference in GC content values were calculated against twelve putatively *E. kobei* genomes available at the Genbank database including two genomes indicated as *E. kobei* type strains: *E. kobei* DSM 13645^T, and *E. kobei* JCM 8580^T. Additionally, MLSA and whole genome analysis through TYGS platform were performed.

Table 12. ANIb and DDH values of *E. kobei* DSM13645^T, *E. kobei* JCM 8580^T and other non-type *E. kobei* strains against *Enterobacter* sp. N10.

Genome (acc. numbers)	ANIb [%]	ANIm [%]	DDH [%]	Difference in G+C	DDH, G+C Interpretation
E. kobei DSM 13645^T (CP017181)	99.25	99.85	98.20	0.50	same species
E. kobei 44593 (JZXR01000000)	98.76	99.36	94.30	0.36	same species
E. kobei GN02825 (LEDC01000000)	98.66	99.22	93.10	0.35	same species
E. kobei 35730 (JZYS01000000)	98.58	99.15	92.40	0.12	same species
E. kobei UCI 29 (KK736271.1)	98.51	99.14	92.20	0.36	same species
E. kobei 8706 (LLXN00000000.1)	98.48	99.13	92.40	0.32	same species
E. kobei 42202 (JZYH01000000)	98.45	99.12	92.30	0.20	same species

E. kobei MGH 23 (KI535658.1)	98.41	99.11	91.90	0.30	same species
E. kobei BIDMC 67 (KK736229.1)	98.39	99.09	91.60	0.33	same species
E. kobei SMART_635 (LPPL01000000)	98.35	99.08	91.80	0.04	same species
E. kobei e1326 (FJYB00000000.1)	98.34	99.08	91.60	0.50	same species
E. kobei JCM 8580^T (MKXD00000000)	79.01	58.76	23.10	1.02	distinct species

Analysis between *Enterobacter* sp. N10 and *E. kobei* DSM 13645^T revealed ANIb and ANIm values of 99.25 % and 99.85 %, respectively, thus above the established threshold of 95-96 (Richter & Rosselló-Móra, 2009) for species delineation. Likewise, the predicted dDDH value of 94.30 % obtained is above the threshold of 70 % (Chun et al., 2018) for species definition based on DDH values. Finally, G+C content differed in 0.01 % between these two genomes, thus in less than 1 % as suggested previously to be within species (Meier-Kolthoff et al., 2014). With exception to *E. kobei* JCM 8580^T, based on values obtained from ANIb, ANIm, difference in G+C and dDDH analysis, all isolates affiliated with *E. kobei*.

On the other hand, analysis between *Enterobacter* sp. N10 and *E. kobei* JCM 8580^T revealed ANIb and ANIm values of 79.01 % and 58.76 %, respectively, thus below the established threshold for species delineation. Additionally, the predicted dDDH value of 23.10 % obtained is below the threshold of 70 % for species definition based on DDH values (Chun et al., 2018). Lastly, G+C content differed in 1.02 % between these two genomes, thus in more than 1% as suggested previously to be distinct species (Meier-Kolthoff et al., 2014). Comparison of *E. kobei* JCM 8580^T against *E. kobei* DSM 13645^T revealed ANIb and ANIm values of 79.26 % and 84.75 %, respectively, thus below the established threshold for species delineation. Additionally, the predicted dDDH value of 23.40 % obtained is below the threshold of 70 % for species definition based on DDH values. Lastly, G+C content differed in 0.52 % between these two genomes, less than 1 % , is insufficient to discriminate these two isolates (Meier-Kolthoff et al., 2014).

All genomes belonging to *E. kobei* deposited in the PATRIC database (total of 55), 48 affiliate with *E. kobei* DSM 13645^T, 2 of them affiliate with the type strain initially deposited, *E. kobei* JCM8580^T and which were also deposited as strain type (JCM8580^T = ATCC BAA-260^T = DSM27110^T) and 5 of the isolates do not belong to *E. kobei* species, since they do not affiliate with either of the two isolates described above (table A7). The only available genomes that affiliate with the type strain JCM 8580^T are those that were deposited as a type strain to different culture collections

The MLSA was performed using *dnaA, fusA, gyrB, leuS, pyrG, rpiB* and *rpoB* concatenated housekeeping genes sequences retrieved from *Enterobacter* sp. N10 and another 18 genomes of *Enterobacter* spp., including *E. kobei* DSM 13645^{T}. Results are shown in figure 24.

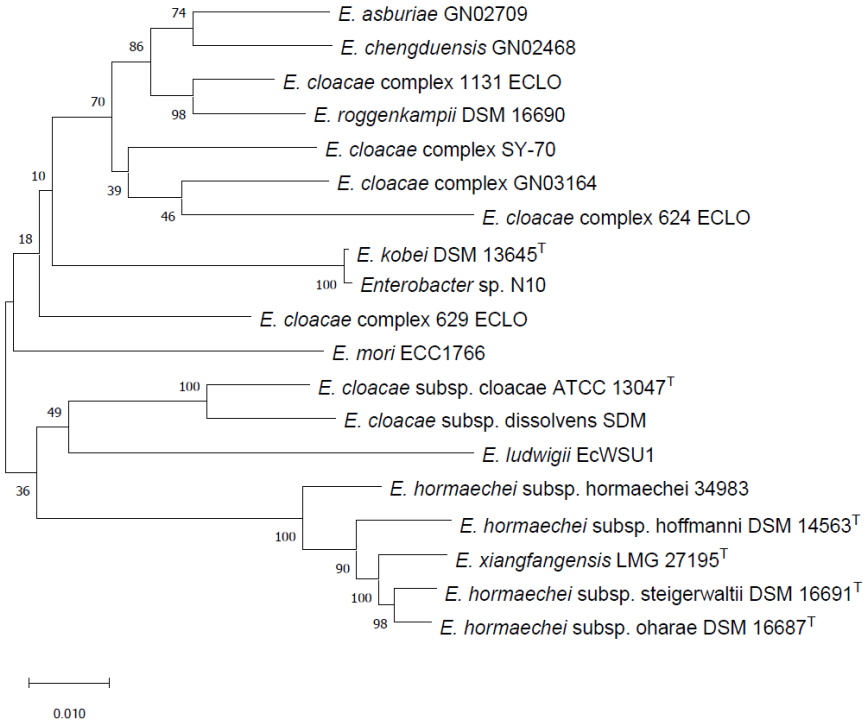

Figure 24. Phylogenetic tree of the MLSA using a bootstrap analysis with 1000 replications based on *dnaA, fusA, gyrB, leuS, pyrG, rpiB* and *rpoB* concatenated housekeeping genes of *Enterobacter kobei* N10 and other representatives of different groups of genus *Enterobacter*. Genbank accession numbers: LMG 27195, NZ_CP017183; DSM 16691, NZ_CP017179; DSM 16687, NZ_CP017180; DSM 14563, NZ_CP017186; 34983, NZ_CP010377; ECC1766, NZ_NEEF01000026.1; ATCC 13047, CP001918.1; SDM, NC_018079.1; EcWSU1, NC_016514.1; GN02709, NZ_LEDH00000000.1; 1131_ECLO, NZ_JWCB00000000.1; GN02468, NZ_LEDT00000000.2; DSM 16690, NZ_CP017184; SY-70, NZ_JALR00000000.1; GN03164, NZ_LECZ00000000.1; 624_ECLO, NZ_JUZQ01000000; DSM 13645, NZ_CP017181; 629_ECLO, NZ_JUZL01000000.

According to MLSA results, *Enterobacter* sp. N10 affiliates to E. *kobei* species, being phylogenetically related to E. *kobei* DSM 13645^{T}.

To support previous results, we performed whole genome based phylogenetic analysis of *Enterobacter* sp. N10 against 18 genomes representing *Enterobacter cloacae* complex group (n=18) (chavda et al., 2016), *Enterobacter kobei* JCM 8580^{T} and *Enterobacter kobei* N10 and submitted to TYGS platform. The results of phylogenetic affiliation of *Enterobacter* sp. N10 based on TYGS analysis are shown in figure 25.

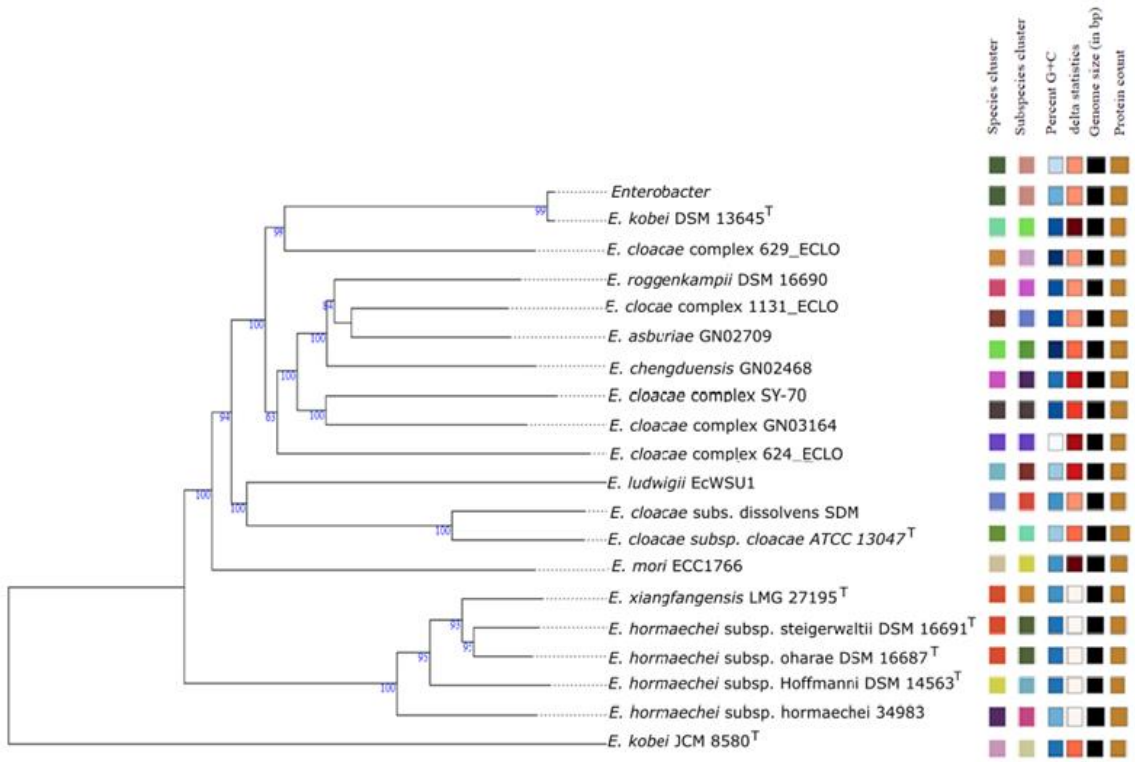

Figure 25. Phylogenetic tree based on whole genome analysis of the *Enterobacter kobei* N10 obtained from TYGS. Species and subspecies cluster colors represent different clusters. Percent of G+C varies from white (64.0) to dark blue (69.3). δ statistic values varies from white (0.1) to brown (0.2). Genome size (bp) varies from (4.2 Mb) to (8.0 Mb), dark rectangle size represents genome size. Protein count varies from (3211) to (7675), golden rectangle size represents number of proteins. Genbank accession numbers: Genbank accession numbers: LMG 27195, NZ_CP017183; DSM 16691, NZ_CP017179; DSM 16687, NZ_CP017180; DSM 14563, NZ_CP017186; 34983, NZ_CP010377; ECC1766, NZ_NEEF01000026.1; ATCC 13047, CP001918.1; SDM, NC_018079.1; EcWSU1, NC_016514.1; GN02709, NZ_LEDH00000000.1; 1131_ECLO, NZ_JWCB00000000.1; GN02468, NZ_LEDT00000000.2; DSM 16690, NZ_CP017184; SY-70, NZ_JALR00000000.1; GN03164, NZ_LECZ00000000.1; 624_ECLO, NZ_JUZQ01000000; DSM 13645, NZ_CP017181; 629_ECLO, NZ_JUZL01000000; JCM 8580T, MKXD00000000.1.

All results, obtained from ANIb, ANIm, difference in GC, dDDH, MLSA and TYGS analysis affiliate *Enterobacter* sp. N10 to *E. kobei* when compared with *E. kobei* DSM 13645^T.

For *E. kobei* N10 multi loci typing we performed MLST analysis using MLST 2.0 tool. New alleles of *fusA*, *leuS* and *rpiB* genes were detected. These HKG sequences were submitted to pubMLST Enterobacter database (https://pubmlst.org/ecloacae/) to attribute a new locus. *fusA* was assigned as 239, *leuS* as 448 and *rpiB* as 169, respectively. After that, a MLST were performed using *dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rpiB* and *rpoB* genes. Finally, a new sequence type (1378) was attributed. Results obtained are shown in table 13.

Table 13. Allelic analysis and sequence type of *E. kobei* N10. *New alleles detected in this isolate.

Locus	Allele	Length	Contig	Start position	End position
dnaA	71	442	3	20536	20977
fusA	239*	646	9	12959	13604
gyrB	87	434	3	24212	24645
leuS	448*	578	1	212404	212981
pyrG	254	259	16	66938	67196
rpiB	169*	607	85	4140	4746
rpoB	3	545	10	24679	25223

To further analyse the differences observed between *E. kobei* N10 and the two *E. kobei* type strains, *E. kobei* DSM13645^{T} and *E. kobei* JCM 8580^{T}, the core and dispensable genome analysis was performed using PGAdb-builder.

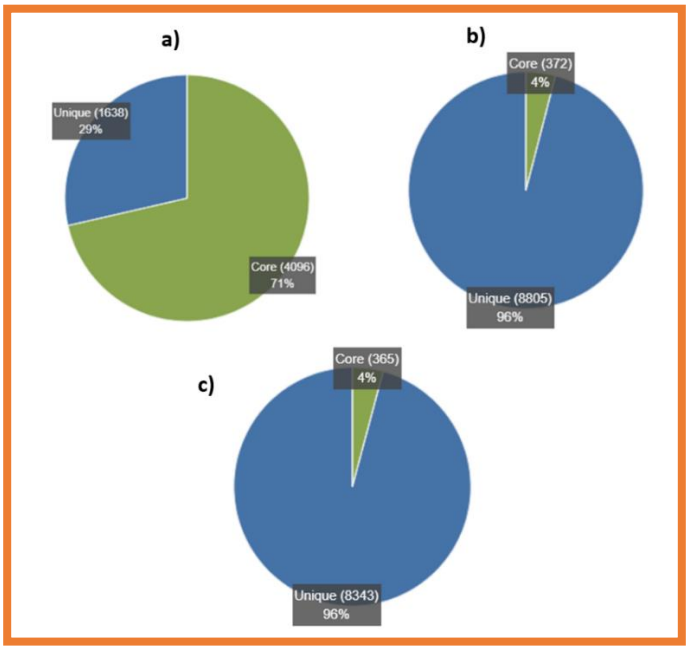

Figure 26. a) *E. kobei* N10 compared with *E. kobei* DSM 13645^{T}; b) *E. kobei* N10 compared with *E. kobei* JCM 8580^{T}. c) *E. kobei* JCM 8580T compared with *E. kobei* DSM 13645T.

E. kobei N10 when compared to *E. kobei* DSM 13645^{T} shares a core genome of 71 % in a total of 5734 genes, whereas when compared to *E. kobei* JCM 8580^{T} only shares 4 % of the core genome in a total of 9177 genes analyzed. The comparison of *E. kobei* JCM 8580T against *E. kobei* DSM 13645T reveals a set of 365 (4 %) shared genes and 8343 (96 %) not shared genes. These results are further evidence that the *E. kobei* N10 is related to *E. kobei* DSM 13645^{T} and different from *E. kobei* JCM 8580^{T}.

7.3. Antibiotic resistance genes and mobilome analysis

***C. freundii* F6**

Results obtained from ARG detection based on WGS analysis are shown in this section. In order to find additional ARG, contigs where ARG were detected using CARD and Resfinder tools were analyzed trough blast searching in NCBI database. The compiled results of ARG detected through the Resfinder, CARD and NCBI in *C. freundii* F6 are described in table 14.

Table 14. Detected ARG in *C. freundii* F6 using Resfinder, CARD and NCBI (*) databases search. Genes were predicted using CARD tool and % similarity refers to protein similarity.

Resistance mechanism	Drug Class	Contig	Protein	Predicted gene	Protein similarity (%)
Drug inactivation	β-lactams	38	Class C β-lactamase	*bla*$_{CMY-152}$	99.74
Drug inactivation	β-lactams	74	Carbapenem-hydrolyzing class A β-lactamase	*bla*$_{KPC-3}$	100
Drug inactivation	β-lactams	82	Class D β-lactamase	*bla*$_{OXA-1}$	100
Drug inactivation	Phenicols	82	Chloramphenicol acetyl transferase	*cat*B3	100
Drug inactivation	Aminoglycosides, fluoroquinolones, ciprofloxacin	82	Aminoglycoside N(6')-acetyltransferase type 1	*aacA4*-cr	100
Drug inactivation	Macrolides	91	macrolide 2'-phosphotransferase I	*mphA*	100
Drug target protection	Fluoroquinolones	92	Fluoroquinolone resistance protein	*QnrS*1	100
Drug target replacement	Sulfonamides	82	Dihydropteroate synthase type-1	*sul*1	100
Drug target replacement	Diaminopyrimidine	115	Dihydrofolate reductase	*dfrA*14	99.34

Drug efflux	Tetracycline	16	Multidrug transporter MdfA	*MdfA*	87.8
Drug efflux	Aminocoumarins	21	Multidrug resistance protein MdtB	*mdtC*	92.69
Drug efflux	Aminocoumarins	21	Multidrug resistance protein MdtC	*mdtC*	92.98
Drug efflux	Fluoroquinolones	54	Multidrug export protein EmrB	*emrB*	95.51
Drug efflux	tetracycline, glycylcycline, rifamycin, cephalosporin, penam, phenicol, triclosan, fluoroquinolone	10	Multidrug efflux pump subunit AcrA	*acrA*	90.18
Drug efflux	tetracycline antibiotic, phenicol antibiotic, rifamycin antibiotic, glycylcycline, penam, cephalosporin, triclosan, fluoroquinolone	10	multidrug efflux RND transporter permease subunit protein	*acrB*	94.57
Drug target alteration	polymyxin and cationic antimicrobial peptides	13	Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	*pmrF*	88.47
Drug efflux	nitroimidazole antibiotic	16	Lipid A export ATP-binding/permease protein MsbA	*msbA*	95.19
Drug efflux	tetracycline antibiotic	93	Tetracycline resistance protein, class C	*tet(A)*	99.74
Drug efflux	macrolides	21	Macrolide export protein MacA	*macA**	99.73
Drug efflux	macrolides	21	Macrolide export ATP-binding/permease protein MacB	*macB**	100
Drug efflux	puromycin	21	Putative multidrug resistance outer	*mdtQ**	100

			membrane protein MdtQ		
Drug efflux	aminocoumarins	21	Multidrug resistance protein MdtA	*mdtA**	100
Drug efflux	fluoroquinolones	50	Multidrug export protein EmrA	*emrA**	100
Drug target alteration	Sulfonamides	5	Dihydropteroate synthase	*folP* with mutation	47.76
antibiotic target alteration	fosfomycin	6	Hexose-6-phosphate:phosphate antiporter	*UhpT* with mutation	95.46
antibiotic target alteration	penam, cephamycin, cephalosporin)	7	Penicillin-binding protein 3	*PBP3 conferring resistance*	52.75
antibiotic target alteration	fosfomycin	13	Glycerol-3-phosphate transporter	*GlpT* with mutation	95.9
antibiotic target alteration	fluoroquinolones	13	DNA gyrase subunit A	*Escherichia coli gyrA with mutation* S83l	90.97
antibiotic target alteration	elfamycin	124	Elongation factor Tu	*Escherichia coli EF-Tu* mutants	97.69
reduced permeability to drug, drug efflux and drug target alteration	tetracycline, phenicol, fluoroquinolone, rifamycin, penam, cephalosporin, glycylcycline, monobactam, penem, triclosan, carbapenem, cephamycin	17	Regulatory protein SoxS	*soxS* with mutation	93.46
drug efflux,drug target alteration	tetracycline, rifamycin, phenicol, penam, cephalosporin, glycylcycline, triclosan, fluoroquinolone antibiotic	39	Multiple antibiotic resistance protein MarR	*Escherichia coli marR* mutant	91.67

This analysis confirmed the presence of carbapenemase-encoding genes previously detected by PCR. Additionally, it was predicted that *C. freundii* F6 harbour genes associated with resistance to several antibiotics, such as resistance to β-lactams (*bla*$_{KPC-3}$, *bla*$_{CMY-152}$, *bla*$_{OXA-1}$), phenicols (*catB3*), aminocoumarins (*mdtB*, *mdtC* and *mdtA*), tetracycline (*tetA* and *mdfA*), aminoglycosides (*aacA4-cr*), fluoroquinolones (*aacA4-cr*, *qnrS1*, *emrA* and *emrB*), ciprofloxacin (*aacA4-cr*), diaminopyrimidine (*dfrA14*), sulfonamides (*sul1*), polymyxin (*pmrF*), nitroimidazole (*mbsA*), macrolides (*macA* and *macB*) and puromycin (*mdtQ*). Mutations in housekeeping genes (HKG) conferring antibiotic resistance were also detected, such as mutations in *gyrA* (fluoroquinolones), in *Uhp*T (fosfomycin), in *EF-Tu* (elfamycin) and in *marR* (several antibiotics). The *pmrF* and *acrA*, genes conferring resistance to several antibiotics were also detected. The resistance phenotype observed can be justified with the detected ARG. The replicons detected in *C. freundii* F6 using PlasmidFinder 2.1 are described in table 15.

Table 15. Detected plasmids in *C. freundii* F6 obtained from PlasmidFinder 2.1.

Replicon	Similarity (%)	Contig	Accession number
IncFIA(HI1)	100	24	AF250878
IncFII(K)	100	24	CP000648
IncM1	100	36	U27345
IncN	100	66	AY046276
pKPC-CAV1321	100	53	CP011611

Citrobacter freundii F6 harbour IncFIA, IncFII, IncM1, IncN and pKPC-CAV1321 replicons. The genetic context analysis of *bla*$_{KPC-3}$ showed that the *bla*$_{KPC-3}$ is flanked by *ISKpn6* and *ISKpn7* (figure 27). In the same contig other insertion sequences were identified, namely *ISPSY42-like* and additional copies of *ISKpn6* and *ISKpn7*. Using Blast searching tool against the NCBI database, the most identical region (99 %; 6632 bp) found is located on pSECR18-1644 plasmid (MT129535.1) of *Klebsiella aerogenes*, which carries a different KPC variant.

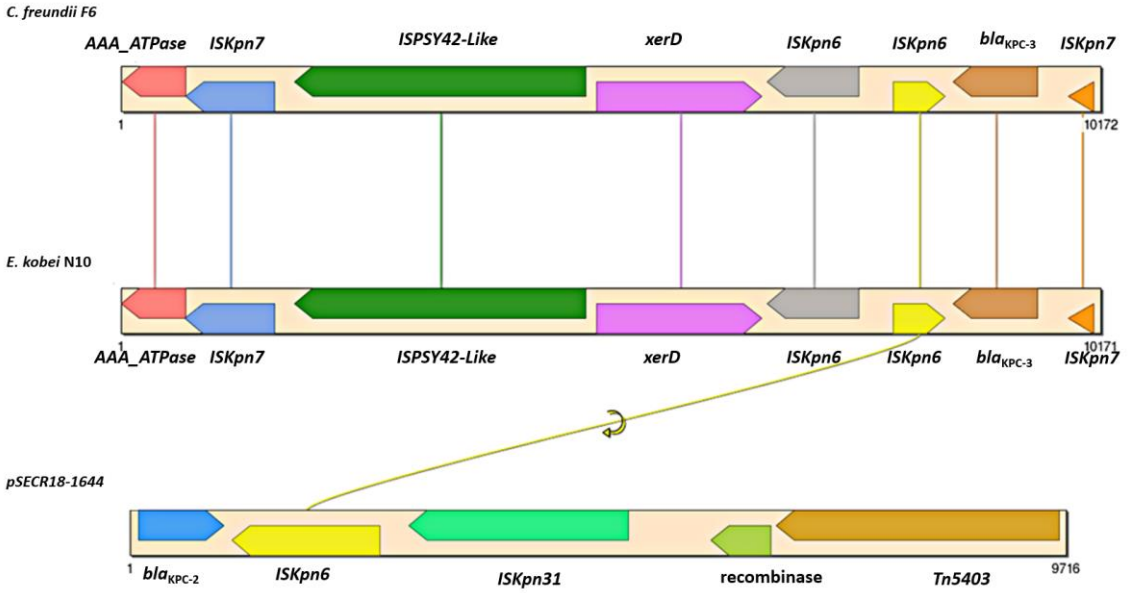

Figure 27. Representation of the genetic context of *bla*$_{KPC-3}$ in *Citrobacter freundii* F6 and *Enterobacter* sp. N10 isolates and the closest region found in genbank – *Klebsiella aerogenes* pSECR18-1644 (Acc. Number MT129535.1).

Also, the presence of integrons and related sequences was inspected in the genome of *C. freundii* F6, thus the class 1 integron contained in a transposon was identified. This integron harbours gene cassettes conferring resistance to several antibiotics, such as, *sul1* (sulfonamides), *bla*$_{OXA-1}$ (some β-lactams), *catB3* (phenicols) and *aacA4-cr* (aminoglycoside, fluoroquinolones and ciprofloxacin). Additionally, it also harbours *qacEΔ1* gene, conferring resistance to quaternary ammonium compounds (detergents) (figure 28).

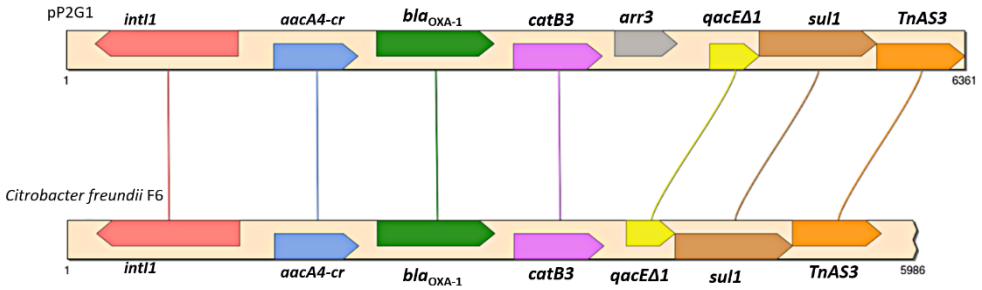

Figure 28. Genetic context of *qacEΔ1*, *sul1*, *bla*$_{OXA-1}$, *catB3* and *aacA4*, found in a transposon-borne class 1 integron and a similar structure present in a region of a pP2G1 plasmid from *Aeromonas rivipollensis* (Genbank acc. number HE616910.2).

***R. ornithinolytica* N9**

In order to find additional ARG, contigs where ARG were detected using CARD and Resfinder tools were analyzed trough blast searching in NCBI database. The compiled results of ARG detected through the Resfinder, CARD and NCBI in *R. ornithinolytica* N9 are described in table 16.

Table 16. Detected ARG in *R. ornithinolytica* N9 using Resfinder, CARD and NCBI (*) databases search. Genes were predicted using CARD tool and % similarity refers to protein similarity.

Resistance mechanism	Drug class	Contig	Protein	Predicted gene	Similarity (%)
Drug inactivation	β-lactams	30	carbapenem-hydrolyzing class A β-lactamase	*bla*$_{GES-5}$	100
Drug inactivation	β-lactams	30	β-lactamase	*bla*$_{ORN-1}$*	-
Drug inactivation	β-lactams	50	carbapenem-hydrolyzing class A β-lactamase	*bla*$_{KPC-3}$	100
Drug inactivation	β-lactams	81	β-lactamase	*bla*$_{MOX-3}$	100
Drug inactivation	β-lactams	136	β-lactamase	*bla*$_{OXA-9}$	100
Drug inactivation	β-lactams	138	β-lactamase	bla $_{OXA-10}$	100
Drug inactivation	Aminoglycoside	167	Aminoglycoside acetyltransferase AAC(6')-Ib	*aacA4*	100
Drug inactivation	aminoglycosides	136	Aminoglycoside adenyltransferase ANT(3')-Ia	not predicted	-
Drug inactivation	aminoglycosides	159	Aminoglycoside adenyltransferase ANT(3')-Ia	*aadA1*	100
Drug inactivation	Fosfomycin	12	Fosfomycin resistance protein *FosA*	*fosA5*	89.93
Drug target replacement	diaminopyrimidine antibiotic	88	Dihydrofolate reductase	*drfA14*	99.38
Drug efflux	Fluoroquinolones	55	*Klebsiella pneumoniae KpnH*	*kpnH*	93.36

Drug efflux	Fluoroquinolones	55	Multidrug export protein *EmrA*	*emrA**	100
Drug efflux	tetracycline, nitrofuran, glycylcycline, fluoroquinolone, diaminopyrimidine	40	Multidrug efflux RND transporter permease subunit OqxB	*adeF*	61.15
Drug efflux	phenicol, diaminopyrimidine, fluoroquinolone	40	Multidrug efflux RND membrane fusion protein MexE	not predicted	-
Drug efflux	streptogramin, pleuromutilin, lincosamide, macrolide, oxazolidinone, tetracycline, phenicol	81	ABC-F type ribosomal protection protein MsrE	*msrE*	100
Drug efflux	amphipathic compounds	23	Multidrug resistance protein D	not predicted	100
Drug efflux	nitroimidazole	5	Lipid A export ATP-binding/permease protein MsbA	*msbA*	92.44
Drug target alteration	fusaric acid	55	Aldehyde dehydrogenase	*kpnG*	94.62
Drug inactivation	macrolides	81	Macrolide 2'-phosphotransferase	*mphE*	100
Drug target protection	streptogramin, pleuromutilin, lincosamide, macrolide, oxazolidinone, tetracycline, phenicol	81	Macrolide efflux protein *msrE*	*msrE*	100
Drug inactivation	β-lactams	99	class A broad-spectrum β-lactamase TEM-1	$bla_{\text{TEM-1}}$	100
Drug inactivation	aminoglycoside	99	aminoglycoside O-phosphotransferase APH(6)-Id	*aph(6)-Id*	99.64
Drug inactivation	aminoglycoside	99	aminoglycoside O-phosphotransferase APH(3'')-Ib	*aph*(3'')-Ib	99.63
Drug target replacement	sulfonamide	99	Dihydropteroate synthase type-2	*sul2*	100

Drug inactivation	streptomycin		(Streptomycin kinase) protein A	*strA**	100
Drug inactivation	streptomycin		(Streptomycin kinase) protein B	*strB**	100
Drug target alteration	sulfonamide antibiotic	2	Dihydropteroate synthase	*folP* with mutation	49.25
Drug target alteration	cephalosporin; cephamycin; penam	7	Penicillin-binding protein 3	*PBP3* conferring resistance	52.02
Drug target alteration	fluoroquinolone antibiotic	18	DNA gyrase subunit A	*Escherichia coli gyrA* conferring resistance S83I	90.95
Drug target alteration	fosfomycin	23	Hexose-6-phosphate:phosphate antiporter	*UhpT* with mutation	96.27
Drug target alteration	elfamycin antibiotic	161	Elongation factor Tu	*Escherichia coli EF-Tu* mutants	97.3
Drug target alteration; Drug efflux	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan	32	Multiple antibiotic resistance protein *MarR*	*Escherichia coli marR* mutant	84.03

The genes detected by WGS confirm the results obtained with ARG screening by PCR. Additionally, it was observed that the isolate *R. ornithinolytica* N9 harbors genes associated with resistance to several antibiotics, such as to β-lactams (*bla*$_{GES-5}$, *bla*$_{KPC-3}$, *bla*$_{ORN-1}$, *bla*$_{MOX-3}$, *bla*$_{OXA-9}$, *bla*$_{OXA-10}$, *bla*$_{TEM-1}$), aminoglycosides (*aacA4*, *aadA1*), fosfomycin (*fosA*), diaminopyrimidine (*drfA14*), fluoroquinolones (*kpnH* and *emrA*), macrolides (*mphE*), nitroimidazole (*msbA*), fusaric acid (*kpnG*) and sulfonamides (*sul2*). In addition to these ARG, it also harbors genes that confer resistance to several antibiotics, such as *adeF* and *msrE* and genes. Genes possessing high similarity with mutated genes which confer antibiotic resistance, such as *UhpT* (fosfomycin), *gyrA* (fluoroquinolones) and *marR* (several antibiotics) were also detected. The resistance phenotype can be justified by detected ARG.

The replicons detected in *R. ornithinolytica* N9 using PlasmidFinder 2.1 are described in table 17.

Table 17. Detected plasmids in *R. ornithinolytica* N9 obtained from PlasmidFinder 2.1.

Replicon	Similarity (%)	Contig	Accession number
Col(MGD2)	100	117	003789
Col(pHAD28)	95,24	103	KU674895
Col(pHAD28)	95,65	107	KU674895
Col440I	95,61	129	CP023920
FIA(pBK30683)	100	93	KF954760
FII(pBK30683)	100	48	KF954760

The cointegrated FIA and FIIA replicons had been already detected by specific multiplex-PCR targeting pBK30683 plasmid. pBK30683 plasmid structure and *R. ornithinolytica* N9 contigs, corresponding to each part of this plasmid are described in figure 29 and table 18.

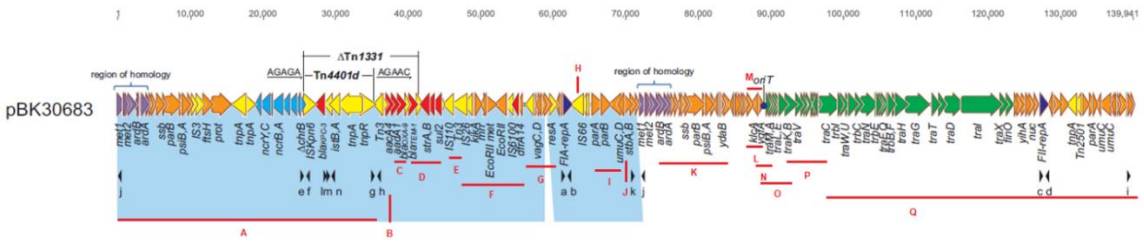

Figure 29. pBK30683 plasmid structure and *R. ornithinolytica* N9 contigs, corresponding to each part of this plasmid.

Table 18. Regions of pbk30683 detected by WGS analysis in *R. ornithinolytica* N9.

contig	contig size	pb match (% identity)	Included genes	Region
50	36174	36174/36174 (100)	*met1; met2; ardB; ardA; ssb; parB; psiB, A; IS3; ftsH; prot; tnpA; tnpA; ncrY, C; ncrB, A; deltachrB; ISKpn6; blaKPC-3; istB, A; tnpA; tnpR*	A
167	572	572/572(100)	*aacA4*	B
136	1342	1342/1342(100)	*blaOXA-9, aadA1*	C
99	4705	4705/4705 (100)	*blaTEM-1; straA, B; sul2; Integrase*	D
128	1927	1927/1927 (100)	*IS110; Tn3; IS26*	E
88	6892	6891/6892 (99)	*IS26; KikA; mrr; EcoRII met; EcoRII; IS6100; dfrA14*	F
93	5422	4470/4493 (99)	*Vag C, D; resA*	G
121	2454	2442/2454 (99)	*IS66*	H
108	3847	3847/3847 (100)	*parA; parB; umuC*	I
64.5	23147	784/926(85)	*stbB*	J
80	10954	10954/10954 (100)	*ardB; ardA; ssb; parB; psiB, A; ydaB*	K
130	1793	1793/1793 (100)	*klcA; ygfA*	L
64.2	23147	1370/1626 (84)	*klcA; ygfA*	M
105	4132	2435/2500 (97)	*klcA; ygfA; traM*	N
64.1	23147	4118/4775 (86)	*traM, A; traL, E; traK, B*	O
102	4206	4204/4206 (99)	*traB; traV; traC*	P
48	41028	41012/41036 (99)	*traC; trbl; traW, U; trbC; traN; trbE; traE, Q; trbB, F; traH; traG; traT; traD; traI; traX; finO; yihA; nuc; FII-repA; tnpA; tn2501; parA; umuC; umuC*	Q

The detected pBK30683-like plasmid, harbours nine ARG, including $bla_{\text{KPC-3}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-9}}$ (β-lactams), *sul2* (sulfonamide), *aacA4*, *aadA1*, *strA*, *strB* (aminoglycosides) and *dfrA14* (trimethoprim). The $bla_{\text{KPC-3}}$ is located on a Tn4401d transposon and is flanked by *ISKpn6* and *istA*, *istB* genes. The *strA* and *strB* genes present in this plasmid were not detected using the CARD and Resfinder tools, however their presence was confirmed through blast search against Genbank database.

Figure 30. Genetic context of $bla_{\text{GES-5}}$ in *R. ornithinolytica* N9 constructed by SimpleSynteny tool.

The analysis of the $bla_{\text{GES-5}}$ gene context showed that this gene was located in a class 3 integron, followed by an *aacA4* gene cassette (figure 30). Since the *aacA4* gene is located at the beginning of this contig, it is not possible to determine the following gene

cassettes of this integron. Due to the presence of the *bla*$_{\text{ORN-1}}$ gene, an intrinsic β-lactamase present in *R. ornithinolytica* chromosome (Walckenaer et al., 2004) and due to remaining genes present in this contig, it is thought that this carbapenemase-harboring integron is located on the chromosome.

***E. kobei* N10**

In order to find additional ARG, contigs where ARG were detected using CARD and Resfinder tools were analyzed trough blast searching in NCBI database.The compiled results of ARG detected through the Resfinder, CARD and NCBI in *E. kobei* N10 are described in table 19.

Table 19. Detected ARG in *E. kobei* N10 using Resfinder, CARD and NCBI (*) databases search. Genes were predicted using CARD tool and % similarity refers to protein similarity.

Resistance mechanism	Drug class	Contig	Protein	Predicted gene	Protein similarity (%)
Drug inactivation	-lactams	105	carbapenem-hydrolyzing class A β-lactamase	bla_{KPC-3}	100
Drug inactivation	β-lactams	116	carbapenem-hydrolyzing class A β-lactamase	bla_{GES-5}	100
Drug inactivation	β-lactams	57	class C β-lactamase	bla_{ACT-9}	100
Drug inactivation	Fosfomycin	21	Fosfomycin resistance protein FosA	*fosA2*	96.45
Drug inactivation	Macrolide	156	macrolide 2'-phosphotransferase I	*mphA*	100
Drug inactivation	Aminoglycoside	209	Aminoglycoside 6'-N-acetyltransferase	*aacA4*	100
Drug target replacement	Sulfonamide	167	Dihydropteroate synthase type-1	*sul1*	100
Drug efflux	Macrolide	27	Macrolide export ATP-binding/permease protein MacB	*macB*	100
Drug efflux	aminocoumarin	33	Multidrug resistance protein MdtA	*mdtA*	100
Drug efflux	Aminocoumarin	33	Multidrug resistance protein MdtB	*mdtB*	100
Drug efflux	Aminocoumarin	33	Multidrug resistance protein MdtC	*mdtC*	99.80
Drug efflux	Quinolones, β-lactams, chloramphenicol, tetracyclines	39	Multidrug resistance protein RomA	*ramA*	92.74
Drug efflux	Fosfomycin and deoxycholate	46	Multidrug resistance protein MdtG	*mdtG**	100
Drug efflux	Fluoroquinolone	52	Multidrug export protein EmrA	*emrA**	99.74
Drug efflux	Fluoroquinolone	52	Multidrug export protein EmrB	*emrB*	92.43

Drug efflux	nitroimidazole	27	lipid A ABC transporter ATP-binding protein/permease MbsA	*msbA*	100
Drug target alteration	fosfomycin	3	Hexose-6-phosphate:phosphate antiporter	*Escherichia coli UhpT* with mutation	93.95
Drug target alteration	sulfonamide	4	Dihydropteroate synthase	*folP* with mutation	48.13
Drug target alteration	cephalosporin; cephamycin; penam	7	Penicillin-binding protein 3	*Haemophilus influenzae* PBP3	53.1
Drug target alteration	elfamycin	9	Elongation factor Tu	*Escherichia coli EF-Tu* mutants	98.48
Drug target alteration	elfamycin	10	Elongation factor Tu	*Escherichia coli EF-Tu* mutants	98.48
Drug target alteration	fluoroquinolone	31	DNA gyrase subunit A	*gyrA* conferring resistance **S83F**	96.24
Drug target alteration; Drug efflux	fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol; triclosan	76	Multiple antibiotic resistance protein MarR	*Escherichia coli marR* mutant	89.58

The genes detected by WGS confirm ARG screening by PCR. Additionally, it was observed that the isolate *E. kobei* N10 harbors genes associated with resistance to several antibiotics, such as β-lactams (*bla*$_{KPC-3}$, *bla*$_{GES-5}$, *bla*$_{ACT-9}$), fosfomycin (*fosA* and *mdtG*), deoxycholate (*mdtG*), aminoglycosides (*aacA4*), macrolide (*mphA* and *macB*), sulfonamides (*sul1*), fluoroquinolones (e*mrA* and *emrB*), nitroimidazole (*msbA*) and aminocoumarin (*mdtA*, *mdtB* and *mdtC*). In addition to these ARG, it also harbors the ramA MDR gene, conferring resistance to quinolones, β-lactams, chloramphenicol and tetracyclines. This isolate also harbors genes with high similarity to mutated genes that confer antibiotic resistance, such as *EF-Tu* conferring resistance to elfamycin, *gyrA* conferring resistance to fluoroquinolones and *UhpT* conferring resistance to fosfomycin. The resistance phenotype can be justified by detected ARG.

The replicons detected in *E. kobei* N10 using PlasmidFinder 2.1 are described in table 20.

Table 20. Detected plasmids in *E. kobei* N10 obtained from PlasmidFinder 2.1

Replicon	Similarity (%)	Contig	Accession number
Col440I	95,61	140	CP023920
IncFIB(pECLA)	100	75	CP001919
IncFII(pECLA)	99,6	162	CP001919
IncM1	100	36	U27345
IncN	99,22	107	AY046276
IncX5	100	44	MF062700
pKPC-CAV1321	100	28	CP011611

A total of 7 replicons were detected, namely Col, IncFIB, IncFII, IncM, IncN, IncX and pKPC-CAV1321.

The genetic context of *bla*$_{KPC-3}$ of *E. kobei* N10 is 100 % identical of *bla*$_{KPC-3}$ genetic context found in *C. freundii* F6 and such as *C. freundii* F6 plasmid, it shares a identical region (99 %; 6632 bp) with pSECR18-1644 plasmid (MT129535.1) of *Klebsiella aerogenes*, which carries a different KPC variant (figure 19). Like in *C. freundii* F6, the genetic context of *bla*$_{KPC-3}$ was analysed and showed that the *bla*$_{KPC-3}$ is flanked by *ISKpn6* and *ISKpn7*. In the same contig other insertion sequences were identified, namely *ISPSY42-like* and another *ISKpn6* and *ISKpn7* sequences.

The *bla*$_{GES-5}$ found in the *E. kobei* N10 is contained in a class 3 integron. In the same contig there are genes associated with mobile genetic elements, namely *repA*, *mobA* and *mobC* and the aminoglycoside resistance gene *aacA4*. This mobile structure is identical

to the plasmid pCR16 found in Rio Lis, Portugal (Teixeira et al., 2020), sharing the *bla*$_{GES-5}$, *intI3*, *repA*, *mobA* and *mobC* genes.

The genetic context of *bla*$_{GES-5}$ found in *E. kobei* N10 is found in figure 31.

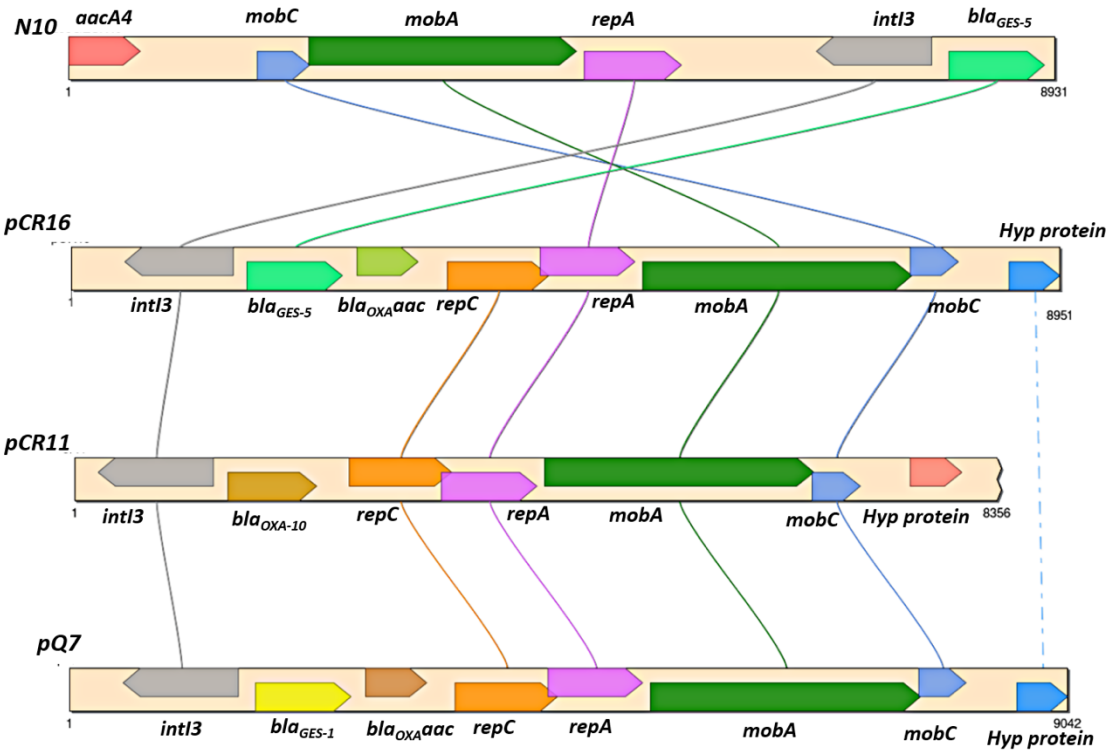

Figure 31. Genetic context of *bla*$_{GES-5}$ found in *E. kobei* N10 and similar genetic contexts found in aquatic environments. plasmid pQ7 (GenBank accession no.FJ696404). CR16 (GenBank accession no. RBWI00000000) and CR11 (GenBank accession no. RBMO00000000).

7.4. *In silico* virulence factors

All genomes were inspected for virulence factors using the VF analyzer againt VFDB database. The results are shown in figure 32 and tables A8, A9 and A10.

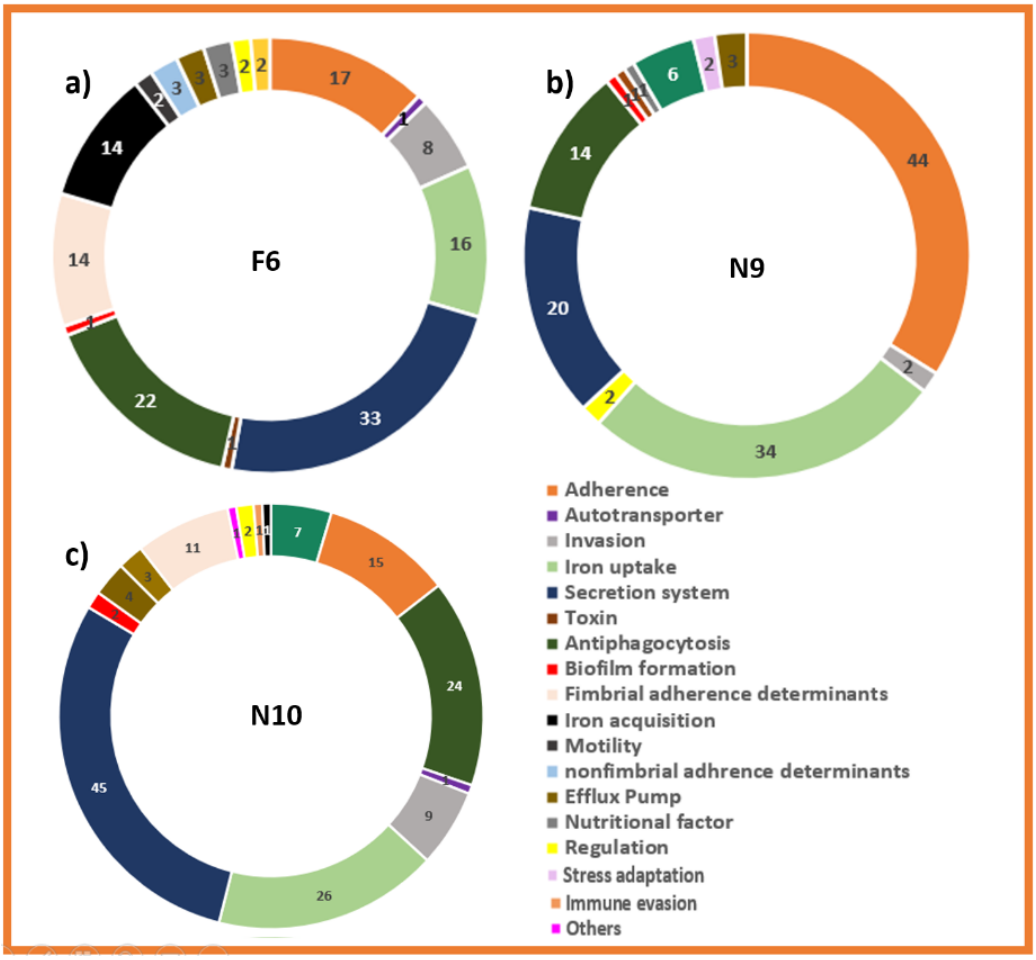

Figure 32. Virulence factors found in *Citrobacter freundii* F6 (a), *Raoultella ornithinolytica* N9 and *Enterobacter* sp. N10 (c) obtained from VFDB.

The total number of virulence factors predicted were 142, 130 and 152, for *Citrobacter freundii* F6, *R. ornithinolytica* N9 and *E. kobei* N10 respectively. For all, the categories with more virulent factors attributed were secretion system, iron uptake, antiphagocytosis and adherence. *C. freundii* motility genes are in higher amount (n=14; 10 %) than in two other genomes. Genes related with stress adaptation were not detected in *R. ornithinolytica* N9.

Pathogenicity analysis was performed for all genomes using PathogenFinder 1.1 tool. The results obtained are shown in tables 21, A11, A12 and A13.

Table 21. Results obtained from PathogenFinder 1.1 for *C. freundii* F6, *Raoultella ornithinolytica* N9 and *E. kobei* N10.

	Probability of being a human pathogen	Input proteome coverage (%)	Matched Pathogenic Families	Matched not Pathogenic Families	Result
C. freundii F6	0.843	1.95	90	7	predicted as human pathogen
R. ornithinolytica N9	0.819	1.65	90	8	predicted as human pathogen
E. kobei N10	0.739	1.75	73	18	predicted as human pathogen

All strains were predicted as human pathogens with a probability varying from 0.739 in *E. kobei* N10 to 0.843 in *C. freundii* F6. The number of proteins related to pathogenic families varied from 73 in *E. kobei* N10 to 90 in *C. freundii* F6 and *R. ornithinolytica* N9.

V. Discussion

Infections caused by carbapenemase-producing bacteria (CPB) are usually associated with hospital settings (Tanner et al., 2019). Despite this, there are several studies reporting the emergence of CPB in the environment, including aquatic environments such as rivers (Aubron et al., 2005; Piedra-Carrasco et al., 2017; Teixeira et al., 2020), coastal waters, namely estuaries (Xin et al., 2019), lakes (Le Terrier et al., 2019), among others. However, little information about the presence of CPB in urban aquatic systems is available. Despite the lack of studies to assess the impact of these environments as antibiotic-resistant bacteria (ARB) and ARG reservoirs and on the spread of ARG, human exposure to these aquatic systems located in recreational areas can occur through direct contact (Hooban et al., 2020; Surette & Wright, 2017;Yang, et al., 2017). This study aimed to assess the presence and characterize CPB, focusing on the *Enterobacteriaceae* family, since it includes some of the most prevalent pathogens in hospital settings and *Enterobacteriaceae* resistant to carbapenems have been considered one of the main public health threats related with antibiotic resistance (Kotb et al., 2020; H. J. Tang et al., 2016, WHO, 2017).

Enterobacteriaceae are part of the group of bacteria commonly named coliforms (Martin et al., 2016). Coliforms are a group of microorganisms that inhabit the gut of both humans and other animals and which are used as indicators of water quality (mishra et al., 2018). In Portugal, as a quality criterion, Decree Law No. 236/98 of August 1, 1998 establishes a maximum recommendable value of 500 CFU/100 mL and a maximum admissible value of 10000 CFU/100 mL of total coliforms in surface water for human usage. In all months and sites sampled, the total coliform counts exceeded the maximum recommended value and in most of the months sampled, Pond 1, Pond 2, Pond 3 and Dos Santos Mártires channel exceeded the maximum admissible value, having in some cases been 1,3 to 50-fold higher, thus indicating that these waters have poor microbiological quality.

Although in this study the cefotaxime-resistant bacteria were not characterized, they represented a significant number comparatively to the total number of CFU found in some sampling sites. Further studies focused on the characterization of these isolates would help to understand whether this resistance is due to natural or acquired mechanisms To slow down the spread of resistance to 3rd generation cephalosporins, implies that these

antibiotics may be used for a longer time and the carbapenems reserved for extreme and punctual cases.

In this study, the percentage of imipenem-resistant bacteria varied from 0 to 1 %. However, though in low amounts, imipenem-resistant bacteria were present in Pond 1, Pond 3 and Dos Santos Mártires channel. In Pond 1 and Dos Santos Mártires channel these bacteria were found during all sampling months. In Pond 3 imipenem-resistant bacteria were found only in November 2019. On average, and over the all sampling months, pond 1 and its estuarine adjacent channel demonstrated the highest values of total coliforms load, imipenem-resistant and cefotaxime-resistant bacteria. Moreover, results demonstrated that over the sampling period there were fluctuations both in the total number and in the percentage of bacteria resistant to cefotaxime and imipenem. This neither tendentious nor stable values may be due to changes in climatic conditions, namely temperature and rainfall which can affect the survival and load of coliforms (Sampson et al., 2006; Tornevi et al., 2014) during sampling period and/or to the input of these bacteria, for instance from urban and hospital wastewaters, which are described in some situations as sources of ARG and ARB to aquatic environments (Rodriguez-Mozaz et al., 2015). Unfortunately, since there is no official source of information available about the route the water takes up to reach these water ponds, it was not possible until the end of this study to determine the primary source of these bacteria. When compared for example with a study by Teixeira et al., 2020 in river Lis, pond 1 reached values of imipenem resistant bacteria (1 %) higher than some sampled sites, demonstrating that in some cases, these environments can reach values higher than aquatic environments which receive wastewaters.

The bacterial abundance and diversity in m-FC agar also varied in each sample, demonstrating a great heterogeneity between sampling periods. Once again, it is not known if this is due to climatic conditions, and/or due to input of bacteria into these environments.

It was also verified that isolates sharing identical genomic characteristics persist in one urban pond (Pond 1) at least for 9 months. For instance, *C. freundii* F6 and *Citrobacter* N6, that were collected 9 months apart, exhibited the same BOX-profile. We also observed isolates affiliated to *Raoutella ornithinolytica* isolated from pond 1 and from its adjacent estuarine channel showing the same BOX-profiles and, suggesting that these bacteria are not limited to urban ponds. It is not known whether this is due to these bacteria being constantly added to urban ponds, or if they survive over time.

Although our aim was to focus on *Enterobacteriaceae* resistant to imipenem (due to the high risk they pose to public health), bacteria belonging to the genus *Aeromonas* were also selected, due to their colonies morphological similarities with *Enterobacteriaceae* colonies in m-FC agar and due to their intrinsic resistance to carbapenems. Although they were not the focus of this study, they were maintained and characterized since the selected isolates harbored genes encoding non-intrinsic carbapenemases.

A considerable diversity of CPB, at species and strain level, with acquired mechanisms was observed, presenting different ARG, mobile genetic elements (MGE) and antimicrobial susceptibility phenotypes.

Analysis of BOX-profiles, 16S rRNA gene sequences, plasmid profiles, antibiotic susceptibility profiles, and detection of carbapenemase-encoding genes and integrase genes, suggests that *R. ornithinolytica* isolated in pond 1 (N1, N8 and N9) and in Dos Santos Mártires channel estuary (N12 and N13) are probably clonal, thus suggesting the transfer of CPB from urban ponds to the estuary. In Ria de Aveiro there are several activities, such as aquaculture, tourism, sports, fishing, among others, thus the spread of these bacteria to these aquatic environments may contribute not only for ARG dissemination but also for direct human exposure (Lillebø et al., 2019). Previous studies performed in Ria de Aveiro revealed the presence of ARG, such as several β-lactamases (*bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-B}}$, *cphA*, etc) (Henriques et al., 2006) and genes conferring resistance to tetracyclines (Henriques et al., 2008), however, as far as we know, there aren't reports of carbapenem resistance genes associated to MGE in this aquatic environment.

Based on the results from the antibiotic susceptibility tests (AST), we observed that more than ¾ of the isolates are MDR, that is, resistant to at least one antibiotic from at least 3 different antibiotic classes. The β-lactams have been used for empirical treatment for more than 70 years (Thakuria & Lahon, 2013). Infections caused by members of the family *Enterobacteriaceae* and the genus *Aeromonas*, in some situations, were already associated to high mortality rates (Rosso et al., 2019; Scarsi et al., 2006). During our study we observed that the majority of isolates were resistant to almost all β-lactams tested, mainly due to the presence of carbapenemase-enconding genes such as *bla*$_{\text{GES-5}}$ and *bla*$_{\text{KPC}}$ conferring resistant to all β-lactams tested. Although *Aeromonas* species harbour an intrinsic carbapenemase (*cphA*) it doesn't confer resistant to other β-lactams such as cefotaxime or aztreonam (Bottoni et al., 2015). A study by Aravena-Román et al., 2012 demonstrated that amoxicillin was effective against almost all *Aeromonas* isolates,

contrary to our results. In the case of *Enterobacteriaceae* family, in the past, almost all isolates were susceptible to carbapenems (Papp-wallace et al., 2011) and until 1990 known carbapenemases were associated to the chromosome of some organisms (Queenan & Bush, 2007), however we observed that almost all isolates were resistant to all β-lactams, including all carbapenems due to the presence of carbapenemases associated to MGE.

To determine which genetic determinants were involved in these resistance phenotypes and whether they were associated with mobile genetic elements, several carbapenemase- and class 1, 2 and 3 integrase encoding genes were screened.

Although with the primers and conditions used it was not possible to detect *cphA* gene in *Aeromonas* isolates, it is known that some of *Aeromonas* species harbor this gene that encodes carbapenems and penems resistance (Chen et al., 2012). Nevertheless, all *Aeromonas* isolated during this study carried $bla_{\text{GES-5}}$, associated to class 3 integron, with the exception of *Aeromonas* O7, where no carbapenem resistance mechanisms were detected. Since among β-lactams antibiotics, this isolate is only resistant to carbapenems, it is thought that this resistance may be due to the presence of the *cphA* gene, once as previously mentioned, this gene confers specific resistance to carbapenems but not to other β-lactam antibiotics. Although $bla_{\text{GES-5}}$ had already been found in *Aeromonas* (Girlich et al., 2012), as far as we know, this is the first report in Portugal of *Aeromonas* carrying $bla_{\text{GES-5}}$. This genus, associated to aquatic environments, is described as a vehicle for the spread of ARG (Piotrowska et al., 2017). Despite its intrinsic resistance to carbapenems, the presence of $bla_{\text{GES-5}}$ in these organisms may represent a risk for the spread of carbapenemases to other susceptible bacteria. Among isolates belonging to *Enterobacteriaceae*, $bla_{\text{GES-5}}$ were already found in *Enterobacter* and *Klebsiella* species (Gomi et al., 2018), however, as far as we know, we report for the first time its presence in *R. ornithinolytica*.

Regarding *Enterobacter* sp. N11 and Enterobacter N15, both isolated in pond 3, none of the targeted carbapenemase-encoding genes was detected. In *Enterobacter* species, imipenem resistance may be due to the derepression of the intrinsic *ampC* gene together with changes in cell membrane permeability (Boyd et al., 2020) or due to the presence of another carbapenemase gene not detected in this study.

For the remaining 27 isolates at least one carbapenemase gene was detected and 7 of them harbored two carbapenemase genes ($bla_{\text{KPC-3}}$ and $bla_{\text{GES-5}}$ or $bla_{\text{KPC-3}}$ and $bla_{\text{VIM-1}}$).

The KPC encoding gene was the carbapenemase-encoding gene more prevalent in the collection, detected in 70 % of the isolates. It was identified in all *Citrobacter* (n=8), all *R. ornithinolytica* (n=11), in the only *Klebsiella* isolate and in one *Enterobacter* sp.

The IncFIA/FII conjugative plasmid pBK30683 has been described in both aquatic environments and hospital settings (Tacão et al., 2017;Teixeira et al., 2020), carrying a *bla*$_{KPC}$ gene but also additional genetic determinants encoding resistance to 4 antibiotic classes, namely β-lactams, aminoglycosides, sulfonamides and trimethoprim (Chen et al., 2014). This plasmid was detected in two different sampling sites and is likely present in 23.81 % of *bla*$_{KPC}$-carrying isolates, belonging to *R. ornithinolytica* species. The resistance phenotype is according to these ARG. In Portugal pBK30683-like plasmids were already found in Lis river (Teixeira et al., 2020), and in hospital settings (Rodrigues et al., 2016; Tacão et al., 2017). Previously mentioned studies reported the presence of this plasmid in *K. pneumoniae* and *E. coli*. We report its presence for the first time in *R. ornithinolytica*, demonstrating that this plasmid can be present in different species. Although conjugation assays were not successful under the experimental conditions used in this study, transference of carbapenemase-encoding genes may be possible, since this plasmid carries the required conjugation machinery/genes. Although not inspected for the presence of this plasmid, N8 and N13 isolates were similar (probably clones) to isolates N1, N9 and N12 *R. ornithinolytica* isolates, so, they are likely to harbor pBK30683 plasmid. Even so this hypothesis should be confirmed since, as we observed in this study for other isolates, strains with identical BOX profiles may harbor distinct plasmids.

During this study it was not possible to fully characterize the *bla*$_{VIM-1}$ class 1 integron, however, from the cassette array detailed so far, this integron seems highly similar to one that was detected previously in *K. pneumoniae* and *E. coli* in a hospital in Spain between 2005 and 2007 (Tato et al., 2010) with cassettes *bla*$_{VIM-1}$, *aacA4*, *dfrII*, *aadA1* and *catB2*. The *bla*$_{VIM}$ variants are often associated to class 1 integrons (Khosravi et al., 2011; Tato et al., 2010; Yatsuyanagi et al., 2004). The *bla*$_{VIM-1}$ is often associated with species of *Enterobacteriaceae* (Bonardi & Pitino, 2019) and it was already described in *C. freundii* (Villa et al., 2017).

The *Raoultella*, *Citrobacter*, *Enterobacter* and *Klebsiella* genera are ubiquitous in nature (Oliveira et al., 2016; Pati et al., 2018; Podschun & Ullmann, 1998; Zamani et al., 2019) and *Aeromonas* genus is well known for inhabiting aquatic environments (Senderovich et al., 2008). So, the presence of these organisms is expectable even in the

absence of anthropogenic activities. However, the presence of integrons and ARG such as *sul1* can be used as indicator of water contamination with ARG (Adelowo et al., 2018). Ponds 1, 2 and 3 seems to be connected, having the same water route. However, all CPB were collected in Pond 1 or in its adjacent estuarine channel, which suggests that there may be an input of contaminated water upstream Pond 1.

Three isolates were selected for whole genome sequencing (WGS). The coverage values, obtained in WGS of *C. freundii* F6, *R. ornithinolytica* N9 and *E. kobei* N10 are higher than 50^{X}, which has been suggested as enough to ensure good genome assembly through Illumina sequencing (Desai et al., 2013). Additionally, according to the information available regarding genomes deposited in the PATRIC database (Gillespie et al., 2011), belonging to the same species, the GC content and genome size of the three sequenced genomes are within the expected values.

The correct phylogenetic affiliation is fundamental, in both clinical and research microbiology laboratories (Franco-Duarte et al., 2019; Y. W. Tang et al., 1998).

Based on ANIb, ANIm, dDDH, MLSA, TYGS and MALDI-TOF, N9 isolate affiliated to *R. ornithinolytica* and based on ANIb, ANIm, dDDH and TYGS, F6 isolate affiliated to *C. freundii*. Moreover *C. freundii* F6 was affiliated to ST 270. This sequence type was assigned to an isolate obtained from a diarrheal patient, in ShiJiaZhuang, China, 2016 (PubMLST, isolate CitroHB2016003, id: 222), demonstrating that this STcrossed continents. Since there is no more information about this strain, genomic comparison between these two isolates is not possible.

Based on MLST analysis and *hsp60* typing, the clonal complex of *Enterobacter* members has been regrouped (Hoffmann & Roggenkamp, 2003; Miyoshi-Akiyama et al., 2013; Paauw et al., 2008). During this study, it was noted that the genomes of the *E. kobei* N10 and *E. kobei* JCM 8580 ^{T}, described as type strain of *E. kobei* species (Kosako et al., 1996) do not belong to the same species. Brady et al., 2013 observed that there are possibly two different strains deposited as type strain, namely *E. kobei* DSM 13645 ^{T} and *E. kobei* JCM 8580^{T} Aditionally, based on whole-genome sequence analysis and the values obtained from ANI and dDDH, *E. kobei* N10 , is phylogenetically close to *E. kobei* DSM 13645^{T} and distant from *E. kobei* JCM 8580 ^{T}. Morand et al., 2009 grouped *Enterobacter* strains in clusters, using *hsp60* partial sequences. One of them was *hsp60* sequence of *E. kobei* ATCC $BAA260^{T}$. However, this sequence, obtained from GenBank, do not correspond to *hps60* sequence of *E. kobei* ATCC BAA260 ^{T} genome, deposited in PATRIC database. The sequence used by Morand et al., 2009 is 100 % identical to *hps60*

sequence of *E. kobei* DSM 13645 ^{T}, suggesting that there are sequences from 2 different organisms deposited with the same strain name. However, our results based on ANIb, ANIm, dDDH, TYGS and MLSA, strongly suggest that *E. kobei* N10 is phylogenetically related with *E. kobei* DSM 13645^{T} and both belong to the *E. kobei* species. Moreover, based on MLST we described a new strain of *E. kobei* N10 with three new genetic alleles (*fusA*, *leuS* and *rplB*) to which a new ST (1378) was assigned.

From whole-genome sequence analysis it was noted that *C. freundii* F6 and *R. ornithinolytica* N9 harbour a higher percentage (5.84 and 5.60 % respectively) of genes related to virulence, disease, defense, phages, prophages, transposable elements and plasmids than their representative type strains (3.85 and 3.38 % respectively). Further studies are needed to compare these strains with other strains of the same species, collected from different settings including hospitals and other environmental compartments.

C. freundii F6 harbors an integron array identical to an integron present in an *Aeromonas rivipollensis* P2G1 plasmid, recovered from Ter River in Ripoll, Spain (Marti & Balcázar, 2012). The presence of this integron, carrying gene cassettes encoding resistance to sulfonamides (*sul1*), phenicols (*catB3*), some β-lactams ($bla_{\text{OXA-1}}$), aminoglycoside (*aacA4-cr*) and fluoroquinolones (*aacA4-cr*) confers resistance to 5 antibiotic classes, thus conferring a MDR phenotype.

Isolates *E. kobei* N10 and *C. freundii* F6 harbour the same $bla_{\text{KPC-3}}$ genetic context, a transposon-borne $bla_{\text{KPC-3}}$. It was not possible to determine if this $bla_{\text{KPC-3}}$-harbouring region is part of a plasmid, to which $bla_{\text{KPC-3}}$ is usually associated (Swathi et al., 2016). The most similar (%) sequence found in NCBI database was a region of *Klebsiella aerogenes* pSECR18-1644 plasmid (MT129535.1). Our results suggest that it can be a new genetic context, however further studies are needed to confirm this hypothesis. Nevertheless, the *ISKpn6* and *ISKpn7* insertion sequences found flanking $bla_{\text{KPC-3}}$ gene have been often described upstream and downstream of $bla_{\text{KPC-3}}$ gene, respectively (Cuzon et al., 2011; Yi Yun Liu et al., 2016; Naas et al., 2008, 2012; D. Wang et al., 2014).

The $bla_{\text{GES-5}}$ was found both on chromosome (in *R. ornithinolytica* N9) and in a plasmid (*E. kobei* N10). The fact that $bla_{\text{GES-5}}$ was found in at least 2 different genetic contexts, namely chromosome and plasmid-borne, always associated to class 3 integrons (*Klebsiella* sp. N14, *Aeromonas* sp. O3, *R. ornithinolytica* N9 and *E. kobei* N10) suggests that class 3 integrons may play an important role in its dissemination. Although this class

of integrons is less prevalent than class 1, some studies suggest that they may be involved in the spread of ARG from hospital to the environment and vice-versa (Barraud et al., 2013; Simo Tchuinte et al., 2016). The genetic context of *E. kobei* N10 *bla*$_{\text{GES-5}}$ is similar to a region found previously in a plasmid identified in a *C. freundii* isolate detected in River Lis water (Teixeira, Tacão, Pureza, et al., 2020). Further studies are necessary to understand if this genetic structure is emerging in Portuguese aquatic environments, representing a threat to ARG dissemination. This can be a worrying situation once Lis River and Pond 1 are around 100 km apart and also because these similar structures were found in two different organisms, demonstrating that it can be present in different species.

In Portugal, *bla*$_{\text{KPC-3}}$ was already found in both clinical settings (Aires-De-Sousa et al., 2019; Caneiras et al., 2018; Manageiro et al., 2015, 2018; Rodrigues et al., 2016) and in the environment (Teixeira et al., 2020). Likewise, *bla*$_{\text{GES-5}}$ (almost always associated with *intI3*) in Portugal was also found in both hospital (Manageiro et al., 2015, 2018; Perdigão et al., 2020), and in the environment ((Manageiro et al., 2014; Teixeira, Tacão, Pureza, et al., 2020). Additionally to studies previously mentioned, a study by Gatica *et al* reported that *bla*$_{\text{GES}}$ is one of the β-lactamases associated to integrons in WWTP from Europe, suggesting that its origin may be wastewater effluent water (Gatica et al., 2016).

We observed that in the environment, at least two isolates (*Citrobacter* F6 and *Citrobacter* N6), collected 9 months apart and with the same BOX-profile, suggesting that they are the same strain, harboured *bla*$_{\text{KPC.}}$ It means that organisms possessing these enzymes either are continuously being introduced in these aquatic environments or can survive for at least 9 months in the environment where, supposedly there isn't selective pressure exerted by carbapenems. Based on literature that we found (above described), in Portugal, *bla*$_{\text{KPC}}$ is more prevalent than *bla*$_{\text{GES-5}}$ and is found mainly in clinical settings.

In this work we also observed that class 1 and 3 integrons can be involved in dissemination of these carbapenemase-enconding genes and other ARG which confer resistance to other antibiotic classes, once we found different ARG linked to them and *bla*$_{\text{GES-5}}$ was always linked to class 3 integrons.

In silico analysis of *C. freundii* F6, *R. ornithinolytica* N9 and *E. kobei* N10 genome sequences suggests they have the potential to cause disease since they carry genes associated with virulence factors and have been predicted as human pathogens. These species have already been described as human pathogens (Hoffmann et al., 2005; L. Liu

et al., 2018; Seng et al., 2016). Despite these results and reports associating these bacteria to infections, further pathogenicity studies are needed.

Further studies are needed to fully elucidate the genetic contexts of the detected ARG, not only those that confer carbapenem resistance, but also genes that confer resistance to other antibiotics, in order to understand how these genes are circulating, disseminating and accumulating in organisms and the real risk they represent to Public Health. Since a significant diversity of ARG associated with different genetic contexts has been detected, which are often found in clinical settings and wastewater treatment plants (WWTP) effluents, it would be important to determine the possible source(s) of environmental contamination in order to stop the spread of ARG, mainly to last-resort antibiotics, since they are the last step that bacteria have to overcome to put the humanity in a scenario similar to that observed in pre-antibiotic era.

VI. Conclusion

Through this study it was possible to conclude that although the amount of CPB in studied aquatic environments is low, a significant diversity of these bacteria was detected, with carbapenemases genes being associated with different genetic contexts, some of them associated to mobile genetic elements which confer MDR phenotypes. It was also concluded that the same CPB strains were found in these aquatic environments for a long period of time and that they were able to spread to other aquatic environments. Further studies are needed to elucidate the source of these ARG and other ARG especially those conferring resistance to last-resort antibiotics, to evaluate which other natural environments can be also contaminated, how can we mitigate their dissemination and the real risk that they represent for Public Health.

VII. References

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VIII. Appendix

Table A1 PCR primers used in bacterial typing, 16S rRNA based affiliation and screening of ARG and integrons.

Target	Primers	Final concentration (µM)	Annealing (ºC)	Program	Amplicon size (bp)	Source
BOX element	BOXA1R: CTACGGCAAGGCGACGCTGACG	0.4	53	Q	variable	Versalovic et al., 1994
16S rDNA	27_F: AGA GTT TGA TCC TGG CTC AG	0.3	52	H	1400	Lane, 1991
	1492_R: GGY TAC CTT GTT AAC GAC TT					
bla$_{IMP}$	IMP_F: GAA TAG AGT GGC TTA ATT GTC	0.3	55	B	232	(Henriques et al., 2006)
	IMP_R: GGT TTA AYA AAA CAA CCA CC					
bla$_{VIM}$	VIM_F: GAT GGT GTT TGG TCG CAT ATC G	0.3	58	B	475	(Henriques et al., 2006)
	VIM_R: GCC ACG TTC CCC GCA GAC G					
bla$_{KPC}$	KPC_F: CAT TCA AGG GCT TTC TTG CTG C	0.3	55	B	538	(Dallenne et al., 2010)
	KPC_R: ACG ACG GCA TAG TCA TTT					
bla$_{GES}$	GES_F: AGT CGG CTA GAC CGG AAA G	0.3	57	D	399	(Dallenne et al., 2010)
	GES_R: TTT GTC CGT GCT CAG GAT					
bla$_{NDM}$	NDM_F: GGT TTG GCG ATC TGG TTT TC	0.3	52	E	621	(Laurent Poirel et al., 2011)
	NDM_R: CGG AAT GGC TCA TCA CGA TC					
bla$_{OXA-48}$	blaOXA4854I_F: AGC AAG GAT TTA CCA ATA AT	0.3	50	I	571	(Zong, 2012)
	blaOXA4854I_R: GGC ATA TCC ATA TTC ATC					

bla$_{CphA}$	AER_F: GCCTTGATCAGCGCTTCGTAGTG AER_R: GCGGGGATGTCGCTGACGCAG	0.3	60	B	670	(Henriques et al., 2006)
bla$_{CTX-M}$ (Lu)	CTX_F: SCV ATG TGC AGY ACC AGT AA CTX_R: GCT GCC GGT YTT ATC VCC	0.3	55	A	600	(Lu et al., 2010)
mcr-1	CLR5_F: CGG TCA GTC CGT TTG TTC CLR5_R: CTT GGT CGG TCT GTA GGG	0.3	58	O	320-350	(Liu et al., 2016)
IntI1	Int1_F: ACA TGC GTG TAA ATC GTC Int1_R: CTG GAT TTC GAT GAC GGC ACG	0.3	55	C	280	(Kraft et al., 1986)
IntI2	Int2_F: ACG GCT ACC CTC TGT TAT Int2_R: TTA TTG CTG GGA TTA GGC	0.3	50	C	233	(Goldstein et al., 2001)
IntI3	IntI3_F: AGT GGG TGG CGA ATG AGT G IntI3_R: TGT TCT TGT ATC GGC AGG TG	0.3	50	C	600	(Goldstein et al., 2001)
IntI1 variable region	Int1_F: ACA TGC GTG TAA ATC GTC VIM_R: GCC ACG TTC CCC GCA GAC G	0.3	50	B	variable	
IntI3 variable region	IntI3_F: AGT GGG TGG CGA ATG AGT G GES_R: TTT GTC CGT GCT CAG GAT	0.3	50	B	variable	
Integron Class 1 Variable region	5'-CS: GGC ATC CAA GCA GCA AG 3'-CS: AAG CAG ACT TGA CCT GA	0.3	55	G	variable	(Levesque et al., 1995)
Integron Class 1 variable region	VIM_F: GAT GGT GTT TGG TCG CAT ATC G 3'-CS: AAG CAG ACT TGA CCT GA	0.3	50	B	variable	
Integron Class 1 variable region	VIM_F: GAT GGT GTT TGG TCG CAT ATC G Sul1_R: AAA AAT CCC ATC CCC GGR TC	0.3	50	B	variable	
Integron Class 1 variable region	VIM_F: GAT GGT GTT TGG TCG CAT ATC G qacE_R: CAA GCT TTT GCC CAT GAA GC	0.3	50	B	variable	

Table A2. PCR programs used in PCR-based molecular typing, 16S rDNA affiliation and screening of ARG genes and integrons.

Program A			Program G		
94 ºC	2 min	× 1	94 ºC	5 min	× 1
94 ºC	15 s	× 30	94 ºC	30 s	× 30
Y ºC	30 s		Y ºC	30 s	
72 ºC	45 s		72 ºC	3 min	
72 ºC	10 min	× 1	72 ºC	10 min	× 1
Program B			**Program H**		
94 ºC	5 min	× 1	94 ºC	3 min	× 1
94 ºC	30 s	× 30	94 ºC	1 min	× 30
Y ºC	30 s		Y ºC	1 min	
72 ºC	1 min		72 ºC	2 min	
72 ºC	7 min	× 1	72 ºC	10 min	× 1
Program C			**Program I**		
94 ºC	5 min	× 1	93 ºC	2 min	× 1
94 ºC	30 s	× 30	93 ºC	30 s	× 40
Y ºC	30 s		Y ºC	30 s	
72 ºC	30 s		68 ºC	1 min	
72 ºC	7 min	× 1	68 ºC	5 min	× 1
Program D			**Program O**		
94 ºC	10 min	× 1	94 ºC	15 min	× 1
94 ºC	40 s	× 30	94 ºC	30 s	× 25
Y ºC	40 s		Y ºC	90 s	
72 ºC	1 min		72 ºC	1 min	
72 ºC	7 min	× 1	72 ºC	10 min	× 1
Program E			**Program Q**		
94 ºC	10 min	× 1	94 ºC	7 min	× 1
94 ºC	30 s	× 36	94 ºC	1 min	× 30
Y ºC	40 s		Y ºC	1 min	
72 ºC	50 s		65 ºC	8 min	
72 ºC	5 min	× 1	65 ºC	16 min	× 1

Table A3. Primers and targets used in replicon typing.

Inc group	Primer name	DNA sequence	Target site	Amplicon size
HI1	HI1 Forward	GGA GCG ATG GAT TAC TTC AGT AC	*parA-parB*	471
	HI1 Reverse	TGC CGT TTC ACC TCG TGA GTA		
HI2	HI2 Forward	TTT CTC CTG AGT CAC CTG TTA ACA C	iterons	644
	HI2 Reverse	GGC TCA CTA CCG TTG TCA TCC T		
I1	I1 Forward	CGA AAG CCG GAC GGC AGA A	RNAI	139
	I1 Reverse	TCG TCG TTC CGC CAA GTT CGT		
X	X Forward	AAC CTT AGA GGC TAT TTA AGT TGC TGA T	*ori γ*	376
	X Reverse	TGA GAG TCA ATT TTT ATC TCA TGT TTT AGC		
L/M	L/M Forward	GGA TGA AAA CTA TCA GCA TCT GAA G	*repA,B,C*	785
	L/M Reverse	CTG CAG GGG CGA TTC TTT AGG		
N	N Forward	GTC TAA CGA GCT TAC CGA AG	*repA*	559
	N Reverse	GTT TCA ACT CTG CCA AGT TC		
FIA	FIA Forward	CCA TGC TGG TTC TAG AGA AGG TG	iterons	462
	FIA Reverse	GTA TAT CCT TAC TGG CTT CCG CAG		
FIB	FIB Forward	GGA GTT CTG ACA CAC GAT TTT CTG	*repA*	702
	FIB Reverse	CTC CCG TCG CTT CAG GGC ATT		
W	W Forward	CCT AAG AAC AAC AAA GCC CCC G	*repA*	242
	W Reverse	GGT GCG CGG CAT AGA ACC GT		
Y	Y Forward	AAT TCA AAC AAC ACT GTG CAG CCT G	*repA*	765
	Y Reverse	GCG AGA ATG GAC GAT TAC AAA ACT TT		
P	P Forward	CTA TGG CCC TGC AAA CGC GCC AGA AA	iterons	534
	P Reverse	TCA CGC GCC AGG GCG CAG CC		
FIC	FIC Forward	GTG AAC TGG CAG ATG AGG AAG G	*repA2*	262

	FIC Reverse	TTC TCC TCG TCG CCA AAC TAG AT		
A/C	A/C Forward	GAG AAC CAA AGA CAA AGA CCT GGA	*repA*	465
	A/C Reverse	ACG ACA AAC CTG AAT TGC CTC CTT		
T	T Forward	TTG GCC TGT TTG TGC CTA AAC CAT	*repA*	750
	T Reverse	CGT TGA TTA CAC TTA GCT TTG GAC		
FIIS	FIIS Forward	CTG TCG TAA GCT GAT GGC	*repA*	270
	FIIS Reverse	CTC TGC CAC AAA CTT CAG C		
F	FrepB Forward	TGA TCG TTT AAG GAA TTT TG	RNAI*/repA*	270
	FrepB Reverse	GAA GAT CAG TCA CAC CAT CC		
K/B	K/B Forward	GCG GTC CGG AAA GCC AGA AAA C	RNAI	160
	K Reverse	TCT TTC ACG AGC CCG CCA AA		
B/O	B/O Reverse	TCT GCG TTC CGC CAA GTT CGA	RNAI	159

Table A4. PCR programs used in replicon typing.

Panel 1-3			Frep		
94 ºC	5 min	× 1	94 ºC	5 min	× 1
94 ºC	1 min	× 30	94 ºC	1 min	× 30
60 ºC	30 s		52 ºC	30 s	
72 ºC	1 min		72 ºC	1 min	
72 ºC	5 min	× 1	72 ºC	5 min	× 1

Table A5. Primers and targets used in screening of pBK30661 and pBK30683 plasmids.

Primer name	Primer sequence	Target	Amplicon size
IA-1f	GCC GTC CTT TCT GTG ACA AAT CA	IncFIA *repA*	516
IA-1r	GGA TGG ACT GTG GGC ACG TT		
IA-2f	CCG TTT CTG TGT CAT TTG CTC CT	Second IncFII *repA*	250
IA-2r	CTT ATA GTG AGA CGG CCG GAA CC		
IA-3f	ATA CCG GTG CCG CCA TGC TGC G	Tn4401 upstream junction between *chrB* gene and *ISKpn6*	213
IA-3r	TCG TCA TGC CGC GGA CCA CCC C		
IA-4f	CCG GCA TCA CCG GCC CTC ACC T	Tn4401 downstream junction between Tn4401 *tnpR* gene and neighboring Tn3 *tnpA* gene	515
IA-4r	ACA CTC CCG GCT GTG CGC CTG A		
IA-5f	CGA TGA CGT GGA GAG CAG TA		534
IA-56r	TCC CGA GAA TGA ATC TGG AC	Region between putative cytoplasmic protein gene and adenine-specific methyltransferase gene (*met1*)	
IA-6f	CGT GCA TTC GGT GAC TAA AA	Region between hypothetical protein gene and adeninespecific methyltransferase gene (*met1*)	768
4401v-r (3781L)	CAC AGC GGC AGC AAG AAA GC	Tn4401d isoform	
4401v-r1	GCA AGC CGC TCC CTC TCC AG		635
4401v-f (3098U)	TGA CCC TGA GCG GCG AAA GC		314

Table A6. PCR programs used in screening of pBK30661 and pBK30683 plasmids.

Duplex I, II, III, IV		
95 ºC	4 min	× 1
95 ºC	30 s	× 35
60 ºC	30 s	
72 ºC	1 min	
72 ºC	7 min	× 1

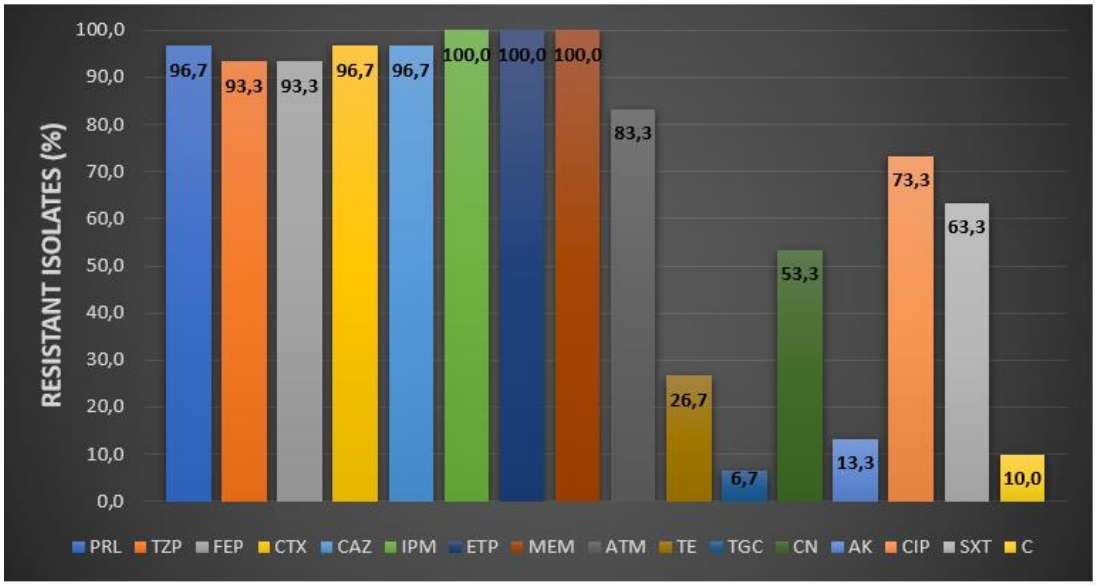

Figure A1. Resistance phenotypes of 30 isolates.

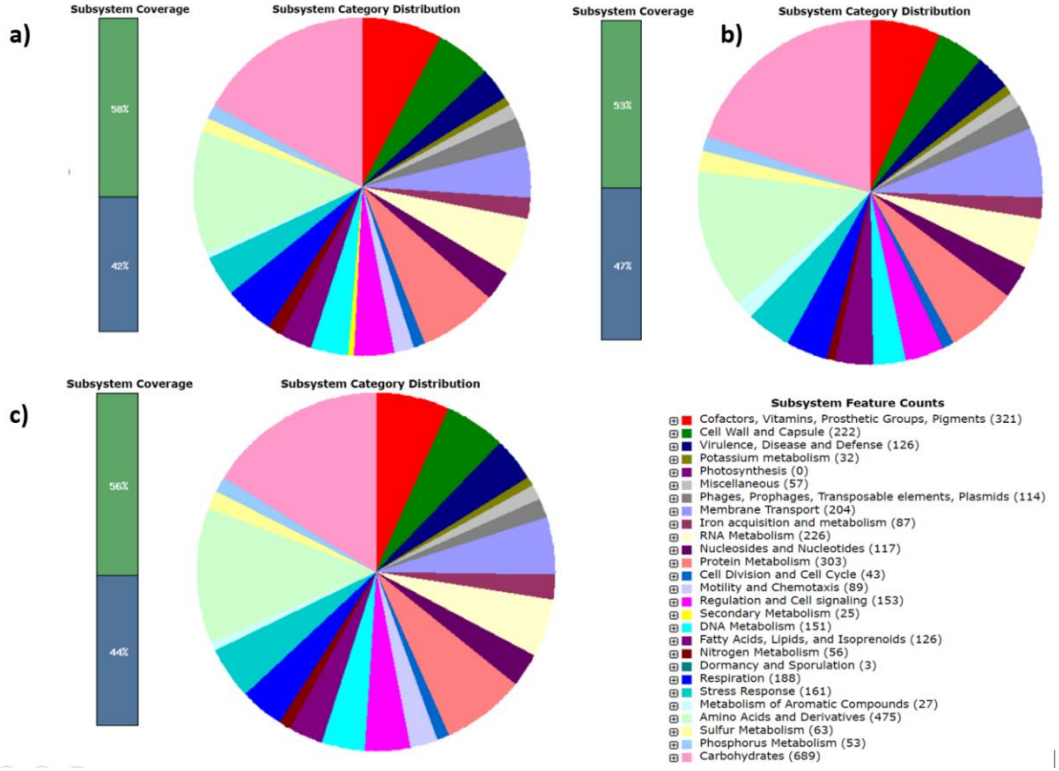

Figure A2. Genes annotated and related subsystems of *C. freundii* F6. (a); *Raoultella ornithinolytica* N9 (b); *Enterobacter kobei* N10 (c).

Table A7. ANIb, ANIm, dDDH and difference in G+C of *E. kobei* deposited in PATRIC database and *E. kobei* N10 against DSM 13645^{T} and JCM 8580^{T} strains.

		against DSM 13645T							against JCM 8580T						
Strain	Acc number	ANIb [%]	Aligned [%]	ANIm [%]	Aligned [%]	dDDH [%]	dif G+C	Same species	ANIb [%]	Aligned [%]	ANIm [%]	Aligned [%]	dDDH [%]	dif G+C	Same species
E. kobei N10		99.55	91.19	99.85	93.30	98.20	0.50	+	79.05	65.64	84.65	35.05	23.10	1.02	-
E. kobei 42-12	BJEX01000000	99.10	87.36	99.43	88.03	95.30	0.05	+	78.68	65.86	84.21	34.42	22.60	0.57	-
E. kobei E14	VTUD01000000	98.83	87.92	99.18	89.41	92.50	0.05	+	78.96	65.73	84.57	35.10	23.00	0.48	-
E. kobei MUGSI_253		98.77	86.57	99.18	87.86	92.80	0.02	+	78.99	65.37	84.50	35.03	23.00	0.55	-
E. kobei MUGSI_253		98.76	86.57	99.18	87.86	92.80	0.06	+	78.99	65.37	84.50	35.04	23.00	0.58	-
E. kobei CRE54	PXKD01000000	98.75	89.05	99.12	90.61	91.70	0.07	+	78.94	66.63	84.62	35.41	23.10	0.59	-
E. kobei CRE71	PXKA01000000	98.62	84.82	99.16	85.92	92.50	0.04	+	79.02	64.96	84.63	34.96	23.10	0.48	-
E. kobei EB_P8_L5_01.19	CP043511	98.61	87.05	99.14	87.77	92.10	0.31	+	79.00	65.86	84.77	34.76	23.30	0.84	-
E. kobei C16	CP042578	98.61	88.33	99.10	89.30	91.60	0.51	+	78.88	66.14	84.68	34.96	23.30	1.03	-
E. kobei WCHEK045523	CP032897	98.59	87.42	99.06	88.20	91.50	0.20	+	78.89	66.20	84.61	35.10	23.30	0.73	-
E. kobei 2485STDY5438361	UNXB01000000	98.59	86.14	98.88	90.22	20.40	0.23	+	79.36	68.09	84.60	42.44	23.20	0.30	-
E. kobei MUGSI_221		87.04	77.24	88.29	74.67	33.50	0.29	-	79.05	66.89	84.44	36.64	22.90	0.23	-

E. kobei MUGSI_221		87.04	77.21	88.29	74.66	33.50	0.41	-	79.05	66.87	84.44	36.63	22.90	0.11	-
E. kobei MER	QGLV01000000	98.86	86.76	99.21	88.55	92.90	0.12	+	78.92	65.60	84.49	34.91	23.00	0.40	-
E. kobei 145e9	QMCN01000000	98.84	88.33	99.19	90.35	92.60	0.02	+	79.02	66.11	84.68	35.48	23.20	0.50	-
E. kobei 121J9	QMCO01000000	98.82	86.29	99.20	89.29	92.70	0.19	+	79.04	65.45	84.68	35.04	23.20	0.33	-
E. kobei 149G8	QMCJ01000000	98.81	87.88	99.21	89.83	92.60	0.04	+	79.05	65.84	84.66	35.31	23.10	0.56	-
E. kobei 151B8	QMCI01000000	98.74	88.20	99.06	90.86	91.30	0.09	+	78.82	66.14	84.47	34.72	22.90	0.43	-
E. kobei 145F2	QMCM01000000	98.69	87.05	99.17	88.85	92.30	0.08	+	78.88	65.16	84.49	34.73	23.00	0.44	-
E. kobei TUM11131	BEGI01000000	98.68	88.18	99.12	89.43	91.70	0.42	+	79.15	67.07	84.85	35.77	23.30	0.94	-
E. kobei ICBEaBL-III-03(2)	NIHL00000000	98.67	86.94	99.08	89.75	91.70	0.53	+	78.83	64.96	84.50	34.58	23.00	1.06	-
E. kobei 149H6	QMCK01000000	98.56	88.66	99.10	90.37	91.50	0.15	+	78.98	66.10	84.63	35.25	23.20	0.67	-
E. kobei 149H5	QMCL01000000	98.55	88.95	99.10	90.38	91.40	0.15	+	78.98	66.14	84.62	35.28	23.10	0.68	-
E. kobei 131G4	QMCU01000000	98.37	87.48	98.96	89.17	89.70	0.35	+	78.73	66.84	84.48	35.10	22.90	0.88	-
E. kobei PECIMP	QHMI01000000	87.13	78.27	88.31	76.12	33.90	0.25	-	79.21	66.17	84.63	36.50	23.10	0.77	-
Enterobacter sp. 44593	JZXR01000000	99.05	89.22	99.42	90.72	94.60	0.14	+	79.22	67.07	84.93	36.42	23.40	0.67	-
Enterobacter sp. GN02366	LEEC01000000	98.80	87.06	99.14	88.27	92.40	0.07	+	78.82	65.83	84.39	34.62	22.80	0.46	-

Enterobacter sp. GN02275	LEEK01000000	98.76	88.20	99.16	89.90	92.20	0.05	+	78.96	66.54	84.62	35.20	23.10	0.48	-
Enterobacter sp. GN02225	LDCL01000000	98.74	87.08	99.15	88.54	92.30	0.09	+	78.89	65.86	84.49	34.98	22.90	0.43	-
E. kobei UTA41	JAAMLZ010000001	98.67	87.86	99.11	89.15	91.60	0.42	+	78.94	66.22	84.59	35.27	23.00	0.94	-
Enterobacter sp. 42202	JZYH01000000	98.67	88.01	99.12	89.37	91.90	0.30	+	78.96	65.74	84.50	35.12	23.00	0.82	-
Enterobacter sp. 35730	JZYS01000000	98.67	88.69	99.11	90.03	91.90	0.62	+	78.95	66.20	84.70	34.98	23.00	1.15	-
E. kobei AS012401	VKUW01000000	98.63	88.88	99.14	90.40	91.60	0.09	+	78.80	65.78	84.48	35.04	23.00	0.43	-
Enterobacter sp. GN02204	LEEQ01000000	98.58	87.71	99.13	88.87	91.70	0.07	+	78.90	66.26	84.49	35.12	22.90	0.45	-
E. kobei 070	CP050073	98.49	87.76	99.03	88.62	90.90	0.18	+	78.88	66.10	84.67	35.16	23.20	0.70	-
Enterobacter sp. GN02186	LDCJ01000000	98.46	85.89	99.10	86.80	90.70	0.13	+	78.90	66.42	84.52	34.88	23.00	0.66	-
E. kobei 2485STDY5438374	UNXN01000000	87.79	80.43	88.68	79.42	35.20	0.19	-	78.85	67.81	84.37	35.81	22.70	0.71	-
Enterobacter sp. GN03191	LECY01000000	98.83	86.99	99.15	87.74	92.30	0.05	+	78.80	66.45	84.38	34.59	22.80	0.48	-
Enterobacter sp. GN02825	LEDC01000000	98.78	89.02	99.14	90.59	92.00	0.15	+	78.94	66.70	84.61	35.41	23.10	0.68	-
Enterobacter sp. GN02454	LEDW01000000	98.57	87.99	99.14	89.26	91.80	0.23	+	78.98	66.09	84.58	35.18	23.00	0.75	-
E. kobei DSM 13645T	CP017181								78.96	65.68	84.75	34.85	23.40	0.52	-
E. kobei BH-18	JSVH00000000	98.79	84.10	99.14	86.07	92.60	0.12	+	78.87	64.19	84.52	34.28	23.10	0.41	-

E. kobei GN06078	LVUX00000000	98.73	87.70	99.09	89.63	91.60	0.01	+	78.89	66.02	84.47	34.92	23.00	0.51	-
E. kobei 3380STDY6027363	FKLS00000000	98.65	88.77	99.11	89.95	92.10	0.60	+	78.96	66.49	84.80	35.20	23.40	1.13	-
E. kobei ECC3026	LYUS00000000	98.64	87.13	99.13	87.99	92.20	0.25	+	79.05	66.08	84.59	35.55	23.00	0.77	-
E. kobei ECC3047	LYUT00000000	98.63	87.15	99.13	88.00	92.20	0.25	+	79.04	66.08	84.59	35.51	23.00	0.77	-
E. kobei GN05680	LVUM00000000	98.62	86.38	99.12	87.40	92.10	0.08	+	78.82	65.84	84.46	34.65	22.90	0.44	-
E. kobei SMART_635	LPPL01000000	98.51	86.59	99.13	87.73	91.80	0.54	+	79.03	65.91	84.69	35.09	23.00	1.06	-
E. kobei EkBL-II-14(1)	NEWG00000000	90.52	80.34	91.24	82.46	43.20	1.17	-	79.15	64.17	84.56	36.85	23.20	0.65	-
E. kobei JCM 8580T	MKXD00000000	79.26	63.91	84.75	34.29	23.40	0.52	-							
E. kobei E. kobei DSM27110T	FYBC00000000	79.24	63.67	84.59	34.13	23.10	0.54	-	100.00	99.49	99.99	99.84	99.00	0.02	+
E. kobei ATCC BAA-260T	FTNJ00000000	79.11	63.07	84.58	33.64	23.00	0.55	-	99.99	98.92	99.98	99.38	99.10	0.03	+

Table A8. Virulence factors and its related genes found in *C. freundii* F6 against *Escherichia coli* and *Klebsiella pneumonieae* databases from VFDB.

Virulence factor	gene	length	contig
Adherence			
Agf/Csg	*csgA*	450	77
E. coli common pilus (ECP)	*ecpA*	588	49
Hemorrhagic *E. coli* pilus (HCP)	*hcpA*	438	7
Hemorrhagic *E. coli* pilus (HCP)	*hcpB*	1383	7
Hemorrhagic *E. coli* pilus (HCP)	*hcpC*	1203	7
P fimbriae	*papC*	2472	49
Type I fimbriae	*fimA*	555	60
Type I fimbriae	*fimC*	693	60
Type I fimbriae	*fimD*	2613	4
Type I fimbriae	*fimD*	2427	7
Type IV pili	*pilW*	927	2
Type IV pili	*pilW*	888	4
Type IV pili	*pilW*	939	6
Type IV pili	*pilW*	885	23
type 3 fimbriae	*mrkC*	2613	4
Curli fibers	*csgG*	684	77
Lateral flagella	*lfhA*	1722	49
Autotransporter			
EhaB, AIDA-I type	*ehaB*	2850	73
Invasion			
Invasion of brain endothelial cells (Ibes)	*ibeB*	1386	44
Invasion of brain endothelial cells (Ibes)	*ibeC*	1734	8
Flagella	*cheB*	1050	1
Flagella	*cheR*	873	1
Flagella	*cheW*	504	1
Flagella	*cheY*	411	1
Flagella	*cheZ*	651	1
Flagella	*motA*	888	1
Iron uptake			
Aerobactin siderophore	*iutA*	2181	22
Hemin uptake	*chuA*	1983	19
Hemin uptake	*chuS*	1032	19
Hemin uptake	*chuT*	840	10
Hemin uptake	*chuU*	1005	10
Hemin uptake	*chuU*	963	19
Hemin uptake	*chuW*	1338	10
Hemin uptake	*chuX*	495	10
Hemin uptake	*chuY*	624	10
Iron/managanease transport	*sitA*	918	47
Iron/managanease transport	*sitB*	816	47
Iron/managanease transport	*sitC*	858	47

Iron/managanease transport	*sitD*	840	47
Heme transport	*shuV*	780	19
Pyoverdine	*pvdH*	1386	51
Heme transport	*shuV*	771	10
Secretion system			
Flagella (cluster I)	*fliS*	408	1
T4SS effectors	-	1125	23
T6SS-II	*clpV*	2574	43
Flagella (cluster I)	*flgB*	417	34
Flagella (cluster I)	*flgC*	405	34
Flagella (cluster I)	*flgD*	681	34
Flagella (cluster I)	*flgE*	1287	34
Flagella (cluster I)	*flgF*	756	34
Flagella (cluster I)	*flgG*	783	34
Flagella (cluster I)	*flgH*	564	34
Flagella (cluster I)	*flgI*	1098	34
Flagella (cluster I)	*flgJ*	951	34
Flagella (cluster I)	*flgK*	1659	34
Flagella (cluster I)	*flgL*	954	34
Flagella (cluster I)	*flgM*	294	34
Flagella (cluster I)	*flhA*	2079	1
Flagella (cluster I)	*flhB*	1152	1
Flagella (cluster I)	*flhC*	582	1
Flagella (cluster I)	*flhD*	342	1
Flagella (cluster I)	*fliA*	720	1
Flagella (cluster I)	*fliE*	315	1
Flagella (cluster I)	*fliF*	1683	1
Flagella (cluster I)	*fliG*	999	1
Flagella (cluster I)	*fliH*	708	1
Flagella (cluster I)	*fliI*	1371	1
Flagella (cluster I)	*fliJ*	444	1
Flagella (cluster I)	*fliL*	468	1
Flagella (cluster I)	*fliM*	1005	1
Flagella (cluster I)	*fliN*	414	1
Flagella (cluster I)	*fliP*	738	1
Flagella (cluster I)	*fliQ*	270	1
Flagella (cluster I)	*fliR*	786	1
Flagella (cluster I)	*fliZ*	462	1
Toxin			
Colicin-like Usp	*usp*	1644	2
Antiphagocytosis			
Capsular polysaccharide	*rmlC*	573	1
Capsule	--	936	56
Capsule	*uge*	807	1
Capsule	*wbaP*	1005	1

Capsule	-	1089	1
Capsule	-	900	1
Capsule	-	1407	1
Capsule	-	1167	1
Capsule	-	906	56
Capsule	-	1395	56
Capsule	-	1371	56
Capsule	-	1212	56
Capsule		480	56
Capsule		966	56
Capsule		1122	56
Capsule		2163	56
Capsule		423	56
Capsule		1047	56
Capsule		1122	56
Capsule		2163	56
Capsule		423	56
Capsule		1047	56
Biofilm formation			
PNAG (Polysaccharide poly-N-acetylglucosamine)	*pgaC*	1335	4
Fimbrial adherence determinants			
Fim	*fimF*	537	60
Fim	*fimI*	384	60
Fim	*fimW*	594	60
Stc	*stcB*	684	21
Agf/Csg	*csgA*	450	77
Agf/Csg	*csgB*	486	77
Agf/Csg	*csgC*	336	77
Agf/Csg	*csgD*	651	77
Agf/Csg	*csgF*	414	77
Fim	*fimA*	555	60
Fim	*fimD*	2622	60
Fim	*fimH*	1008	60
Fim	*fimZ*	633	60
Stc	*stcC*	2445	21
Iron acquisition			
Aerobactin	*iutA*	2181	22
Ent siderophore	*entA*	756	2
Ent siderophore	*entB*	858	2
Ent siderophore	*entC*	1188	2
Ent siderophore	*entD*	654	2
Ent siderophore	*entE*	1611	2
Ent siderophore	*entF*	3891	2
Ent siderophore	*entS*	1236	2

Ent siderophore	*fepA*	2274	2
Ent siderophore	*fepB*	930	2
Ent siderophore	*fepC*	798	2
Ent siderophore	*fepD*	972	2
Ent siderophore	*fepG*	993	2
Ent siderophore	*fes*	1218	2
Motility			
Flagella	*flaA*	1272	1
Flagella	*motB*	930	1
nonfimbrial adhrence determinants			
MisL	*misL*	2811	6
RatB	*ratB*	6381	9
SinH	*sinH*	2172	9
Efflux Pump			
AcrAB	*acrA*	1194	10
AcrAB	*acrB*	3114	5
AcrAB	*acrB*	3150	10
Nutritional factor			
Allantoin utilization	*allA*	582	10
Allantoin utilization	*allR*	693	10
Allantoin utilization	*allS*	927	10
Regulation			
RcsAB	*rcsA*	552	1
RcsAB	*rcsB*	642	13
Serum resistance			
LPS rfb locus	-	810	1
LPS rfb locus	-	1440	56

Table A9. Virulence factors and its related genes found in *R. ornithinolytica* N9 against *Escherichia coli* and *Klebsiella pneumonieae* databases from VFDB.

Virulence factor	gene	length	contig
Adherence			
Type 3 fimbriae	*mrkA*	582	20
Type 3 fimbriae	*mrkA*	618	44
Type 3 fimbriae	*mrkB*	702	20
Type 3 fimbriae	*mrkB*	702	44
Type 3 fimbriae	*mrkC*	2439	20
Type 3 fimbriae	*mrkC*	2487	44
Type 3 fimbriae	*mrkD*	996	20
Type 3 fimbriae	*mrkD*	741	44
Type 3 fimbriae	*mrkF*	588	20
Type 3 fimbriae	*mrkF*	597	44
Type 3 fimbriae	*mrkH*	588	20
Type 3 fimbriae	*mrkI*	516	20
Type 3 fimbriae	*mrkI*	585	44
Type 3 fimbriae	*mrkI*	585	65

Type 3 fimbriae	*mrkJ*	582	20
Type 3 fimbriae	*mrkJ*	645	44
Type I fimbriae	*fimA*	549	20
Type I fimbriae	*fimB*	606	20
Type I fimbriae	*fimC*	657	20
Type I fimbriae	*fimD*	2535	2
Type I fimbriae	*fimD*	2526	20
Type I fimbriae	*fimD*	2511	44
Type I fimbriae	*fimE*	597	20
Type I fimbriae	*fimF*	534	20
Type I fimbriae	*fimG*	504	20
Type I fimbriae	*fimH*	906	20
Type I fimbriae	*fimI*	531	20
Type I fimbriae	*fimK*	1449	20
Hemorrhagic E.coli pilus (HCP)	*hofC*	1206	7
The tad locus	*tadA*	1302	30
Type IV pili	*pilW*	954	48
E. coli common pilus (ECP)	*ecpA*	636	31
E. coli common pilus (ECP)	*ecpB*	669	31
E. coli common pilus (ECP)	*ecpC*	2454	31
E. coli common pilus (ECP)	*ecpD*	1623	31
E. coli common pilus (ECP)	*ecpE*	645	31
Hemorrhagic *E.coli* pilus (HCP)	*hcpA*	432	7
Hemorrhagic *E.coli* pilus (HCP)	*hcpB*	1386	7
Hemorrhagic *E.coli* pilus (HCP)	*hcpC*	1206	7
Type I fimbriae	*fimD*	2469	40
Tap type IV pili	*tapD*	798	7
The tad locus	*tadA*	1302	30
Type IV pili	*pilW*	837	12
Type IV pili	*pilW*	978	79
Invasion			
Invasion of brain endothelial cells (Ibes)	*ibeB*	1386	73
Invasion of brain endothelial cells (Ibes)	*ibeB*	1386	77
Iron uptake			
Aerobactin siderophore	*iutA*	2193	15
Hemin uptake	*chuS*	1032	13
Hemin uptake	*chuU*	996	13
Iron/managanease transport	*sitA*	918	13
Iron/managanease transport	*sitB*	822	13
Iron/managanease transport	*sitC*	852	13
Iron/managanease transport	*sitD*	858	13
Salmochelin siderophore	*iroB*	1113	31
Salmochelin siderophore	*iroD*	1236	31
Salmochelin siderophore	*iroE*	900	21
Salmochelin siderophore	*iroN*	2184	16

Yersiniabactin siderophore	*fyuA*	1980	3
Yersiniabactin siderophore	*irp1*	9492	3
Yersiniabactin siderophore	*irp2*	6024	3
Yersiniabactin siderophore	*ybtA*	960	3
Yersiniabactin siderophore	*ybtE*	1578	3
Yersiniabactin siderophore	*ybtP*	1749	3
Yersiniabactin siderophore	*ybtQ*	1803	3
Yersiniabactin siderophore	*ybtS*	1311	3
Yersiniabactin siderophore	*ybtT*	681	3
Yersiniabactin siderophore	*ybtU*	1059	3
Yersiniabactin siderophore	*ybtX*	1260	3
Acinetobactin	*basG*	1137	43
Pyoverdine	*pvdH*	1386	10
Ent siderophore	*entA*	756	1
Ent siderophore	*entB*	852	1
Ent siderophore	*entC*	1188	1
Ent siderophore	*entE*	1611	1
Ent siderophore	*entS*	1242	1
Ent siderophore	*fepA*	2232	70
Ent siderophore	*fepB*	960	1
Ent siderophore	*fepC*	795	1
Ent siderophore	*fepD*	987	1
Ent siderophore	*fepG*	993	1
Regulation			
RcsAB	*rcsA*	519	3
RcsAB	*rcsB*	642	18
Secretion system			
T6SS-I	*clpV/tssH*	2601	54
T6SS-I	*dotU/tssL*	690	54
T6SS-I	*hcp/tssD*	432	2
T6SS-I	*hcp/tssD*	492	54
T6SS-I	*icmF/tssM*	3291	75
T6SS-I	*impA/tssA*	1602	75
T6SS-I	*ompA*	1482	54
T6SS-I	*sciN/tssJ*	498	75
T6SS-I	*tssF*	1755	75
T6SS-I	*tssG*	1086	75
T6SS-I	*vasE/tssK*	1305	54
T6SS-I	*vgrG/tssI*	2331	54
T6SS-I	*vipA/tssB*	498	54
T6SS-I	*vipB/tssC*	1491	54
T6SS-II	*clpV*	2472	28
T6SS-III	*impF*	471	75
SCI-I T6SS	--	471	75
T2SS	*exeF*	1215	7

T2SS	*exeI*	366	7
T6SS-III	--	924	1
Antiphagocytosis			
Capsule	--	630	6
Capsule		891	21
Capsule		1395	21
Capsule		909	21
Capsule		1434	21
Capsule	*wzc*	2166	21
Capsule	-	849	6
Capsule	-	1167	21
Capsule	-	555	21
Capsule	-	870	21
Capsule	-	1065	21
Capsule	-	1377	21
Capsule	-	1134	21
Capsule	-	1068	24
Biofilm formation			
AdeFGH efflux pump/transport autoinducer	*adeG*	3153	40
Endotoxin			
LOS	*lgtF*	747	4
Nutritional factor			
Allantoin utilization	*allS*	927	17
Serum resistance			
LPS rfb locus	--	1305	21
LPS rfb locus		891	21
LPS rfb locus		1155	21
LPS rfb locus		1893	21
LPS rfb locus		1011	21
LPS rfb locus		960	29
Stress adaptation			
Catalase	*katA*	1395	92
Manganese transport system	*mntB*	864	13
Efflux pump			
AcrAB	*acrA*	1086	1
AcrAB	*acrB*	3081	1
AcrAB	*acrB*	3147	1

Table A10. Virulence factors and its related genes found in *E. kobei* N10 against *Escherichia coli* and *Klebsiella pneumonieae* databases from VFDB.

Virulence factor	gene	length	contig
Serum resistance			
LPS rfb locus	--	3567	44
LPS rfb locus	--	1332	44
LPS rfb locus	--	912	44
LPS rfb locus	--	1005	66
LPS rfb locus	*rmlD*	891	44
LPS rfb locus	*wbbL*	831	44
LPS rfb locus	-	1398	33
Adherence			
Type I fimbriae	*fimA*	564	2
CFA/I fimbriae	*cfaB*	507	15
Curli fibers	*csgA*	390	45
Hemorrhagic *E.coli* pilus (HCP)	*hcpA*	402	7
Hemorrhagic *E.coli* pilus (HCP)	*hcpB*	1365	7
Hemorrhagic *E.coli* pilus (HCP)	*hofB*	1365	7
P fimbriae	*papC*	2442	34
Type I fimbriae	*fimC*	666	2
Type I fimbriae	*fimC*	672	41
Type I fimbriae	*fimD*	2511	1
Type I fimbriae	*fimD*	2409	3
Type I fimbriae	*fimD*	2409	5
Type I fimbriae	*fimD*	2436	23
Type I fimbriae	*fimD*	2535	32
Curli fibers	*csgF*	351	15
Antiphagocytosis			
Capsule	*cpsG_1*	1269	30
Capsule	*wzb*	399	104
Capsule	-	1389	33
Capsule	-	900	33
Capsule	-	1341	33
Capsule	-	1392	33
Capsule	-	1365	33
Capsule	-	1224	33
Capsule	-	966	33
Capsule	-	1122	33
Capsule	-	2163	33
Capsule	-	444	33
Capsule	-	1047	33
Capsule	-	555	44
Capsule	-	891	44

Capsule	-	867	44
Capsule	-	1065	44
Capsule	-	1323	66
Capsule	-	1065	66
Capsule	-	1407	66
Capsule	-	1167	66
Capsule	-	969	104
Capsule	--	897	66
Capsule	--	2220	104
Autotransporter			
EhaB, AIDA-I type	*ehaB*	2775	46
Invasion			
Invasion of brain endothelial cells (Ibes)	*ibeB*	1386	8
Invasion of brain endothelial cells (Ibes)	*ibeB*	1386	71
Flagella	*cheA*	2031	5
Flagella	*cheB*	1008	5
Flagella	*cheR*	723	5
Flagella	*cheW*	504	5
Flagella	*cheY*	390	5
Flagella	*cheZ*	630	5
Flagella	*motA*	834	5
Iron uptake			
Aerobactin siderophore	*iucA*	1743	12
Aerobactin siderophore	*iucB*	948	12
Aerobactin siderophore	*iucC*	1743	12
Aerobactin siderophore	*iucD*	1326	12
Aerobactin siderophore	*iutA*	2145	12
Hemin uptake	*chuA*	1983	64
Hemin uptake	*chuS*	1029	64
Hemin uptake	*chuU*	993	64
Iron/managanease transport	*sitA*	894	101
Iron/managanease transport	*sitB*	807	101
Iron/managanease transport	*sitC*	858	101
Iron/managanease transport	*sitD*	840	101
Heme transport	*shuV*	792	64
Pyoverdine	*pvdH*	1377	104
Ent siderophore	*entA*	762	58
Ent siderophore	*entB*	855	58
Ent siderophore	*entC*	1188	58
Ent siderophore	*entE*	1611	58
Ent siderophore	*entF*	3744	101
Ent siderophore	*entS*	1236	58
Ent siderophore	*fepA*	2214	101

Ent siderophore	*fepB*	960	58
Ent siderophore	*fepC*	774	58
Ent siderophore	*fepD*	1017	58
Ent siderophore	*fepG*	993	58
Ent siderophore	*fes*	1197	101
Secretion system			
EPS type II secretion system	*epsE*	1476	9
(Vibrio)	*epsE*	1479	59
Hcp secretion island-1 encoded type VI secretion system (H-T6SS)	--	537	35
Hcp secretion island-1 encoded type VI secretion system (H-T6SS)	--	1500	35
Hcp secretion island-1 encoded type VI secretion system (H-T6SS)	*clpV1*	2628	35
T2SS	*exeD*	1677	9
(*Aeromonas*)	*exeD*	1908	59
T2SS	*exeF*	1209	59
TTSS (SPI-1 encode)	*iagB*	441	59
T6SS-I	*clpV/tssH*	2850	60
T6SS-I	*vipA/tssB*	480	93
T6SS-I	*vipB/tssC*	1539	93
T6SS-II	*clpV*	2574	102
T6SS-III	*impA*	1152	93
Flagella (cluster I)	*flgB*	417	45
Flagella (cluster I)	*flgC*	405	45
Flagella (cluster I)	*flgD*	609	45
Flagella (cluster I)	*flgE*	1209	45
Flagella (cluster I)	*flgF*	732	45
Flagella (cluster I)	*flgG*	783	45
Flagella (cluster I)	*flgH*	564	45
Flagella (cluster I)	*flgI*	1098	45
Flagella (cluster I)	*flgJ*	954	45
Flagella (cluster I)	*flgK*	1641	45
Flagella (cluster I)	*flgL*	936	45
Flagella (cluster I)	*flgM*	294	45
Flagella (cluster I)	*flhA*	2079	5
Flagella (cluster I)	*flhB*	1149	5
Flagella (cluster I)	*flhC*	579	5
Flagella (cluster I)	*flhD*	336	5
Flagella (cluster I)	*fliA*	690	12
Flagella (cluster I)	*fliE*	315	12
Flagella (cluster I)	*fliF*	1632	12
Flagella (cluster I)	*fliG*	999	12
Flagella (cluster I)	*fliH*	708	12
Flagella (cluster I)	*fliI*	1401	12

Flagella (cluster I)	*fliJ*	444	12
Flagella (cluster I)	*fliL*	471	12
Flagella (cluster I)	*fliM*	1005	12
Flagella (cluster I)	*fliN*	405	12
Flagella (cluster I)	*fliP*	738	12
Flagella (cluster I)	*fliQ*	252	12
Flagella (cluster I)	*fliR*	786	12
Flagella (cluster I)	*fliS*	411	12
Flagella (cluster I)	*fliZ*	552	12
Biofilm formation			
AdeFGH efflux pump/transport autoinducer	*adeG*	3153	47
PNAG (Polysaccharide poly-N-acetylglucosamine)	*pgaC*	1272	29
Efflux pump			
AcrAB	*acrA*	1068	2
AcrAB	*acrB*	3147	2
AcrAB	*acrB*	3114	51
AcrAB	*acrB*	1995	20
Endotoxin			
LOS	*htrB*	858	8
LOS	*lgtF*	771	3
LOS	*wbaP/rfbP*	1323	66
Fimbrial adherence determinants			
Agf/Csg	*csgG*	711	15
Fim	*fimD*	2562	2
Fim	*fimF*	522	2
Fim	*fimH*	1008	2
Fim	*fimI*	507	2
Sti	*stiB*	672	41
Sti	*stiC*	2238	41
Stj	*stjB*	2295	4
Stj	*stjB*	2376	14
Stj	*stjC*	621	4
Stj	*stjC*	624	14
Others			
O-antigen	--	1197	33
Regulation			
RcsAB	*rcsA*	477	12
RcsAB	*rcsB*	651	31
Immune evasion			
LPS glucosylation	*gtrB*	918	19
Motility			
Flagella	*motB*	930	5

Table A11. Proteins associated to pathogenesis found in *C. freundii* F6 using PathogenFinder 1.1.

Contig	Accession ID	Organism	Protein function	Identity (%)
64	CP001138	*Salmonella enterica* subsp. enterica serovar Agona str. SL483, complete genome.	type I site-specific deoxyribonuclease, HsdR family	97.84
1	CP001127	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome.	ATP-dependent protease	98.39
108	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	99.77
1	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.1
43	CU928158	*Escherichia fergusonii* ATCC 35469 chromosome, complete genome.	inorganic polyphosphate/ATP-NAD kinase	98.63
11	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.74
89	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	ISEc8 transposase	99.61
1	CP001127	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome.	phage portal protein, lambda family	98.43
100	CU928164	*Escherichia coli* IAI39 chromosome, complete genome.	Group II intron-encoded reverse transcriptase/maturase	100.0
3	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	L-carnitine/gamma-butyroβine antiporter	98.22
29	XXX	*Salmonella enterica* subsp. enterica serovar Typhi str. CT18	XXX	97.85
4	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.11
6	CP000886	*Salmonella enterica* subsp. enterica serovar Paratyphi B str. SPB7, complete genome.	hypothetical protein	97.49
48	CP000243	*Escherichia coli* UTI89, complete genome.	Nucleoside permease NupC	98.75
55	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	100.0
56	AP010960	*Escherichia coli* O111:H- str. 11128 DNA, complete genome.	predicted colanic acid polymerase WcaD	98.27
24	CP000966	*Klebsiella pneumoniae* 342 plasmid pKP91, complete sequence.	plasmid partition protein A	100.0
93	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	100.0

32	CP001138	*Salmonella enterica* subsp. enterica serovar Agona str. SL483, complete genome.	secretion protein HlyD family protein	97.88
6	XXX	*Salmonella enterica* subsp. enterica serovar Typhi str. CT18	XXX	97.76
6	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.88
1	CP000886	*Salmonella enterica* subsp. enterica serovar Paratyphi B str. SPB7, complete genome.	hypothetical protein	97.66
27	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	98.36
24	CP000966	*Klebsiella pneumoniae* 342 plasmid pKP91, complete sequence.	plasmid partition parB protein	100.0
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	mediator of plasmid stability	99.38
67	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative membrane protein	97.83
47	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.72
7	CP000243	*Escherichia coli* UTI89, complete genome.	30S ribosomal protein S2	97.51
6	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative carbohydrate kinase	98.69
6	CP001127	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome.	PTS system mannose/fructose/sorbose family IID component	97.89
17	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.45
56	AP010960	*Escherichia coli* O111:H- str. 11128 DNA, complete genome.	predicted glycosyl transferase WcaA	98.93
11	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	100.0
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	98.82
25	CP001127	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633, complete genome.	nitrite transporter NirC	99.26
75	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative elongation factor	98.42
3	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.23
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	99.57
6	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative PTS system protein	99.2
43	CP000038	*Shigella sonnei* Ss046, complete genome.	conserved hypothetical protein	98.37
9	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	ribonuclease III	99.12
40	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.52

24	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	putative plasmid SOS inhibition protein A	99.17
15	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.93
119	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	100.0
93	CP000604	*Salmonella enterica* subsp. enterica serovar Newport str. SL254 plasmid pSN254, complete sequence.	tetracycline repressor protein R, class A	100.0
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	mediator of plasmid stability	99.55
6	CP000857	*Salmonella enterica* subsp. enterica serovar Paratyphi C strain RKS4594, complete genome.	two-component system response regulator protein	97.46
2	CP000800	*Escherichia coli* E24377A, complete genome.	flavodoxin	98.3
82	CP000863	*Acinetobacter baumannii* ACICU, complete genome.	aminoglycoside 6'-N-acetyl transferase type Ib	97.49
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
9	CP000243	*Escherichia coli* UTI89, complete genome.	RNA polymerase sigma E	98.95
65	CP001063	*Shigella boydii* CDC 3083-94, complete genome.	IS1 protein InsB	97.73
7	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.34
26	AP010960	*Escherichia coli* O111:H- str. 11128 DNA, complete genome.	predicted acyl transferase WcaF	98.91
27	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	YfbU domain protein	97.56
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	99.4
5	CP000243	*Escherichia coli* UTI89, complete genome.	50S ribosomal subunit protein L13	97.89
42	FN554766	*Escherichia coli* 042 complete genome.	conserved hypothetical protein	98.15
14	CP000468	*Escherichia coli* APEC O1, complete genome.	conserved hypothetical protein	97.69
38	CP000857	*Salmonella enterica* subsp. enterica serovar Paratyphi C strain RKS4594, complete genome.	co-chaperonin GroES	100.0
63	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative phage tail completion protein	97.97
42	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-6 prophage; inner membrane lipoprotein	98.64

38	CP000604	*Salmonella enterica* subsp. enterica serovar Newport str. SL254 plasmid pSN254, complete sequence.	quaternary ammonium compound-resistance protein SugE1	100.0
23	CP000247	*Escherichia coli* 536, complete genome.	thioredoxin 1	100.0
63	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative phage tail protein	100.0
24	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	putative plasmid SOS inhibition protein B	99.3
24	CP000650	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN5, complete sequence.	putative antirestriction protein	98.59
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	99.28
10	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative methylated-DNA methyltransferase	99.03
4	CP001654	*Dickeya dadantii* Ech703, complete genome.	conserved hypothetical protein	99.21
43	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.11
22	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	histidine triad nucleotide-binding protein 2	97.48
7	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	cell division protein FtsL	99.17
40	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	preprotein translocase auxillary subunit	99.09
89	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	ISEc8 transposase	100.0
20	AE017220	*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67, complete genome.	Transposase insN for insertion sequence element IS	99.0
19	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.11
24	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	Hypothetical protein	100.0
34	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	100.0
29	AE014075	*Escherichia coli* CFT073, complete genome.	Unknown pentitol phosphotransferase enzyme II, B component	99.01
2	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	100.0
19	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	98.99
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	putative cytoplasmic protein	100.0

5	CP000034	*Shigella dysenteriae* Sd197, complete genome.	conserved hypothetical protein	98.51
2	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.96
10	CP000647	Klebsiella pneumoniae subsp. pneumoniae MGH 78578, complete sequence.	haemolysin expression modulating protein	100.0
15	FN392235	*Erwinia pyrifoliae* DSM 12163 complete genome, culture collection DSM:12163.	hypothetical protein	97.56
93	CP000604	*Salmonella enterica* subsp. enterica serovar Newport str. SL254 plasmid pSN254, complete sequence.	conserved hypothetical protein	100.0
42	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-57 prophage; predicted inner membrane protein	97.44
1	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	100.0
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
26	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	conserved hypothetical protein	98.65
3	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	putative amino acid/amine transport protein	98.11
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
24	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
22	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.33

Table A12. Proteins associated to pathogenesis found in *R. ornithinolytica N9* using PathogenFinder 1.1.

Contig	Accession ID	Organism	Protein function	Identity (%)
48	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	oriT nicking-unwinding	98.97
48	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	F pilus assembly and aggregate stability protein	100.0
2	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	96.58
21	CP000970	*Escherichia coli* SMS-3-5, complete genome.	glycosyl transferase family 8	98.89
27	CP000964	*Klebsiella pneumoniae* 342, complete genome.	anthranilate synthase, component II	97.18
12	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	4-hydroxyphenylacetate 3-hydroxylase	99.04
123	CU928164	*Escherichia coli* IAI39 chromosome, complete genome.	Group II intron-encoded reverse transcriptase/maturase	100.0
47	CP000604	*Salmonella enterica* subsp. enterica serovar Newport str. SL254 plasmid pSN254, complete sequence.	transposase InsA	99.8

33	CP000964	*Klebsiella pneumoniae* 342, complete genome.	ascorbate-specific permease IIC component	96.78
59	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	galactose-proton symport of transport system	96.98
69	CP000964	*Klebsiella pneumoniae* 342, complete genome.	phage portal protein, HK97 family	99.76
54	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	nucleoside permease	98.0
108	CP000650	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN5, complete sequence.	plasmid partition protein A	99.25
78	CP000965	*Klebsiella pneumoniae* 342 plasmid pKP187, complete sequence.	protein SopA	97.94
2	CP000964	*Klebsiella pneumoniae* 342, complete genome.	autoinducer-2 ABC transporter, periplasmic autoinducer-2-binding protein LsrB	96.14
3	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	catabolic threonine dehydratase	96.05
99	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	100.0
18	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	acetyl-CoA carboxylase β subunit	97.54
150	CP000650	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN5, complete sequence.	mediator of plasmid stability	98.6
3	AE017042	*Yersinia pestis* biovar Microtus str. 91001, complete genome.	transcriptional regulator YbtA	96.87
5	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	outer membrane protein 1a	100.0
7	CP000243	*Escherichia coli* UTI89, complete genome.	30S ribosomal protein S2	97.1
23	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	putative carbohydrate kinase	97.39
12	CP000964	*Klebsiella pneumoniae* 342, complete genome.	3,4-dihydroxyphenylacetate 2,3-dioxygenase	97.19
9	AE017220	*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67, complete genome.	transcriptional regulation of aerobic, anaerobic respiration, osmotic balance (CRP family)	96.8
50	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	98.43
99	CP000641	*Shigella sonnei* Ss046 plasmid pSS04	pA, complete sequence.	100.0
12	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	2-oxo-hepta-3-ene-1,7-dioic acid hydratase	99.63
12	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	deoxyribose-phosphate aldolase	97.68
21	CP000970	*Escherichia coli* SMS-3-5, complete genome.	O-antigen export system permease protein RfbA	99.61
50	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	99.03

36	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	96.4
56	CP000964	*Klebsiella pneumoniae* 342, complete genome.	thiazole biosynthesis protein ThiG	96.88
21	CP000970	*Escherichia coli* SMS-3-5, complete genome.	O-antigen export system ATP-binding protein RfbB	100.0
35	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	ribonuclease III	96.46
34	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	99.03
5	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	arginine 3rd transport system periplasmic binding component	97.12
39	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	putative regulator	96.28
48	CP000966	*Klebsiella pneumoniae* 342 plasmid pKP91, complete sequence.	type-F conjugative transfer system pilin acetylase TraX	97.1
47	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	F pilin acetylation	97.1
6	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	uridine kinase	96.24
156	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	100.0
44	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	96.14
75	BA000007	*Escherichia coli* O157:H7 str. Sakai DNA, complete genome.	putativa replication protein	99.53
154	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	mediator of plasmid stability	96.91
19	CP000783	*Enterobacter sakazakii* ATCC BAA-894, complete genome.	hypothetical protein	96.53
95	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	hypothetical protein	100.0
62	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
35	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	RNA polymerase sigma-70 factor	98.95
68	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	flavodoxin	96.59
174	CP000057	*Haemophilus influenzae* 86-028NP, complete genome.	transposon Tn3 resolvase	100.0
88	CP001063	*Shigella boydii* CDC 3083-94, complete genome.	IS1 protein InsB	96.59
7	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.35
26	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.39
13	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	96.79
20	CP000964	*Klebsiella pneumoniae* 342, complete genome.	type-1 fimbrial protein	97.25
48	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	surface exclusion	100.0

99	FM180570	*Escherichia coli* 0127:H6 E2348/69 plasmid pE2348-2, strain E2348/69.	UNKNOWN	100.0
28	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	putative prophage protein for establishment of lysogeny	98.82
80	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	99.4
28	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	putative prophage phage head completion protein	98.21
2	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	ribosomal protein L13	98.59
7	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	conserved hypothetical protein	96.05
45	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	transcriptional repressor for methionine biosynthesis	98.1
57	CP000857	*Salmonella enterica* subsp. enterica serovar Paratyphi C strain RKS4594, complete genome.	co-chaperonin GroES	96.91
48	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	hypothetical protein	99.32
2	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	96.99
24	CP000247	Escherichia coli 536, complete genome.	thioredoxin 1	96.33
139	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-6 prophage; partial transposase of insertion element IS911A	100.0
50	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	97.12
121	CP000244	*Escherichia coli* UTI89 plasmid pUTI89, complete sequence.	hypothetical protein	97.76
102	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
102	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
7	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	cell division protein	99.17
14	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	preprotein translocase auxillary subunit	97.27
69	CP000964	*Klebsiella pneumoniae* 342, complete genome.	HNH endonuclease domain protein	97.87
121	CP001063	*Shigella boydii* CDC 3083-94, complete genome.	IS66 family element, orf2	100.0
15	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	100.0
59	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	putative ferredoxin	100.0
16	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	cellobiose-specific PTS family enzyme IIB component	99.06
33	CP000243	*Escherichia coli* UTI89, complete genome.	putative PTS system IIB protein; subunit of SgaTBA, a putative PTS permease	98.02

102	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
24	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	twin arginine translocase protein A	96.39
1	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	cold shock protein	97.1
62	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	putative cytoplasmic protein	100.0
25	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	conserved hypothetical protein	96.63
92	CP000948	Escherichia coli str. K12 substr. DH10B, complete genome.	CP4-6 prophage; IS1 repressor protein InsA	100.0
33	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	conserved hypothetical protein	97.06
48	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	inner membrane protein	100.0
3	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	conserved hypothetical protein	96.39
1	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	haemolysin expression modulating protein	97.22
48	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	F pilin synthesis	100.0
154	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
90	CP000824	*Citrobacter koseri* ATCC BAA-895 plasmid pCKO2, complete sequence.	hypothetical protein	97.3
12	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	putative amino acid/amine transport protein	96.23
102	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0
77	AP006726	*Klebsiella pneumoniae* NTUH-K2044 plasmid pK2044 DNA, complete genome.	hypothetical protein	100.0
80	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	hypothetical protein	100.0

Table A13. Proteins associated to pathogenesis found in *E. kobei* N10 using PathogenFinder 1.1.

Contig	Accession number	Organism	Protein function	Identity (%)
34	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	96.74
65	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-6 prophage; predicted dehydratase	97.71
123	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	99.83
87	CU928158	*Escherichia fergusonii* ATCC 35469 chromosome, complete genome.	inorganic polyphosphate/ATP-NAD kinase	96.92

161	AP006726	*Klebsiella pneumoniae* NTUH-K2044 plasmid pK2044 DNA, complete genome.	hypothetical protein	99.62
21	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	4-hydroxyphenylacetate 3-hydroxylase	97.69
60	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	ISEc8 transposase	98.83
165	CU928164	*Escherichia coli* IAI39 chromosome, complete genome.	Group II intron-encoded reverse transcriptase/maturase	99.8
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	RND efflux system, outer membrane lipoprotein, NodT family	99.8
6	CP000964	*Klebsiella pneumoniae* 342, complete genome.	4-hydroxybenzoate decarboxylase, subunit C	97.26
53	AE014075	*Escherichia coli* CFT073, complete genome.	Galactose-proton symporter	95.7
65	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-6 prophage; predicted sugar transporter	98.91
50	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.03
49	CP001120	*Salmonella enterica* subsp. enterica serovar Heidelberg str. SL476, complete genome.	integrase	96.05
9	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.73
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	protein of unknown function DUF214	99.75
26	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	96.08
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	efflux transporter, RND family, MFP subunit	98.69
22	AP010960	*Escherichia coli* O111:H- str. 11128 DNA, complete genome.	hypothetical protein	99.15
3	CP001063	*Shigella boydii* CDC 3083-94, complete genome.	DNA replication and repair protein RecF	95.52
1	CP000468	*Escherichia coli* APEC O1, complete genome.	UDP-galactose-4-epimerase	95.56
66	CP000886	*Salmonella enterica* subsp. enterica serovar Paratyphi B str. SPB7, complete genome.	hypothetical protein	95.65
18	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	3-phenylpropionate dioxygenase, alpha subunit	95.59
4	CP000243	*Escherichia coli* UTI89, complete genome.	malate dehydrogenase	95.83
22	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.67
75	CP000649	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN4, complete sequence.	mediator of plasmid stability	97.52
11	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	95.38
23	CP000243	*Escherichia coli* UTI89, complete genome.	30S ribosomal protein S2	97.93
172	CP001383	*Shigella flexneri* 2002017, complete genome.	ISEhe3, transposase orfB	97.55
136	CP001339	*Thioalkalivibrio* sp. HL-EbGR7, complete genome.	ABC-type phosphate/phosphonate transport system, periplasmic component	98.61

83	CP000266	*Shigella flexneri* 5 str. 8401, complete genome.	conserved hypothetical protein	95.75
43	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	98.0
44	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	Glycosyltransferase	98.55
136	CP001339	*Thioalkalivibrio* sp. HL-EbGR7, complete genome.	ATP-binding protein of phosphonate ABC transporter	99.64
136	CP001339	*Thioalkalivibrio* sp. HL-EbGR7, complete genome.	phosphate ABC transporter permease	100.0
27	CP000800	*Escherichia coli* E24377A, complete genome.	pyruvate formate-lyase 1-activating enzyme	96.34
1	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	96.8
65	U00096	*Escherichia coli* str. K-12 substr. MG1655, complete genome.	CP4-6 prophage; predicted DNA-binding transcriptional regulator	96.03
17	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	ribonuclease III	95.4
2	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	98.55
22	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	TerY1	98.58
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	ABC transporter related	99.58
28	CP001125	*Salmonella enterica* subsp. enterica serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	98.44
79	CP000964	*Klebsiella pneumoniae* 342, complete genome.	3-dehydro-L-gulonate-6-phosphate decarboxylase	96.76
15	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.0
9	CP000783	*Enterobacter sakazakii* ATCC BAA-894, complete genome.	hypothetical protein	98.27
1	CP000800	*Escherichia coli* E24377A, complete genome.	flavodoxin	95.45
20	CP000647	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence.	hypothetical protein	100.0
22	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	TerX	100.0
209	CP000863	*Acinetobacter baumannii* ACICU, complete genome.	aminoglycoside 6'-N-acetyl transferase type Ib	98.91
70	CP000243	*Escherichia coli* UTI89, complete genome.	RNA polymerase sigma E	98.95
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	transcriptional regulator, TetR family	99.5
23	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	97.35
6	CP001144	*Salmonella enterica* subsp. enterica serovar Dublin str. C	2021853, complete genome.	96.79
11	CP000886	*Salmonella enterica* subsp. enterica serovar Paratyphi B str. SPB7, complete genome.	hypothetical protein	96.45

73	AP010960	*Escherichia coli* O111:H- str. 11128 DNA, complete genome.	hypothetical protein	98.33
48	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	hypothetical protein	99.34
4	CP000243	*Escherichia coli* UTI89, complete genome.	50S ribosomal subunit protein L13	97.89
35	CP000783	*Enterobacter sakazakii* ATCC BAA-894, complete genome.	hypothetical protein	96.88
24	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	transcriptional repressor for methionine biosynthesis	97.14
22	AP006726	*Klebsiella pneumoniae* NTUH-K2044 plasmid pK2044 DNA, complete genome.	Tn903 transposase	100.0
129	CP000514	*Marinobacter aquaeolei* VT8, complete genome.	conserved hypothetical protein	100.0
57	CP000857	*Salmonella enterica* subsp. enterica serovar Paratyphi C strain RKS4594, complete genome.	co-chaperonin GroES	96.91
22	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	TerW	100.0
161	AP006726	*Klebsiella pneumoniae* NTUH-K2044 plasmid pK2044 DNA, complete genome.	hypothetical protein	98.63
30	CP000247	*Escherichia coli* 536, complete genome.	thioredoxin 1	99.08
92	CP000784	*Enterobacter sakazakii* ATCC BAA-894 plasmid pESA2, complete sequence.	hypothetical protein	100.0
66	CP000036	*Shigella boydii* Sb227, complete genome.	putative alpha helix protein	95.5
7	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	cell division protein FtsL	95.87
2	CP000243	*Escherichia coli* UTI89, complete genome.	hypothetical protein YajC	99.09
69	CP000034	*Shigella dysenteriae* Sd197, complete genome.	IS911 ORF1	99.0
92	CP000784	*Enterobacter sakazakii* ATCC BAA-894 plasmid pESA2, complete sequence.	hypothetical protein	100.0
161	AP006726	*Klebsiella pneumoniae* NTUH-K2044 plasmid pK2044 DNA, complete genome.	hypothetical protein	100.0
82	CU928162	*Escherichia coli* ED1a chromosome, complete genome.	Putative transcriptional regulator	100.0
198	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R, complete sequence.	ISEc8 transposase	100.0
114	CP000648	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence.	Hypothetical protein	96.3
41	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.44
10	CP000822	*Citrobacter koseri* ATCC BAA-895, complete genome.	hypothetical protein	96.04
79	CP001113	*Salmonella enterica* subsp. enterica serovar Newport str. SL254, complete genome.	ascorbate-specific phosphotransferase enzyme IIB component	98.02
16	CP000880	*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--, complete genome.	hypothetical protein	97.98

149	CP000243	*Escherichia coli* UTI89, complete genome.	conserved hypothetical protein YihD	95.51
1	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	cold shock protein	100.0
124	AE014073	*Shigella flexneri* 2a str. 2457T, complete genome.	IS1 orfB	100.0
22	CP000034	*Shigella dysenteriae* Sd197, complete genome.	IS1 ORF1	97.8
54	CP000800	*Escherichia coli* E24377A, complete genome.	IS1, transposase orfA	97.8
124	CP000948	*Escherichia coli* str. K12 substr. DH10B, complete genome.	CP4-6 prophage; IS1 repressor protein InsA	100.0
26	CP000026	*Salmonella enterica* subsp. enterica serovar Paratyphi A str. ATCC 9150, complete genome.	conserved hypothetical protein	98.53
4	CP000034	*Shigella dysenteriae* Sd197, complete genome.	conserved hypothetical protein	97.01
6	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	putative cytoplasmic protein	95.89
14	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA, complete genome.	putative amino acid/amine transport protein	98.11
71	CP000783	*Enterobacter sakazakii* ATCC BAA-894, complete genome.	hypothetical protein	100.0