Universidade de São Paulo
Faculty of Pharmaceutical Sciences
Pharmacy Postgraduate Program
Clinical Analysis Area

Comparative analysis of *Klebsiella pneumoniae* belonging to the endemic high-risk clonal
group CG258

Louise Teixeira Cerdeira

Thesis to obtain the title of DOUTOR
Supervisor: Prof. Dr. Nilton Erbet Lincopan Huenuman

São Paulo
2019

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Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Farmácia
Área de Análises Clínicas

Análise comparativa de *Klebsiella pneumoniae* pertencente ao grupo clonal endêmico de alto
risco GC258

Louise Teixeira Cerdeira

Tese para obtenção do Título de DOUTOR
Orientador: Prof. Dr. Nilton Erbet Lincopan Huenuman

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Louise Teixeira Cerdeira

Comparative analysis of *Klebsiella pneumoniae* belonging to the endemic high-risk clonal
group CG258

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São Paulo, 28 de maio de 2019.

"The scientist is not the man who provides the real answers; is the one who asks the real questions".

(Claude Lévi-Strauss)

DEDICATION

I dedicate this thesis to my parents, Gilberto de Lima Cerdeira and Maria Luiza Teixeira Cerdeira. For all dedication, trust, unconditional support throughout this journey and for encouraging from childhood to study and believe in the premise that through education we can change the world. Father, 40 years sailing on the high seas and being absent from the family were not in vain. Mom, my heroine, my foundation, my source of inspiration. I love you unconditionally.

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ABSTRACT

CERDEIRA, L. T. **Comparative analysis of *Klebsiella pneumoniae* belonging to the endemic high-risk clonal group CG258**. 2019. 165f. Thesis (Doctorate) – Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, 2019.

The rapid spread of carbapenem-resistant lineages of *Klebsiella pneumoniae*, clustered within the clonal group CG258, is a growing public health problem associated with healthcare-associated infections. The objective of this study was to perform a genomic analysis of KPC-2 and/or CTX-M β -lactamase-producing strains of *K. pneumoniae* belonging to CG258 (ST11, ST258, ST340, ST437) circulating at the human-animal-environment interface, in Brazil and South America. The analysis was conducted to characterize the antimicrobial resistome, virulome, genetic elements of transfer and mobilization associated with the dissemination of the *bla*_{KPC-2} gene, and to perform a detailed comparative genomic analysis of the CG258; with subsequent pathogenicity evaluation in an invertebrate (*Galleria mellonella*) model of infection, aiming to identify biomarkers of virulence. The main results are presented in the format of six manuscripts. **Manuscript I:** New draft genome sequence of a *Klebsiella pneumoniae* strain 1194/11, belonging to ST340, showing a wide resistome. **Manuscript II:** The first draft genome sequence of a *Klebsiella pneumoniae* 606B ST340 carrying *bla*_{CTX-M-15} in food-producing animal isolated in Brazil. **Manuscript III:** The first draft genome sequence of a *Klebsiella pneumoniae* strain Kp171, recovered from a water sample collected in an urban river in Brazil, demonstrating that anthropogenic activities, including the release of wastewater and sewage from hospitals, may have contributed to the contamination of aquatic environments, raising a concern to public health. **Manuscript IV:** Identification and complete sequence analysis of an IncX3 plasmid carrying a non-Tn4401 genetic element (NTE_{KPC-1c}), originating from a hospital associated lineage of *K. pneumoniae* ST340, showing the spread of *bla*_{KPC-2} in new Incompatibility group. **Manuscript V:** Dissemination of *bla*_{KPC-2} in novel non-Tn4401 Element (NTE_{KPC-1Id}) carry by new small IncQ1 and Col-Like plasmids in lineages of *Klebsiella pneumoniae* ST11 and ST340. **Manuscript VI:** Yersiniabactin, colibactin and wider resistome contribute to enhanced virulence and persistence of KPC-2-producing *K. pneumoniae* CG258 in South America. The results obtained in the present study allow us to obtain a first genomic landscape of *K. pneumoniae* lineages of the CG258, circulating at the human-animal-environment interface, in Brazil and South America. In this regard, most likely the interplay of yersiniabactin and/or colibactin, and resistance to clinically significant antibiotics (as carbapenems and polymyxins) are contributing to the emergence of highly virulent and MDR lineages that pose great risk to human health. On the other hand, the wide antimicrobial resistome (antibiotics, disinfectants and heavy metals) could be contributing to adaptation of KPC-2- and/or CTX-M-producing *K. pneumoniae* CG258 in the human-animal-environment interface, highlighting the urgent need for enhanced control efforts. In conclusion, these findings could contribute to the development of strategies for prevention, diagnosis and treatment of *K. pneumoniae* infections.

Keywords: *Klebsiella pneumoniae*, KPC-2, virulome, resistome, genomic analysis.

RESUMO

CERDEIRA, L. T. **Análise comparativa de *Klebsiella pneumoniae* multiresistente pertencente ao grupo clonal endêmico de alto risco GC258**. 2019. 165f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

A rápida disseminação de linhagens de *Klebsiella pneumoniae* resistentes aos carbapenêmicos, agrupadas dentro do grupo clonal GC258, é um crescente problema de saúde pública associado com infecções relacionadas à assistência à saúde. O objetivo deste estudo foi realizar uma análise genômica de cepas de *K. pneumoniae* produtoras de β -lactamases KPC-2 e/ou CTX-M, pertencentes ao GC258 (ST11, ST258, ST340, ST437), circulando na interface humana-ambiente-animal, no Brasil e na América do Sul. A análise foi direcionada para caracterizar o resistoma e viruloma, elementos genéticos de transferência e mobilização associados com a disseminação de genes *bla*_{KPC-2}, e realizar uma análise de genômica comparativa detalhada do GC258, com posterior avaliação da patogenicidade em modelo invertebrado (*Galleria mellonella*) de infecção, visando identificar biomarcadores de virulência. Os principais resultados são apresentados na forma de seis manuscritos. **Manuscrito I:** Nova sequência “draft” do genoma de *K. pneumoniae* 1194/11 isolado de amostra clínica, pertencente ao ST340, mostrando um amplo resistoma. **Manuscrito II:** O reporte da primeira sequência “draft” do genoma de *K. pneumoniae* 606B (ST340), contendo *bla*_{CTX-M-15} em animais de produção isolados no Brasil. **Manuscrito III:** O primeiro esboço da sequência do genoma de *K. pneumoniae* Kp171, recuperado de uma amostra de água coletada em um rio urbano no Brasil, demonstrando que atividades antrópicas, incluindo a liberação de esgoto e esgoto de hospitais, podem ter contribuído para a contaminação ambientes aquáticos, levantando uma preocupação para a saúde pública. **Manuscrito IV:** Identificação e análise de sequência completa de um plasmídeo IncX3 portador de um elemento genético não Tn4401 (NTE_{KPC-1c}), originado de uma linhagem hospitalar associada a *K. pneumoniae* ST340, mostrando a disseminação de *bla*_{KPC-2} no novo grupo Incompatibilidade. **Manuscrito V:** Disseminação de *bla*_{KPC-2} no novo elemento non-Tn4401 (NTE_{KPC-1ld}) portado por novos pequenos plasmídeos IncQ1 e Col-Like em linhagens de *K. pneumoniae* ST11 e ST340. **Manuscrito VI:** Os resultados obtidos no presente estudo permitem gerar um panorama genômico das linhagens de *K. pneumoniae* do GC258, circulando na interface humana-animal-ambiente, no Brasil e na América do Sul. De principal interesse, a convergência da virulência associada com genes codificando yersiniabactina e/ou a colibactina e a resistência a antibióticos clinicamente significativos (como carbapenêmicos e polimixinas), estão contribuindo para o aparecimento de linhagens altamente virulentas e multiresistentes que apresentam um grande risco à saúde humana. Por outro lado, a ampla resistência aos antimicrobianos (antibióticos, desinfetantes e metais pesados) poderia estar contribuindo para a adaptação de estirpes de *K. pneumoniae* do GC258, produtoras de KPC-2-e/ou CTX-M, na interface humana-ambiente-animal, destacando a necessidade urgente de medidas para o controle de disseminação. Em conclusão, esses achados poderiam contribuir para o desenvolvimento de estratégias de prevenção, diagnóstico e tratamento das infecções por *K. pneumoniae*.

Palavras-chaves: *Klebsiella pneumoniae*, KPC-2, resistoma, viruloma, análise comparativa.

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1. INTRODUCTION

Over the years, the overuse and misuse of antimicrobial compounds have contributed to the increase of resistance in clinically important bacterial species, being an endemic cosmopolitan phenomenon. In this regard, different genera and species have acquired and/or developed mechanisms of resistance to last generation antibiotics, compromising therapeutic activity with a further negative prognostic impact (BALSALOBRE et al., 2014; DRAENERT et al., 2015). In fact, annually, infections caused by multidrug-resistant (MDR) bacteria kill thousands of people around the world. According to the World Health Organization (WHO), if no restrictive measures are taken, it is estimated that by 2050 the number of MDR-related deaths will increase to 10 million annually, and milder infections may be fatal (O'NEILL, 2014).

Worryingly, the antimicrobial resistance problem is not restricted to hospital settings. In fact, aquatic environments, food (fruits, vegetables and meat) and animals (livestock, pets and wildlife) have been directly or indirectly exposed to antimicrobial compounds, including antibiotics, disinfectants, herbicides and/or pesticides. The main concern is the wide use of antibiotics in farm animals (as growth promoter or for prophylaxis/therapeutic purposes), which has contributed to the establishment of resistant bacteria in their microbiota (VAN BOECKEL et al., 2015, MONTE et al., 2017). In addition, anthropogenic activities can contaminate the environment by improperly disposing of waste in sewage, which may contain toxic substances (e.g., heavy metals), antimicrobial agents and/or resistant bacteria, contributing to the alteration of the various ecosystems and creating environmental reservoirs of resistance (CHENG et al., 2015; HALL et al., 2014; ROCA et al., 2015; WOOLHOUSE et al., 2015; CERDEIRA et al., 2016a; CERDEIRA et al., 2017a).

It is well known that bacterial adaptability to survival in hostile environments is facilitated by acquisition of new genes and/or mobile elements via genetic recombination, and less frequently by point mutations (BLAIR et al., 2015). Thus, horizontal gene transfer (THG) occurs dynamically between bacteria of the same species or different genera, by conjugation, transformation, transduction, and/or homologous recombination (FROST et al., 2005; SLATER et al., 2008).

Currently, the spread of bacterial resistance among different ecosystems and hosts has been studied within a “One-Health” concept that portrays the interaction between humans, animals, and the environment. This strategy has become essential to ensure the application of correct practices related to the prevention, surveillance and detection of zoonoses/anthropozoonoses.

The proposed intention is to raise awareness about the risks associated with the misuse of antibiotics in breeding companion and production animals, which consequently reflects on human and environment health (CONRAD et al., 2013).

1.1 Antibacterial agents

Antibacterial agents are chemical compounds produced by microorganisms or synthetically, with the ability to inhibit or kill bacteria (ROSSI & ANDREAZZI, 2005). They are classified into the following classes: beta-lactams, glycopeptides, aminoglycosides, macrolides, lincosamide, tetracyclines, phenicols, quinolones, sulphonamides and polymyxins, based on the mechanism of action on the bacterial cell and its chemical structure (ROSSI & ANDREAZZI, 2005). The classes most used for the treatment of Gram-negative bacilli (GNB) infections are beta-lactams, aminoglycosides, quinolones and polymyxin. The main mechanisms of action of antimicrobials are: effect on cell wall synthesis, inhibition of protein synthesis, effect on cell membrane structure and function, interference in nucleic acid synthesis and antimetabolic activity or antagonistic competitiveness (ROSSI & ANDREAZZI, 2005).

Beta-lactams have an effect on the cell wall, by binding to PBPs (penicillin binding proteins), preventing the formation of the cell wall and causing lysis of the bacterial cell (SPRATT, 1988). Aminoglycosides prevent protein synthesis by binding to the A (aminoacyl) site of 16S rRNA on the 30S subunit of the ribosome by interfering with the translocation process (MAGNET & BLANCHARD, 2005). The quinolones target the topoisomerases, leading to the blockade of DNA replication (ROSSI & ANDREAZZI, 2005). Polymyxins cause rupture of cell membrane integrity by lipopolysaccharide (LPS) binding, competitively displacing calcium and magnesium ions and/or disrupting membrane integrity (FALAGAS & KASIAKOU, 2005).

Microorganisms have developed mechanisms of resistance to antimicrobials over time, these mechanisms may be intrinsic or acquired. The intrinsic mechanisms are natural to each species and may even be used to identify the bacterial species and the acquired ones can be by mutation or transmission of external genetic material (ROSSI & ANDREAZZI, 2005). Mutations are alterations in DNA sequences that result in changes in the structure of a gene and may be spontaneous or induced. Spontaneous mutations occur due to an error during the DNA replication process or by interference of environmental agents, whereas induced mutations can occur by a deliberate action in which the organism is exposed to genotoxic agents (ROSSI &

ANDREAZZI, 2005; PADILLA & COSTA, 2015). The main mechanisms of antimicrobial resistance that the microorganisms have developed are: i) alteration in membrane permeability, ii) overexpression of efflux pumps, iii) genetic alteration of the target and, iv) enzymatic action.

1.2 β -lactams and β -lactamases

β -lactams (eg, penicillins, cephalosporins, monobactams and carbapenems) are among the most clinically prescribed antibacterial agents (HUTTNER et al., 2015; Eiamphungporn et al., 2018). Among Gram-negative bacilli, β -lactam resistance is mediated mainly by the production of enzymes, called β -lactamases (BONOMO, 2017; BUSH, 2018), which catalyze the hydrolysis of the β -lactam ring, inactivating, the action of several antimicrobials belonging to this group (BONOMO, 2017; BUSH, 2018).

β -lactamase classifications have been based on sequence similarity (Ambler structural classification), which defines 4 classes. Enzymes belonging to classes A, C and D are called serine- β -lactamases, since present a serine amino acid residue at the active site of the enzyme. Those belonging to class B, named as metallo- β -lactamases (MBL), are zinc-dependent enzymes (Zn^{+2}). On the other hand, a functional classification scheme for β -lactamase and its correlation with molecular structure was propoused by Bush, Jacoby and Medeiros (1995), which defines different groups of β -lactamases (groups 1 to 4, with subdivisions) according to the substrate of the enzyme and the inhibition profile by β -lactamases inhibitors.

Extend-Spectrum β -lactamases (ESBLs) are enzymes capable of hydrolyzing penicillin, cephalosporins (third and fourth generation) and monobactam (i.e., aztreonam), which can be inactivated by specific inhibitors, such as clavulanic acid, sulbactam and tazobactam (RAHMAN et al., 2018; BUSH, 2018). They are identified in several members of the Enterobacteriales family and some non-fermenting gram-negative bacilli, however in clinical infectious diseases the main problem has been related to the ESBL production by *Klebsiella pneumoniae* and *Escherichia coli* (PITOUT et al., 2005; ADEOLU et al., 2016). In this regard, ESBL-producing *K. pneumoniae* are often involved in infections related to health care in ICUs (HENDRIK; VOOR IN 'T HOLT; VOS, 2015), and in South America, especially in Brazil, the predominant ESBL belongs to the family CTX-M (CHAGAS et al., 2011; ROCHA; PINTO; BARBOSA, 2015).

The global spread of genes encoding CTX-M-like enzymes is considered to be one of the fastest and most successful phenomena of microbial resistance in the antimicrobial era

(CANTÓN et al., 2012; D'ANDREA et al., 2013), more than 200 variants have been identified worldwide (BUCKNER; CIUSA; PIDDOCK, 2018). CTX-M variants can be divided into five groups according to similarities in their amino acid sequences: CTX-M-1 (e.g., *bla*_{CTX-M-15}), CTX-M-2 (e.g., *bla*_{CTX-M-2}), CTX-M-8 (e.g., *bla*_{CTX-M-8}), CTX-M-9 (e.g., *bla*_{CTX-M-14}) and CTX-M-25 (PITOUT; LAUPLAND, 2008; PEIRANO; PITOUT, 2010). CTX-M-2, CTX-M-15 and CTX-M-14 are the worldwide predominant genotypes (BEVAN et al., 2017).

In South America, especially in Brazil, the predominant ESBL belongs to the CTX-M family (CHAGAS et al., 2011; ROCHA; PINTO; BARBOSA, 2015), mainly CTX-M-2, CTX-M-8, CTX-M-14 and CTX-M-15 (ROCHA et al., 2016; CERDEIRA et al., 2018). In this regard, it is estimated that approximately 50% of the strains of *K. pneumoniae* are ESBL-producing in this country (ROCHA et al., 2016), increasing the risk of therapeutic failures and mortality, which end up impacting global public health (PITOUT; LAUPLAND, 2008; MASLIKOWSKA et al., 2016).

Plasmids of different incompatibility groups are involved in the rapid spread of *bla*_{CTX-M} genes (CANTÓN et al., 2012; ROCHA et al., 2016), and may also carry other genes that confer resistance to aminoglycosides, carbapenems and fluoroquinolones (CANTÓN et al., 2012), contributing to an uncontrolled pandemic scenario. This may be partly justified because these enzymes may coexist with other β -lactamases (eg, KPC, NDM and IMV) in the same strains of *K. pneumoniae*, a common bacterial strategy to increase antimicrobial resistance (CANTÓN et al., 2012).

Several factors are related to the success of ESBLs, such as efficient capture and dispersion of the *bla*_{CTX-M} gene by mobile genetic elements, association with high-risk bacterial clones and high selective pressure caused by the indiscriminate use of extended-spectrum cephalosporins and fluoroquinolones in clinical and veterinary environments (D'ANDREA et al., 2013).

Although CTX-M has played an important role over the years (BEVAN, et al., 2017), other ESBL enzymes such as TEM and SHV have also been associated with significant outbreaks of *K. pneumoniae* (CANTÓN et al., 2012; PATERSON; BONOMO, 2005). Therefore, carbapenems are used as a therapeutic choice for treatment of severe infections by ESBL-producing microorganisms (TAMMA; RODRIGUEZ-BAÑO, 2017). However, this practice has led to the selection of resistant strains producing carbapenemases, such as *Klebsiella pneumoniae* carbapenemase (KPC) and metallo- β -lactamases (MBL) (VAN BOXTEL et al., 2016).

Carbapenemases comprise a heterogeneous group of β -lactamases belonging to class A (penicillinases), B (metalloenzymes) or D (oxacillinases) enzymes, that hydrolyze carbapenem antibiotics, such as ertapenem, imipenem and/or meropenem, as well as other penicillins (QUEENAN; BUSH, 2007; BERTONCHELI; HÖRNER, 2008; PITOUT, NORDMANN, POIREL, 2015).

KPC-type carbapenemases are frequently reported in *K. pneumoniae*, although it has also been described in other members of the Enterobacteriaceae family and non-fermenting Gram-negative bacilli, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (ROBLEDO et al., 2011; MUNOZ-PRICE et al., 2013; CHEN et al., 2014; PITOUT; NORDMANN; POIREL, 2015; DAI et al., 2016).

KPC-type carbapenemases belongs to class A of Ambler and functional group 2f of Bush, Jacoby and Medeiros (QUEENAN, BUSH, 2007; BUSH, JACOB, 2010), and confers resistance to all active β -lactams used against infections caused by *K. pneumoniae* (SHANMUGAM; MEENAKSHISUNDARAM; JAYARAMAN, 2013).

This enzyme was first found in a clinical strain of *K. pneumoniae* in North Carolina (USA) in 1996 (YIGIT et al., 2001) and is currently worldwide spread (BEIRÃO et al., 2011; CASTANHEIRA et al., 2013; RUIZ-GARBAJOSA et al., 2013; LEE et al., 2016; VAN DUIN; DOI, 2017). Currently, more than 40 KPC variants have been published (NAAS; DORTET; IORGA, 2016; LEE et al., 2016; PALZKILL, 2018) and/or deposited in NCBI database, being reported as endemic in the USA, Greece, Poland, Italy, China, Taiwan. KPC-2 and KPC-3 variants are the most frequently identified in the European Union, in Asia, and in Oceania (LEE et al., 2016), Israel, Colombia, Argentina and Brazil. (PITOUT; NORDMANN; POIREL, 2015).

In addition to KPC, other carbapenemases have assumed epidemiological importance due to their association with transposons, plasmids and integrons (PARTRIDGE et al., 2018). Thus, the emphasis is given to metallo- β -lactamases, which are currently classified as IMP (*Imipenemase*), VIM (*Verona Imipenemase*), SPM-1 (*São Paulo MBL*); GIM (*German Imipenemase*) and SIM-1 (*Seoul Imipenemase*) (MENDES et al., 2006), and more recently AIM-1 (*Australian Imipenemase*) (YONG et al., 2007), KHM (*Kyorin University Hospital*) (SEKIGUCHI et al. (New Delhi MBL) (YONG et al., 2009) and DIM-1 (*Dutch imipenemase*) (POIREL et al., 2010).

In addition to the carbapenemases mentioned above, other important enzymes have been identified in Gram-negative pathogens, such as oxacillinase-like carbapenemases (OXA),

which include enzymes with lower hydrolytic capacities against carbapenems, however not less important (PEREIRA et al., 2015). In 2001, the enzyme OXA-48 was isolated for the first time in Turkey in *K. pneumoniae* strains and is currently observed worldwide (SHANTHI et al., 2013, PEREIRA et al., 2015), as well as other allelic variants described, such as the OXA-181 found in several microorganisms of the Enterobacteriaceae family, especially *K. pneumoniae* (SHANTHI et al., 2013). More recently, a new allelic variant, OXA-370, has been reported in a strain of *Enterobacter hormaechei* in southern Brazil (PEREIRA et al., 2015). The importance of these enzymes has been associated with the fact that these genes are also found as gene cassettes in integrons, transposons or associated with insertion sequences, located on conjugative or non-conjugative plasmids (QUEENAN; BUSH, 2007; NOLL et al., 2018).

Figure 1. Worldwide distribution of KPC-producing *K. pneumoniae*. (1) USA; (2) Colombia; (3) Brazil; (4) Argentina; (5) Italy; (6) Greece; (7) Poland; (8) Israel; (9) China; (10) Taiwan; (11) Canada; (12) Spain; (13) France; (14) Belgium; (15) Netherlands; (16) Germany; (17) UK; (18) Ireland; (19) Sweden; (20) Finland; (21) Hungary; (22) India; (23) South Korea; (24) Australia; (25) Mexico; (26) Cuba; (27) Puerto Rico; (28) Uruguay; (29) Portugal; (30) Switzerland; (31) Austria; (32) Czech Republic; (33) Denmark; (34) Norway; (35) Croatia; (36) Turkey; (37) Algeria; (38) Egypt; (39) South Africa; (40) Iran; (41) United Arab Emirates; (42) Pakistan; (43) Russia; (44) Japan. Adapted from Lee et al., 2016.

1.3. *Klebsiella pneumoniae* producing KPC-2-type carbapenemases

Klebsiella pneumoniae is a ubiquitous gram-negative bacterium belonging to the family Enterobacteriaceae, being isolated from mammals and various ecological environments. As a cosmopolitan pathogen, the species has been the etiological agent of nosocomial and community infections, mainly associated with pneumonia and urinary tract infections. The main

problem of *K. pneumoniae* is the expression of resistance for commercially available antibiotics, mainly to broad-spectrum cephalosporins and carbapenems. This resistance has resulted from acquisition of genes mobilized by plasmids, encoding extended-spectrum beta-lactamases (ESBL) and *Klebsiella pneumoniae* carbapenemases (KPCs).

K. pneumoniae outbreaks associated with KPC production were first described in New York (BRATU et al, 2005; WOODFORD et al., 2004), however epidemiological surveillance reports indicate that these bacteria are widespread around the world. Nowadays, the production of KPC in Enterobacteriaceae has been described in several countries, including American (USA, Canada, Colombia, Argentina, Uruguay), European, Asian and Oceanic countries (LEE et al., 2016) (Figure 1).

Currently, more than 44 *bla*_{KPC} variants have been registered in NCBI database, of which only *bla*_{KPC-2} and *bla*_{KPC-3} have been identified in Brazil, so far (SAMPAIO & GALES, 2016; RIBEIRO et al., 2016). In this regard, the first description of *K. pneumoniae* producing KPC was published in 2009, in strains isolated in 2006, in Recife (MONTEIRO et al, 2009); however, a later publication showed that *K. pneumoniae* strains producing KPC-2 appeared in Brazil in 2005, in the city of São Paulo (PAVEZ et al, 2009). Since then a large number of publications, including multicenter studies such as SENTRY (GALES et al, 2012) have reported the rapid dissemination and endemicity of this type of bacteria in Brazilian hospitals, associated with outbreaks of Health Care-Related Infections (ROCHE et al., 2007). An aggravating factor has been the dissemination of *K. pneumoniae* strains producing KPC-2 to urban aquatic environments and recreational waters in metropolitan areas of Brazil (NASCIMENTO et al., 2017; OLIVEIRA et al, 2014; MONTEZZI et al., 2015; CERDEIRA et al., 2017; PASCHOAL et al., 2017; FRANCISCO et al., 2019), raising a public health concern, since it could indicate a direct contamination by hospital sewage (CHAGAS et al, 2011; PICÃO et al., 2013).

1.4 Polymyxins

The difficulty in treating severe infections caused by MDR Gram-negative bacilli has pushed the reintroduction of polymyxins (polymyxin B and colistin) as a last resort therapy (FALAGAS; KASIAKOU, 2005; LI et al., 2006; BISWAS et al., 2012, POIREL, JAYOL, NORDMANN, 2017). However, due to the increased use of these antibiotics, resistance to polymyxins has been reported by bacteria that are normally susceptible to these drugs (BARON et al., 2016; FERNANDES et al., 2016; FERNANDES et al., 2017; MONTE et al., 2017;

SELLERA et al., 2017; OLIVEIRA et al., 2018). Similarly, the number of reports of infections caused by bacteria naturally resistant to polymyxins, such as *Proteus spp.*, *Providencia spp.*, *Morganella morganii* and *Serratia marcescens* have increased (OLAÏTAN; MORAND; ROLAIN, 2014; POIREL; JAYOL; NORDMANN, 2017).

Polymyxin resistance in *K. pneumoniae* results from the addition of L-Ara4N (4-amino-4-deoxy-l-arabinose) and/or pEtN (phosphoethanolamine) to the lipid A. The *pmrHFIJKLM* (or *arnBCADTEF*) operon and the *pmrC* gene that promote these modifications, respectively, are under positive control of the two-component systems (TCSs) PhoPQ and PmrAB. So, single point mutations in both TCSs have been held responsible for polymyxin resistance in clinical *K. pneumoniae*. On the other hand, the *mgrB* gene, which encode a small regulatory transmembrane protein (MgrB), negatively regulates the kinase activity of PhoQ, increasing colistin resistance. Alteration of the *mgrB* gene by insertion sequences (IS5-like, *IS1F*, *ISKpn13*, *ISKpn14*, *IS10R*), and/or point mutations represents the most common cause of polymyxin resistance in *K. pneumoniae*, whereas mutations in the genes *phoPQ*, *pmrAB* or *mgrB* suggests a role for other genetic loci in resistance. Mutations (L94M, Q10L, Y31H, W140R, N141I, P151S and S195) in CrrB, the sensor kinase partner of the TCS CrrAB (for Colistin resistance regulation), were recently shown to confer elevated resistance to colistin. Curiously, *crrAB* genes that code for these proteins are not harboured by all *K. pneumoniae* isolates. Indeed, *crrB* mutations have been detected in strains belonging to sequence types ST11, ST29 and ST258, so far (JEANNOT et al., 2017).

In addition to the mutation-based mechanisms, acquisition of plasmid-borne *mcr*-type genes constitutes a major cause of polymyxin resistance in *E. coli* and *K. pneumoniae*. Phosphoethanolamine transferases encoded by *mcr*-type genes use the the same strategy based on the addition of pEtN to LPS (JEANNOT et al., 2017).

The polymyxyn resistance issue create a great threat with regard to intractable infections (Olaitan; Morand; Rolain, 2014), and the most frightening aspect is the ease with which these microorganisms are able to transfer resistance determinants to other bacterial species, favoring this scenario (WANG et al., 2018).

1.5 Heavy metal compounds

Heavy metals (HM) are highly toxic to most microorganisms, however there are bacteria that have a variety of resistance or tolerance mechanisms that make them capable of handling

high concentrations of these metals, so these microorganisms can be used as agents of removal of metals by absorption and adsorption mechanisms (CONGEEVARAM et al., 2007; GAYLARDE et al., 2005). In addition, the use of absorption and adsorption mechanisms has been shown to increase the adsorption capacity of these compounds. Resistant bacterial species can live in environments contaminated by heavy metals regardless of contamination levels, some bacteria adapt in the presence of these metals in concentrations higher than 1 ppm, becoming dominant in these environments. In relation to the tolerance to these metals, the cell uses some mechanisms to prevent the entry of metal into the cytoplasm. Resistance is related to more specialized mechanisms, such as the internalization of the metal (some microorganisms exhibit one or more combinations of resistance mechanisms, but the main mechanism that regulates intracellular metal concentrations is related to membrane transport (DOPSON et al., 2014).

The environments that are contaminated by toxic metals are usually derived from agricultural, industrial or domestic activities, allowing the accumulation of these metals, since they are not degraded. Several studies have presented data showing that an increase in the concentration of metals in the environment causes an increase in the tolerance of the community of microorganisms, especially bacteria. Thus, according to the various selective pressures (which includes contaminants), many organisms with tolerance/resistance genes to different molecules are selected. Tolerance/resistance mechanisms include efflux pumps, active transport, permeation barrier exclusion, enzymatic detoxification, and even a reduction in the sensitivity of cell targets to metal ions. These tolerance mechanisms are often transmitted by plasmids through horizontal gene transfer, leading to adaptations by the bacterial community. (CONGEEVARAM et al., 2007; DOPSON et al., 2014). Additionally, the presence of HMs genes most common found are the: silver (sil), copper (pco), arsenic (ars) and mercury (mer); rarely tellurite (ter) (CERDEIRA et al., 2018).

1.6 Quaternary ammonium compounds (QACs)

QACs are cationic surfactants or surfactants, widely known for their potent biocidal property. These compounds are generally found in disinfectants and antiseptics, and most formulations containing QACs in their composition do not require rinsing after use. The term disinfectant refers to all formulations that are applied to inert surfaces or inanimate articles for the purpose of removing pathogenic microorganisms. The term antiseptic is used for all formulations that contain a germicidal, microbicidal or bactericidal agent, safe for use in living

organisms, and can be used for hand hygiene, wound antisepsis, among other applications. (OBLAK et al., 2018).

The phenomenon of resistance to QACs has been known since the 1950s and 1960s and, as with antibiotics, is based on the mechanisms of intrinsic resistance and acquired resistance. Currently, several studies described in the literature report increased bacterial resistance to these compounds (RUSSELL et al., 2002). Intrinsic resistance is generally more evident in Gram-negative bacteria due to the presence of the outer membrane and *Mycobacterium* spp. Due to their structural composition of the cell wall. Additionally, bacterial spores are also intrinsically resistant. On the other hand, this resistance may also be related to baseline efflux pump activity (HEGSTAD et al., 2010). Acquired resistance can occur when bacteria are exposed for long periods to formulations containing QACs. Sub-inhibitory concentrations are not rapidly neutralized, and the environment may select bacterial populations resistant to high concentration rates of these compounds, as with antibiotics. Acquired resistance is caused by mutation or overexpression of endogenous chromosomal genes, or by the acquisition of mobile genetic elements, such as plasmids, integrons, and transposons, which harbor resistance genes (VIJAYAKUMAR et al., 2018). Overexpression of efflux pumps is also a very common mechanism and confers resistance to antibiotics, QACs, and toxic compounds, as their primary function is to expel harmful compounds into the bacterial cell (JIANG et al., 2017). There are five main classes of efflux systems, (i) ATP-binding cassette carriers (ABC), (ii) drug metabolite carriers, including the small family of multidrug resistance (SMR), (iii) the large major facilitator superfamily (MFS), (iv) the resistance-nodulation-division (RND) family, and (v) the toxic multi-compound expulsion protein (MATE) (AHMAD et al., 2018). Mobile genetic elements, such as large plasmids carrying integron resistance determinants, are responsible for conferring resistance to both antibiotics and QACs. Class 1 integrons usually carry the gene, *qacEΔ1*, which encodes a partially deleted but functional efflux pump, in addition to the *sul1* gene, which confers resistance to sulfonamides. This fact points to the likelihood of cross-resistance between antibiotics and QACs, since these genes, which are in the same conserved segment of DNA, confer resistance to ATBs and QACs simultaneously (AMOS et al., 2018).

Benzalkonium chloride It is a cationic surfactant compound, also known as ADBACs are a mixture of (alkylbenzyltrimethylammonium chlorides. Structurally they consist of one hydrophobic and one hydrophilic region, and are easily soluble in water, alcohol and acetone.

Its use as biocide is very common in hospital environments (human-veterinary), in the disinfection of surfaces, skin and mucous membranes (BEIER et al., 2015). In the food processing area these compounds are used in order to ensure the microbiological safety of food products, however, the wide use in this area has raised great concern about the possible role of QACs in the dissemination of resistant isolates. In recent years, this problem has been frequently reported in gram-positive and gram-negative bacteria isolated from food (JIANG et al., 2015).

Cetyl-pyridinium chloride (CPC) is a quaternary ammonium compound recognized for its broad-spectrum antimicrobial activity and potent activities against fungi such as *Candida albicans*. Initially, the CPC was intended only as a germicidal agent for skin application, such as preparing patients for later major surgical procedures. Over time, new properties of CPC were discovered, widening its field of application (HAGAN et al., 1946; EDLIND et al., 2005). Currently, CPC is often used as an important component in the preparation of various personal care products such as toothpastes, lozenges and mouthwashes; in order to prevent plaque and treat gingivitis, as well as antiseptic in nasal decongestants (ZHANG et al., 2018; BEIER et al., 2016).

Cetyltrimethylammonium bromide (Cetrimide) or CTAB is a chemically surfactant or quaternary ammonium cationic surfactant compound. In the industrial area, CTAB is applied in the manufacture of hair conditioners and conditioners (JENNINGS et al., 2015). Due to its potent antibacterial property, it is used in water disinfection (JIN et al., 2015). In the field of molecular biology inputs, CTAB is a surfactant used for DNA isolation from tissues containing high amounts of polysaccharides (CLARKE, 2009).

1.7 Resistome and Pan-Resistome

Complete genome sequencing provides a range of applications, allowing for a more comprehensive analysis of the structure and content of microbial genomes (FORDE & O'TOOLE, 2013). This type of sequencing is useful for investigating the transmission of a microorganism, but also allows the investigation of the dissemination of the plasmids involved in the transmission of resistance genes (HAZEN et al, 2014), and the genetic differentiation that makes it possible to occupy these microorganisms in different ecological niches (RAMOS et al, 2014).

For *K. pneumoniae*, the first complete genome was performed in 2006 at the Genome Sequencing Center at the University of Washington in St. Louis. The genome was named *K. pneumoniae* subsp. *pneumoniae* MGH 78578. It includes a 5.6 Mbp chromosome, with a GC content of 57% (accession number CP000647.1) containing 5 plasmids. In addition, a number of studies with complete genome analysis of *K. pneumoniae* have been performed (WU et al., 2009, KUMAR et al., 2011, SNITHKIN et al., 2012, DOI, 2014, LEE et al., 2004, MATHERS et al. 2015), in which different objectives such as epidemiological analysis, resistance, clonality and *in vivo* evolution were proposed.

In Brazil, the complete genome of a *K. pneumoniae* producing KPC-2 (Kp13 - CP003999.1) was sequenced, but this strain was not related to the main clones disseminated in the country (e.g., ST11, ST340 and ST258) (RAMOS et al., 2014).

Resistance genes are DNA sequences that can be found in the bacterial chromosome or extrachromosomal elements (plasmids, integrons, transposons), conferring resistance to antimicrobials of various classes. These resistance genes may exist as cassette genes, which have a sequence that functions as a recombination site necessary for their incorporation into integrons. Its incorporation in this last element favors its expression, since the cassette gene lacks its own promoter (HALL, 2012; PARTRIDGE et al., 2009).

With advances in genomics new concepts have been established and incorporated into the area of bacterial resistance. For example, the study that comprises the identification and analysis of bacterial resistance determinants in a given ecosystem is called a resistor (PERRY & WRIGHT, 2014; VAN SCHAIK, 2015), whereas pan-resistance could be defined as the analysis of resistance genes, to different classes of antimicrobials present in bacteria belonging to the same genus and species, in different environments (FONDI et al., 2010; GILLINGS, 2013). In practice, the resistome provides us with information about the predominant resistance genotypes established by a bacterial population that makes up a particular environment that has been subjected to selective antimicrobial pressure (PERRY & WRIGHT, 2014; VAN SCHAIK, 2015). The earliest studies investigating bacterial resistance began to be performed in different parts of the world. For *K. pneumoniae* producing KPC the first resistomes have compared strains not belonging to CG258, isolated in Korea, USA and Italy (LEE et al., 2014) and specifically for CG258, the analysis of the resistance has not been done, the comparative analysis of the complete genome of *K. pneumoniae* strains belonging to CG258 (ST11, ST258, ST437, ST442), isolated in China, USA and Brazil, respectively (LIU et al., 2012; RAMOS et

al. 2014; CHEN et al., 2014; BOWERS et al., 2015; DELEO et al., 2014; CERDEIRA et al., 2018).

1.8 Transfer and mobilization of resistance genes

The dynamics of antimicrobial resistance involves numerous factors that interact and contribute to the adaptation of bacterial populations (MUNITA; ARIAS, 2016; PARTRIDGE et al., 2018). However, as key to the evolution of these microorganisms, one of the main factors involved is the ability of bacteria to mobilize resistance genes by plasmids, integrative conjugative elements, transposons, gene cassettes in integrons and insertion sequences (PARTRIDGE et al., 2018).

Plasmids that confer antimicrobial resistance were first described in 1960 by Watanabe and Fukasawa (1960) in Japan. Subsequently, numerous reports associated the worldwide spread of resistance genes to plasmids (RAMIREZ et al., 2014; CARATTOLI et al., 2015; FERNANDES et al., 2016; HARDIMAN et al., 2016; SUN et al., 2018). One of the ways to identify and classify these plasmids is based on the incompatibility, in which the plasmids belonging to the same incompatibility group (Inc) can not be stably propagated in the same cell line (DATTA; HUGHES, 1983; COUTURIER et al., 1988, ORLEK et al., 2017).

Nowadays, the genomic study has allowed to refine the identification of these groups of plasmid incompatibility, in which more than 110 groups and subgroups of incompatibility have already been reported based on the plasmid database of Enterobacteriaceae (PlasmidFinder) (CARATTOLI et al. 2014; ZETNER et al., 2017). Interest in the study of *K. pneumoniae* plasmids has increased, as the literature has focused attention on their sequencing (ZHAO et al., 2010; ORLEK et al., 2017), particularly of those plasmids associated with the presence of genes which encode the production of CTX-M extended spectrum β -lactamases (KIM; KO, 2018; SHEN et al., 2019) and carbapenemases (eg, KPC, IMP and NDM) (FENG et al., 2016; CERDEIRA et al., 2017; PASKOVA et al., 2018; CERDEIRA et al., 2019). In *K. pneumoniae* strains producing the *bla*_{KPC-2} gene, major incompatibility groups include: IncFII, IncFIA, IncFIB, IncN, IncX, IncR, IncHI1, IncI, IncA/C, IncP, IncU, IncW, IncL and ColE (CHEN et al., 2014; NAVON-VEENZIA; KONDRATYEVA; CARATTOLI, 2017).

The literature has also shown that these plasmids are undergoing rearrangement, which may favor the evolutionary success of the strain (HE et al., 2016; MANGAT et al., 2017). Indeed, the *bla*_{KPC-2} gene has been found in a hybrid incompatibility group, IncX3-IncU, as well as a set of genes involved in replication, mobilization, conjugation and other accessory genes,

similar to other hybrid plasmids (IncX3-IncU) identified in Brazil (AINODA et al., 2019). Other multireplicon plasmids have also been documented in these microorganisms carrying the *bla_{KPC-2}* gene, such as IncR-IncFII plasmids (FENG et al., 2018).

Such genomic approaches also revealed a great diversity of plasmids in Gram-negative and Gram-positive bacteria (ORLEK et al., 2017). Research on bacterial adaptation phenomena, both in antimicrobial resistance and fitness cost, shows that bacteria can carry small plasmids containing resistance genes, and the presence of these plasmids may in some way benefit their success in a given ecosystem (SAN MILLAN et al., 2015). Although adaptive phenomena have not been evaluated, studies have shown important examples of small plasmids associated with resistance to carbapenems (GALETTI et al., 2016; STOESEER et al., 2017; CERDEIRA et al., 2019).

Recently, small plasmids IncQ1 and Col-like (14,873 bp and 9,548 bp, respectively) carrying the *bla_{KPC-2}* gene were identified in clinical strains of *K. pneumoniae* recovered in 2011 and 2015, respectively, in Brazil (CERDEIRA et al., 2019).

The *bla_{KPC}* gene can be found in transposons located on plasmids, ensuring their dispersion not only among *K. pneumoniae* species, but also in other bacterial genera (CUZON; NAAS; NORDMANN, 2011). The transposon Tn4401 (transposon of the Tn3 family) is considered the most important epidemiologically carrying the gene *bla_{KPC}* (CUZON; NAAS; NORDMANN, 2011; CHERUVANKY et al., 2017). This transposon is composed of a gene encoding a transposase (*tnpA*), a resolvase (*tnpR*) and the *bla_{KPC}* gene flanked by the IS*Kpn7* (upstream) and IS*Kpn6* (downstream) insertion sequences. Between IS*Kpn7* and *bla_{KPC}* deletions of 68-255-bp have determined variants of this transposon (CHERUVANKY et al., 2017). To date, nine isoforms of Tn4401 (Tn4401*a*-Tn4401*i*) have been reported in the literature (CHEN et al., 2012; BRYANT et al., 2013; PECORA et al., 2015; BARANIAK et al., 2015; CHERUVANKY et al. 2017; ARAÚJO et al., 2018), of which Tn4401*b* and Tn4401*b* are the most widespread (MUNOZ-PRICE et al., 2013, CHERUVANKY et al., 2017).

In Brazil, the isoforms "a", "b", "c" and "i" have already been found (ANDRADE et al., 2011, ALMEIDA et al., 2012, PEREIRA et al. al., 2014; PÉREZ-CHAPARRO et al., 2014; ARAÚJO et al., 2018), however, the dissemination of the *bla_{KPC-2}* gene occurs mainly due to the dispersion of Tn4401*b* (PEREIRA et al., 2013).

Although Tn4401 is the most reported in association with the *bla_{KPC}* gene in strains of *K. pneumoniae* (CUZON; NAAS; NORDMANN, 2011; CHERUVANKY et al., 2017), other studies have increasingly demonstrated the frequency of the non-Tn4401 (NTE_{KPC}), also

associated with the dispersion of this resistance gene (CERDEIRA et al., 2017, ARAÚJO et al., 2018a, DE BELDER et al., 2018; MANAGEIRO et al., 2018; et al., 2019). These elements were first described in 2006 in China (SHEN et al., 2009), and were thereafter reported in South America (Brazil and Argentina) (GOMEZ et al., 2011, CERDEIRA et al., 2017).

Based on the insertion sequences located upstream to the *bla*_{KPC} gene, the NTE_{KPC} element can be divided into three groups: NTE_{KPC}-I (no insert), NTE_{KPC}-II (Δ *bla*_{TEM} gene insert) and NTE_{KPC}-III (Tn5563/IS6100 insert) (CHEN et al., 2014). The NTE_{KPC}-I element may further be classified according to upstream and / or downstream insertion sites of the IS26 sequence and in the presence of IS*Kpn8*, such as NTE_{KPC}-Ia, NTE_{KPC}-Ib, NTE_{KPC}-Ic and NTE_{KPC}-Id (CHEN et al., 2014). In the same way, NTE_{KPC}-II can be subdivided based on differences in Δ *bla*_{TEM} size and deletions in NTE_{KPC}-IIa, NTE_{KPC}-IIb, NTE_{KPC}-IIc and NTE_{KPC}-IId (CHEN et al., 2014; CERDEIRA et al. 2019).

The role of NTE_{KPC} in the global dissemination of KPC is still unknown; however, it is believed that this element has evolved from Tn4401 by recombination and/or insertion of other smaller mobile genetic elements since all the NTE_{KPC} structures described so far contain genetic remnants of Tn4401 (CHEN et al., 2014; CERDEIRA et al., 2019).

In addition to the important involvement of plasmids from different incompatibility groups and transposon Tn4401 in high-risk clones of *K. pneumoniae* (CHEN et al., 2014), other elements, such as integrons, have been associated with the acquisition and dissemination of resistance to antimicrobials (GILLINGS, 2014). Although integrons are not considered mobile elements in itself, because it does not present functions for automobility (DOMINGUES; SILVA; NIELSEN, 2012), this elements are able to incorporate ORFs (open reading frames), making them functional genes in the bacterial genome (ROWE-MAGNUS, 2009; CAMBRAY; GUEROUT; MAZEL, 2010). In general, the integron structure consists of a gene encoding an integrase (*intI*), a recombination site (*attI*) and a promoter, called P1 or P_{ant}, or more rarely two promoters (P1 and P2) (LÉVESQUE et al., 1995; DAVIES, 2007; GILLINGS, 2014). Integrase catalyzes the recombination between the *attI* site of the integron and the *attC* site associated with the ORFs, allowing the insertion or excision of one or more gene cassettes in the integron variable region (GILLINGS, 2014). In addition, some integrons may contain the *qacEΔ1* gene, which encodes an incomplete version of a protein that mediates resistance to certain detergents, the *sulI* gene encoding sulfonamide resistance, and an ORF, of unknown function (GILLINGS, 2014). A number of integron classes were identified based on the amino acid sequence of the integrases (*intI1*, *intI2*, *intI3*, *intI4* and *intI5*) (Mazel, 2006; Cambray; Guerout; Mazel, 2010),

which is the most prevalent and clinically important in studies of multidrug resistant bacteria (CAMBRAY; GUEROUT; MAZEL, 2010; ESCUDERO et al., 2015). The strains of *K. pneumoniae* carrying class 1 integrons became increasingly common (SANTOS et al., 2011), and their role in the spread of drug resistance became expressive (GHALY et al., 2017). Some reports have associated class 1 integrons with the presence of genes encoding extended-spectrum β -lactamases (eg, *bla*_{CTX-M-15}) (RUI et al., 2018), metallo- β -lactamases and oxacillinase (*bla*_{OXA}) (STALDER et al., 2012; WU et al., 2012) and resistance genes (e.g., *aac(6')Ib-cr*, *qnrA*, *qnrB*) (LI et al., 2013; RUI et al., 2018).

K. pneumoniae producing carbapenemases and ESBLs commonly present a multi-resistant phenotype and harbor several mobile genetic elements (NAVON-VENEZIA; KONDRATYEVA; CARATTOLI, 2017), disseminating antimicrobial resistance for two main reasons: (i) the genetic elements mentioned above can be transmitted vertically, and (ii) microorganisms have numerous opportunities to horizontally transfer these elements to other bacteria, not limited to intraspecific dissemination (NAVON-VENEZIA; KONDRATTAVA; CARATTOLI, 2017; PARTRIDGE et al., 2018).

1.9 *Klebsiella pneumoniae* belonging to the CG258

The plasmid-mediated *bla*_{KPC} gene has been widely spread among clinically significant members of the Enterobacteriaceae family, contributing with the establishment of predominant clones identified worldwide (MATHERS et al., 2015). Although the term "clone" represents the progeny of a bacterial cell that originates from asexual reproduction, implying that the same clonal lineage is formed by closely related bacterial isolates, which has recently diverged of a common ancestor (DIJKSHOORN et al., 2000), for prokaryotes we must consider that there is genomic plasticity due to deletion rearrangements, insertion sequences and genetic recombination. Thus, bacteria classified within the same clone could not be strictly genetically alike. Therefore, a bacterial clone could be better defined if we consider isolates indistinguishable or highly similar when characterized by molecular typing tools (SPRATT, 2004).

In order to characterize clonal lineages with epidemiological regards, use of DNA sequences of internal fragments of multiple housekeeping genes has been the base of the multilocus sequence typing (MLST) method, for typing of multiple loci. MLST database define sequence types (STs) using similarity in allelic profiles, whereas a clonal complex is a set of STs that are all believed to be descended from the same founding genotype (a single common

ancestor). Using the stringent group definition (e.g., 6/7 shared alleles), isolates in the group are considered to belong to a single clonal complex. More recently, the term clonal group has been used to define a group of unrelated STs forming smaller CCs connected through single locus variants (SLVs) (EWERS et al., 2014).

Pandemic KPC has occurred primarily by the dissemination of KPC-producing *K. pneumoniae* isolates belonging to the CG258, which include the sequence types ST258, ST11, ST340, ST437 and ST512 (MUNOZ-PRICE et al., 2013; CHEN et al., 2014; MATHERS et al., 2015). Specifically, in Brazil, more prevalent KPC-2-positive isolates have been clustered within ST11, ST340 and ST437, being considered endemic (ANDRADE et al., 2011). More recently, these STs have been isolated from farm animals and from urban aquatic environments (OLIVEIRA et al., 2014; CERDEIRA et al., 2016; CERDEIRA et al., 2017a, NASCIMENTO et al., 2017). Others KPC-2-producing *K. pneumoniae* STs reported in this region are ST16, ST17, ST25, ST70, ST101, ST133, ST423, ST442, ST443 and ST617 (SEKI et al., 2011; ANDRADE et al. 2011).

Most *K. pneumoniae* strains belonging to ST258, ST147, ST37 and ST14 have been reported as high-risk clones, since clinical, microbiological and genomic characteristics are associated with a MDR profile, global distribution, persistence for long periods of time, rapid dissemination among hosts, increased pathogenicity, ability to cause severe and/or recurrent infections, and unfavourable outcomes (Baquero et al., 2013, Mathers et al., 2015). Most likely high-risk clones have a genetic architecture that enhances their fitness by contributing to the Darwinian principle of survival of the fittest (HACKER & CARNIEL, 2001). High-risk clones in *K. pneumoniae* have a diverse arsenal of *bla* genes (for carbapenemases and/or ESBL) and other genes encoding to resistance to different antibiotics, a genetic repertoire for recombination and horizontal gene transfer of genetic elements, such as plasmids and transposons, as well as multiple types of plasmids; reflecting the complex epidemiology associated with *K. pneumoniae* (NAVON-VENEZIA; KONDRATYEVA; CARATTOLI, 2017). Therefore, understanding unique characteristics of CG258 and each of the epidemic STs may clarify the success of these clones and help in the elaboration of measures to prevent and control their dissemination (NAVON-VENEZIA; KONDRATYEVA; CARATTOLI, 2017).

1.10 Virulence factors in *Klebsiella pneumoniae*

Recently, the term “virulome” has been widely used *in silico* analyzes and is composed by main virulence factors genes. In this regard, in Gram-negative pathogens virulence factors are numerous and diverse (CLEGG; MURPHY, 2016; GIRAUD; YCHLIK; CLOECKAERT, 2017; KIM et al., 2019). Among these virulence factors, for *K. pneumoniae* the importance of fimbriae, adhesins, lipopolysaccharides (LPS), ureases, siderophores, polysaccharide capsules and biofilm formation are emphasized (CLEGG; MURPHY, 2016).

The polysaccharide capsule has represented the main virulence mechanism of *Klebsiella pneumoniae*, being associated with the evasion of the host immune system (LEE et al., 2017). Currently there are 79 described capsular types, which have been used to discriminate strains during clinical infections (PAN et al., 2015; CLEGG; MURPHY, 2016). The identification of capsular types can be performed by genotyping methods, through the characterization of genes responsible for polysaccharide capsule synthesis (*galF*, *cpsACP*, *wzi*, *wza*, *wzb* and *wzc*) (PAN et al., 2015). Serotypes K1, K2, K4 and K5 are highly virulent and are often associated with severe infections in humans and animals (BRISSE et al., 2009). Additionally, the importance of the antigenic portion of lipopolysaccharides as a virulence factor in *K. pneumoniae* should also be considered, especially in relation to severe diseases such as sepsis and pneumonia (EVRARD et al., 2010; HSIEH et al., 2012).

The type of capsular polysaccharide (CPS) is also a virulence-associated trait. CPS genes in *K. pneumoniae* strains are chromosomally encoded, and clustered in the *cps* genomic locus (BRISSE et al., 2013; WYRES et al., 2016). *K. pneumoniae* have been classified into distinct capsular (K) types, and currently over 77 K serotypes have been described (STRUVE et al., 2015). *K. pneumoniae* use a Wzy-dependent capsule synthesis process based on genes arrayed in a synthesis locus (K-locus), which is 10–30 kbp in size (PAN et al., 2015; CHUANG et al., 2006; WYRES et al., 2016). The K-locus includes a set of genes in the terminal regions encoding for the core capsule biosynthesis machinery (i.e., *galF*, *wzi*, *wza*, *wzb*, *wzc*, *gnd* and *ugd*). The central region is highly variable, encoding for specific sugar synthesis of the capsule, processing and export proteins, plus the core assembly components Wzx (flippase) and Wzy (capsule repeat unit polymerase) (PAN et al., 2015; WYRES et al., 2016).

The fimbriae also have great relevance in the development of infections, since they are related to the capacity of a bacterium to adhere in the surfaces of the host tissues (CLEGG; MURPHY, 2016; MARTIN; BACHMAN, 2018). In addition to all the virulence factors that make the microorganisms have a greater ability to cause damage, this fact can be more

complicated, since these strains may also present hypervirulent and hypermucoviscous characteristics (CLEGG; MURPHY, 2016; CATALÁN-NÁJERA; GARZA-RAMOS; BARRIOS-CAMACHO, 2017; SHANKAR et al., 2018; TABRIZI et al., 2018). The hypermucoviscosity of a bacterium is characterized by the formation of a viscous filament ≥ 5 mm after the elongation (with a loop) of a colony in an *in vitro* experiment called a string test (CATALÁN-NÁJERA; GARZA-RAMOS; BARRIOS-CAMACHO, 2017). In addition to hypermucoviscose, the strain may also be hypervirulent. Although there is no consensus among researchers on the definition of these strains, many consider that hypervirulent strains present at least 2 of the 3 microbiological characteristics: (i) positive string test, (ii) presence of the *rmpA* gene, and/or (iii) presence of aerobactin gene (CATALÁN-NÁJERA; GARZA-RAMOS; BARRIOS-CAMACHO, 2017).

Thus, it can be observed that virulence factors are encoded by important genes, such as those that act as transcriptional regulators of capsular polysaccharide synthesis (*rmpA*), lipopolysaccharide nucleus biosynthesis (*wabG*), allantoin metabolism (*allABCDRS*), urease (*ureADE*), hemolysin (*khe*), fimbriae type 1 (*fimABCDEFGHIK*) and type 3 (*mrkABCDEFHLJ*), aerobactin (*iucABCD/iutA*) and enterobactin (CATALÁN-NÁJERA; GARZA-RAMOS; BARRIOS-CAMACHO, 2017; WANG et al., 2018; CANEIRAS et al., 2018).

1.11 *Galleria mellonella* as model for *in vivo* virulence assay

Galleria mellonella, also known as large wax moth, has a wide geographic distribution and is now considered a pest for beekeeping, feeding on pollen and wax produced and stored in bee hives. *Galleria mellonella* is an invertebrate animal that possesses an immune system constituted by innate and humoral response, presenting several elements similar to the vertebrate immune system, including phagocytic cells and the production of antimicrobial peptides, becoming a good model of infections for evaluation of the activity, *in vivo*, of antibacterials and the interaction between host and antibiotic (LEUKO AND RAIVO, 2012; BENTHALL et al., 2015). In addition, the use of *G. mellonella* presents advantages over other animal models, due to the low maintenance cost, easy handling, the possibility of the larvae being maintained at 37°C, an important characteristic for pathogen evaluation and apparently related to the minimization of ethical factors related to manipulation and experimental animals (PELEG et al., 2009). Currently, this animal model has been used for *in vivo* evaluation of the

efficacy of antibiotics to multiresistant bacteria (MRs), virulence, compounds with a synergistic potential for antibiotic use, and comparative evaluation of susceptibility of strains MRs *in vitro* versus *in vivo* (BENTHALL et al, 2015; MOURA et al, 2017).

In Brazil, antimicrobial resistance in clinically significant bacterial pathogens has been documented to be more challenging than in developed countries (ROSSI, 2011). On the other hand, convergence of virulence and resistance has begun to be reported, mainly in endemic lineages of *K. pneumoniae* belonging to the CG258. Therefore, in this investigation, using a genomic approach, we have performed a meticulous virulome and resistome analysis of KPC-2- and CTX-M-producing *K. pneumoniae* strains belonging to CG258, circulating at the human-animal-environment interface, with further *in vivo* virulence evaluation using a *Galleria mellonella* infection model. Considering that MDR *K. pneumoniae* is a transboundary problem, a further investigation was conducted in deep, using a comparative genomic analysis that included data from *K. pneumoniae* circulating in South America countries.

1.12 Comparative genomics

Comparative genomics is the direct comparison of the genetic material of one organism against another in order to obtain a better biological understanding between species (SIVASHANKARI & SHANMUGHAVEL, 2007). This approach has been widely used in the determination of the function of genes and non-coding regions of the genome, resistome, virulome, as well as to characterize the occurrence of evolutionary events and establish phylogenetic relationships (RUST et al., 2002; HOLT et al., 2015; WYRES et al., 2016; CERDEIRA et al., 2018; LAM et al., 2018; HEINZ, et al., 2019).

2. OBJECTIVES

The aim of this study was to perform a genomic comparative analysis of KPC-2 and/or CTX-M-type-producing *Klebsiella pneumoniae* isolates belonging to the endemic high-risk clonal group CG258, circulating at the human-animal-environment interface, in Brazil and South America.

1. To perform the assembly and annotation of *K. pneumoniae* genomes obtained by long- and/or -short reads sequencing technology;
2. To characterize plasmids carrying *bla*_{KPC-2} and *bla*_{CTX-M} genes harbored by *K. pneumoniae* of the CG258;
3. To identify and characterize genetic elements contributing to mobilization and trasnposition of *bla*_{KPC-2} genes in *K. pneumoniae* of the CG258;
4. To identify and analyze the antimicrobial resistome of *K. pneumoniae* lineages belonging to the CG258;
5. To identify and analyze the virulome of *K. pneumoniae* lineages belonging to the CG258;
6. To evaluate, *in vivo*, the virulent behavior of *K. pneumoniae* lineages belonging to the CG258 in invertebrate infection model;
7. To identify virulence biomarkers for servere *K. pneumoniae* infections.

3. PUBLISHED WORKS

Results and discussion of data obtained from this thesis are presented in the format of manuscripts, which were published in scientific journals. Six manuscripts were published, as main author: i) **Manuscript I** (Genome Announcement) – Draft Genome Sequence of a Hospital-Associated Clone of *Klebsiella pneumoniae* ST340/CC258 Coproducing RmtG and KPC-2 Isolated from a Pediatric Patient; ii) **Manuscript II** (Journal Global of Antimicrobial Resistance) – Draft genome sequence of a CTX-M-15-producing *Klebsiella pneumoniae* sequence type 340 (clonal complex 258) isolate from a food-producing animal; iii) **Manuscript III** (Journal Global of Antimicrobial Resistance) – Draft genome sequence of an environmental multidrug-resistant *Klebsiella pneumoniae* ST340/CC258 harbouring *bla*_{CTX-M-15} and *bla*_{KPC-2} genes; iv) **Manuscript IV** (Diagnostic Microbiology and Infectious Diseases) – IncX3 plasmid harbouring a non-Tn4401 genetic element (NTE_{KPC}) in a hospital-associated clone of KPC-2-producing *Klebsiella pneumoniae* ST340/CG258; v) **Manuscript V** (Antimicrobial Agents and Chemotherapy) – Small IncQ1 and Col-Like Plasmids Harboring *bla*_{KPC-2} and Non-Tn4401 Elements (NTE_{KPC}-IId) in High-Risk Lineages of *Klebsiella pneumoniae* CG258; vi) **Manuscript VI** (BioRxiv) – Yersiniabactin, Colibactin and Wider Resistome Contribute to Enhanced Virulence and Persistence of KPC-2-Producing *Klebsiella pneumoniae* Clonal Group 258 in South America.

Six further manuscripts were published as co-author: i) **Appendix D** – Draft genome sequences of KPC-2- and CTX-M-15-producing *Klebsiella pneumoniae* ST437 isolated from a clinical sample and urban rivers in São Paulo (Journal Global of Antimicrobial Resistance); ii) **Appendix E** – Insights into a novel Tn4401 deletion (Tn4401i) in a multidrug-resistant *Klebsiella pneumoniae* clinical strain belonging to the high-risk clonal group 258 producing KPC-2 (International Journal of Antimicrobial Agents); iii) **Appendix F** – Hypervirulence and biofilm production in KPC-2-producing *Klebsiella pneumoniae* CG258 isolated in Brazil (Journal of Medical Microbiology); iv) **Appendix G** – Detection of ISEcp1-associated *bla*_{CTX-M-15} -mediated resistance to colistin in KPC-producing *Klebsiella pneumoniae* isolates (International Journal of Antimicrobial Agents); v) **Appendix H** – Draft genome sequence of a KPC-2-producing *Klebsiella pneumoniae* ST340 carrying *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes: a rich genome of mobile genetic elements and genes encoding antibiotic resistance (Journal Global of Antimicrobial Resistance); vi) **Appendix I** – International high-risk clones of *Klebsiella pneumoniae* KPC-2/CC258 and *Escherichia coli* CTX-M-15/CC10 in urban lake waters (Science of the Total Environment).

Additionally, tree manuscripts are in submission process for *Microbial Genomics*, *Frontiers Microbiology* and *Nature Microbiology* journals. Finally, others 39 manuscripts were published during the period of this study.

3.1 Manuscript I

Draft Genome Sequence of a Hospital-Associated Clone of *Klebsiella pneumoniae* ST340/CC258 Coproducing RmtG and KPC-2 Isolated from a Pediatric Patient

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We report here the draft genome sequence of a *Klebsiella pneumoniae* strain 1194/11, belonging to the hospital-associated sequence type 340 (ST340; clonal complex CC258), isolated from a catheter tip culture from a pediatric patient. The multidrug-resistant strain coproduced the 16S rRNA methyltransferase rRNA RmtG and β -lactamases KPC-2 and CTX-M-15.

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Klebsiella pneumoniae is a leading cause of nosocomial infection. In this regard, clinical strains exhibiting a multidrug-resistant (MDR) profile have been associated with the persistence of clonally related sequence types (STs), clustered in high-risk complexes (1). In this regard, most KPC-2-producing *K. pneumoniae* strains belong to the clonal complex 258 (CC258), which includes ST11, ST258, ST340, and ST437 (1). In Brazil, strains of *K. pneumoniae* ST340 coproducing KPC-2 and CTX-M-15 have been found in clinical settings and aquatic ecosystems (2–4). Here, we present the draft genome sequence of an MDR ST340 *K. pneumoniae* strain coproducing the 16S rRNA methyltransferase rRNA RmtG and β -lactamases KPC-2 and CTX-M-15.

K. pneumoniae strain 1194/11 was isolated in 2011 from a catheter tip culture from a pediatric patient admitted to a children's hospital in São Paulo, southern Brazil (5). The strain was collected at the Instituto Adolfo Lutz (IAL), which serves as a state reference laboratory and receives multidrug-resistant Gram-negative pathogens on an ongoing basis. The isolate belonged to ST340/CC258 and displayed resistance to gentamicin, tobramycin, amikacin, arbekacin, neomycin, apramycin, ceftazidime, cefotaxime, cefepime, ertapenem, and meropenem (5).

The genomic DNA of *K. pneumoniae* strain 1194/11 was sequenced using an Illumina MiSeq sequencing platform, which produced 1,824,432 paired-end reads at 100× total coverage. *De novo* assembly was performed using the A5-MiSeq pipeline (6), and the assembled contigs were annotated using NCBI Prokaryotic Genome Annotation Pipeline version 3.2. In total, there were 5,643 protein-coding genes, 123 RNA-coding genes (81 tRNAs, 27 rRNAs, and 15 noncoding RNAs [ncRNAs]), and 108 pseudogenes, with a G+C content of 56.7% and estimated 5,702,010-bp genome size.

The resistome of *K. pneumoniae* strain 1194/11 was analyzed using ResFinder 2.1 (7), identifying *aac(3)-IId*, *rmtG*, and *aadA2* aminoglycoside resistance genes; *bla*_{CTX-M-15}, *bla*_{SHV-11},

*bla*_{KPC-2}, and *bla*_{TEM-1B} β -lactam resistance genes; the *sul1* sulfonamide resistance gene; the *tet(D)* gene (conferring resistance to tetracycline); trimethoprim resistance genes *dfrA12* and *dfrA25*; the fosfomycin resistance *fosA*-like gene, and plasmid-mediated quinolone resistance (PMQR) genes *oqxA*, *oqxB*, and *qnrB2*.

Accession number(s). The genome sequence of *Klebsiella pneumoniae* 1194/11 has been deposited in the GenBank database under the accession number [LYZC000000000](https://www.ncbi.nlm.nih.gov/nuclseq/LYZC000000000).

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Genome Note

Draft genome sequence of a CTX-M-15-producing *Klebsiella pneumoniae* sequence type 340 (clonal complex 258) isolate from a food-producing animal Louise Cerdeira^a, Ketrin C. Silva^b, Miriam R. Fernandes^a, Susan lenne^c, Tiago A. de Souza^c, Doroti de Oliveira Garcia^d, Andrea M. Moreno^b, Nilton Lincopan^{a,e,*}^a Department of Clinical Analysis, School of Pharmacy, Universidade de São Paulo, São Paulo, Brazil^b Department of Preventive Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil^c Genome Investigation and Analysis Laboratory (GENIAL), Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil^d Center of Bacteriology, Instituto Adolfo Lutz, São Paulo, Brazil^e Department of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil

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ABSTRACT

Klebsiella pneumoniae carrying bla_{CTX-M-15} have been widely disseminated in hospital settings. In this regard, most clinically important strains belong to clonal complex 28 (CC258), which includes sequence type 340 (ST340). In this study, we present the draft genome sequence of a CTX-M-15-producing ST340 *K. pneumoniae* strain isolated from a food-producing animal in Brazil.

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Klebsiella pneumoniae is a leading cause of nosocomial infection worldwide. In this regard, their occurrence has been associated with the ability to acquire resistance to broad-spectrum cephalosporins and carbapenems, mediated by the production of extended-spectrum β -lactamases (ESBLs), mostly CTX-M-15, and *K. pneumoniae* carbapenemases (KPCs), respectively [1]. Indeed, the production of these enzymes has contributed to the wide dissemination of international high-risk clonal lineages among *K. pneumoniae* isolates, such as clonal complex 258 (CC258), which includes the predominant sequence type 258 (ST258) and, to a lesser extent, ST11, ST340, ST437 and ST512, which are single-locus variants of ST258 [2]. In Brazil, the pandemic high-risk CC258 has contributed to the nationwide dissemination of multidrug-resistant *K. pneumoniae* strains in

hospital settings, and their extrahospital spread to urban rivers has recently been documented [1].

In 2012, during a surveillance study conducted to assess the occurrence of ESBL-producing bacteria in swine production, a ceftiofur-resistant CTX-M-15-producing *K. pneumoniae* (strain 606B), belonging to ST340, was recovered from a faecal swab collected from a 45-day-old pig with diarrhoea on a commercial farm located in Minas Gerais State, southeastern Brazil. Total genomic DNA of *K. pneumoniae* 606B was sequenced using the Illumina MiSeq sequencing platform (Illumina Inc.). Here we present the first draft genome sequence of a CTX-M-15-producing ST340 *K. pneumoniae* strain isolated from a food-producing animal in Brazil, which can be used as a reference sequence for comparative analysis among high-risk clonal lineages of *K. pneumoniae* isolated from humans and animals.

A total of 2811266 paired-end reads (300 bp read length) were generated with 150 $\times$ coverage. The sequence reads were initially de novo assembled using A5-MiSeq pipeline [3] and SPAdes v.3.5 [4]. The draft genome sequence of *K. pneumoniae*

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6068 consisted of 91 contigs, which were merged and manually curated using Geneious software v.R9 (Biomatters Ltd., Auckland, New Zealand) [5]. The genome sequence was automatically annotated using the NCBI Prokaryotic Genome Annotation Pipeline v.3.2. We identified 5238 coding sequences, 45 pseudo-genes, 82 tRNA, 26 rRNA and 57.4% GC content. The resistome was analysed using ResFinder 2.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>) and the aminoglycoside resistance genes *aadA2*, *aac(3)-IId*, *strA*, *aph(3')-Ia* and *strB*, the β -lactam resistance genes *bla_{TEM-1A}*, *bla_{SHV-11}* and *bla_{CTX-M-15}*, the sulphonamide resistance genes *sul1* and *sul2*, the tetracycline resistance genes *tetA* and *tetD*, the trimethoprim resistance genes *dfrA14* and *dfrA12*, the fosfomycin resistance gene *fosA*, the phenicol resistance gene *florR*, the macrolide resistance gene *mphA*, and the fluoroquinolone resistance genes *qnrS1*, *oqxB* and *oqxA* were identified. Plasmid classification was carried out in silico by blastn against the PlasmidFinder 1.3 database (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) and two plasmids belonging to IncR and ColRNAI incompatibility groups were detected.

In summary, these findings confirm the presence of multidrug-resistant CTX-M-15-producing *K. pneumoniae* belonging to the high-risk CC258 in food-producing animals in Brazil. To our knowledge, this is the first draft genome sequence of a CTX-M-15-producing *K. pneumoniae* ST340 isolated from a food-producing animal. Further genomic analysis of clonally related ST340 *K. pneumoniae* strains isolated from humans and urban rivers in Brazil are currently underway to elucidate epidemiological aspects regarding dissemination and mobilisation of clinically important antibiotic resistance genes among different hosts and ecosystems.

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under accession no. LYMZ000000000. The version described in this paper is the first version: LYMZ010000000.

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Competing interests

None declared.

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Genome Note

Draft genome sequence of an environmental multidrug-resistant *Klebsiella pneumoniae* ST340/CC258 harbouring *bla*_{CTX-M-15} and *bla*_{KPC-2} genes Louise Cerdeira^{a,*}, Miriam R. Fernandes^a, Susan lenne^b, Tiago A. Souza^b,
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ABSTRACT

Anthropogenic activities, including the release of wastewater and sewage from hospitals, have contributed to the contamination of aquatic environments, raising a concern to public health. In this study, we present the first draft genome sequence of a *Klebsiella pneumoniae* strain (Kp171, TIET-4200) belonging to the high-risk hospital-associated clonal lineage ST340/CC258, which was recovered from a water sample collected in an urban river in Brazil.

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Dissemination of hospital-associated multidrug-resistant (MDR) bacteria to the environment is a public health issue worldwide. In this regard, several human activities have contributed to the release of chemical and biological agents into aquatic environments, mainly in metropolitan areas. Indeed, MDR bacteria have been identified in urban rivers, most likely due to hospitals not treating their wastewater before disposing of it into the general sewage collecting system, raising a concern to public health [1–3]. Therefore, in urban areas, rivers can act as reservoirs of MDR pathogens, facilitating their transmission to humans and animal-exposed through drinking water and/or recreational activities. We have recently reported the isolation of KPC-2-producing *Klebsiella pneumoniae* strains belonging to the high-risk clonal complex CC258 in urban rivers in Brazil [4]. In this study, we present the first draft genome sequence of the KPC-2/CTX-M-15-

producing *K. pneumoniae* Kp171 (TIET-4200) strain, belonging to the high-risk hospital-associated clonal lineage ST340/CC258.

K. pneumoniae Kp171 displayed resistance to several antibiotics, including cefotaxime, ceftriaxone, ceftazidime, cefepime, ertapenem, imipenem, meropenem, ciprofloxacin, trimethoprim/sulfamethoxazole and gentamicin. Total genomic DNA was sequenced using an Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA), which produced 700 444 paired-end reads with a 50× total coverage. De novo assembly was performed using the A5-miseq pipeline [5] and Geneious v.R9 (Biomatters Ltd., Auckland, New Zealand). A total of 273 contigs were generated; following improved genome assembly by removing redundant contigs, the number of contigs was reduced to 201, with a G + C content of 57.2% and ca. 5.5 Mb genome size. The NCBI Prokaryotic Genome Annotation Pipeline v.3.2 was used for genome annotation, resulting in 5665 protein-encoding genes, 123 RNA-encoding genes (78 tRNAs, 31 rRNAs and 14 ncRNAs) and 291 pseudogenes.

The resistome was analysed using ResFinder 2.1. (<https://cge.cbs.dtu.dk/services/ResFinder/>) and the following resistance genes were identified: *aac(3)-IId* and *aadA2* (aminoglycoside resistance); *bla*_{CTX-M-15}, *bla*_{SHV-11}, *bla*_{KPC-2} and *bla*_{TEM-1B} (β-lactam

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resistance); *sul1* (sulphonamide resistance); *tet(D)* (tetracycline resistance); *dfrA12* and *dfrA25* (trimethoprim resistance); *fosA*-like (fosfomycin resistance); and *oqxA*, *oqxB* and *qnrB2* (fluoroquinolone resistance). Multilocus sequence typing (MLST) using MLST 1.8 (<https://cge.cbs.dtu.dk/services/MLST/>) identified sequence type ST340. Four plasmid incompatibility groups [IncR, IncN, IncFIB(K) and IncFII(K)] were identified by PlasmidFinder 1.3. (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>).

To our knowledge, this is the first draft genome sequence of an environmental KPC-2/CTX-M-15-producing *K. pneumoniae* ST340/CC258 isolated from an urban river. Further genomic analyses of clonally related (ST340) *K. pneumoniae* strains isolated from humans and animals in Brazil are currently underway in order to elucidate epidemiological aspects regarding the dissemination and mobilisation of clinically important antibiotic resistance genes among different hosts and ecosystems.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession no. MJBQ00000000. The version described in this paper is version MJBQ01000000.

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Competing interests

None declared.

Ethical approval

Not required.

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3.4 Manuscript IV

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IncX3 plasmid harboring a non-Tn4401 genetic element (NTE_{KPC}) in a hospital-associated clone of KPC-2-producing *Klebsiella pneumoniae* ST340/CG258

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ABSTRACT

IncX-type plasmids have achieved clinical significance for their contribution in the dissemination of genes conferring resistance to carbapenems (most *bla*_{KPC}- and *bla*_{NDM}-type genes) and polymyxins (*mcr*-type genes), both antibiotics considered last resort for multidrug-resistant Gram-negative infections. In this study, we report the identification and complete sequence analysis of an IncX3 plasmid (designated pKP1194a) carrying a non-Tn4401 genetic element (NTE_{KPC}) of *trpR-trpA* (partial)-*bla*_{KPC-2}-*ΔISKpn6/traN*, originating from a hospital-associated lineage of *K. pneumoniae* belonging to the ST340/CG258, with epidemiological link to Brazil.

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The plasmid-mediated *bla*_{KPC} gene has been widely spread among clinically significant members of the Enterobacteriaceae family, contributing with the establishment of predominant clones identified worldwide (Mathers et al., 2015). In this regard, pandemic KPC has occurred primarily by the dissemination of KPC-producing *K. pneumoniae* isolates belonging to the clonal group (CG) CG258, which include the sequence types ST258, ST11, ST340, ST437, and ST512 (Chen et al., 2014; Mathers et al., 2015). Dissemination of *bla*_{KPC} has also been supported by the horizontal transfer of Tn4401-type transposons and plasmids. Tn4401 harbors *trpA* encoding a transposase, *trpR* encoding resolvase, and 2 insertion sequence elements (ISKpn7 and ISKpn6) bracketing the *bla*_{KPC} gene (Naas et al., 2008). To date, 8 Tn4401 isoforms from Tn4401a to Tn4401h have been identified, which differ by a 68–255-bp deletions upstream of *bla*_{KPC} relative to Tn4401b as the prototype sequence (Cheruvanky et al., 2017; Kim et al., 2017; Naas et al., 2012). In addition, other non-Tn4401 elements (NTE_{KPC}) have been also reported (Chen et al., 2014). Although, several different incompatibility groups of KPC-

containing plasmids have been identified in the CG258, including, IncA/C, IncF, IncI, IncN, IncP, IncR, IncX and ColE1, molecular studies have showed that IncF plasmids have been key vectors responsible for spreading KPC genes among *K. pneumoniae* isolates, contributing to the global success of high-risk clones (Chen et al., 2014; Mathers et al., 2015). More recently, IncX-type plasmids have achieved clinical significance worldwide for their contribution in the dissemination of genes conferring resistance to carbapenems (most *bla*_{KPC}- and *bla*_{NDM}-type genes) and polymyxins (*mcr*-type genes), both antibiotics considered last resort for multidrug-resistant Gram-negative infections (Bustamante and Iredell, 2017; Campos et al., 2015; Dobiasova and Dolejska, 2016; Fernandes et al., 2016; Fortini et al., 2015; Ho et al., 2013; Kim et al., 2017; Partridge et al., 2015). In this study, we report the identification and complete sequence analysis of an IncX3 plasmid (designated pKP1194a) carrying *bla*_{KPC-2}, originating from a hospital-associated lineage of *K. pneumoniae* belonging to the ST340/CG258, with epidemiological link to Brazil (Pereira et al., 2013).

During a Brazilian surveillance study conducted to investigate antibiotic resistance determinants for broad-spectrum beta-lactams and aminoglycosides in clinical isolates of *K. pneumoniae* collected at the

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Table 1
Genetic characteristics of *K. pneumoniae* strain 1194/11 (ST340) carrying IncX3 plasmid-mediated bla_{KPC-2}.

Parameters analyzed	Genetic characteristics
Genome size (bp)	5,702,010
GC content %	56.7
CDS	5643
rRNA (n)	27
tRNA (n)	81
scgMLST 634 genes % ID (diff %)	99.7% (0.3%)
Resistance genes	bla _{KPC-2} , bla _{SHV-11} , bla _{TEM-1A} , bla _{TEM-1B} , bla _{CTX-M-15} , bla _{OXA-48}
	β-lactams
	Phenicol
	Rifampicin
	Sulphonamides
	Tetracyclines
	Trimethoprim
	Aminoglycoside
	Fluoroquinolone
	Fosfomycin
	QRDR mutation
	Heavy metal
Efflux pumps and regulators	acrA, acrB, acrR, envR, fs, marA, marR, oqxA, oqxB, oqxC, ramA, ramR, raxA, rob, sdiA, soxR, soxS
Virulence genes	fyuA, irp1, irp2, mrkA, mrkB, mrkC, mrkD, mrkF, mrkH, mrkI, mrkJ, ybtA, ybtE, ybtF, ybtG, ybtH, ybtI, ybtJ, ybtK, ybtL, ybtM, ybtN, ybtO, ybtP, ybtQ, ybtR, ybtS, ybtT, ybtU, ybtV, ybtW, ybtX, ybtY, ybtZ
Associated K type	50
Incompatibility group	ColRNAI, IncHI1B, IncR, IncU, IncX3

Instituto Adolfo Lutz (a state reference laboratory in São Paulo), a MDR *K. pneumoniae* strain (1194/11) isolated from a catheter tip culture of a pediatric patient admitted to a children's hospital was characterized as KPC-2 producer, belonging to the ST340 (CG258) (Bueno et al., 2013). The total genomic DNA of strain 1194/11 was extracted to construct a Nextera XT DNA library, which was sequenced using the MiSeq platform (Illumina, San Diego, CA, USA). The sequence reads were *de novo* assembled using A5-MiSeq pipeline and Geneious version 8.1.8 (<http://www.geneious.com>). Data from whole-genome sequence analysis are summarized in Table 1 (Cerdeira et al., 2016a). In this regard, WGS revealed that bla_{KPC-2} was carried on an IncX3 plasmid (pKP1194a) (Fig. 1A) (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>), harbored on a non-Tn4401 element (NTE_{KPC}), similar to NTE_{KPC}-Ic (Chen et al., 2014), presenting a partial ISKpn6 (ΔISKpn6/traN) with the associated left IR (IRL), a partial trpA gene and an intact trpR gene, upstream and downstream of the bla_{KPC-2} gene, respectively (Fig. 1B). Interestingly, in a recent study reporting the emergence of KPC-2-producing *K. pneumoniae*

ST340/CG258 in Peru, the bla_{KPC-2} gene was also located within a non-classical Tn4401 structure (Homa et al., 2017), which confirms previous descriptions regarding the diversity of genetic environments surrounding bla_{KPC} genes (Wang et al., 2016), thereby highlighting the ease of dissemination of this gene worldwide owing to its ability to be spread through different genetic supports (Homa et al., 2017). Indeed, different elements harboring the bla_{KPC-2} gene, carried by IncX3 plasmids, have been reported in *K. pneumoniae* strains isolated from patients in United States (Ho et al., 2013) and Korean (Kim et al., 2017) hospitals, as compared to classical Tn4401 structure (Fig. 1B).

The IncX3 pKP1194a plasmid (GenBank accession no. KX756453) is 45,674 bp in size, with G + C content of 45.9%, 78 protein-encoding genes, and a basic core structure that include sequences encoding replication, partitioning and type IV secretion system (T4SS), which are known to allow their own conjugative transfer. Indeed, the pKP1194a plasmid was successfully transferred to *E. coli* C600STR strain by conjugation (broth mating method), where transconjugants were selected on

Fig. 1. In A, structure of the IncX3 pKP1194a plasmid (GenBank accession no. KX756453), identified in a high-risk hospital-associated clone of *Klebsiella pneumoniae* sequence type 340 (CG258), with epidemiological link to Brazil. The pKP1194a plasmid harbored a non-Tn4401 genetic element (NTE_{KPC}) presenting a partial ISKpn6 (ΔISKpn6/traN) with the associated left IR (IRL), a partial trpA gene and an intact trpR gene, upstream and downstream of the bla_{KPC-2} gene, respectively. In B, comparative analysis of Transposon Tn4401, ΔTn4401a and NTE_{KPC} elements carried by IncX3 plasmids identified in KPC-2-producing *K. pneumoniae* strains isolated in Brazil (NTE_{KPC} in pKP1194a), United States (ΔTn4401a in pKPC-NY79) and Korea (ΔTn4401a in pKPC_Kp01).

Fig. 2. Comparison of IncX3 plasmid carrying the *bla*_{KPC-2} gene from hospital-associated lineages of *K. pneumoniae* ST340 (purple), *K. pneumoniae* ST442 (green) and *E. coli* ST224 (pink), isolated in Brazil. High levels of sequence identity between plasmid sequences are indicated as ring with colored blocks, whereas no or weak sequence identity is shown as white blocks.

MacConkey agar plates containing streptomycin (2 µg/mL) and ertapenem (2 µg/mL), and no other resistance genes were identified. For the assembly of the pKP1194a plasmid, we have performed a hybrid *de novo* strategy, as previously described (Cerdeira et al., 2011), combining 2 different sets of contigs generated from 2 assembler software (i.e., SPAdes 3.5.1 and Velvet 1.2.12), then the result was blasted in NCBI confirming 99% sequence identity with a *bla*_{KPC-2}-harboring plasmid, with a no defined typing, designed pKP13d (GenBank accession no. CP003997), previously found in a *Klebsiella pneumoniae* ST442 (Ramos et al., 2014). We have used this sequence as reference, confirming circular plasmid structure *in silico*, whereas plasmid size was confirmed by PFGE. Additionally, pKP1194a plasmid shares >95% sequence identity with plasmid pECO37P1 (GenBank accession no. KU963389), recently identified in a KPC-2-producing *E. coli* belonging to ST224/CC58, in Brazil (Fig. 2), which supports the hypothesis that members of this plasmid lineage might play an important role in mediating the horizontal transmission of *bla*_{KPC-2} among hospital-associated clones of *K. pneumoniae* and *E. coli*. In fact, IncX3 plasmids carrying *bla*_{KPC} genes have been reported in *K. pneumoniae* ST258 from United States (Ho et al., 2013) and Australia (Partridge et al., 2015), and in *K. pneumoniae* ST512 from Italy (Fortini et al., 2015). More recently, the clonal spread of *K. pneumoniae* ST307 carrying self-transferable IncX3-type plasmid harboring *bla*_{KPC-2} was reported in South Korea (Kim et al., 2017). On the other hand, IncX3-type plasmids have been associated with the dissemination of the *bla*_{NDM-1} gene in China (Yang et al., 2015). So, most likely IncX3-type plasmids have contributed with the predominance of ST340 in Brazil and other countries in South America (Pereira et al., 2013; Martins et al., 2015; Horna et al., 2017). In fact, the ST340 (a single-locus variant of ST258) is a high-risk lineage with epidemiological link to Brazil (Pereira et al., 2013; Martins et al., 2015), since it has been not limited to nosocomial settings, being further identified in urban rivers and food producing-animals (Oliveira et al., 2014; Cerdeira et al., 2016b).

IncX plasmids are self-transmissible replicons that belong to an underestimated plasmid family that include at least 6 subtypes (IncX1 to IncX6) (Du et al., 2016). In Brazil, although the epidemiology of IncX-type plasmids has been unknown, recent sequencing studies have identified IncX3 and IncX4 as plasmids responsible for mobilization of *bla*_{NDM-1} and *mcr-1* genes, respectively, in *Escherichia coli*, suggesting a broad-host range of IncX plasmids in this region (Campos et al., 2015; Fernandes et al., 2016, 2017; Monte et al., 2017; Sellera et al., 2017). So, IncX3 plasmids might play an important role in potentiating the spread of *bla*_{KPC} genes and other pandemic genes, in clinically significant members of the Enterobacteriaceae family.

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Competing Interests

None declared.

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Small IncQ1 and Col-Like Plasmids Harboring *bla*_{KPC-2} and Non-Tn4401 Elements (NTE_{KPC}-Ild) in High-Risk Lineages of *Klebsiella pneumoniae* CG258

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A retrospective genomic study led to the identification of two carbapenem-resistant *Klebsiella pneumoniae* isolates (KPN535 and KPC45) carrying *bla*_{KPC-2} genes on nonconjugative plasmids. These isolates were recovered in 2011 and 2015 from rectal swab cultures of inpatients from two hospitals in Brazil and belonged to the hospital-associated lineages ST340 and ST11 (CG258).

For both *K. pneumoniae* strains, total genomic DNA was extracted and sequenced using long-read (PromethION; Oxford Nanopore) and short-read (NextSeq; Illumina) sequencing technologies, with further hybrid *de novo* assembly using Unicycler (v0.4.0), which resolved complete circularized sequences of chromosome and plasmids (1, 2).

Interestingly, in KPN535 and KPC45, the *bla*_{KPC-2} gene was found on small IncQ1 and Col-like (Col-KPC) plasmids named pKPN535a and pKPC45a, respectively (Fig. 1A and B). The pKPN535a plasmid is 14,873 bp in size, with a G+C content of 54.6%, containing the *higA* antitoxin-encoding gene, genes encoding ParE/RelE-superfamily toxins, and the *aph(3')*-Vla aminoglycoside resistance gene. On the other hand, Col-KPC is 9,548 bp in size (with a G+C content of 52.3%), sharing >90% identity with the Col (MGD2) plasmid (NC_003789) (3), and carrying *relaxase* and *mobC* genes.

Both plasmids contain a variant of non-Tn4401 elements (NTE_{KPC}), designated NTE_{KPC}-Ild, with the gene array *tnpR*-*Δbla*_{TEM}-*bla*_{KPC-2}-*ΔISKpn6*/*traN* (Fig. 1C). Interestingly, in the two plasmids, NTE_{KPC}-Ild elements were flanked by two identical 243-bp direct repeats, whereas pKPN535a carries a third 243-bp repeat downstream *repC*. The NTE_{KPC}-Ild have been separated in three groups according to the absence or presence of *bla*_{TEM}, where the second group (NTE_{KPC}-II) includes variants that have a truncated *bla*_{TEM} gene (4, 5); in contrast, all NTE_{KPC} structures described to date (including NTE_{KPC}-Ild) contain genetic remnants of Tn4401, which is consistent with their having evolved from Tn4401 by recombination and/or insertion of other smaller mobile genetic elements. By using NCBI blast against the NR database, we noted that similar NTE_{KPC}-Ild structures (100% identity) have been recently identified in *Klebsiella aerogenes* from Brazil (GenBank accession numbers MG786907 and MH000708). Therefore, although no additional information is available, the possibility that *Enterobacteriales* carrying *bla*_{KPC-2} on NTE_{KPC}-Ild elements have spread in Brazil and into other countries is deeply concerning. In fact, NTE_{KPC} elements have been described in China, Argentina, Brazil, and Russia (4–7). Therefore, the role of NTE_{KPC} elements in global dissemination of *bla*_{KPC} deserves additional investigation.

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FIG 1 Genetic structures of small IncQ1 pKPN535a (MH595533) (A) and Col-KPC pKPC45a (MH595534) (B) plasmids harboring the *bla*_{KPC-2} gene and non-Tn4401 elements (NTE_{KPC-II}d) identified in *K. pneumoniae* strains belonging to ST11 and ST340 (CG258), respectively. Protein coding sequences are represented by the arrows and labeled with gene name or product. (C) Alignment of Tn4401 and NTE_{KPC} genetic elements harboring *bla*_{KPC} genes identified in Brazil. NTE_{KPC} genetic elements encompass NTE_{KPC-I}c associated with *bla*_{KPC-3} carried by IncX3 plasmids (4), and the two NTE_{KPC-II}d elements identified in this study. Based on the insertion of *Δbla*_{TEM} upstream of the *bla*_{KPC} gene, NTE_{KPC} elements have been classified as NTE_{KPC-II}, whereas NTE_{KPC-II} variants are based on the differences to the length of *Δbla*_{TEM} and deletions between *Δbla*_{TEM} and *bla*_{KPC-2} (4). In both plasmids, NTE_{KPC-II}d elements were flanked by two identical 243-bp direct repeats (DR [open circles], GGGGTCGTCTCAGAATTCGGAAAAATAAGACGCTAGCGGGTGTATCTGTCAAGGTTGAAGCCTGAGAGGCGCAGCGCAGATCGTCAGAAAAAGGCCAAAAA CGATCCTAATCTGACGCAACATAGGTGGGGTGCCTGACGCCGGTTGAGGCGTACTTCAACTGGACACATTCCAGAAAGCCAAAGCATGGCATGGCCTGCCCGCTGTCTTACC GTGCTTATTTCGGTTTTCTCATCGACC). Protein coding sequences are represented by the arrows and labeled with gene name or product. Light blue shading denotes shared regions of homology (>95%).

Plasmids have played a key role in the horizontal spread of antibiotic resistance genes, promoting the survival and selection of clonal lineages among clinically significant pathogens (8). IncQ plasmids are of particular interest since they are highly mobilizable, being stably maintained, and transferred among a wide range of Gram-negative bacteria (9, 10). On the other hand, Col-like plasmids are mobilizable vectors that have been increasingly reported as antibiotic resistance carriers, in members of the *Enterobacteriaceae* family, being postulated as versatile gene capture platforms (11). These novel groups of IncQ1 and Col-KPC plasmids, identified in this study, might have originated through independent recombination events between NTE_{KPC}-IId and a recipient IncQ1 or Col-type plasmid backbone, which is consistent with independent recombination events generating the variability among members of this group of plasmids (10, 12). Interestingly, large direct repeats could flank genomic rearrangements between NTE_{KPC} elements and small mobilizable plasmids. In fact, recent studies have reported the presence of these small plasmids in KPC-2-producing *Pseudomonas aeruginosa* and *Escherichia coli* and in BKC-positive *Klebsiella pneumoniae* isolates (12–15).

In summary, we report here the identification and complete sequence of two plasmids, pKPN535a (MH595533) and pKPC45a (MH595534), which represent new groups of small IncQ1 and Col-KPC vectors conferring carbapenem resistance in high-risk lineages of *K. pneumoniae* CG258, representing a novel mechanism for dissemination of carbapenem resistance that may carry lower fitness costs and could potentially result in increased persistence and wider dissemination.

Data availability. The nucleotide sequences of the pKPN535a and pKPC45a plasmids were deposited at GenBank under accession numbers MH595533 and MH595534, respectively.

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1 **Yersiniabactin, Colibactin and Wider Resistome Contribute to Enhanced Virulence and** 2 **Persistence of KPC-2-Producing *Klebsiella pneumoniae* CG258 in South America**

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23
24 **Keywords:** *K. pneumoniae*; ST11; ST340; CG258; hvKp; virulence; carbapenemase, KPC-2, KPC-3,
25 CTX-M; multidrug-resistance; *Galleria mellonella*; Latin America; One Health.

26
27 **Abbreviations:** CR-hvKp, carbapenem-resistant hypervirulent *Klebsiella pneumoniae*; KPC, *Klebsiella*
28 *pneumoniae* carbapenemase; CTX, cefotaximase; CG, clonal group; ST, sequence type; QACs, quaternary
29 ammonium compounds; KL, K-locus; ybt, yersiniabactin; clb, colibactin; ICEKp, integrative conjugative
30 element *K. pneumoniae*; pLVPK, large virulence plasmid of *K. pneumoniae*; CPS, capsular
31 polysaccharides; MLST, multilocus sequence typing; YbSTs, yersiniabactin sequence types; CbSTs,
32 colibactin sequence types; CR-Kp, carbapenem-resistant *K. pneumoniae*; MIC, minimum inhibitory
33 concentration; ESBL, extended-spectrum beta-lactamase; HM, heavy metal; ML, maximum likelihood;
34 MDR, multidrug resistance; PDR, pandrug resistance; Inc, incompatibility; IS, insertion sequence; KPZM,
35 Zn2+/Mn2+-metabolism module; QRDR, quinolone-resistance determining region; PMQR, plasmid-
36 mediated quinolone resistance.

37 Abstract

38 The emergence and dissemination of carbapenem-resistant hypervirulent *Klebsiella*
39 *pneumoniae* (CR-hvKp) is a worrisome public health issue compromising the treatment and
40 outcome of infections caused by this pathogen. We performed a detailed virulome and
41 resistome analysis of representative KPC- and/or CTX-M-producing *K. pneumoniae*
42 belonging to clonal group (CG) 258 (sequence types ST11, ST258, ST340, ST437),
43 circulating in Argentina, Brazil, Chile, Colombia and Peru; with further evaluation of the
44 virulence behavior using the *Galleria mellonella* infection model. Genomic analysis of *K.*
45 *pneumoniae* strains recovered from the human-animal-environment interface revealed a wide
46 resistome characterized by the presence of genes and mutations conferring resistance to
47 human and veterinary antibiotics, quaternary ammonium compounds (QACs) and heavy
48 metals. Plasmid Inc typing revealed the presence of a wide diversity of replicon types with
49 IncF, IncN, IncR and Col-like being frequently detected. Moreover, KPC-2-producing *K.*
50 *pneumoniae* belonging to ST11 (KL-64 and KL-105) and ST340 (KL-15) carried multiple
51 variants of distinct yersiniabactin siderophore (*ybt*) and/or genotoxic colibactin (*clb*) genes. In
52 this regard, ICEKp3, ICEKp4 and ICEKp12 were identified in strains belonging to ST11 and
53 ST340, recovered from Argentina, Brazil, Chile and Colombia; whereas *ybt* 17 and a novel
54 *ybt* sequence type (YbST346) were identified together with *clb* in ICEKp10 structures from
55 ST11 and ST258, from Brazil and Colombia, respectively. *K. pneumoniae* ST11
56 (ICEKp10/YbST346 and ICEKp4/*ybt* 10) strains killed 100% of wax moth larvae, in a similar
57 way to hypervirulent K1/ST23 strain (*ybt*- and *clb*-negative) carrying the pLVPK-like
58 plasmid, indicating enhanced virulence. In summary, our results indicate that yersiniabactin,
59 colibactin and an expanded resistome have contributed to enhanced virulence and persistence
60 of KPC-2-producing *K. pneumoniae* CG258 in South America. Therefore, active surveillance
61 of hospital-associated lineages of *K. pneumoniae* should not only focus on clonal origin and
62 antimicrobial resistance, but also on the virulence factors *ybt* and *clb*.

63 INTRODUCTION

64 Carbapenem resistance is a major public health concern worldwide, and currently *Klebsiella*
 65 *pneumoniae* belonging to the clonal group CG258 (which include the sequence types ST11,
 66 ST258, ST340, ST437, and ST512) seem to be the main culprits for the spread of *bla*_{KPC}
 67 genes (Bowers et al., 2015; Chen et al., 2014; Holt et al. 2015; Mathers et al., 2015; Paczosa
 68 and Meccas 2016; Wyres and Holt, 2016). This problem has been further exacerbated by the
 69 convergence of KPC-2 production and hypervirulence, resulting in the emergence of
 70 carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKp) lineages, particularly in Asian
 71 countries (Chen et al., 2017; Lee et al., 2017; Dong et al., 2018a; 2018b; Du et al., 2018; Gu
 72 et al., 2018; Wang et al., 2018). In these countries hypervirulence has been associated with
 73 the appearance and dissemination of a pLVPK-like plasmid harbouring two capsular
 74 polysaccharides (CPS) upregulator genes (*rmpA* and *rmpA2*) and several siderophore gene
 75 clusters (*iroBCDN*, *iucABCD* and *iutA*) (Struve et al., 2015; Chen et al., 2017; Du et al.,
 76 2018; Gu et al., 2018). However, the acquisition of integrative conjugative elements (ICEKp)
 77 harbouring yersiniabactin siderophore (*ybt*) is also associated with enhanced virulence,
 78 whereas carriage of the genotoxic colibactin (*clb*) genes (in ICEKp10structures) has been
 79 associated with invasive disease and colorectal cancer (Holt et al., 2015; Lam et al., 2018a).

80 Based on gene content variation, genomic investigation has allowed the identification
 81 of 14 different structural ICEKp variants, constituting a novel target that deserves further
 82 analysis for evolutionary and genomic surveillance studies (Wu et al., 2009; Lam et al.,
 83 2018a; Lin et al., 2008). A MLST-style approach based on diversity in eleven *ybt* locus genes
 84 has defined yersiniabactin sequence types (YbSTs) by unique combinations of *ybt* gene
 85 alleles and showed that YbST sequences were clustered into 17 distinct *ybt* lineages (Lam et
 86 al., 2018a). In a similar way, variations in the *clb* locus genes have allowed definition of
 87 colibactin sequence types (CbSTs), whereas phylogenetic analysis of the *clb* locus has

88 revealed three lineages that have each associated with a different *ybt* lineage [i.e., *clb* 1 (*ybt*
89 12), *clb* 2A (*ybt* 1) and *clb* 2B (*ybt* 17)] within the same overall structure (ICEKp10).

90 Virulence in CR-Kp strains has also been associated with the type of capsular
91 polysaccharide (Cortés et al., 2002; Diago-Navarro et al., 2014; Gomez-Simmonds and
92 Uhlemann, 2017; Liu et al., 2017). In this regard, over 79 capsule (K) serotypes have been
93 described in the international K serotyping scheme (Brisse et al., 2004; Pan et al., 2015;
94 Struve et al., 2015). More recently, diversity of the capsule synthesis locus (K-locus), which
95 is 10–30 kbp in size, has been used as a novel typing method for genomic surveillance and
96 epidemiological investigations of this pathogen, and identified 134 distinct K-loci, which are
97 predictive of K serotype (Wyres et al., 2016).

98 In South American countries, KPC-2-producing *K. pneumoniae* has been circulating
99 in Colombia, Brazil and Argentina since at least 2005 (Villegas et al., 2006; Pavez et al.,
100 2009; Gomez et al., 2011), and has more recently been reported in Ecuador, Chile,
101 Venezuela, Paraguay, Uruguay and Peru (Cifuentes et al., 2012; Zurita et al., 2013; Marquez
102 et al., 2014; Falco et al., 2016; Gomez et al., 2016; Homa et al., 2017). Although, molecular
103 epidemiology studies have confirmed predominance of the CG258 among KPC-2-producing
104 *K. pneumoniae* isolates collected in this region (Andrade et al., 2011; Cejas et al., 2012;
105 Pereira et al., 2013; Gomez et al., 2016; Barria-Loaiza et al., 2017; Homa et al., 2017), few
106 studies have focused on virulence determinants in these strains (Andrade et al., 2018; Araújo
107 et al., 2018). In fact, studies of biofilm formation and identification of a common set of
108 virulence genes have been restricted to KPC-2-producing *K. pneumoniae* from Brazil,
109 whereas sporadic identification of hypervirulent *K. pneumoniae* (hvKp) isolates belonging to
110 K1/ST23 and K19/ST29 have been reported in infected patients from Argentina and Brazil
111 (Cejas et al., 2014; Coutinho et al., 2014; Moura et al., 2017), and also in non-human
112 primates from Brazil (Anzai et al., 2017). In this study, using a genomic approach, we have
113 performed a detailed virulome and resistome analysis of KPC- and CTX-M-producing *K.*

114 *pneumoniae* strains belonging to CG258, recovered from the human-animal-environment
115 interface in Latin America, with further *in vivo* virulence evaluation using a *Galleria*
116 *mellonella* infection model.

117

118 METHODS

119 *K. pneumoniae* strains and genome collection

120 Laboratory studies included 19 KPC-2- and/or CTX-M-producing *K. pneumoniae* isolates
121 belonging to CG258 (ST11, ST258, ST340, ST437), representative of local surveillance
122 studies performed in Brazil, Peru, Chile and Argentina, between 2010 to 2016; recovered
123 from human, food-producing animals (chicken and swine) and environmental (urban rivers
124 and urban lake) samples (Oliveira et al., 2014; Martins et al., 2015; Cerdeira et al.,
125 2016a;2016b; Cerdeira et al., 2017; Horna et al., 2017; Nascimento et al., 2017). *K.*
126 *pneumoniae* ATCC 13883 and hvKp K1/ST23 A58300 (Coutinho et al., 2014) were used as
127 control strains. For genome analysis, all publicly available genomes from 36 *K. pneumoniae*
128 CG258 strains isolated in South America were included, of which 23 were previously
129 published (Bowers et al., 2015; Araújo et al., 2018; Casella et al., 2018; Dalmolin et al.,
130 2018; Pitt et al., 2018). For all 55 genomes included in this work, accession numbers are
131 listed in Table S1.

132

133 Antibiotic susceptibility patterns and hypermucoviscosity phenotypical identification

134 Resistance phenotypes were determined by Kirby-Bauer method, against 30 different human
135 and veterinary antibiotics, and the results were interpreted using the Clinical and Laboratory
136 Standards Institute guidelines (CLSI, 2015; 2017) and The European Committee on
137 Antimicrobial Susceptibility Testing (EUCAST, 2017). Additionally, minimum inhibitory
138 concentrations (MICs) for ertapenem, imipenem, meropenem, enrofloxacin, ciprofloxacin,
139 levofloxacin and polymyxin B, were determined by microdilution or Etest methods (CLSI,

2017; EUCAST, 2017). Production of ESBL and carbapenemase enzymes was confirmed by growth on CHROMagar ESBL and CHROMagar KPC, respectively.

Hypermucoviscosity phenotypes of *K. pneumoniae* isolates were determined by the string test as previously described (Moura et al., 2017). Briefly, a positive string test was defined by the formation of a viscous string > 5 mm in length when a colony was grown on a blood agar plate at 37 °C overnight and stretched by an inoculation loop.

Sequencing of *K. pneumoniae* strains

For 12 *K. pneumoniae* strains, total genomic DNA was extracted using PureLink™ Genomic DNA Mini Kit according to the Manufacturer's instructions. Library preparation was performed using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Sequencing was performed using the Illumina NextSeq platform with paired-end reads (150bp). Additionally, 10 *K. pneumoniae* isolates selected from the 19 KPC-2 and/or CTX-M producers (Table S1) were subjected to further sequencing using a PromethION R9.4.1 flow cell (Oxford Nanopore Technologies). A 2D MinION library was generated from 1.5 µg purified genomic DNA using the Nanopore Sequencing Kit (SQK-NSK007). DNA was repaired (NEBNext FFPE RepairMix), prepared for ligation (NEBNextUltra II End-Repair/dA-tailing Module) and ligated with adapters (NEB Blunt/TA Ligase Master Mix).

Bioinformatic analysis

Forty-one *K. pneumoniae* CG258 genomes with available short-read sequence data (including genomes obtained in this study and publicly available in the GenBank) were subjected for *de novo* assemblies using Unicycler (v0.4.0) (Wick et al., 2017). For ten genomes obtained with Nanopore reads, scaffold bridging was performed, building a high-quality finished genome sequence. The contigs were annotated by Prokka v1.12 (<https://github.com/tseemann/prokka>). MLSTs, YbSTs, CbSTs (Lam et al., 2018a; Diancourt et al., 2005), virulome, resistome and

166 plasmid replicon genes were screened by SRST2 (Inouye et al., 2014), using BIGSdb
167 (Bialek-Davenet et al., 2014), ARG-Annot (Gupta et al., 2014), and PlasmidFinder (Carattoli
168 et al., 2014) databases. On the other hand, since 14 of the publicly available genomes (used in
169 this study) were only available as pre-assembled sequences, Kleborate
170 (<https://github.com/katholt/Kleborate>) and PlasmidFinder were used to identify MLST,
171 resistome, virulome and plasmid replicon genes. Kleborate was further used to predict
172 yersiniabactin ICEKp structures in all 55 genomes.

173 ResFinder 3.0 database was used to confirm resistomes (Zankari et al., 2012), and
174 while capsule and O-antigen biosynthesis loci were identified using Kaptive (Wick et al.,
175 2018), heavy metal (HM) and QAC genes were screened using BLASTN against local
176 HM/QAC and BIGSdb databases.

177 Single nucleotide variants were identified using RedDog v1beta.10.3
178 (<https://github.com/katholt/RedDog>), with the reference genome of *K.*
179 *pneumoniae*30660/NJST258_1 (CP006923) (Bowers et al., 2015). Gubbins v.2.1.0 (Croucher
180 et al., 2014) was used to identify and exclude recombination imports. Maximum likelihood
181 (ML) trees were inferred from the recombination-masked alignment by running RaxML
182 v8.2.9 (Stamatakis et al., 2006) five times, selecting the final tree with the highest likelihood.
183 To assess branch support, we conducted 100 non-parametric bootstrap replicates using
184 RAxML.

185

186 *Galleria mellonella* killing assays

187 In order to evaluate the virulence behavior of KPC-2- and/or CTX-M-15-producing *K.*
188 *pneumoniae* strains, *in vivo* experiments were carried out using a *Galleria mellonella*
189 infection model (Junqueira 2012; Insua et al. 2013), with the non-virulent *K. pneumoniae*
190 strain ATCC 13883 and the clinical hvKp K1/ST23 strain A58300 (Coutinho et al. 2014), as
191 comparative strains. Fourteen *K. pneumoniae* strains of different lineages of CG258 and

origins (i.e., human, animal or environmental), circulating in Latin America, were evaluated. For each experiment, a control group containing five larvae was inoculated with sterile PBS in order to discard death due to physical trauma. In all experiments, groups of 250 to 350 mg *G. mellonella* larvae were inoculated with 10^6 CFU, and survival analysis was evaluated during 96h (Moura et al. 2017). Survival curves were plotted using the Kaplan-Meier method, and data were analyzed by the Fisher's exact test, with $P < 0.001$ indicating statistical significance. The statistical software used was Prism7 (Graph Pad Software, San Diego, CA, USA). *G. mellonella* larvae that did not demonstrate a response to physical stimulation and had body melanization were considered dead. All experiments were performed in independent triplicate assays.

202

203 RESULTS

204 Antimicrobial resistance profiles, resistome and plasmid populations

All 19 *K. pneumoniae* evaluated, *in vitro*, exhibited resistance to multiple antibiotics and were classified as MDR or PDR phenotypes (Magiorakos et al., 2012) (Table 1). In fact, resistome analysis revealed the presence of genes conferring resistance to aminoglycosides, quinolones, sulphonamides, tetracycline, phenicols, fosfomycin and beta-lactam antibiotics (*bla_{OXA-1}*, *bla_{OXA-2}*, *bla_{OXA-9}*, *bla_{OXA-10}*, *bla_{CTX-M-15}*, *bla_{SHV-11}*, *bla_{SHV-12}*, *bla_{LAP-2}*, *bla_{TEM-1A}*, *bla_{TEM-1B}*, *bla_{TEM-55}*) (Figure 1; Table S2). Moreover, point mutations in GyrA (Ser-83-Ile), GyrB (Asp-466-Glu) and ParC (Ser-80-Ile) were associated with quinolone resistance. Polymyxin resistance in 5 of the 19 (26%) human and environmental isolates was associated to *mgrB* mutations (i.e., Gly-28-Cys or Tn3 insertion at position 134) (Table S2). Additionally, the presence of genes conferring resistance to silver (*sil*), copper (*pco*), arsenic (*ars*), mercury (*mer*), tellurite (*ter*), and quaternary ammonium compounds (*qacA*, *qacE*, *qacEΔ1*, *qacL* and *sugE*) supported a wider resistome (Figure 1, Table S2), which could contribute to the apparent high versatility, persistence and adaptation of CG258 to various

ecosystems and hosts (Navon-Venezia et al., 2017; Dong et al., 2018). Notably, the presence of tellurite resistance has also been associated with hypervirulent clonal groups of *K. pneumoniae* (Passet and Brisse, 2015; Martin et al., 2018).

Additional *in silico* analysis of 36 genome sequences obtained from GenBank confirmed the wider resistome of *K. pneumoniae* strains circulating in Latin America. However, others CTX-M gene variants, such as *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, *bla*_{CTX-M-9}, *bla*_{CTX-M-14} and *bla*_{CTX-M-59} could be identified, as well as the *bla*_{KPC-3} carbapenemase gene identified in two Colombian *K. pneumoniae* strains (Colombia-2009a, Colombia-2009b) alone (Table S2). Moreover, presence of *armA*, *rmtB*, *rmtD* and *rmtG*16S rRNA methyltransferases encoding genes, conferring resistance to most aminoglycosides, was confirmed in 6 of the 55 (11%) analyzed genomes. Worryingly, a third of isolates were predicted to be polymyxin resistant based on deletions in *mgrB* and/or *pmrB* genes in 20 of the 55 analyzed genomes; whereas the *mcr-1* gene was identified in a human strain isolated in Brazil (Table S2).

Plasmid incompatibility (Inc) typing revealed the presence of a wide diversity of plasmid replicon types harbored by KPC-2- and/or CTX-M-type-producing *K. pneumoniae* (Figure 1, Table S2). Among 55 genomes analyzed, IncF, IncN, IncR and Col-like replicons were over-represented (96.4%, 54.5%, 27.3% and 27.3% respectively), whereas other Inc types identified were: X3 (*n*= 8), HI1B (*n*= 8), L/M (*n*= 6), U (*n*= 5), Q1 (*n*= 4), P6 (*n*= 3), A/C2 (*n*= 2), I1 (*n*= 1), I2 (*n*= 1), HI1A (*n*= 1) and X4 (*n*= 1). However, *bla*_{KPC} plasmids belonging to the IncN incompatibility group were identified in 30 of the 55 (54%) carbapenem-resistant *K. pneumoniae* strains.

Virulome, yersiniabactin, colibactin and integrative conjugative elements (ICEs)

Among genomes analysed, lineages belonging to ST11 and ST340 carried multiple variants of distinct yersiniabactin siderophore (*ybt*) and/or genotoxic colibactin (*clb*) genes from distinct *ybt/clb* lineages and ICE_{Kp} variants. In this regard, we detected *ybt* lineage 9

(ICEKp3), *ybt* 10 (ICEKp4) and *ybt* 16 (ICEKp12) in nine *ybt*⁺ *K. pneumoniae* strains belonging to ST11 and ST340, isolated from human samples collected in Argentina, Brazil and Chile (Figure 2A, Table S3); whereas *ybt* 17 and a novel *ybt* sequence type YbST346, that does not belong to any of the 17 previously described *ybt* lineages (Lam et al, 2018a), were identified together with *clb* lineage 2B in ICEKp10 structures, in eight Brazilian *K. pneumoniae* ST11 strains recovered from human and environmental samples (Figure 2B). ST258 lineages from Colombia harbored the classical ICEKp10 with the *ybt* 17 and *clb* 2B.

The alignment between the classical ICEKp10/*ybt* 17 and that of ICEKp10 with the novel YbST346 shows that the main difference is the insertion sequence ISEc21 (IS110 family) located within the Zn²⁺/Mn²⁺ metabolism module (KPZM) (Figure 2B, Table S3).

Regarding other virulence determinants, the presence of the aerobactin locus (*iuc*) was only identified in a human KPC-2-positive *K. pneumoniae* strain ST11 from Brazil (Figure 1).

***In silico* serotyping, capsule locus (KL) analysis and string test**

In silico serotyping of 55 genomes analyzed showed a predominance of O4 [K36, K15, K-non-typeable (NT)], O2v2 (K8, K27, K-NT) and O2v1 (K64) serotypes, which were associated with ST340 (O4/K15, O4/K-NT), ST437 (O4/K36), ST11 (O2v2/K8, O2v2/27, O2v2/K-NT, O2v1/K64), and ST258 (O2v2/K-NT) (Figure 1, Table S2). On the other hand, we investigated the diversity of capsule synthesis loci using full locus information extracted from whole genome sequences. These results show that K-loci were diverse in human and environmental *K. pneumoniae* ST11 (i.e., KL-8, KL-27, KL-64, KL-105, KL-107, KL-127), in this region (Figure 1, Table S2). Interestingly, in *K. pneumoniae* belonging to ST340, KL-15 was assigned to human and environmental strains collected in Argentina, Peru and Brazil, respectively; whereas KL-151 was only identified in animal strains from Brazil. On the other hand, human and environmental *K. pneumoniae* ST437 (from Brazil) were typed as KL-36;

whereas KL-106 and KL-107 accounted for strains of ST258, in Brazil and Colombia, respectively.

KL-64/ST11 and KL-105/ST11 showed a high virulence behavior in the *G. mellonella* model, the latter being identified in clinical samples from Chile and Brazil. In this regard, KL-64 has been previously associated with strains from invasive *K. pneumoniae* infections (Follador et al., 2016).

The genetic structure of the *cps* synthesis loci across the virulent ST11 (KL-105 and KL-64) and ST340 (KL-15) was distinct from the K-loci from hvKpK1 (KL-1) and K2 (KL-2) (Figure 3). In this concern, for these K-loci, a conserved genetic organization at the 5' end of the *cps* locus was observed from *galF* to *wzc* genes, whereas *wzc-gnd* and *gnd-ugd* regions were variable. Moreover, while in KL-64 the *gnd-ugd* region is composed of genes involved in GDP-D-mannose synthesis (*manB* and *manC*) and deoxythymidine diphosphate (dTDP)-L-rhamnose synthesis (*rmlA*, *rmlB*, *rmlC* and *rmlD*), in KL-105 the *gnd-ugd* region is only composed of operon *manCB* (Figure 3) (Pan et al., 2015; Wyres et al., 2015; Wyres et al., 2016). Interestingly, dTDP-L-rhamnose is the precursor of L-rhamnose, a saccharide required for the virulence of some pathogenic bacteria, being essential for resistance to serum killing and for colonization (Giraud et al., 2000).

To investigate the hypermucoviscosity phenotype, all the isolates were subjected to the string test. Among the 19 KPC-2 and/or CTX-M-15 producers, only one CTX-M-15-producing *K. pneumoniae* ST340/KL-151 strain (FA64), isolated from a healthy chicken sample, in Brazil, showed hypermucoviscosity. However, neither of the known hypermucoviscosity encoding genes (*rmpA* or *rmpA2*) were detected in its genome.

***In vivo* virulence behavior of *K. pneumoniae* CG258**

Using the *G. mellonella* virulence model, ST11 CR-KP strains ($n=2$, KL-64/*ybt*⁺/*clb*⁺; $n=2$, KL-105/*ybt*⁺) killed 100% of wax moth larvae inoculated with 1×10^6 colony-forming units of

the bacterial specimens, within 96 h, in a similar way to the known hypermucoviscous hvKp K1/ST23 strain which is *ybt*⁻ and *clb*-negative and carries the pLVPK-like plasmid ($P > 0.9999$) (Figure 4A). KPC-2- and/or CTX-M-15-producing *K. pneumoniae* strains belonging to ST340 killed >60% of wax moth larvae ($n=2$, KL-15/*ybt*⁺; $n=3$, KL-15/*ybt*⁻; $n=1$, KL-151/*ybt*⁻). One ST340 KL-15/*ybt*⁺ strain isolated from a human infection killed 100% of *G. mellonella* (Figure 4B). *K. pneumoniae* belonging to ST437 ($n=3$ strains, all KL-36/*ybt*⁻/*clb*⁻) killed ~50% of *G. mellonella*, compared to *K. pneumoniae* ATCC 13883 control (Figure 4D).

Overall, among 14 *K. pneumoniae* strains evaluated, 4/6 (83%) *ybt*⁺ isolates (4/4 ST11, 1/2 ST340) killed all wax moth larvae within 96 h, compared to only 1/8 (13%) *ybt*⁻ strains (1/1 ST258, 0/4 ST340, 0/3 ST437) ($P = 0.03$, Fisher's exact test), suggesting that in the absence of pLVPK-like plasmids, the presence of *ybt* could be enough to confer enhanced virulence. However, the single ST258 strain (*ybt*⁻ and *clb*-negative), isolated from a human clinical sample also killed 100% of *G. mellonella* within 96 h (Figure 4C), suggesting that other factors, not elucidated in this study, may also be contributing to the virulence phenotype of CG258 (Araújo et al., 2018; Hennequin and Robin, 2016; Shah et al., 2017; Fu et al., 2018; Marcoleta et al., 2018; Zheng et al., 2018).

DISCUSSION

In South American countries, antimicrobial resistance has long been documented to be more challenging than in developed ones (Gales et al., 2012; Sampaio and Gales, 2016). In this regard, the high prevalence of carbapenem resistance in this region has occurred primarily by the dissemination of KPC-producing *K. pneumoniae* isolates belonging to CG258, which have been identified beyond the hospital setting, constituting a One Health problem (Andrade et al., 2011; Gomez et al., 2011; Cejas et al., 2012; Oliveira et al., 2014; Barría-Loaiza et al., 2016; Rojas et al., 2017; Homa et al., 2017; Nascimento et al., 2017). In this study, we performed a resistome and virulome analysis of KPC-and/or CTX-M-producing *K.*

322 *pneumoniae* lineages belonging to CG258, circulating in hospital settings. Additionally,
323 environmental and animal *K. pneumoniae* isolates, recovered in Brazil, were also
324 investigated.

325 Genome analysis revealed a wider resistome, which includes genetic determinants
326 conferring resistance to human and animal antibiotics, QACs and HMs, supporting
327 persistence and adaptation of CG258 to different hosts and anthropogenically affected
328 environments. Among MDR and PDR lineages, the presence of mutations in *mgrB/pmrB*
329 genes and in the quinolone resistance-determining region; as well as acquisition of 16s rRNA
330 methylases- and β -lactamases-encoding genes (including *bla_{ESBL}* and *bla_{KPC-2}*) have
331 contributed with resistance to polymyxins, fluoroquinolones, aminoglycosides and broad-
332 spectrum β -lactam antibiotics. Moreover, we have identified, for the first time, the presence
333 of the narrow-spectrum β -lactamase encoding gene *bla_{LAP-2}* (GenBank accession number
334 EU159120) and *bla_{TEM-55}* ESBL gene (GenBank accession number DQ286729) in *K.*
335 *pneumoniae* strains ST340 recovered from swine and human hosts, respectively, in Brazil,
336 confirming versatility of this lineage to acquire novel genetic determinants of resistance.

337 We have identified regional *bla_{KPC}* spread consistent with high prevalence of IncN
338 plasmids, previously associated with the global spread of these genes (Stoesser et al., 2017).
339 On the other hand, the wide diversity of Inc-type plasmids, found in this study, including
340 small mobilizable Col-like replicons could be associated with the acquisition of multiple
341 resistance mechanisms, contributing to the wider resistome. Therefore, the presence of *K.*
342 *pneumoniae* in a wide range of environmental reservoirs and hosts, with plasmids that have
343 been shown to facilitate the dissemination of successful resistance genes, even in the absence
344 of selection pressures, may represent a difficult situation to control (Stoesser et al., 2017).
345 Another important issue is the identification of IncHI1-type plasmids, which have been
346 associated with the dissemination of *mcr-1* and *bla_{CTX-M}*-type genes in Colombia and
347 Uruguay, respectively (Saavedra et al., 2017; Garcia-Fulgueiras et al., 2017).

Hypervirulent *K. pneumoniae* strains have been sporadically reported in Argentina and Brazil, being associated with remarkable mortality and the production of a hypermucoviscous phenotype in lineages belonging to ST23 with capsular serotype K1, and ST29/K19 (Cejas et al., 2014; Coutinho et al., 2014; Moura et al., 2017). In this study, virulome analysis revealed that *ybt* and *clb* genes have been acquired by strains of CG258 in South America, highlighting the need to also consider these additional virulence factors rather than the presence of pLVPK-like plasmids and hypermucoviscous phenotypes alone, in the establishment of hypervirulence in carbapenem-resistant lineages of *K. pneumoniae*.

Notably, there have been increasing reports of highly virulent *K. pneumoniae* strains carrying *ybt* belonging to the international clone ST11. The emergence of CR-hvKp strains carrying *ybt* plus a deletion variant of the pLVPK-like plasmid belonging to ST11 has been associated with outbreak of fatal nosocomial infection in China (Gu et al., 2018), raising an epidemiological alert in response to the increased number of cases reported, in the last year (Lee et al., 2017; Zhan et al., 2017; Chen and Kreiswirth, 2018; Du et al., 2018; Wong et al., 2018; Yao et al., 2018).

In summary, these results (available for interactive exploration in Microreact at <https://microreact.org/project/H1LSZsRz7>) confirm the enhanced virulence of KPC-2- and/or CTX-M-producing *K. pneumoniae* belonging to the international high-risk clone CG258 in South America, where acquisition of ICEKp encoding yersiniabactin and colibactin, and wider resistome have likely contributed to enhanced virulence and persistence of ST11 (KL-64 and KL-105) and ST340 (KL-15) lineages, in the human-environment interface. While capsule composition deserves further investigation, active surveillance should not only focus on clonal origin, antimicrobial resistance and presence of pLVPK-like plasmids, but also the virulence associated with yersiniabactin and colibactin, as well as other biomarkers for differentiation of hvKp from classical *K. pneumoniae* (Russo et al., 2018); and control measures should be conducted to prevent the global dissemination of these lineages.

374 CONCLUSION

375 Our study points out several important issues. Firstly, interplay of yersiniabactin and/or
376 colibactin and KPC-2 production has become to be identified among *K. pneumoniae*
377 belonging to CG258, in South America, contributing to the emergence of highly virulent
378 lineages that pose great risk to human health (Lam et al. 2018a). Second, in South America
379 ICEKp3, ICEKp4 and ICEKp10 carrying *ybt* and/or *clb* circulate among KPC-2-producing *K.*
380 *pneumoniae* belonging to ST11 (KL-64 and KL-105), where multiple distinct K-loci often
381 indicates distinct sublineages that may correlate with independent ICEKp acquisitions
382 (supported by our phylogenetic analysis shown in Figure 1); being associated with enhanced
383 virulence, these should be considered a target for genomic surveillance along with
384 antimicrobial resistance determinants. Third, the wide resistome could be contributing to
385 adaptation of KPC-2- and/or CTX-M-producing *K. pneumoniae* CG258 in the human-animal-
386 environment interface, highlighting the urgent need for enhanced control efforts. Finally,
387 these findings could contribute to the development of strategies for prevention, diagnosis and
388 treatment of *K. pneumoniae* infections.

389

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Table 1. Resistance profile of KPC-2- and/or CTX-M-producing *K. pneumoniae* strains belonging to CG258 circulating in South America*

Strain	MICs (µg/ml)							Kirby-Bauer
	POL	ETP	IMP	MER	ENO	CIP	LVX	
KP171	2	8	16	8	8	8	32	CTX, CAZ, CPM, CRO, NAL, GEN, SUT, ATM, TET, CLO, FOS
1194	2	4	8	8	8	8	32	CTX, CAZ, CPM, CRO, NAL, AMI, GEN, SUT, ATM, TET, CLO, FOS
606B	2	1	1	0.25	4	4	16	CTX, CAZ, CPM, CRO, NAL, GEN, SUT, ATM, TET, CLO, FOS
KPN535	16	4	8	4	8	8	32	CTX, CAZ, CPM, CRO, NAL, GEN, SUT, ATM, CLO, FOS
KPC45	32	4	8	4	4	4	16	CTX, CAZ, CPM, CRO, NAL, SUT, ATM
IBL2.4	8	4	8	8	4	8	32	CTX, CAZ, CPM, CRO, CFO, NAL, ATM, TET
KP488	>32	4	8	8	4	4	32	CTX, CAZ, CPM, CRO, CFO, NAL, GEN, SUT, ATM, CLO, FOS
196	0.25	>32	8	>32	8	>32	>32	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM
148	32	>32	>32	>32	4	>32	>32	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET, CLO, FOS
314	2	>32	>32	>32	4	>32	>32	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET
KP411	0,5	>32	>32	>32	8	8	8	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET, CLO
KP337	0,5	>32	>32	>32	4	4	8	CTX, CAZ, CPM, CRO, CFO, NAL, ATM, TET, CLO
KP326	0,5	>32	8	8	8	8	16	CTX, CAZ, CPM, CRO, NAL, SUT, ATM, TET, CLO, GEN
KP515	0,5	>32	>32	>32	8	8	16	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET, CLO, FOS
KP870	0,5	>32	>32	>32	16	8	16	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET, CLO, FOS
FA64	1	1	0.5	0.5	4	8	4	CTX, CAZ, CPM, CRO, NAL, SUT, ATM
2KP	1	8	8	8	8	8	8	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET
1ECKPC	1	4	8	8	8	8	8	CTX, CAZ, CPM, CRO, CFO, NAL, SUT, ATM, TET, CLO, GEN
N7	1	8	8	8	4	8	8	CTX, CAZ, CPM, CRO, NAL, SUT, ATM, TET, CLO, GEN, AMI

*POL – polymyxin B, ETP – ertapenem, IMP – imipenem, MER- meropenem, ENO – enrofloxacin, CIP – ciprofloxacin, LVX – levofloxacin, CTX – cefotaxime, CAZ – ceftazidime, CPM – cefepime, CRO – ceftriaxone, CFO – ceftiofloxacin, NAL – nalidixic acid, AMI - amikacin, GEN – gentamicin, SUT – sulfamethoxazole/trimethoprim, ATM - aztreonam, TET – Tetracycline, CLO – chloramphenicol, FOS – fosfomicin.

Figure legends

Fig. 1. Virulome, resistome and plasmid population of KPC- and/or CTX-M-producing *K. pneumoniae* belonging to CG258 in Latin America. Tracks indicate: (1) country, (2) strain source, (3) sequence type (ST), (4) ICEKp structure. The red/black/orange regions represent the presence of the gene, and blank regions represent their absence. Amg, aminoglycosides resistance genes (i.e., transferases and 16S rRNA methylases); Col, polymyxin resistance genes (including *mgtB/pmrB* mutations and *mcr-1*); Flq, fluoroquinolone resistance genes (i.e., QRDR mutations and PMQR); MLS, macrolides resistance genes (*mphA*, *erm*); Phe, phenicols resistance genes (*cat*, *cml*, *flor*) (Table S2).

Fig. 2. Comparative analysis of integrative and conjugative elements ICEKp3, ICEKp4, ICEKp10, and ICEKp12. In A, ICEKp mobilizing yersiniabactin identified in *K. pneumoniae* CG258 in South America. In B, alignment of ICEKp10/ybt 17 against ICEKp10 carrying a novel yersiniabactin sequence YbST346, identified in lineages belonging to ST11, isolated in Brazil. Blue blocks represent yersiniabactin synthesis locus *ybt*, labelled with the associated *ybt* lineage. Orange represents the mobilization module. Light orange represents KPZM (Zn^{2+}/Mn^{2+}) metabolism module. Light blue shading denotes shared regions of homology (>95%), where the main difference between the two ICEKp10 is the presence of a novel *ybt* lineage, and the insertion sequence *ISEc21* (*IS110* family) located inside the Zn^{2+}/Mn^{2+} metabolism module (KPZM). The key differences between ICEKp3, ICEKp4 and ICEKp12 have been previously defined and are restricted to a single variable region, where ICEKp3 was constituted by restriction endonuclease, DUF4917 domain containing protein, ATP/GTP phosphatase, reverse transcriptase, DDE endonuclease, and five hypothetical proteins; whereas ICEKp4 was formed by transposase, ABC transporter, type I restriction endonuclease, DNA methyltransferase and hypothetical protein; and ICEKp12 contained an additional Zn^{2+}/Mn^{2+} metabolism module (KPZM) (Marcoleta et al. 2016; Lam et al., 2018a).

Fig. 3. K-loci (KL-1, K-L2, KL-105, KL-64 and KL-15) structures of CR-KP lineages belonging to CG258. In *K. pneumoniae*, K-locus includes a set of genes in the terminal regions encoding for the core capsule biosynthesis machinery (i.e., *galF*, *wzi*, *wza*, *wzb*, *wzc*, *gnd* and *ugd*). The central region is highly variable, encoding for specific sugar synthesis of the capsule, processing and export proteins, plus the core assembly components Wzx (flippase) and Wzy (capsule repeat unit polymerase) (Pan et al., 2015; Wyres et al., 2016). Protein coding sequences are represented as arrows colored by predicted function of the protein product and labelled with gene names where known.

Fig. 4. *In vivo* virulence behavior of CR-KP belonging to ST11, ST340, ST437 and ST258 in a *Galleria mellonella* infection model. The virulence behavior of 1×10^6 colony-forming units of representative *K. pneumoniae* strains on *G. mellonella* survival was assessed using both, non-virulent (ATCC 13883, ST not determined in this study) and hypervirulent (A58300 K1/ST23) *K. pneumoniae* control strains. In A, *K. pneumoniae* KPC45, KP488, IBL2.4 and N7 belonging to ST11, recovered from human and environmental samples. In B, *K. pneumoniae* 1194, KP870, 1ECKPC, 2KP, FA64, KP171 strains belonging to ST340, recovered from human, animal and environmental samples. In C, *K. pneumoniae* KP337 strain belonging to ST258, recovered from a clinical sample. In D, *K. pneumoniae* KP196, KP411 and 314 strains belonging to ST437, recovered from human and environmental samples. Clinical and epidemiological characteristics of *K. pneumoniae* strains are quoted in Table S1. * $P > 0.9999$, indicates no statistically significant difference with respect to the hypervirulent A58300 K1/ST23 *K. pneumoniae* control strain.

Figure 1

Figure 2

Figure 3

Figure 4

Supplementary material

Table 1

Strain	Accession number	Origin	Isolation source	Country	City	Year	Sequence type	Reference
1714	GCF_002104175.1	Human	Blood	Brazil	São Paulo	2013	ST340	NCBI
1721	GCF_002192975.1	Human	Blood	Brazil	São Paulo	2013	ST340	NCBI
1787	GCF_002104355.1	Human	Blood	Brazil	São Paulo	2013	ST11	NCBI
1799	GCF_002104315.1	Human	Blood	Brazil	São Paulo	2013	ST11	NCBI
1849	GCF_002104265.1	Human	Blood	Brazil	São Paulo	2013	ST258	NCBI
1853	GCF_002104705.1	Human	Blood	Brazil	São Paulo	2013	ST11	NCBI
4182	GCF_002104215.1	Human	Blood	Brazil	São Paulo	2014	ST11	NCBI
4281	GCF_002104255.1	Human	Blood	Brazil	São Paulo	2014	ST340	NCBI
4448	GCF_002104295.1	Human	Blood	Brazil	São Paulo	2015	ST258	NCBI
4477	GCF_002104365.1	Human	Blood	Brazil	São Paulo	2014	ST340	NCBI
4490	GCF_002104185.1	Human	Blood	Brazil	São Paulo	2016	ST258	NCBI
11_BR_13	GCF_001701915.1	Human	Blood	Brazil	São Paulo	2013	ST437	Pitt et al., 2018
1194 ^{ab}	LYZC000000000	Human	Catheter tip	Brazil	São Paulo	2011	ST340	Cerdeira et al., 2016b
12_BR_13	GCF_001701895.1	Human	Urine	Brazil	São Paulo	2013	ST11	Pitt et al., 2018
148 ^{bc}	LZCY000000000	Environment	Water	Brazil	São Paulo	2011	ST437	This study
196 ^a	LZCZ000000000	Environment	Water	Brazil	São Paulo	2011	ST437	This study
1ECKPC ^b	PISH000000000	Human	Blood	Peru	Lima	2013	ST340	This study
2KP ^b	NSLE000000000	Human	Blood	Argentina	Buenos Aires	2015	ST340	This study
3111F	GCF_002251715.1	Human	Rectal swab	Brazil	Porto Alegre	2014	ST437	Dalmolin et al., 2017
314 ^a	MAOP000000000	Human	Blood	Brazil	São Paulo	2011	ST437	This study
606B ^{bc}	LYMZ000000000	Animal - Swine	Rectal swab	Brazil	Minas Gerais	2012	ST340	Cerdeira et al., 2016a
B03	NTHT000000000	Human	Blood	Brazil	Porto Alegre	2013	ST437	Araújo et al., 2018
B04	NTGK000000000	Human	Blood	Brazil	Recife	2009	ST11	Araújo et al., 2018
B11	NTHU000000000	Human	Blood	Brazil	Brasília	2016	ST11	Araújo et al., 2018
B16	NTCW000000000	Human	Peritoneal secretion	Brazil	Beilo Horizonte	2009	ST437	Araújo et al., 2018
B30	NTHX000000000	Human	Blood	Brazil	João Pessoa	2016	ST437	Araújo et al., 2018
B35	NTHY000000000	Human	Blood	Brazil	Santa Juliana	2016	ST11	Araújo et al., 2018
Brazil-2006	SRR1561199	Human	Unknown	Brazil	Brasília	2006	ST11	Bowers et al., 2015
Brazil-2007b	SRR1561201	Human	Unknown	Brazil	Brasília	2007	ST258	Bowers et al., 2015
Brazil-2008a	SRR1561202	Human	Unknown	Brazil	Brasília	2008	ST437	Bowers et al., 2015
Brazil-2008b	SRR1561203	Human	Unknown	Brazil	Brasília	2008	ST437	Bowers et al., 2015
Brazil-2009a	SRR1561204	Human	Unknown	Brazil	Brasília	2009	ST11	Bowers et al., 2015
Brazil-2009b	SRR1561205	Human	Unknown	Brazil	Brasília	2009	ST437	Bowers et al., 2015
Brazil-2010a	SRR1561206	Human	Unknown	Brazil	Brasília	2010	ST340	Bowers et al., 2015
Brazil-2010b	SRR1561207	Human	Unknown	Brazil	Brasília	2010	ST340	Bowers et al., 2015
Brazil-2010e	SRR1561210	Human	Unknown	Brazil	Brasília	2010	ST11	Bowers et al., 2015
CCBH17440	GCF_001715215.1	Human	Blood	Brazil	Rio de Janeiro	2014	ST437	NCBI
Colombia-2009a	SRR1561215	Human	Unknown	Colombia	Bogotá	2009	ST258	Bowers et al., 2015
Colombia-2009b	SRR1561216	Human	Unknown	Colombia	Bogotá	2009	ST258	Bowers et al., 2015
FA64 ^b	LXTP000000000	Animal - Chicken	Rectal swab	Brazil	Minas Gerais	2015	ST340	This study
IBL2.4 ^a	NSLG000000000	Environment	Water	Brazil	São Paulo	2013	ST11	Nascimento et al., 2017
KP171 ^{bc}	MJBQ000000000	Environment	Water	Brazil	São Paulo	2011	ST340	Cerdeira et al., 2017
KP337	LXTP000000000	Human	Wound secretion	Brazil	São Paulo	2010	ST258	This study
KP411	NTGJ000000000	Human	Rectal swab	Brazil	São Paulo	2010	ST437	This study
KP417 ^b	NSLE000000000	Human	Unknown	Brazil	São Paulo	2011	ST258	This study
KP488	NSLF000000000	Human	Rectal swab	Brazil	São Paulo	2011	ST11	This study
KP515	NTGJ000000000	Human	Urine	Brazil	São Paulo	2011	ST340	This study
KP870	NSLE000000000	Human	Blood	Brazil	São Paulo	2011	ST340	This study
KPC02	NTCV000000000	Human	Blood	Brazil	Araguari	2013	ST340	Araújo et al., 2018
KPC05	NTGJ000000000	Human	Rectal swab	Brazil	Uberlândia	2014	ST11	Araújo et al., 2018
KPC40	GCF_002209465.1	Human	Blood	Brazil	Uberlândia	2015	ST11	NCBI
KPC45 ^b	LXTP000000000	Human	Blood	Brazil	Uberlândia	2015	ST11	This study
KPN535 ^b	NSLE000000000	Human	Rectal swab	Brazil	Recife	2011	ST340	This study
N7	LXTP000000000	Human	Urine	Chile	Osorno	2016	ST11	This study
SJRP-KP10	MUDD000000000	Human	Urine	Brazil	São José do rio Preto	2011	ST340	Caseira et al., 2018

^a Previously sequenced genomes from our own studies

^b Samples with Nanopore reads

Table 2

Strain	Origin	Isolation source	Year	Sequence type	K_type	K_locus	O_locus	Aminoglycoside resistance genes	Colistin resistance	Fosfomycin	Fluoroquinolone (PMQR, QRDR)	Macrolides, lincosamides, streptogramins	Phenicol resistance	Rifampicin
148	Environment	Water	2011	ST437	K36	KL36	O4	Aph3-1a	MgrB-85%	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
196	Environment	Water	2011	ST437	K36	KL36	O4	Aph3-1a	-	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
314	Human	Blood	2011	ST437	K36	KL36	O4	Aph3-1a;AadA2	mgrB SNP	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
1194	Human	Catheter tip	2011	ST340	K15	KL15	O4	RmtG;AadA2;AadA1-pm;Aac6-lb;Aac6-lb;StrB;StrA;Sat-2A;Aac3-1ld	-	fosA	QnrB5;ParC-80i;GyrA-83i	-	CatA1	Arr6
1714	Human	Blood	2013	ST340	K15	KL15	O4	AadA2;Aac3-1ld;StrB;StrA;AphA6	-	fosA	QnrB19;ParC-80i;GyrA-83i	-	-	-
1721	Human	Blood	2013	ST340	K107	KL107	OL102	Aac3-1ld;AphA6;AadA2;StrB;StrA	PmrB-65%	fosA	QnrB19;ParC-80i;GyrA-83i	-	-	-
1787	Human	Blood	2013	ST11	K64	KL64	O2v1	Aac3-1la	-	fosA	ParC-80i;GyrA-83i	-	CatA1	-
1799	Human	Blood	2013	ST11	K64	KL64	O2v1	Aac3-1la;Aph3-1a;AadA2	-	fosA	ParC-80i;GyrA-83i	MphA	CatA1	-
1849	Human	Blood	2013	ST258	K107	KL107	O2v2	StrB;StrA;RmtB;Sat-2A;Aac3-1ld;Aph3-1a;AadA2	MgrB-74%	fosA	ParC-80i;GyrA-83i	Errm42;MphA	CatA1	-
1853	Human	Blood	2013	ST11	K27	KL27	O2v2	Aph3-1a;AadA1-pm	MgrB-85%;PmrB-54%	fosA	ParC-80i;GyrA-83i;GyrA-87Y	MphA	CatA1	-
4182	Human	Blood	2014	ST11	K64	KL64	O2v1	Aac3-1la;AadA2	MgrB-85%	fosA	ParC-80i;GyrA-83i	-	CatA1	-
4281	Human	Blood	2014	ST340	K15	KL15	O4	StrB;StrA;Aph3-1a;Aac3-1la	-	fosA	QnrB1 ou QnrB66;ParC-80i;GyrA-83i	-	CatB4	-
4448	Human	Blood	2015	ST258	K107	KL107	O2v2	StrB;StrA;Sat-2A;RmtB;Aac3-1ld;AadA2	-	fosA	ParC-80i;GyrA-83i	Errm42;MphA	CatA1	-
4477	Human	Blood	2014	ST340	K15	KL15	O4	Aph3-1a	-	fosA	ParC-80i;GyrA-83i	-	-	-
4490	Human	Blood	2016	ST258	K107	KL107	O2v2	StrB;StrA;RmtB;Aac3-1ld;AadA2;Sat-2A	MgrB-83%	fosA	ParC-80i;GyrA-83i	Errm42;MphA	CatA1	-
11_BR_13	Human	Blood	2013	ST437	K36	KL36	O4	-	-	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
12_BR_13	Human	Urine	2013	ST11	K64	KL64	O2v1	Aac3-1la;AadA2;Aph3-1a	MgrB-57%	-	ParC-80i;GyrA-83i	MphA	CatA1	-
1ECKPC	Human	Blood	2013	ST340	K15	KL15	O4	AadA2;AadA16	-	fosA	QnrB2;ParC-80i;GyrA-83i	MphA;MphA	-	Arr3
2KP	Human	Blood	2015	ST340	K15	KL15	O4	Aac3-1ld;Aph3-1a	-	fosA	ParC-80i;GyrA-83i	MphA	CatB3	Arr3
3111F	Human	Rectal swab	2014	ST437	K36	KL36	O4	Aac3-1ld;Aph3-1a	Mcr1	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
606B	Animal - Swine	Rectal swab	2012	ST340	K151	KL151	O4	Aph3-1a;AadA2;StrB;StrA;Aac3-1ld	-	fosA	QnrS1;ParC-80i;GyrA-83i	MphA	FloR;CatA2	-
B03	Human	Blood	2013	ST437	K36	KL36	O4	Aac3-1ld;Aph3-1a	-	fosA	QnrB1;ParC-80i;GyrA-83i	-	CatB4	-
B04	Human	Blood	2009	ST11	K64	KL64	O2v1	AadA1-pm;Aac6-lq;AphA6	-	fosA	ParC-80i;GyrA-83i	-	CatA1	-
B11	Human	Blood	2016	ST11	K107	KL107	OL102	Aac3-1ld	-	fosA	ParC-80i;GyrA-83i	-	CatB4	-
B16	Human	Peritoneal secretion	2009	ST437	K36	KL36	O4	Aph3-1a;Aac3-1ld	-	fosA	QnrB1;ParC-80i;GyrA-83i	MphA	CatB4	-
B30	Human	Blood	2016	ST437	K36	KL36	O4	Aac3-1ld;AadA2	-	fosA	ParC-80i;GyrA-83i	-	CatB4	-
B35	Human	Blood	2016	ST11	K64	KL64	O2v1	-	MgrB-57%	fosA	ParC-80i;GyrA-83i	-	-	-
Brazil-2006	Human	Unknown	2006	ST11	K8	KL8	O2v2	AadA1-pm;Aac6-lq	-	fosA	ParC-80i;GyrA-83i	-	CatA2;CmiA5	-
Brazil-2007b	Human	Unknown	2007	ST258	K106	KL106	O2v2	Aac6-lb;AadA2	-	fosA	ParC-80i;GyrA-83i	MphA	CatA1	-
Brazil-2008a	Human	Unknown	2008	ST437	K36	KL36	O4	AadA2	-	fosA	QnrB1;ParC-80i;GyrA-83i	-	-	-
Brazil-2008b	Human	Unknown	2008	ST437	K36	KL36	O4	Aph3-1a;AadA2;AadA1-pm;Aac6-lb;StrB;StrA	-	fosA	ParC-80i;GyrA-83i	MphA	FloR	-
Brazil-2009a	Human	Unknown	2009	ST11	K127	KL127	OL101	Aph3-1a;AadA1-pm;AadA2	PmrB-65%	fosA	ParC-80i;GyrA-83i	-	-	-
Brazil-2009b	Human	Unknown	2009	ST437	K36	KL36	O4	AadA1-pm;Aac3-1la;Aac6-lq;Aac6-lb;StrB;StrA	-	fosA	QnrA1;ParC-80i;GyrA-83i	-	CatA1;FloR	-
Brazil-2010a	Human	Unknown	2010	ST340	K15	KL15	O4	RmtD;AadA1-pm;Aac6-lb;AphA6	PmrB-60%	fosA	ParC-80i;GyrA-83i	-	CatA1	Arr4
Brazil-2010b	Human	Unknown	2010	ST340	K15	KL15	O4	StrB;StrA;Aac3-1ld;AphA6	-	fosA	ParC-80i;GyrA-83i	-	CatA2	-
Brazil-2010e	Human	Unknown	2010	ST11	K64	KL64	O2v1	AphA6;Aac6-lq	MgrB-85%	fosA	ParC-80i;GyrA-83i	-	-	-
CCBH17440	Human	Blood	2014	ST437	K36	KL36	O4	ArmA	MgrB-57%	fosA	QnrA1;ParC-80i;GyrA-83i	MsrE;MphE	CatB3;CatA1	Arr3
Colombia-2009a	Human	Unknown	2009	ST258	K107	KL107	O2v2	Aph4-1a;Aac3-1va;RmtG;AadA1-pm;AadA2;AadA16;Aac6-lb	-	fosA	ParC-80i;GyrA-83i;GyrA-87N	-	CmiA1	Arr3
Colombia-2009b	Human	Unknown	2009	ST258	K107	KL107	O2v2	Aph4-1a;Aac3-1va;AadA2;AadA1-pm	-	fosA	ParC-80i;GyrA-83i;GyrA-87N	-	CmiA1;CatA1	-
FA84	Animal - Chicken	Rectal swab	2015	ST340	K151	KL151	O4	Aac3-1ld;AadA2	-	fosA	QnrB5;ParC-80i;GyrA-83i	MphA	-	-
IBL2.4	Environment	Water	2013	ST11	K64	KL64	O2v1	Aph3-1a, Aac3-1la, Aph3-lb, Aph6-ld, Aac6-lb	mgrB SNP	fosA	QnrB66;ParC-80i;GyrA-83i;GyrA-87G	-	-	-
KP171	Environment	Water	2011	ST340	K15	KL15	O4	AadA2	-	fosA	QnrB2;ParC-80i;GyrA-83i	-	-	-
KP337	Human	Wound secretion	2010	ST258	K106	KL106	O2v2	AadA2;Aac6-lb	-	fosA	ParC-80i;GyrA-83i	MphA	CatA1	-
KP411	Human	Rectal swab	2010	ST437	K36	KL36	O4	AadA3;Aph3-1a	-	fosA	ParC-80i;GyrA-83i	MphA	CatB4	-
KP417	Human	Unknown	2011	ST258	K106	KL106	O2v2	AadA2;Aac6-lb	MgrB-34%	fosA	ParC-80i;GyrA-83i	MphA	-	-
KP488	Human	Rectal swab	2011	ST11	K64	KL64	O2v1	Aac3-1la	mgrB SNP	fosA	ParC-80i;GyrA-83i	-	CatA1	-
KP515	Human	Urine	2011	ST340	K15	KL15	O4	AadA2	-	fosA	ParC-80i;GyrA-83i	-	-	-
KP870	Human	Blood	2011	ST340	K15	KL15	O4	Aac3-1ld;AadA2	-	fosA	QnrB2;ParC-80i;GyrA-83i	-	CatA2	-
KPC02	Human	Blood	2013	ST340	K15	KL15	OL102	StrB	-	fosA	ParC-80i;GyrA-83i	-	-	-
KPC05	Human	Rectal swab	2014	ST11	K105	KL105	O2v2	Aph3-1a;AphA6	-	fosA	QnrB5;ParC-80i;GyrA-83i	MphA	CatB3	Arr3
KPC40	Human	Blood	2015	ST11	K105	KL105	O2v2	-	MgrB-87%	fosA	ParC-80i;GyrA-83i	-	-	-
KPC45	Human	Blood	2015	ST11	K105	KL105	O2v2	-	Tn2 disrupted position	fosA	ParC-80i;GyrA-83i	-	-	-
KPN535	Human	Rectal swab	2011	ST340	K15	KL15	O4	AadA2;Aph3-1a;AadA1-pm;AadA2;Aac3-1ld;AphA6	mgrB SNP	fosA	QnrB19;ParC-80i;GyrA-83i	-	CmiA1	-
IN7	Human	Urine	2016	ST11	K105	KL105	O2v2	AadA2;AadA1-pm;Aac6-lb	-	fosA	ParC-80i;GyrA-83i	-	CmiA5;CatA2	Arr2
SJRP-KP10	Human	Urine	2011	ST340	K15	KL15	O4	AadA2;Aac3-1ld;AadA1-pm	MgrB-66%	fosA	QnrB2;ParC-80i;GyrA-83i	-	-	-

Table 2 – Continuation

Strain	Sulfonamide resist	Tetracycline	Trimethoprim resistanc	Beta-lactam resistance	Carbapenemases	Extended-spectrum beta-lactamases	Beta-lactamase	Beta-lactamase
148	Sull	TetA	DfrA30	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
196	Sull	-	DfrA30	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
314	Sull	-	-	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
1194	Sull; SullI	TetB	DfrA8; DfrA15b; DfrA14	AmpH; OXA-9	KPC-2	CTX-M-15, TEM-55	SHV-11	TEM-1A
1714	Sull; SullI	TetD; TetB	DfrA12	AmpH	KPC-2	CTX-M-15	SHV-11	TEM-1B
1721	Sull; SullI	TetB; TetD	DfrA12	AmpH	-	CTX-M-15	SHV-11	-
1787	Sull	-	-	AmpH; OXA-2	KPC-2	CTX-M-8; CTX-M-2	SHV-11	-
1799	Sull	-	DfrA12	AmpH; OXA-2	KPC-2	CTX-M-2	SHV-11	TEM-1B
1849	SullI	TetG	DfrA12	AmpH	KPC-2	CTX-M-14	SHV-11	-
1853	Sull	-	-	AmpH; OXA-2	KPC-2	CTX-M-2	SHV-11	-
4182	Sull	-	DfrA12	AmpH; OXA-2	KPC-2	CTX-M-2	SHV-11	TEM-1B
4281	SullI	TetD	DfrA14	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	TEM-1B
4448	SullI; Sull	TetG	DfrA12	AmpH	KPC-2	CTX-M-14	SHV-11	-
4477	-	-	-	AmpH	KPC-2	CTX-M-15	SHV-11	-
4490	SullI; Sull	TetG	DfrA12	AmpH	KPC-2	CTX-M-14	SHV-11	-
11_BR_13	Sull	TetA	DfrA30	AmpH; OXA-1	KPC-2	-	SHV-11	-
12_BR_13	Sull; Sull	-	DfrA12	AmpH	KPC-2	CTX-M-2	SHV-11	TEM-1B
1ECKPC	Sull; Sull; Sull; Sull	TetD	DfrA27	AmpH; OXA-2	KPC-2	-	SHV-11	-
2KP	Sull	-	DfrA14	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
3111F	Sull	TetA; TetD	DfrA30	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
606B	Sull; SullI	TetA; TetD	DfrA12; DfrA14	AmpH; LAP-2	-	CTX-M-15	SHV-11	TEM-1A
B03	-	TetD	-	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	TEM-1B
B04	SullI; Sull	-	DfrA15b	AmpH	KPC-2	CTX-M-2	SHV-11	-
B11	-	TetD	DfrA14	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	TEM-1B
B16	Sull	TetD	DfrA30	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	TEM-1B
B30	-	-	-	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	TEM-1B
B35	-	TetA	-	AmpH	KPC-2	-	SHV-11	-
Brazil-2006	Sull; SullI	TetB; TetD	DfrA15b	AmpH	-	CTX-M-2	SHV-11	TEM-1B
Brazil-2007b	Sull	-	DfrA12	AmpH; OXA-9	-	-	-	TEM-1A
Brazil-2008a	Sull	TetA	-	AmpH	KPC-2	-	-	-
Brazil-2008b	Sull; SullI	TetA	-	AmpH; OXA-9	KPC-2	-	SHV-11	TEM-1A
Brazil-2009a	Sull	-	DfrA12	AmpH; OXA-9	KPC-2	-	-	TEM-1A
Brazil-2009b	Sull; SullI	TetB	DfrA8	AmpH; OXA-9	KPC-2	CTX-M-9	SHV-11	-
Brazil-2010a	Sull	TetB	DfrA8; DfrA14	AmpH; OXA-9	KPC-2	SHV-12; CTX-M-9	-	TEM-1A
Brazil-2010b	SullI	TetD	-	AmpH	KPC-2	CTX-M-15	-	TEM-1B
Brazil-2010e	SullI	-	DfrA15b	AmpH	KPC-2	-	-	-
CCBH17440	-	-	DfrA8	AmpH; OXA-1	KPC-2	CTX-M-9	SHV-11	-
Colombia-2009a	SullI; Sull; SullI	-	DfrA12; DfrA27	AmpH	KPC-3	CTX-M-12	SHV-11	TEM-1A
Colombia-2009b	Sull; SullI	-	DfrA12	AmpH	KPC-3	SHV-12	-	-
FA64	Sull	TetD	DfrA12; DfrA14	AmpH	-	CTX-M-15	SHV-11	TEM-1B
IBL2.4	-	TetA	DfrA30	AmpH	KPC-2	-	SHV-11	-
KP171	Sull; Sull; Sull	-	DfrA12; DfrA25	AmpH	-	-	SHV-11	-
KP337	Sull	-	DfrA12	AmpH; OXA-9	KPC-2	SHV-12	-	TEM-122
KP411	Sull	TetA	DfrA30	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
KP417	Sull	-	-	AmpH; OXA-9	KPC-2	-	SHV-11	-
KP488	Sull	-	DfrA30	AmpH; OXA-2	KPC-2	-	SHV-11	TEM-1B
KP515	Sull	-	DfrA12	AmpH	KPC-2	-	SHV-11	-
KP870	Sull	TetD	DfrA12; DfrA25	AmpH	KPC-2	CTX-M-15	SHV-11	TEM-1B
KPC02	-	-	-	AmpH	KPC-2	-	SHV-11	TEM-1A
KPC05	Sull	-	-	AmpH; OXA-1	KPC-2	CTX-M-15	SHV-11	-
KPC40	-	-	-	AmpH	-	CTX-M-15	SHV-11	-
KPC45	-	-	-	AmpH	KPC-2	CTX-M-15; CTX-M-15	SHV-11	-
KPN535	Sull; SullI	-	DfrA12; DfrA14	AmpH	KPC-2	CTX-M-15	SHV-11	-
N7	SullI	TetA	-	AmpH; OXA-10; OXA-9	KPC-2	SHV-182	SHV-11	-
SJRP-KP10	Sull	TetD	DfrA12; DfrA25	AmpH; OXA-9; OXA-2	KPC-2	CTX-M-15; CTX-M-59	-	-

Table 2 – Continuation

Strain	Quaternary ammonium compounds resistance	Heavy metal resistance genes	Plasmids population (incompatibility group)
148	QacA, SugE	merA, merB, merC, pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS	IncFIB, IncFII, IncN
196	QacE Δ 1, QacE, QacA	arsA, arsB, arD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS, silR, silS, silC, silE	IncFIB, IncFII, IncN
314	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncN
1194	QacE, QacA	terF, terA, terC, terZ, terX, terW, pcoA, pcoB, pcoC, pcoD	IncU, IncX3, IncR, IncHI1B, IncFII, IncFIA, ColpVC, ColRNAI
1714	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD, pcoE, pcoR-S, silC, silE, silR, silS	IncFIB
1721	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, merC, pcoA, pcoB, pcoC, pcoD	IncFIB
1787	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncL/M, IncN
1799	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD	IncFIB
1849	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncU, IncX3
1853	QacE Δ 1, QacE	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD	IncFIB, IncN
4182	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD	IncFIB, IncN
4281	QacE, QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD, silC, silE, silR, silS	IncFIB
4448	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncU, IncX3
4477	QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD, pcoA-E, pcoR-S, silC, silE	IncFII, IncFIB, IncN
4490	QacE Δ 1, QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncU, IncX3
11_BR_13	QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncN
12_BR_13	QacE, QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD	IncFIB, IncN
1ECKPC	QacE, QacA, sugE	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncP6
2KP	QacA, SugE	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFIA, IncFII, IncR, IncP6
3111F	QacE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncX4
606B	QacE, QacA	pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS, silC, silE, silR-S, MerC, arsB, arsD, arsH	ColRNAI, IncFIA, IncFIB, IncFII, IncR
B03	QacA, SugE	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncN
B04	QacE Δ 1, QacE, QacA, SugE	terF, terA, terC, terZ, terX, terW, merC, merA, merE	IncFIB, IncFII, IncN, IncQ1, IncR, HI1B
B11	QacE, QacA, SugE	silE, arsB, arsH, pcoA, pcoB, pcoC, pcoD	IncFIA, IncFIB, IncR, IncX3
B16	QacE Δ 1, QacE, QacA, SugE	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncN
B30	QacE, QacA, SugE	pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncN
B35	SugE, QacA	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColRNAI, IncFIB, IncFII, IncN
Brazil-2006	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, HI1B
Brazil-2007b	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColRNAI, IncFIB, IncFII, IncX3
Brazil-2008a	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	Col156, IncFIB, IncFII, IncN
Brazil-2008b	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncA/C2, IncFIB, IncFII, IncN
Brazil-2009a	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIA, IncFIB, IncFII, HI1B, IncN
Brazil-2009b	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII
Brazil-2010a	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColFST258, IncFIA, IncFIB, IncFII, HI1B, IncN
Brazil-2010b	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, HI1A, HI1B, IncR, IncN
Brazil-2010e	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, HI1B, IncQ1, IncR, IncL/M
CCBH17440	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, HI1B, IncN
Colombia-2009a	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColRNAI, IncFIB, IncFII, IncN, IncR, IncL/M, IncI2
Colombia-2009b	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColRNAI, IncFIB, IncFII, IncN, IncR, IncI2
FA64	SugE, QacA	silE, arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	ColFST258, IncFIA, IncFIB, IncFII, HI1B
IBL2.4	QacA, QacE, SugE	pcoA-E, pcoR-S, silC, silE, silR-S	ColRNAI, IncFIB, IncFII, IncN
KP171	QacA	arsB, arsD, arsH, merC, merA, merE, pcoA, pcoB, pcoC, pcoD, pcoR, silC	Col440I, IncFIA, IncFII, IncFIB, IncN, IncR
KP337	SugE, QacA	silE, arsB, arsH, pcoA, pcoB, pcoC, pcoD	IncFII, IncFIB, IncX3
KP411	SugE, QacA	silE, arsB, arsH, pcoA, pcoB, pcoC, pcoD	IncFII, IncFIB, IncN
KP417	SugE, QacA	arsA-D, arsH, pcoA-E, pcoR-S, silE	IncFII, IncFIB, IncL/M, IncX3
KP488	QacA, QacE, SugE	pcoA-E, pcoR-S, silC, silE, silR-S	ColRNAI, IncFIB, IncFII, IncN
KP515	SugE, QacA	silE, arsA, arsB, arsH, pcoA, pcoB, pcoC, pcoD	IncFII, IncFIB, IncN
KP870	QacC, SugE	silE, arsA, arsB, arsH, pcoA, pcoB, pcoC, pcoD	IncFIA, IncFII, IncFIB, IncN, IncR
KPC02	SugE, QacA	arsB, merC, merA, merE, pcoA, pcoB, pcoC, pcoD	ColIMGS31, IncFII, IncN
KPC05	QacE Δ 1, QacE, QacA, SugE	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD, pcoA-E, pcoR-S, silE	IncFIB, IncL/M, IncQ1, IncR
KPC40	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncFIB, IncFII, IncN
KPC45	QacA	pcoA-E, pcoR-S	Col(MGD2), IncR
KPN535	QacL, QacA, QacE Δ 1, SugE	arsA-D, arsH, merA-C, pcoA-E, pcoR-S, silR-S, silC, silE	Col440I, Col440II, ColRNAI, IncFIA, IncFIB, IncI1, IncQ1, IncR
N7	SugE, QacA	arsB, arsD, arsH, pcoA, pcoB, pcoC, pcoD	IncA/C2, IncR, IncP6
SJRP-KP10	QacE Δ 1, QacE	arsA-D, arsH, pcoA-E, pcoR-S, silE	IncFIA, IncFII, IncFIB, IncFIB, IncR, IncHI1B, IncFIA, IncL/M, IncU

Table 3

Strain	Country	Sequence type (ST)	Insertion sequence (IS)	Yersiniabactin	ICEKp	YbST	Colibactin	CbST	ybtS	ybtX	ybtQ	ybtP	ybtA	irp2	irp1	ybtU	ybtT	ybtE	fyuA
B04	Brazil	ST11	-	9	ICEKp3	344	-	-	ybtS_5	ybtX_11	ybtQ_6	ybtP_90	ybtA_1	irp2_187	irp1_217	ybtU_9	ybtT_5	ybtE_5	fyuA_11
2KP	Argentina	ST340	-	9	ICEKp3	345	-	-	ybtS_5	ybtX_11	ybtQ_6	ybtP_5	ybtA_1	irp2_171	irp1_192	ybtU_9	ybtT_5	ybtE_5	fyuA_11
1194	Brazil	ST340	-	9	ICEKp3	183	-	-	ybtS_5	ybtX_11	ybtQ_6	ybtP_5	ybtA_1	irp2_25	irp1_37	ybtU_9	ybtT_5	ybtE_5	fyuA_11
KPC40	Brazil	ST11	-	10	ICEKp4	26	-	-	ybtS_16	ybtX_12	ybtQ_4	ybtP_3	ybtA_3	irp2_35	irp1_33	ybtU_3	ybtT_10	ybtE_9	fyuA_2
KPC45	Brazil	ST11	-	10	ICEKp4	26	-	-	ybtS_16	ybtX_12	ybtQ_4	ybtP_3	ybtA_3	irp2_35	irp1_33	ybtU_3	ybtT_10	ybtE_9	fyuA_2
KPC05	Brazil	ST11	-	10	ICEKp4	26	-	-	ybtS_16	ybtX_12	ybtQ_4	ybtP_3	ybtA_3	irp2_35	irp1_33	ybtU_3	ybtT_10	ybtE_9	fyuA_2
B11	Brazil	ST11	-	10	ICEKp4	26	-	-	ybtS_16	ybtX_12	ybtQ_4	ybtP_3	ybtA_3	irp2_35	irp1_33	ybtU_3	ybtT_10	ybtE_9	fyuA_2
Brazil-2010e	Brazil	ST11	-	16	ICEKp12	277	-	-	ybtS_6	ybtX_15	ybtQ_5	ybtP_4	ybtA_11	irp2_33	irp1_23	ybtU_2	ybtT_11	ybtE_4	fyuA_2
1853	Brazil	ST11	-	17	ICEKp10	265-3LV	2B	19-1LV	ybtS_6	ybtX_15	ybtQ_5	ybtP_13	ybtA_11	irp2_153*	irp1_29*	ybtU_2	ybtT_11	ybtE_4	fyuA_2*
Colombia-2009a	Colombia	ST258	-	17	ICEKp10	265	2B	19-1LV	ybtS_6	ybtX_15	ybtQ_5	ybtP_13	ybtA_11	irp2_279	irp1_29	ybtU_2	ybtT_11	ybtE_4	fyuA_2
Colombia-2009b	Colombia	ST258	-	17	ICEKp10	265	2B	19	ybtS_6	ybtX_15	ybtQ_5	ybtP_13	ybtA_11	irp2_279	irp1_29	ybtU_2	ybtT_11	ybtE_4	fyuA_2
12_BR_13	Brazil	ST11	IS110	18	ICEKp10	346	2B	17	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
4182	Brazil	ST11	IS110	18	ICEKp10	346	2B	17	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
1799	Brazil	ST11	IS110	18	ICEKp10	346	2B	17-1LV	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
1787	Brazil	ST11	IS110	18	ICEKp10	346	2B	17-1LV	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
KP488	Brazil	ST11	IS110	18	ICEKp10	346	2B	17	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
IBL2.4	Brazil	ST11	IS110	18	ICEKp10	346	2B	17	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
B35	Brazil	ST11	IS110	18	ICEKp10	346	2B	17	ybtS_48	ybtX_15	ybtQ_5	ybtP_94	ybtA_60	irp2_181	irp1_200	ybtU_50	ybtT_11	ybtE_4	fyuA_84
N7	Chile	ST11	-	10	ICEKp4	95-1LV	-	-	ybtS_3	ybtX_12	ybtQ_4	ybtP_66	ybtA_3	irp2_137	irp1_139	ybtU_3	ybtT_10	ybtE_60	fyuA_2

* Represent the presence of SNPs.

Note: After underline "_" represent the allele number

Table 3 – Continuation

Strain	clbA	clbB	clbC	clbD	clbE	clbF	clbG	clbH	clbI	clbJ	clbK	clbL	clbM	clbN	clbO	clbP	clbQ	clbR
B04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2KP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1194	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KPC40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KPC45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KPC05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brazil-2010e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1853	clbA_2	clbB_10*	clbC_2	clbD_2	-	clbF_4	clbG_2	clbH_8	clbI_2	clbJ_6*	clbK_11*	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	-
Colombia-2009a	clbA_2	clbB_10	clbC_2	clbD_2	clbE_2	clbF_4	clbG_2	clbH_8	clbI_2	clbJ_6	clbK_6	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	clbR_2
Colombia-2009b	clbA_2	clbB_10	clbC_2	clbD_2	clbE_2	clbF_4	clbG_2	clbH_8	clbI_2	clbJ_7	clbK_6*	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	clbR_2
12_BR_13	clbA_2	clbB_10	clbC_2	clbD_2	-	clbF_2	clbG_2	clbH_8	clbI_2	clbJ_6	clbK_6	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	-
4182	clbA_2	clbB_10	clbC_2*	clbD_2	-	clbF_2	clbG_2	clbH_8	clbI_2	clbJ_4	-	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	-
1799	clbA_2	clbB_10	clbC_2	clbD_2	-	clbF_2	clbG_2	clbH_8*	clbI_2	clbJ_6	clbK_6	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	-
1787	clbA_2	clbB_10	clbC_2	clbD_2	-	clbF_2	clbG_2	clbH_8	clbI_2*	clbJ_6	clbK_6	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	-
KP488	clbA_2	clbB_10	clbC_2	clbD_2	clbE_2	clbF_2	clbG_2	clbH_8	clbI_2	clbJ_4	clbK_11	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	clbR_2
IBL2.4	clbA_2	clbB_10	clbC_2	clbD_2	clbE_2	clbF_2	clbG_2	clbH_8	clbI_2	clbJ_4	clbK_11*	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	clbR_2
B35	clbA_2	clbB_10	clbC_2	clbD_2	clbE_2	clbF_2	clbG_2	clbH_8	clbI_2	clbJ_7	clbK_6*	clbL_2	clbM_2	clbN_2	clbO_2	clbP_2	clbQ_2	clbR_2
N7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Represent the presence of SNPs.

Note: After underline "_" represent the allele number

4. CONCLUSION

To conclude, our study points out several important issues. Firstly, assembly and annotation of *K. pneumoniae* genomes obtained by long-and/or -short reads sequencing technology contributed for the first draft genomes of *K. pneumoniae* CG258 isolated from environment and food-producing animal (swine and chicken) samples, in Brazil. In this regard, these data confirm a wide antimicrobial resistome (antibiotics, disinfectants and heavy metals), and diversity of replicons types and plasmid content, among predominant ST11 and ST340 clones. Second, characterization of plasmids carrying *bla*_{KPC-2} and *bla*_{CTX-M} genes revealed the presence of Col-like > IncN (ST15) > IncX3-U > IncQ1 > IncF > IncU harboring *bla*_{KPC-2}, and IncF > IncR > harboring *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, *bla*_{CTX-M-9}, *bla*_{CTX-M-14}, and *bla*_{CTX-M-15} genes in *K. pneumoniae* of the CG258, in Brazil. Third, Tn4401b, Tn4401i, NTE_{KPC}^{-Ic} and NTE_{KPC}^{-IIId} genetic elements contributed to mobilization and transposition of *bla*_{KPC-2} genes in *K. pneumoniae* of the CG258. Fourth, antimicrobial resistome of *K. pneumoniae* lineages belonging to the CG258 revealed presence of genes conferring a MDR phenotype for beta-lactams (*bla*_{CTX-M-type}, *bla*_{KPC-2}, *bla*_{TEM-55}, *bla*_{SHV-182-like}, aminoglycosides (16S rRNA methylases and transferases), fluoroquinolones [PMQR and QRDR mutations (GyrA 83I and ParC 80I presented in all strains, and novel GyrA 87Y and 87N mutations)], tetracyclines, sulphas and polymyxins (deletions/insertions/mutations in *mgrB* and/or *pmrB* genes) antibiotics, as well as for QACs and heavy metals (mainly mercury, arsenic, silver, copper and tellurium). Fifth, analyze of virulome of *K. pneumoniae* lineages belonging to the CG258 revealed the presence of yersiniabactin, colibactin, aerobactin, and ICEKp3, ICEKp4, ICEKp10, and ICEKp12 carrying *ybt* and/or *clb*. A novel *ybt* was identified (designated *ybt18*), which belonged to YbST346; whereas KL64 was predominant among *K. pneumoniae* carrying ICEKp elements. Sixth, presence of yersiniabactin was associated with an enhanced virulent behavior of *K. pneumoniae* lineages belonging to ST11 and ST340 lineages. Seventh, identification of *ybt* and *clb* gene clusters, and KL64 gene markers could be used as biomarkers for severe *K. pneumoniae* infections.

The results obtained in the present study allow us to obtain a first genomic landscape of *K. pneumoniae* lineages of the CG258, circulating at the human-animal-environment interface, in Brazil and South America. In this regard, most likely the interplay of yersiniabactin and/or colibactin, and resistance to clinically significant antibiotics (as carbapenems and polymyxins) are contributing to the emergence of highly virulent and MDR lineages that pose great risk to human health. On the other hand, the wide antimicrobial resistome (antibiotics, disinfectants and

heavy metals) could be contributing to adaptation of KPC-2- and/or CTX-M-producing *K. pneumoniae* CG258 in the human-animal-environment interface, highlighting the urgent need for enhanced control efforts. In conclusion, these findings could contribute to the development of strategies for prevention, diagnosis and treatment of *K. pneumoniae* infections.

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APPENDIX A – Bioinformatic pipeline (PIPA) developed during this study

**29º Congresso Brasileiro
de Microbiologia**

De 22 a 25 de Outubro de 2017 / Foz do Iguaçu-Paraná-Brasil

"60 anos da Sociedade Brasileira de Microbiologia"

Certificado

Certificamos que o trabalho intitulado "SUCCESSFUL DISSEMINATION OF KLEBSIELLA PNEUMONIAE CC258 (ST11, ST437 AND ST340) IN BRAZIL IS SUPPORTED BY AMR GENES, UBIQUITY, PLASMID STABILITY AND VIRULENT BEHAVIOR " com autoria de "LOUISE CERDEIRA; MIRIAM R. FERNANDES; FERNANDA ESPÓSITO; RALF LOPES; GABRIELA FRANCISCO RODRIGUES ; MARIA FERNANDA C. BUENO; DOROTI DE OLIVEIRA GARCIA; NILTON LINCOPAN " foi o 1º colocado como o melhor trabalho na área de " Microbiologia Clínica e Infecção Hospitalar ", durante 29º Congresso Brasileiro de Microbiologia no Rafain Palace Hotel e Convention Center , na cidade de Foz do Iguaçu, PR, no período de 22 a 25 de outubro de 2017.

Prof. Dr. Carlos Pelleschi Taborda
Presidente da SBM

Prof. Dr. Jorge Luiz Mello Sampaio
1º Secretário da SBM

ORGANIZAÇÃO E REALIZAÇÃO:

APOIO:

Eventos simultâneos: XV Encontro Nacional de Microbiologia Ambiental – ENAMA / XIV International Meeting on Paracoccidioidomycosis – PCM

APPENDIX C - *Curriculum Vitae*

Louise Teixeira Cerdeira

Curriculum Vitae

Nome civil

Nome Louise Teixeira Cerdeira

Dados pessoais

Filiação Gilberto de Lima Cerdeira e Maria Luiza Teixeira Cerdeira

Nascimento 23/11/1981 - Belém/PA - Brasil

Carteira de Identidade 3001017 SSP - PA - 20/07/2005

CPF 655.999.852-53

Formação acadêmica/titulação

- 2015** Doutorado em Ciências.
Universidade de São Paulo, USP, São Paulo, Brasil
com período sanduíche em The University of Melbourne (Orientador : Kathryn Elizabeth Holt)
Título: Análise comparativa do resistoma de *Klebsiella pneumoniae* XDR (NDM-1/KPC-2) pertencente ao complexo clonal (CC) endêmico de alto risco CC258
Orientador: Nilton Erbet Lincopan Huenuman
Bolsista do(a): Fundação de Amparo à Pesquisa do Estado de São Paulo
- 2009 - 2011** Mestrado em Genética e Biologia Molecular (Bioinformática).
Universidade Federal do Pará, UFPA, Belém, Brasil
Título: Montagem ab initio de um genoma procarioto utilizando sequências curtas de uma biblioteca pareada: caso de estudo *Corynebacterium pseudotuberculosis*, linhagem I19, Ano de obtenção: 2011
Orientador: Artur Luiz da Costa da Silva
Bolsista do(a): Fundação Amazônia Paraense de Amparo à Pesquisa
- 2015 - 2016** Especialização em MBA EM GESTÃO ESTRATÉGICA E ECONÔMICA DE PROJETOS.
Fundação Getúlio Vargas, FGV-SP, Brasil
Título: Gestão Estratégica de Inovação em Empresas de Diagnóstico
- 2004 - 2005** Especialização em Redes de Computadores.
Universidade da Amazônia, UNAMA, Belém, Brasil
Título: Wireless - Acesso a Internet baseado em GPRS
Orientador: Ricardo Souza Cabeça
- 2000 - 2004** Graduação em Bacharelado em Ciência da Computação.
Centro Universitário do Estado do Pará, CESUPA, Belém, Brasil
Título: Agentes Racionais Reativos: Arquitetura, Exemplos, Comparações e Opções
Orientador: Gustavo Campos

Formação complementar

- 2015 - 2015** Extensão universitária em Medical Device Development. (Carga horária: 20h).
Harvard University, HARVARD, Cambridge, Estados Unidos
- 2014 - 2014** Extensão universitária em Exome Sequencing and Analysis. (Carga horária: 40h).
Baylor College of Medicine, BCM, Houston, Estados Unidos

2013 - 2013	Curso de curta duração em The Genome Access Course. (Carga horária: 30h). Cold Spring Harbor Laboratory, CSHL, Cold Spring Harbor, Estados Unidos
2012 - 2012	Curso de curta duração em Direito autoral. (Carga horária: 8h). Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, IFES, Vitoria, Brasil
2012 - 2012	Curso de curta duração em Revisão de Textos - Word. (Carga horária: 8h). Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, IFES, Vitoria, Brasil
2011 - 2012	Curso de curta duração em Práticas da Educação a Distância - Tutor 2011 - 2. (Carga horária: 100h). Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, IFES, Vitoria, Brasil
2010 - 2010	Extensão universitária em Curso online de formação em tutoria. (Carga horária: 80h). Universidade Federal do Pará, UFPA, Belem, Brasil
2010 - 2010	Curso de curta duração em Plataforma de Sequenciamento de Nova Geração. (Carga horária: 90h). Universidade Federal do Pará, UFPA, Belem, Brasil
2009 - 2009	Extensão universitária em V Curso de Verão em Bioinformática. (Carga horária: 70h). Universidade de São Paulo, USP, Sao Paulo, Brasil
2009 - 2009	Curso de curta duração em Workshop de Bioinformática. (Carga horária: 4h). Life Technologies , LIFE TEC, Sao Paulo, Brasil
2007 - 2007	Curso de curta duração em Treinamento introdutório para bancários. (Carga horária: 51h). Banco do estado do para, BANPARA, Brasil
2007 - 2007	Curso de curta duração em Licitações e Contratos na Administração Pública. (Carga horária: 20h). Escola de Governo do Estado do Pará, EGPA, Brasil
2007 - 2007	Curso de curta duração em Gerenciamento de requisitos baseado em use case. (Carga horária: 10h). TotalBanco, TOTALBANCO, Brasil
2006 - 2006	Curso de curta duração em Certificação Cisco 4.0. (Carga horária: 52h). Lanlink Informática Ltda, LANLINK, Brasil
2006 - 2006	Curso de curta duração em ES Router Configuration. (Carga horária: 40h). Lan Professional, LANPRO, Brasil
2005 - 2005	Extensão universitária em FCP Fundamental. (Carga horária: 40h). Flycomm Training Network, FLYCOMM, Brasil
2005 - 2005	Extensão universitária em Prática em montagem de rack. (Carga horária: 4h). Flycomm Training Network, FLYCOMM, Brasil
2005 - 2005	Curso de curta duração em Capacitação On-line para Gestores e Educadores TIN. (Carga horária: 9h). Ministério do Desenvolvimento, Indústria e Comércio Exterior, MID, Brasil
2004 - 2004	Curso de curta duração em Introdução ao MATlab. (Carga horária: 10h).

	XI Semana de Informática da UFPa, SEMINF, Brasil
2004 - 2004	Curso de curta duração em PHP / MySQL. (Carga horária: 10h). XI Semana de Informática da UFPa, SEMINF, Brasil
2004 - 2004	Extensão universitária em Java Fundamentos. (Carga horária: 40h). Argos Tecnologia, ARGOS, Brasil
2002 - 2002	Extensão universitária em Sgta Sistema Gerenciador de Telecomunicações Aer. (Carga horária: 21h). Nibble Informática, NIBBLE, Brasil
2002 - 2002	Extensão universitária em Instalação e Administração de Redes Ms Windows Nt. (Carga horária: 20h). Starlink Conectividade, STARLINK, Brasil
2002 - 2002	Extensão universitária em Delphi 6 Desenvolvimento de Aplicações. (Carga horária: 40h). Borland Learning Partner, BORLAND, Brasil
2002 - 2002	Extensão universitária em Delphi 6 Criação de Componentes. (Carga horária: 24h). Borland Learning Partner, BORLAND, Brasil
2000 - 2000	Extensão universitária em Formação de Agentes de Direitos Humanos. (Carga horária: 20h). Centro de Assessoramento a Programas de Educação Para a Cidadania, CAPEC, Brasil
2000 - 2000	Extensão universitária em Delphi 5. (Carga horária: 20h). Centro Universitário do Estado do Pará, CESUPA, Belem, Brasil
1999 - 1999	Extensão universitária em Manutenção de Computadores. (Carga horária: 50h). Modelagem e Projeto de Sistemas de Computação, MPS, Brasil
1997 - 1997	Curso de curta duração em OPER. MICROINFORMÁTICA. (Carga horária: 40h). Associação Profissional dos Técnicos Industriais do Pará e Amapá, APTIPA, Brasil

Atuação profissional

1. Grupo Fleury - FLEURY

Vínculo institucional

2012 - 2015	Vínculo: Celetista , Enquadramento funcional: Assessora Científica - Bioinformática , Carga horária: 40, Regime: Dedicação exclusiva
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Atividades

11/2012 - 02/2015 Serviço Técnico Especializado, Inovação, Pesquisa & Desenvolvimento - IP&D

Especificação:

Coordenação da área de Bioinformática , Desenvolvimento de aplicações e automatização de processos de rotina clínica , Desenvolvimento de estratégias e Soluções de Bioinformática para diagnóstico utilizando NGS - Exoma, Painel e NIPT , Desenvolvimento e administração de banco de dados biológicos

11/2012 - 02/2015 Pesquisa e Desenvolvimento, Inovação, Pesquisa & Desenvolvimento - IP&D

Linhas de pesquisa:

2. Instituto Federal de São Paulo - IFSP

Vínculo institucional

2014 - 2014 Vínculo: Servidor público , Enquadramento funcional: Professor , Carga horária: 20, Regime: Parcial

Atividades

08/2014 - 12/2014 Graduação, Análise e Desenvolvimento de Sistemas

Disciplinas ministradas:

Sistema de Gerenciamento de Banco de dados 1 , Sistemas Operacionais 1 , Teoria do Design e Computação Gráfica , Conectividade 2 , Informática aplicada , Segurança de Dados 2

03/2014 - 07/2014 Graduação, Análise e Desenvolvimento de Sistemas

Disciplinas ministradas:

Conectividade 2 , Introdução ao software , Linguagem de programação 1 usando C++ , Segurança de dados 1 , Segurança de dados 2 , Sistemas operacionais

3. Sociedade Brasileira de Microbiologia - SBM

Vínculo institucional

2014 - 2014 Vínculo: Contratado , Enquadramento funcional: Professor, Regime: Parcial

Atividades

08/2014 - 12/2014 Aperfeiçoamento

Especificação:

Acabamento de genomas , Alinhamento de sequências , Anotação de sequências , Depósito de genomas , Montagem de novo , Sistema operacional Linux

4. Instituto Evandro Chagas - IEC

Vínculo institucional

2012 - 2014 Vínculo: Colaborador , Enquadramento funcional: Pesquisador , Carga horária: 10, Regime: Parcial

Outras informações:

Atualmente vice-coordenadora do Núcleo de Bioinformática do CIT-IEC
<http://www.iec.pa.gov.br/index.php/gcPagina/index/66>

Atividades

11/2012 - Atual Pesquisa e Desenvolvimento, Conselho Técnico Científico

Linhas de pesquisa:

Genômica , Transcriptômica , Metagenômica , Mirnômica

5. Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo - IFES

Vínculo institucional

2012 - 2014 Vínculo: Bolsista Capes , Enquadramento funcional: Professora Orientadora de TCC, Regime: Parcial

2012 - 2013 Vínculo: Bolsista Capes , Enquadramento funcional: Tutor à distância,

Regime: Parcial

Atividades

- 05/2013 - Atual** Especialização
Especificação:
Trabalho de Conclusão de curso
- 02/2013 - 06/2013** Graduação, Licenciatura em Informática
Disciplinas ministradas:
Técnicas de programação , Redes de Computadores
- 12/2012 - 02/2013** Graduação, Licenciatura em Informática
Disciplinas ministradas:
Monografia I
- 08/2012 - 12/2012** Graduação, Licenciatura em Informática
Disciplinas ministradas:
Fundamentos de Sistema de Informação , Linguagem de Programação I
- 02/2012 - 07/2012** Graduação, Licenciatura em Informática
Disciplinas ministradas:
Algoritmos I , Laboratório de Montagem e Manutenção
- 01/2012 - 02/2013** Especialização
Especificação:
Trabalho de Conclusão de Curso

6. Universidade Federal do Pará - UFPA

Vínculo institucional

2008 - 2012 Vínculo: Colaborador , Enquadramento funcional: Colaborador, Regime: Parcial

7. Laboratório Nacional de Computação Científica - LNCC

Vínculo institucional

2012 - 2012 Vínculo: Celetista formal , Enquadramento funcional: Analista de Sistema Sênior - Bioinformática , Carga horária: 40, Regime: Integral

8. Sociedade Objetivo de Ensino Superior (UNIP-PA) - SOES - UNIP

Vínculo institucional

2007 - 2010 Vínculo: Celetista formal , Enquadramento funcional: Tutor , Carga horária: 10, Regime: Parcial

Atividades

02/2007 - 12/2010 Graduação, Gestão em Sistemas de Informação
Disciplinas ministradas:
Administração de Banco de Dados , Fundamentos de Sistemas Operacionais , Gerenciamento de Projetos de Tecnologias da Informação , Organização de Computadores

9. Associação para o desenvolvimento Educacional do Pará (Faculdade Ipiranga) - ADEPA

Vínculo institucional

2007 - 2009 Vínculo: Celetista formal , Enquadramento funcional: Professor horista , Carga horária: 20, Regime: Parcial

Atividades

02/2007 - 12/2008 Graduação, Administração

*Disciplinas ministradas:
Informática Básica*

02/2007 - 12/2008 Graduação, Turismo

*Disciplinas ministradas:
Informática Básica*

02/2007 - 12/2008 Graduação, Jornalismo

*Disciplinas ministradas:
Editoração eletrônica , Informática Básica*

10. Escola Agrotécnica Federal de Castanhal - EAFC-PA

Vínculo institucional

2005 - 2005 Vínculo: Servidor público , Enquadramento funcional: Professora Substituta , Carga horária: 40, Regime: Integral

Atividades

01/2005 - 12/2005 Ensino médio

*Especificação:
Informática Avançada , Informática Básica*

11. Banco do Estado do Pará - BANPARÁ

Vínculo institucional

2007 - 2012 Vínculo: Celetista formal , Enquadramento funcional: Técnico de Informática nível superior , Carga horária: 30, Regime: Parcial

12. BS SERVICES LTDA (COBRA TECNOLOGIA) - BS

Vínculo institucional

2006 - 2007 Vínculo: Celetista formal , Enquadramento funcional: Analista de Suporte IV , Carga horária: 40, Regime: Integral

13. Tim Celular S A - TIM

Vínculo institucional

2003 - 2004 Vínculo: Celetista , Enquadramento funcional: Assistente I , Carga horária: 40, Regime: Integral

Atividades

10/2003 - 04/2004 Serviço Técnico Especializado, Varejo

*Especificação:
Elaboração de relatórios analíticos de venda de toda região Norte, acompanhamento dos resultados das vendas através de análises concretas e objetivas possibilitando melhorias contínuas no gerenciamento, nos pontos de venda e nos promotores. Lançamento de*

14. Shalom S. de Processamentos de Dados Ltda (INFRAERO) - SHALOM

Vínculo institucional

2002 - 2002 Vínculo: Celetista formal , Enquadramento funcional: Técnica de Produção , Carga horária: 40, Regime: Integral

Atividades

01/2002 - 10/2002 Serviço Técnico Especializado, Informática

Especificação:

Instalação, Controle e Monitoramento da Infra - estrutura física e lógica da rede do Aeroporto Internacional de Belém e suas regionais

15. Centro Brasileiro-Argentino de Biotecnologia - MCT - CBAB

Vínculo institucional

2010 - 2010 Vínculo: Colaborador , Enquadramento funcional: Monitor de Bioinformática , Carga horária: 45, Regime: Integral

16. Universidade do Estado do Pará - UEPA

Vínculo institucional

2004 - 2004 Vínculo: Colaborador , Enquadramento funcional: Monitora de Informática , Carga horária: 10, Regime: Parcial

Atividades

07/2004 - 07/2004 Treinamento, Departamento de Educação

Especificação:

Informática Básica

17. The University of Melbourne - UNIMELB

Vínculo institucional

2018 - 2018 Vínculo: Bolsista , Enquadramento funcional: Phd Student, Regime: Parcial

18. Universidade de São Paulo - USP

Vínculo institucional

2016 - Atual Vínculo: Bolsista , Enquadramento funcional: Phd Student, Regime: Parcial

Linhas de pesquisa

1. Big data for healthcare
2. Bioinformática
3. Informática médica
4. Genômica

- 5. Metagenômica
- 6. Mirnômica
- 7. Transcriptômica

Revisor de periódico

1. BIOMED RESEARCH INTERNATIONAL

Vínculo

2019 - Atual Regime: Parcial

2. Frontiers in Microbiology

Vínculo

2018 - Atual Regime: Parcial

3. Journal of Global Antimicrobial Resistance

Vínculo

2018 - Atual Regime: Parcial

4. Journal of Infection and Public Health

Vínculo

2017 - Atual Regime: Parcial

5. Journal of Clinical Bioinformatics

Vínculo

2015 - Atual Regime: Parcial

6. Applied Bioinformatics

Vínculo

2015 - Atual Regime: Parcial

Membro de corpo editorial

1. Ecronicon Microbiology

Vínculo

2017 - Atual Regime: Parcial

Produção

Produção bibliográfica

Artigos completos publicados em periódicos

1. RUMI, M. V.; **Cerdeira, L.**; LINCOPAN, N.

Co-occurrence of clinically relevant β -lactamases and MCR-1 encoding genes in *Escherichia coli* from companion animals in Argentina. VETERINARY MICROBIOLOGY. , v.230, p.228 - 234, 2019.

2. GONZALEZ-ROCHA, G.; **Cerdeira, L.**; OPAZO-CAPURRO, A.; BELLO-TOLEDO, H.; MORALES-LEON, F.; LINCOPAN, N.

Evolutionary dynamics of carbapenem-resistant *Acinetobacter baumannii* circulating in Chilean hospitals. INFECTION GENETICS AND EVOLUTION. , v.73, p.93 - 97, 2019.

3. **Cerdeira, Louise T.**; LAM, MARGARET M. C.; WYRES, KELLY L.; WICK, RYAN R.; JUDD, LOUISE M.; LOPES, RALF; RIBAS, ROSINEIDE M.; MORAIS, MARCIA M.; HOLT, KATHRYN E.; LINCOPAN, NILTON

Small IncQ1 and Col-like Plasmids Harboring *bla*_{KPC-2} and non-Tn *4401* Elements (NTE *KPC*-IIId) in High-Risk Lineages of *Klebsiella pneumoniae* CG258. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. , v.1, p.1 - , 2019.

4. SACRAMENTO, ANDREY G.; FERNANDES, MIRIAM R.; SELLERA, FÁBIO P.; DOLABELLA, SILVIO S.; ZANELLA, ROSEMEIRE C.; **Cerdeira, Louise**; LINCOPAN, NILTON

VanA-type vancomycin-resistant *Enterococcus faecium* ST1336 isolated from mussels in an anthropogenically impacted ecosystem. MARINE POLLUTION BULLETIN. , v.142, p.533 - 536, 2019.

5. FUENTES-CASTILLO, D.; MOURA, Q.; LOPES, R.; FERNANDES, M.; CARDOSO, B.; ESPOSITO, F.; **Cerdeira, L.**; LINCOPAN, N.

Wild owls colonized by international clones of extended-spectrum β -lactamase (CTX-M)-producing *Escherichia coli* and *Salmonella* *Infantis* in the Southern Cone of America. SCIENCE OF THE TOTAL ENVIRONMENT. , v.674, p.554 - 562, 2019.

6. FERREIRA, M. L.; FUGA, B.; CAMPOS, P.; **CERDEIRA, L.**; RIBAS, R.

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7. FERNANDES, M.; MOURA, Q.; **Cerdeira, L.**; LINCOPAN, N.

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8. CASELLA, TIAGO; DE MORAIS, ANDRESSA BATISTA ZEQUINI; DE PAULA BARCELOS, DIEGO DINIZ; TOLENTINO, FERNANDA MODESTO; **Cerdeira, Louise Teixeira**; BUENO, MARIA FERNANDA CAMPAGNARI; FRANCISCO, GABRIELA RODRIGUES; DE ANDRADE, LEONARDO NEVES; DA COSTA DARINI, ANA LUCIA; DE OLIVEIRA GARCIA, DOROTI; LINCOPAN, NILTON; NOGUEIRA, MARA CORRÊA LELLES

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9. TAMARIZ, J.; ROSALES, C. L.; RAMOS, C. S.; BELTRAN, P. M.; CABRERA, J. L.; FERNANDES, M.; **Cerdeira, L.**; LINCOPAN, N.

Draft Genome Sequence of the First New Delhi Metallo- β -Lactamase (NDM-1)-Producing *Escherichia coli* Strain Isolated in Peru. GENOME ANNOUNCEMENTS. , v.6, p.e00199-18 - , 2018.

10. FILL, TAÍCIA PACHECO; BARETTA, JÉSSICA FERNANDA; DE ALMEIDA, LUIZ GONZAGA PAULA; MALAVAZI, IRAN; **Cerdeira, Louise Teixeira**; SAMBORSKY, MARKIYAN; DE VASCONCELOS, ANA TEREZA RIBEIRO; LEADLAY, PETER; RODRIGUES-FILHO, EDSON

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20. FERNANDES, M.; **Cerdeira,L**; SELLERA, F.; SILVA, M.; LINCOPAN, N.
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24. MONTE, D.; MEM, A.; FERNANDES, M.; **Cerdeira, Louise**; ESPOSITO, F.; GALVAO, J.; FRANCO, B. D.; LINCOPAN, N.; LANDGRAF, M.
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26. SELLERA, F.; FERNANDES, M.; ESPOSITO, F.; **Cerdeira, Louise**; LINCOPAN, N.
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27. CUNHA, M. V.; **Cerdeira, L.**; LINCOPAN, N.; KNOBL, T.
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29. FERNANDES, M.; **Cerdeira, L.**; LINCOPAN, N.
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Draft genome sequence of an aminoglycoside-resistant RmtD-2-producing *Enterobacter cloacae* subsp. *cloacae* (ST)395 in Brazil. *Journal of Global Antimicrobial Resistance*. , p.1 - 1, 2017.
31. BUENO, M. F.; FRANCISCO, G.; **Cerdeira, L.**; LINCOPAN, N.; GARCIA, D. O.
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32. **Cerdeira, Louise**; FERNANDES, M.; FRANCISCO, G.; BUENO, M. F.; GARCIA, D. O.; LINCOPAN, N.
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33. SELLERA, F.; FERNANDES, M.; **Cerdeira, Louise**; LINCOPAN, N.
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34. CASELLA, T.; **Cerdeira, Louise**; ANDRADE, L.; DARINI, A. L.; LINCOPAN, N.; NOGUEIRA, M.
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36. MOURA, Q.; FERNANDES, M.; **Cerdeira, L.**; GALES, A. C.; SILVA, R. M.; LINCOPAN, N.
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isolated from bloodstream infection. Journal of Global Antimicrobial Resistance. , p.1 - , 2017.

37. MONTE, D.; FERNANDES, M.; **Cerdeira, Louise**; LOPES, T. S.; LINCOPAN, N.
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39. LOPES, RALF; **Cerdeira, Louise**; TAVARES, GRACE S.; RUIZ, JERONIMO C.; BLOM, JOCHEN; HORÁCIO, ELVIRA C. A.; MANTOVANI, HILÁRIO C.; QUEIROZ, MARISA VIEIRA DE
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Orientações e supervisões

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4. Lais Mozer Santos. **IMPLANTAÇÃO DO SISTEMA DE VIDEOCONFERENCIA APLICADA A FORMAÇÃO CONTINUADA DE PROFISSIONAIS DA EDUCAÇÃO.** 2013. Monografia (Pós-graduação em Informática e Educação) - Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo
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7. Alessandra Fontoura dos Santos. **TECNOLOGIA DA INFORMAÇÃO E COMUNICAÇÃO – O IMPACTO DA IMPLANTAÇÃO DO LAPTOP EDUCACIONAL NO COTIDIANO ESCOLAR.** 2013. Monografia (Informática e Educação) - Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo
8. Queila Gomes Zorzanelli. **UM COMPUTADOR POR ALUNO – UCA - OS DESAFIOS DO PROJETO NA ESCOLA ESTADUAL DE ENSINO FUNDAMENTAL E MÉDIO DE BAIXO QUARTEL – LINHARES - ES.** 2013. Monografia (Pós-graduação em Informática e Educação) - Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo
9. Carlos Roberto Coutinho. **UTILIZAÇÃO DE SOFTWARES DE SIMULAÇÃO DE CIRCUITOS NO ENSINO DE ELETRICIDADE E ELETRÔNICA.** 2013. Monografia (Informática e Educação) - Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo

APPENDIX D - Student History

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9136 - 7409871/1 - Louise Teixeira Cerdeira

Email:	lcerdeira@usp.br
Data de Nascimento:	23/11/1981
Cédula de Identidade:	RG - 3001017 - PA
Local de Nascimento:	Estado do Pará
Nacionalidade:	Brasileira
Graduação:	Bacharel em Ciência da Computação - Centro Universitário do Estado do Pará - Pará - Brasil - 2004
Mestrado:	Mestra em Genética e Biologia Molecular (1) - Universidade Federal do Pará - Brasil - 2011

Curso:	Doutorado
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Análises Clínicas
Data de Matrícula:	21/09/2015
Início da Contagem de Prazo:	21/09/2015
Data Limite para o Depósito:	23/09/2019
Orientador:	Prof(a). Dr(a). Nilton Erbet Lincopan Huenuman - 21/09/2015 até o presente. Email: lincopan@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 08/09/2015
Data de Aprovação no Exame de Qualificação:	Aprovado em 27/10/2017
Estágio no Exterior:	The University of Melbourne, Austrália - Período de 12/02/2018 até 13/04/2018
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 21/09/2015

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).

Última ocorrência: Matrícula de Acompanhamento em 04/02/2019

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Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
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9136 - 7409871/1 - Louise Teixeira Cerdeira

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
IBI5035-1/2	Biologia Molecular Computacional (Curso Interinstitucional: Bioinformática - Universidade de São Paulo) (2)	01/08/2013	10/12/2013	120	8	100	B	N	Concluída
BMM5902-1/1	Agentes Antimicrobianos e Quimioterápicos (Instituto de Ciências Biomédicas - Universidade de São Paulo)	05/10/2015	13/11/2015	60	4	75	B	N	Concluída
FBA5728-4/1	Aprimoramento Pedagógico	03/11/2015	30/11/2015	60	0	-	-	N	Pré-matricula indeferida
FBC5792-3/3	Tópicos em Análises Clínicas III	08/03/2016	20/06/2016	15	1	75	B	N	Concluída
FBC5748-4/2	Trabalhos Científicos: da Elaboração à Publicação	05/04/2016	17/05/2016	60	0	-	-	N	Matrícula cancelada
FBC5766-4/3	Tópicos em Análises Clínicas IV	02/08/2016	14/11/2016	15	1	90	A	N	Concluída
Atividade do Programa	Trabalho Publicado no Journal of Global Antimicrobial Resistance - J Glob Antimicrob Resist, com o trabalho intitulado: "Draft genome sequence of a CTX-M-15 producing Klebsiella pneumoniae sequence type 340 (clonal complex 258) isolate from a food-producing animal", páginas 108 a 109, Estado Unidos da América - 2016 (3)	12/09/2016	12/09/2016	-	1	-	-	-	-
Atividade do Programa	Trabalho Publicado no Genome Announcment - Genome Announc, com o trabalho intitulado: "Draft Genome Sequence of a Hospital-Associated Clone of Klebsiella pneumoniae ST340/CC258 Coproducing RmtG and KPC-2 Isolated from a Pediatric Patient", Volume 4, página 1130, Estado Unidos da América - 2016 (3)	03/11/2016	03/11/2016	-	1	-	-	-	-
FBC5751-6/1	Métodos Básicos e Avançados no Monitoramento da Resistência Bacteriana	03/11/2016	18/11/2016	60	0	-	-	N	Turma cancelada
Atividade do Programa	Trabalho Publicado no Journal of Global Antimicrobial Resistance - J Glob Antimicrob Resist, com o trabalho intitulado: "Draft Genome Sequence of an environmental multidrug-resistant Klebsiella pneumoniae ST340/CC258 harbouring blaCTX-M-15 and blaKPC-2 genes", páginas 67 a 68, Estado Unidos da América - 2017 (3)	07/01/2017	07/01/2017	-	1	-	-	-	-
Atividade do Programa	Trabalho Publicado na Science of the Total Environment, com o trabalho intitulado: "International high-risk clones of Klebsiella pneumoniae KPC-2/CC258 and Escherichia coli CTX-M-15/CC10 in urban lake waters", páginas 910 a 915, Volume 598 - 2017 (4)	27/04/2017	27/04/2017	-	1	-	-	-	-
Atividade do Programa	Trabalho Publicado na Diagnostic Microbiology and Infectious Disease, com o trabalho intitulado: "IncX3 plasmid harboring a non-Tn4401 genetic element (NTEKPC) in a hospital-associated clone of KPC-2 producing Klebsiella pneumoniae ST340/CG258", páginas 164 a 167 - 2017 (4)	01/07/2017	01/07/2017	-	2	-	-	-	-

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	20

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Estágios:			
Total:	0	20	20

Créditos Atribuídos à Tese: 167

Observações:

- 1) Curso com validade nacional, de acordo com o disposto na Portaria MEC nº 1.077, de 31.08.2012..
- 2) Disciplina(s) cursada(s) isoladamente e aceita(s) pelo(a) orientador(a) do(a) candidato(a).
- 3) Créditos atribuídos de acordo com o Artigo 64 do Regimento de Pós-Graduação e aprovados pela Comissão de Pós-Graduação, em Sessão de 08/03/2017.
- 4) Créditos atribuídos de acordo com o Artigo 64 do Regimento de Pós-Graduação e aprovados pela Comissão de Pós-Graduação, em Sessão de 10/10/2017.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 04/02/2019

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Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

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FICHA DO ALUNO

9136 - 7409871/1 - Louise Teixeira Cerdeira

Comissão julgadora da tese de doutorado:			
NUSP	Nome	Vínculo	Função
2552149	Nilton Erbet Lincopan Huenuman	ICB - USP	Presidente

Última ocorrência: Matrícula de Acompanhamento em 04/02/2019

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Genome Note

Draft genome sequences of KPC-2- and CTX-M-15-producing *Klebsiella pneumoniae* ST437 isolated from a clinical sample and urban rivers in Sao Paulo, Brazil Gabriela Rodrigues Francisco^a, Maria Fernanda C. Bueno^a, Louise Cerdeira^b, Nilton Lincopan^{b,c}, Susan Ienne^d, Tiago A. Souza^d, Doroti de Oliveira Garcia^{a,*}^a Center of Bacteriology, Instituto Adolfo Lutz, Sao Paulo, Brazil^b Department of Clinical Analysis, School of Pharmacy, Universidade de Sao Paulo, Sao Paulo, Brazil^c Department of Microbiology, Institute of Biomedical Sciences, Universidade de Sao Paulo, Sao Paulo, Brazil^d Core Facility for Scientific Research—Universidade de Sao Paulo (CEFAP-USP/GENIAL), Sao Paulo, Brazil

ARTICLE INFO

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Urban river

Clinical sample

ABSTRACT

Objectives: KPC-producing *Klebsiella pneumoniae* is considered one of the most worrisome multidrug-resistant micro-organisms in nosocomial infections. It has also been reported in wastewater and urban rivers in the city of Sao Paulo, Brazil. Here we report the draft genome sequences of three KPC-2- and CTX-M-15-producing *K. pneumoniae* sequence type 437 (ST437) isolates obtained from two urban rivers and from a clinical sample of a patient in Sao Paulo.

Methods: A genomic library was constructed using a Nextera XT Kit. An Illumina platform was used to perform whole-genome sequencing (WGS).

Results: WGS of environmental isolates Kp148/PINH-4900 and Kp196/TIET-4200 and clinical isolate Kp314/11 resulted in estimated genome sizes of 5 464 058, 5 437 723 and 5 319 218 bp, respectively. Resistome analysis of the environmental and clinical strains revealed the presence of resistance genes to the following antimicrobials in all strains: aminoglycosides [*aac(6′)-Ib-cr*]; β -lactams (*bla_{OXA-1}*, *bla_{SHV-11}*, *bla_{CTX-M-15}* and *bla_{KPC-2}*); fluoroquinolones [*aac(6′)-Ib-cr*, *oqxA* and *oqxB*]; fosfomycin (*fosATM*); macrolides [*mph(A)*]; phenicols (*catB4*); sulfonamides (*sulI*); and trimethoprim (*dfrA30*). The tetracycline resistance gene *tetA* was identified in Kp148/PINH-4900 and Kp314/11 only; the aminoglycoside resistance gene *aph(3′)-Ia* was found only in environmental isolates, and *aadA2* only in Kp314/11; and the phenicol resistance gene *catA1* was identified only in Kp148/PINH-4900.

Conclusions: The draft genome sequences of these strains help us to elucidate the dissemination of resistance genes in micro-organisms inside and outside the hospital and are useful for further comparisons between clinical and environmental strains.

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1. Introduction

KPC-producing *Klebsiella pneumoniae* of clonal complex 258 (CC258), comprising sequence types (STs) ST11, ST258, ST340 and ST437, are disseminated worldwide and are one of the most worrisome multidrug-resistant (MDR) micro-organisms in nosocomial infections. Isolates of ST437 and ST11 are predominant in Brazil [1,2].

The presence of KPC-2-producing *K. pneumoniae* ST437 has also been reported in urban rivers in the city of Sao Paulo, Brazil [3]. Here we report the draft genome sequences of three strains of KPC-2- and CTX-M-15-producing *K. pneumoniae* ST437 isolated from two urban rivers and from a clinical sample obtained from a patient in Sao Paulo.

2. Materials and methods

A local study to monitor the occurrence of carbapenemase-producing Gram-negative bacteria from urban rivers in Southeast Brazil was conducted from January to June 2011 in which two

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strains of *K. pneumoniae* ST437 positive for both *bla*_{KPC-2} and *bla*_{CTX-M-15} were isolated [3]. The first environmental isolate (Kp148/PINH-4900) was isolated from the Pinheiros River and showed resistance to β -lactams (ceftazidime, cefotaxime, cefepime, aztreonam, ticarcillin/clavulanate, ertapenem, imipenem and meropenem), quinolones, trimethoprim/sulfamethoxazole, chloramphenicol, fosfomycin and polymyxin B, and susceptibility to aminoglycosides (amikacin and gentamicin) and tigecycline. The second environmental isolate (Kp196/TIET-4200), was isolated from the Tietê River and showed the same resistance profile as Kp148/PINH-4900, except for susceptibility to chloramphenicol and polymyxin B [3]. In the same year, a *K. pneumoniae* ST437 isolate positive for both *bla*_{KPC-2} and *bla*_{CTX-M-15} (Kp314/11) was obtained from a blood culture of a patient in a hospital located 4 miles from these rivers.

The three abovementioned isolates were selected for whole-genome sequencing. A PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) was used to extract genomic DNA, and a genomic library was constructed using a Nextera XT Kit (Illumina Inc., San Diego, CA). Total genomic DNA of *K. pneumoniae* Kp148/PINH-4900 and Kp196/TIET-4200 was sequenced using a MiSeq platform (Illumina Inc.), whilst strain Kp314/11 was sequenced using a NextSeq platform (Illumina Inc.). In brief, sequence reads were initially assembled de novo using the A5-misec pipeline [4] or Velvet v.1.2.10 (<https://www.ebi.ac.uk/~zerbino/velvet/>), and contigs were curated using Geneious v.R9 (Biomatters Ltd., Auckland, New Zealand). The draft genome sequences were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v.3.2.

Multilocus sequence typing (MLST), antimicrobial resistance genes and plasmid replicons were identified using the services MLST, ResFinder and PlasmidFinder, respectively, available from the Center for Genomic Epidemiology (<http://genomicepidemiology.org/>).

3. Results and discussion

For environmental isolate Kp148/PINH-4900, a total of 1463504 paired-end reads (estimated size 5464058bp) were generated with 78× coverage. A total of 53 contigs were annotated, resulting in 5549 protein-coding genes, 121 RNA-encoding genes (81 tRNAs, 28 rRNAs and 12 ncRNAs) and 159 pseudogenes, with a G+C content of 57.4%. For environmental isolate Kp196/TIET-4200, 1024364 paired-end reads (estimated size 5437723bp) were generated with 54× coverage. A total of 95 contigs were generated. Annotation resulted in 5506 protein-coding genes, 121 RNA-encoding genes (80 tRNAs, 29 rRNAs and 12 ncRNAs) and 154 pseudogenes, with a G+C content of 57.4%. Finally, clinical isolate Kp314/11 generated 11865174 paired-end reads (estimated size 5319218bp), assembled into 375 contigs, with 635.6× coverage. Annotation resulted in 5525 protein-coding genes, 75 RNA-encoding genes (59 tRNAs, 3 rRNAs and 13 ncRNAs) and 272 pseudogenes, with a G+C content of 57.3%.

Resistome analysis both of environmental and clinical strains revealed the presence of resistance genes to the following antimicrobial agents in all strains: aminoglycosides [*aac*(6′)-Ib-cr]; β -lactams (*bla*_{OXA-1}, *bla*_{SHV-11}, *bla*_{CTX-M-15} and *bla*_{KPC-2});

fluoroquinolones [*aac*(6′)-Ib-cr, *oqx*A and *oqx*B]; fosfomycin (*fos*A^{KP}); macrolides [*mph*(A)]; phenicols (*cat*B4); sulfonamides (*sul*1); and trimethoprim (*dfr*A30). The tetracycline resistance gene *tet*A was identified in Kp148/PINH-4900 and Kp314/11 only; the aminoglycoside resistance gene *aph*(3′)-Ia was found only in environmental isolates, and *aad*A2 only in Kp314/11; and the phenicol resistance gene *cat*A1 occurred only in strain Kp148/PINH-4900.

All strains were confirmed as ST437 according to MLST. PlasmidFinder identified four plasmids belonging to the IncFIB (pQil), IncFII (K), IncFIB (K) and IncN in all strains [4].

Surveillance of resistance genes outside of the hospital is becoming critical, mainly in regions where domestic sewage and hospital wastewater are directly discharged into rivers.

The draft genome sequences of these strains can be used for further comparisons of *K. pneumoniae* genome sequences both between clinical and environmental isolates, helping to elucidate the dissemination of antimicrobial resistance genes occurring in micro-organisms inside and outside the hospital environment. This information can also be used for public-health strategies to reduce the environmental spread of MDR micro-organisms.

These Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under accession nos. **LZCY00000000**, **LZCZ00000000** and **MAOP00000000** for strains Kp148, Kp196 and Kp314, respectively. The versions described in this paper are versions **LZCY01000000**, **LZCZ01000000** and **MAOP01000000**.

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Competing interests

None declared.

Ethical approval

Not required.

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Letter to the Editor

Insights into a novel Tn4401 deletion (Tn4401i) in a multidrug-resistant *Klebsiella pneumoniae* clinical strain belonging to the high-risk clonal group 258 producing KPC-2

Sir,

In Brazil and other lower- and middle-income countries, the emergence and spread of high-risk clones of CTX-M-15- and KPC-2-producing *Klebsiella pneumoniae* has been observed in clinical settings at increasingly alarming frequencies [1,2]. The potential for global dissemination of resistance genes in *K. pneumoniae* is of concern because it is related to high-risk clones, i.e. clonal group 258 (CG258), and the presence of mobilised plasmids carrying Tn4401 [2].

This study aimed to investigate the presence of the Tn4401 transposon and the spread of clinical lineages of multidrug-resistant KPC-2-producing *K. pneumoniae* isolates belonging to the high-risk clone CG258 in different regions in Brazil.

A total of 33 carbapenem-resistant *K. pneumoniae* strains were recovered from various clinical specimens from patients admitted to different-sized hospitals in four regions of Brazil (Northeast: Natal, João Pessoa and Recife; Central-West: Brasília and Goiânia; Southeast: Uberlândia, Uberaba and Belo Horizonte; and South: Porto Alegre and Maringá). Species identification was confirmed in the Laboratory of Molecular Microbiology of the Federal University of Uberlândia (Uberlândia, Brazil). Presence of the *bla*_{KPC} gene was evaluated by PCR for all samples according to a previously described method [3].

Whole-genome sequencing (WGS) was carried out on ten representative strains from ten different cities in Brazil using an Illumina NextSeq 500 Sequencer (Illumina Inc., San Diego, CA). Sequence reads were de novo assembled using Velvet v.5.0.1 [4] and Geneious v.R9 (Biomatters Ltd., Auckland, New Zealand). This assembly was submitted to the National Center for Biotechnology Information (NCBI) and was annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) [5]. In silico comparative analyses were performed using the Center for Genomic Epidemiology pipelines (<http://genomicepidemiology.org/>). BLASTn and nucleotide pairwise alignment of Geneious allowed comparison with previously published Tn4401 isoforms (Tn4401a–Tn4401h) [6–10], considering the high coverage of the sequences, and also analysis of the genetic environment of *bla*_{CTX-M-15}. One strain could not be analysed after sequencing as the data generated by the WGS technique did not allow assembly. The NCBI accession numbers previously published [11] of the sequenced strains are shown in Supplementary Table S1.

PCR amplification results revealed 21 distinct KPC-2-producing strains among the 33 carbapenem-resistant *K. pneumoniae* isolates from different regions of Brazil. The ten sequenced KPC-producing

K. pneumoniae isolates were grouped into four sequence types (STs): ST11 (five isolates); ST437 (three isolates); ST340 (one isolate); and ST443 (one isolate) (Supplementary Table S1). According to the literature, ST11 is involved in the spread of *K. pneumoniae* [1,12], and in the current study this ST was detected in different time periods (since 2009). These high-risk clones were previously linked to the global expansion of different carbapenemases (i.e. *bla*_{KPC} and *bla*_{NDM}) and extended-spectrum β -lactamases (ESBLs) (i.e. *bla*_{CTX-M-15}) [12,13] because they are better adapted in hospital environments and are associated with plasmids [12]. Isolates belonging to ST11, ST437 and ST340 clones co-produced the CTX-M-15 ESBL in 70% of strains. In silico restriction analysis of the *bla*_{CTX-M} genetic environment showed identical profiles for all strains, with the *ISEcp1*–*bla*_{CTX-M-15} element (2971 bp) flanked by a 5-bp target-site duplication and probably inserted within a Tn2-derived sequence.

In this study, although the *bla*_{KPC-2} gene was most frequently located on non-Tn4401 elements (NTE_{KPC}), its detection was also evidenced in the Tn4401b isoform, which is still considered the most important epidemiologically related transposon in *K. pneumoniae* [6]. In addition, the genomic study led to the identification of a carbapenem-resistant *K. pneumoniae* isolate carrying the *bla*_{KPC-2} gene in a new variant of Tn4401 (Tn4401i), in a sample belonging to clone ST11/CG258 collected from blood and isolated from a hospitalised patient in the Southeastern region of Brazil in 2016. Alignment of the sequences of this element revealed the *bla*_{KPC-2} gene flanked by *ISKpn6* downstream and *ISKpn7* upstream, with a 203-bp deletion between this IS and *bla*_{KPC-2} (Fig. 1). Structural variation between Tn4401b and the novel Tn4401i isoform, including variation in the putative promoter regions, is also shown in Fig. 1. The role of this new Tn4401 isoform in the global dissemination of the *bla*_{KPC-2} gene deserves to be better investigated from this report. These findings raise important concerns about the possible contribution of this new variant for the dissemination of resistance to carbapenems, as only a few antibiotic options are still available for appropriate treatment [12]. Therefore, efforts still should focus on implementing effective infection prevention and control measures.

In summary, this study provides further evidence that novel variants of the Tn4401 element in high-risk clones of KPC-2-producing *K. pneumoniae* can play a key role in the spread of this gene among clinically significant pathogens selected from environments with intense antibiotic consumption.

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Fig. 1. Structural variation in Tn440Ib transposon (GenBank accession no. [KX276209](#)) and the novel variant Tn440Ii (GenBank accession no. [MH047294](#)), showing a 203-bp deletion between ISKpn7 and the *bla*_{KPC-2} gene. Comparative analysis of the alignment of the promoter regions of *bla*_{KPC-2} isoforms with putative promoters is also represented. The -35 and -10 regions are highlighted in grey and the deletion in isoform 'i' is underlined.

Competing interests

None declared.

Ethical approval

Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijantimicag.2018.08.011](https://doi.org/10.1016/j.ijantimicag.2018.08.011).

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Hypervirulence and biofilm production in KPC-2-producing *Klebsiella pneumoniae* CG258 isolated in Brazil

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Abstract

In this study, we describe the frequency of virulence genes in *Klebsiella pneumoniae* carbapenemase-2-producing *Klebsiella pneumoniae* (KPC-KP), including hypervirulent (hv) and hypermucoviscous (hm) strains by whole-genome sequencing. We also evaluate the capacity for biofilm formation by using phenotypic techniques. The occurrence of several virulence genes (*fimABCDEFHJK*, *mrkABCDHFJ*, *ecpA*, *wabG*, *entB*, *ugE*, *irp1*, *irp2*, *traT*, *iutA* and *ureADE*) and a high frequency of hvhmKPC-KP isolates was found. Most hospital-associated lineages of KPC-KP belong to the international clonal group 258 (CG258). Biofilm formation was a constant feature among 90.9% of KPC-KP strains. This report suggests a close relationship between ST437 and weak biofilm production, given that all weakly biofilm-producing strains belonged to this sequence type. This also supports the dissemination of KPC-KP containing numerous virulence determinants belonging to the biofilm-producing CG258 type in Brazil, including hv and hm strains. These factors allow this pathogen to cause infections, leading to its rapid expansion and persistence in hospital settings.

Klebsiella pneumoniae carbapenemase-2-producing *Klebsiella pneumoniae* (KPC-KP) isolates have become one of the most important human pathogens [1]. The prevalence of this phenotype is advancing worldwide, and it has become a major public health problem, particularly in endemic areas in low- and middle-income countries [2, 3].

There has been increasing concern over the emergence of hypervirulent (hv) and hypermucoviscous (hm) KPC-KP (hvhmKPC-KP) strains due their association with serious infections, in addition to their ability to carry several virulence and resistance genes that represent major threats to public health [4–6].

We investigated the prevalence of hvhmKPC-KP isolates obtained from different regions of Brazil and here we describe the molecular characteristics of virulence based on whole-genome sequencing (WGS). Additionally, biofilm production was evaluated by phenotypic techniques.

In 2016, a total of 33 carbapenem-resistant *K. pneumoniae* strains were obtained from various specimens of patients hospitalized in north-east, centre-west, south-east and southern Brazil. These isolates were identified as *K. pneumoniae* in their place of origin and the species was confirmed in the Laboratory of Molecular Microbiology at the Federal University of Uberlandia, Brazil.

The presence of *bla*_{KPC}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{GES} and *bla*_{NDM} genes was evaluated by PCR, and these are further described in Table S1 (available in the online version of this article). Eleven KPC-KP strains were tested for phenotypic identification of hvhmKPC-KP, biofilm production and WGS. These isolates were selected as representative of different cities and states of Brazil.

The phenotypic identification of hmKPC-KP was determined based on the formation of a viscous filament ≥ 5 mm long after stretching a *K. pneumoniae* colony with a loop on an agar plate (string test) [5]. hvhmKPC-KP strains were

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Keywords: *Klebsiella pneumoniae*; biofilm; KPC-2; WGS; hypervirulence.

Abbreviations: CGE, Center for Genomic Epidemiology; hm, hypermucoviscous; hv, hypervirulent; hvhmKPC-KP, hypervirulent and hypermucoviscous *Klebsiella pneumoniae* carbapenemase-2-producing *Klebsiella pneumoniae*; KPC-KP, *Klebsiella pneumoniae* carbapenemase-2-producing *Klebsiella pneumoniae*; ODC, optical density cut-off value; ST, sequence type; TSA, tryptic soy agar; TSB, tryptic soy broth; WGS, whole-genome sequencing. Sequence accession numbers: NTH000000000, NTGK000000000, NTGJ000000000, NTHU000000000, NTCW000000000, NTHV000000000, NTHW000000000, NTHX000000000, NTHY000000000 and NIRG000000000.

One supplementary table is available with the online version of this article.

defined based on the presence of at least two of the following indicators: a positive string test, amplification of the *rmpA* gene and/or amplification of the aerobactin gene [5].

Total genomic DNA of the selected strains was sequenced using an Illumina NextSeq 500 sequencer, and the sequence reads were *de novo* assembled using Velvet pipeline version 5.0.1 and Geneious version 9.1.8. Pairwise alignment was performed by BLASTn homology searches (<http://blast.ncbi.nlm.nih.gov>), and an *in silico* comparative analysis, including microbial identification, detection of virulence genes (the *fimABCDEFHIK*, *mrkABCDEFHJ*, *ureA*, *UreG*, *iucABC*, *iutA*, *allABCDERS*, *kfuABC* and *ecpABC* gene clusters, and *rmpA*, *rmpB*, *wabG*, *ugE*, *entB*, *traT*, *iroB*, *iroC*, *iroD*, *iroN*, *irp1*, *irp2* and *pdl1* genes) and antimicrobial genotypes, using the Center for Genomic Epidemiology (CGE) pipelines.

Investigation of biofilm formation was performed according to the protocol established by Cassat *et al.* [7] with modifications as suggested by Campos *et al.* [8]. Briefly, the strains were grown for 18 h at 37 °C with shaking at 120 r.p.m. in 20 ml of tryptic soy broth (TSB) using bacteria grown on tryptic soy agar (TSA) plates. Cells were harvested by centrifugation (6000 r.p.m., 10 min, 4 °C) and suspended in saline (0.9 % NaCl prepared in distilled water) at a concentration of approximately 1×10^9 cells ml^{-1} , prior to use in biofilm and adhesion assays. *K. pneumoniae* NTUH-K2044 was used as a control.

Initial adhesion assays were performed as described below. A 200 μl aliquot of a cell suspension, containing 1×10^7 cells ml^{-1} prepared in TSB, was added to 96-well polystyrene plates for 2 h at 37 °C with shaking at 120 r.p.m. The 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and harvested by scraping of wells for 90 s. The cell suspension obtained was plated on TSA for enumeration of colony-forming units (c.f.u.). All experiments were done in quadruplicate, on three independent occasions.

Briefly, 200 μl of a cell suspension containing 1×10^7 cells ml^{-1} prepared in TSB was added to 96-well polystyrene plates. Bacteria grown for 24 h at 37 °C with shaking at 120 r.p.m. in 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and allowed to dry in an inverted position. The total biomass was measured by using methanol fixation, crystal violet staining and acetic acid elution. The absorbance for the eluted dye was read on an ELISA plate reader (Polaris Celer) at 570 nm. The experiments were done with eight replicates for each strain, on six independent occasions. TSB without bacteria was used as a negative control. Biofilm production was interpreted according to the criteria of Saxena *et al.* [9]. The optical density cut-off value (ODc) was established as three standard deviations (SD) above the mean of the optical density of the negative control: ODc = average OD of negative control + 3SD of negative control. For easier interpretation of the results, the strains were divided into the following categories according to their optical densities (ODi): ODi $\leq$ ODc = non-biofilm producer; ODc < ODi $\leq$ 2 $\times$ ODc = weak biofilm producer;

2 $\times$ ODc < ODi < 4 $\times$ ODc = moderate biofilm producer; 4 $\times$ ODc < ODi = strong biofilm producer.

Biofilm cell number was determined based on enumeration of colony-forming units. After biofilm formation, the 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and the biofilm was harvested after scraping the wells for 90 s. The cell suspension obtained was plated onto TSA plates. All experiments were done in quadruplicate, on three independent occasions.

To evaluate the ultrastructure of biofilms, a scanning electron microscopy assay was performed. Two standard glass beads of 0.5 cm diameter were used for each sample, one per well (96-well plates). After biofilm formation, the beads were fixed in glutaraldehyde/paraformaldehyde (2.5 %) for 18 h at 4 °C and washed in PBS solution (pH 7.4) three times. The samples were then maintained in osmium tetroxide (1 %) for 2 h at room temperature and washed again in PBS solution (pH 7.4) three times. The samples were dehydrated in an alcohol series of 30, 40, 50, 60, 70, 80, 90 and 100 % (three times) for 20 min each at room temperature and then critical-point dried. The beads were metallized with a 20 nm thickness of gold, visualized and photographed using a Zeiss scanning electron microscope.

Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software). Quantitative assays were compared using the Kruskal–Wallis test, applying Dunn's multiple comparison test. All tests were performed with a confidence level of 95 % and statistical significance was defined as $P < 0.05$.

Overall, from 33 carbapenem-resistant *K. pneumoniae* strains analysed, 64 % (21/33) harboured the *bla*_{KPC} gene and none harboured the *bla*_{IMP}, *bla*_{VIM}, *bla*_{GES} or *bla*_{NDM} genes. Among 11 KPC-KP isolates (11/21), two (18.2 %) were positive for the string test and were identified as hmKPC-KP (Table 1). Interestingly, both strains were from blood obtained from different regions of the country, central-west and north-east Brazil.

WGS analysis of 11 KPC-KP strains confirmed the species identification, the presence of the KPC-2 variant, and also revealed four different sequence types (STs) among the strains (ST11, ST340, ST437 and ST443). The predominant ST in Brazil was ST11 (54.5 %), followed by ST437 (27.3 %), whereas ST340 and ST443 were identified in only one isolate each (Table 1). One isolate could not be analysed after sequencing because the data generated by the technique (WGS) did not allow assembly. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accessions NTHU000000000 (strain B03), NTGK000000000 (B04), NTGJ000000000 (B05), NTHU000000000 (B11), NTCW000000000 (B16), NTHV000000000 (B17), NTHW000000000 (B29), NTHX000000000 (B30), NTHY000000000 (B35) and NIRG000000000 (B40).

All strains had in common genes encoding type 1 fimbriae (*fimABCDEFHIK*), type 3 fimbriae (*mrkABCDEFHJ*),

Table 1. KPC-2-producing *Klebsiella pneumoniae* strains used in the study of adhesion and biofilm formation

Strain	Locality	Source	Year of isolation	ST*	Virulence-associated genes†										KPC-KP phenotype‡	Mean OD§ value	Biofilm formation		
					<i>fim</i> cluster	<i>mrk</i> cluster	<i>mrkE</i>	<i>ecpA</i>	<i>walK</i>	<i>ureAD</i>	<i>ureEG</i>	<i>ugE</i>	<i>entB</i>	<i>irp1,2</i>				<i>intA</i>	<i>traT</i>
B03	Porto Alegre	Blood	2013	437	+	+	+	+	+	+	+	+	+	+	+	+	KPC-KP	0.32	Weak producer
B04	Recife	Blood	2009	11	+	+	+	+	+	+	+	+	+	+	+	+	hnhvKPC-KP	0.21	Non-producer
B05	Uberlândia	Rectal swab	2014	11	+	+	+	+	+	+	+	+	+	+	+	+	KPC-KP	0.59	Moderate producer
B11	Brasília	Blood	2016	11	+	+	+	+	+	+	+	+	–	+	+	–	KPC-KP	0.68	Moderate producer
B16	Belo Horizonte	Peritoneal secretion	2009	437	+	+	+	+	+	+	+	+	+	+	–	+	KPC-KP	0.28	Weak producer
B17	Goiânia	Blood	2010	443	+	+	+	+	+	+	+	+	+	+	–	+	hnhvKPC-KP	0.61	Moderate producer
B29	Natal	Urine	2010	340	+	+	+	+	+	+	+	+	+	+	–	+	KPC-KP	0.58	Moderate producer
B30	João Pessoa	Blood	2016	437	+	+	+	+	+	+	+	+	+	+	–	+	KPC-KP	0.39	Weak producer
B35	Uberaba	Blood	2016	11	+	+	–	+	+	+	+	–	+	+	+	–	KPC-KP	0.61	Moderate producer
B40	Uberlândia	Liquor	2015	11	+	+	+	+	+	+	+	+	+	+	+	–	KPC-KP	0.70	Moderate producer
B15	Maringá	Urine	2016	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.65	Moderate producer

*ST: sequence type.

†*fim* cluster: *fimABCDGHIK*, *mrk* cluster: *mrkABCDHJL*.

‡hnhvKPC-KP: hypermucoviscous and hypervirulent.

§Optical density.

||Not determined.

The virulence genes *iucABC*, *aiiABCDRS*, *kfuABC*, *ecpBC*, *rmpAB*, *iroBCDN* and *pdf1* were not detected in the strains.

Fig. 1. Evaluation of the number of adhered cells, biomass and biofilm formation in *K. pneumoniae* strains. (a) Number of cells adhering to glass beads plates after 2 h of contact, expressed as log c.f.u. ml⁻¹. (b) Biofilm cellular concentration expressed as log c.f.u. ml⁻¹. (c) Biofilm biomass expressed as crystal violet optical density (OD at 570 nm). Results are the means plus standard deviations (error bars) of three independent experiments. *P<0.01, **P<0.001, ***P<0.0001, using Kruskal-Wallis test with Dunn's multiple comparison test.

Escherichia coli common pilus (*ecpA*), outer core lipopolysaccharide (*wabG*), enterobactin (*entB*), aerobactin (*iutA*) and urease synthesis associated genes (*ureADE*). The virulence genes *iucABC*, *allABCD*, *kfuABC*, *ecpBC*, *mmpA*, *mmpB*, *iroB*, *iroC*, *iroD*, *iroN* and *pdl1* were not detected in the strains surveyed (Table 1). After analysis of the aerobactin gene, the two strains characterized as hmKPC-KP were also defined as hvKPC-KP.

All KPC-KP strains assessed were able to adhere to an unmodified polystyrene surface (Fig. 1a), and 63.6% (strains B04, B11, B15, B16, B29, B30 and B35) of them were able to adhere significantly more compared to the positive control ($P<0.001$). Regarding the number of cells present on each biofilm, 72% (8/11) of the strains had significantly higher numbers of cells than the positive control ($P<0.001$), and only strains B04, B05 and B40 produced biofilms similar to the

control (Fig. 1b). Six strains produced more biofilm mass than the control ($P < 0.001$) including strain B04, which was classified as a non-biofilm producer (Fig. 1c). Quantitatively, seven strains (63.7 %) were moderate biofilm producers, three (27.3 %) were weak producers and one was a non-biofilm producer (Table 1). Interestingly, all *K. pneumoniae* strains that weakly produced biofilm belonged to ST437. These characteristics were confirmed by scanning electron microscopy (data not shown).

The potential of KPC-producers to disseminate, together with the difficulty of their detection and control, raises the need to develop appropriate and effective policies to reduce infection by the strains in low- and middle-income countries [10]. In this study, we detected the presence of *Klebsiella pneumoniae* carbapenemase-2-producing *K. pneumoniae*. The prevalence of this subtype in Brazil has been described previously and it is an example of a successful antimicrobial drug-resistant pathogen; it is one of the most frequently occurring pathogens worldwide [11, 12].

In addition to the problem of dissemination of KPC in Brazil, KPC-KP ST11 strains are prototypes of a well-adapted high-risk clone and have been largely responsible for the global spread of carbapenem resistance [13]. In this report, we performed WGS on a Brazilian collection of KPC-KP isolates and showed the presence of ST11, closely related to CG258, which is the major clonal group among *K. pneumoniae* harbouring the *bla*_{KPC} gene from Latin America [13]. Two other STs, ST340 and ST437, were also found and, according to previous research, have endemic potential similar to ST11 [14]. ST443, belonging to the unusual CG1345 group, was also identified, and its presence in Brazil has already been reported [15].

Additionally, the literature shows that plasmids containing the *bla*_{KPC} gene are not necessarily associated with increased virulence, and that isolates belonging to ST11 do not always have greater virulence than other isolates of *K. pneumoniae* [16–18]. Despite this, the strains analysed in this study contained several virulence genes (*fim*ABCDEFGHJK, *mrk*ABCDHFJ, *ecpA*, *wabG*, *entB*, *ugE*, *irp1*, *irp2*, *traT*, *iutA* and *ureADE*). Recent research has shown that the presence of these genes in *K. pneumoniae* strains (mainly capsular type, LPS, adhesins and iron-scavenging systems) may be responsible for increasing the virulence potential of these micro-organisms and the development of more invasive diseases [18].

In this study, the prevalence of hvhmKPC-KP isolates was high (18.2 % of the total KPC-KP strains). The virulence and resistance context found in these micro-organisms represent a problem for therapy, because these factors increase the likelihood of colonization and persistence of highly resistant phenotypes in hospitalized patients [18]. These findings are alarming because these strains may be resistant to multiple drugs and may be highly transmissible, which presents an imminent threat to public health [19].

In addition, biofilm-associated infection is very difficult to treat due to high resistance to antibiotics [20]. With the

worldwide increase in the occurrence of KPC-KP, data on its ability to form biofilms in nosocomial strains are crucial [21]. Our data indicate that individual *K. pneumoniae* strains had distinct abilities to attach to polystyrene plates and form biofilms. Biofilm formation was a constant feature among 90.9 % of all KPC-KP strains. We found that all the isolates that formed significantly more biofilm than the control belonged to the ST11 endemic lineage. Interestingly, this study also suggests a close relationship between ST437 and weak biofilm production, given that all weakly biofilm-producing strains belonged to this ST. However, there are no data in the literature regarding biofilm formation of *K. pneumoniae* ST437. Due to the lack of data in the literature regarding the production of biofilms by ST437 strains, this study is unique in clarifying biofilm prediction in strains belonging to this ST.

In conclusion, our study has described the important association between KPC-2, biofilm formation and virulence factors in *K. pneumoniae* ST11, an international high-risk clone, in addition to the detection of infections caused by hvhmKPC-KP strains. Systemic surveillance should focus on assessing the dissemination of this virulent, multidrug-resistant and biofilm-producing clone to provide advantages in relation to this global threat to public health and future related challenges.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

No authorization was required from the Ethics Committee for Human Research of Federal University of Uberlândia, because we used a bacteriological collection from the Laboratory of Molecular Microbiology at the Federal University of Uberlândia, without any contact with the patients and their data.

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quantified in parallel by plating serial dilutions on LB agar plates and transformation events were determined by calculating the number of kanamycin-resistant colonies per CFU. Experiments were performed in at least triplicate, statistical analysis (Mann-Whitney Test) was performed using PrismPad (GraphPad software, San Diego, CA, USA) and a *P*-value < 0.05 was considered significant.

MMC functions by cross-linking complementary DNA strands and has been shown to induce the DNA repair (SOS) response pathway in several bacteria, to increase transformation frequencies in *Legionella pneumophila* and to increase genes associated with stress response in *A. baumannii* and *Acinetobacter baylyi* [7]. The minimum inhibitory concentration (MIC) of MMC on *A. baumannii* A118 cells was determined to be 3.125 µg/mL and a concentration of 0.2 µg/mL was used during transformation assays. While transformation frequencies increased with all strains and DNA types under MMC induction, strain ATCC 17978 and ATCC 19606 showed significant increases, with ATCC 17978 showing the most dramatic increases of 19.83-fold with plasmid DNA and 13.04-fold with genomic DNA (Fig. 1A). NAL is a synthetic quinolone antibiotic that functions by inhibiting DNA gyrase and/or topoisomerase. The MIC of NAL was determined as 5 µg/mL and it was tested at a concentration of 1.25 µg/mL. As with MMC, NAL increased transformation frequencies in all strains with both DNA types but only significantly increased transformation frequencies in strain A42, transformed with plasmid DNA (Fig. 1B). MEM is a carbapenem that functions by inhibiting the formation of peptidoglycan in the cell wall and is considered the last line of defense in treating infections caused by Gram-negative multidrug-resistant bacteria. The MIC of MEM was determined to be 0.25 µg/mL and a concentration of 0.063 µg/mL was used for transformation. As with the previously described antibiotics, MEM increased transformation frequencies in all strains tested with both DNA types. However, it significantly increased transformation frequencies by 12.12-fold in ATCC 17978, when transformed with plasmid DNA, and 3.14-fold in A42, when transformed with genomic DNA (Fig. 1C).

As MMC and NAL target the bacterial genome, it is likely that they induce competence via the unique DNA damage stress response pathway of *A. baumannii*. In many bacterial species, DNA damage triggers RecA-dependent autolysis of the LexA repressor, but this does not occur in *A. baumannii* because of the absence of several crucial genes, including *lexA* [8]. Instead, we hypothesize that *A. baumannii*, like *A. baylyi*, responds to DNA damage in a RecA-independent manner, in which RecA is involved in DNA repair as a member of the recombination repair pathway, and that competence developed as a DNA damage response mechanism in this species. The role of MEM is not as clear. Although carbapenems like MEM have been shown to increase plasmid stability in *Klebsiella pneumoniae* by inducing expression of the CRISPR-cas activity regulator, H-NS, the CRISPR-cas system was not identified in any of the tested *A. baumannii* strains [9]. As such, carbapenems may interact with the H-NS regulator to induce competence via a novel pathway. Together, these results indicate there are multiple pathways to competence induction in *A. baumannii* and that further analysis of these pathways is crucial to understanding how competence functions in this species.

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Detection of ISEcp1-associated bla_{CTX-M-15}-mediated resistance to colistin in KPC-producing *Klebsiella pneumoniae* isolates

Sir,

The difficulty in treating severe infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-KP) has led to the worldwide reoccurrence of the use of polymyxins (polymyxin B and colistin) as a last treatment option [1]. Unfortunately, the increasing use of polymyxins has led to reports of colistin-resistant KPC-producing isolates around the world [1,2]. Polymyxin resistance is associated with specific modifications of the lipid A component of bacterial lipopolysaccharide (LPS) that reduce the negative charge of the outer membrane. These modifications have been reported to be because of specific alterations in the pmrA/B and phoP/Q two-component systems [3]. Recent reports indicate that the main colistin resistance mechanism in *K. pneumoniae* is associated with inactivation of the *mcrB* gene [4].

In this study, we investigated whether there were changes in the *mcrB* gene that justified the high level of colistin resistance found in a dominant clone of KPC-KP isolates that caused a major outbreak in Brazil.

A total of 13 colistin-resistant KPC-KP isolates were collected during a major outbreak in July 2015 in the adult intensive care unit of a Brazilian teaching hospital [2]. Species identification and antimicrobial susceptibility were performed on the Vitek-2® system (bioMérieux, Brazil). Colistin susceptibility was determined by the broth microdilution method according to EUCAST guidelines and >2 µg/mL was considered as resistance to colistin. The study conducted by Rossi et al confirmed clone A to be dominant among the outbreak isolates [2]. All isolates were tested for the presence

Fig. 1. Schematic representation of the *mgtB* gene by the ISEcp1-*bla*_{CTX-M-15} in *K. pneumoniae* isolate KPC40. Arrows indicate genes and their respective transcription orientations. IRL, inverted repeated left; IRR, inverted repeated right. The *mgtB* gene truncation is represented by diagonal dashes. *tnpA* corresponds to the transposase-encoding gene of ISEcp1.

of the plasmid-mediated *mcr-1* gene by polymerase chain reaction (PCR), and none were shown to harbor this gene.

The whole genome of the 40KPC strain (colistin-resistant KPC-KP strain; MIC of colistin, 128 µg/mL; date of infection, 03/18/2015) was sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA), and the genome library was constructed using the Nextera XT DNA sample prep kit (Illumina).

The analysis revealed that the strain belonging to the sequencing type (ST) 11 was closely related to the clonal complex (CC) 258, which is the main clonal group among *K. pneumoniae* harboring *bla*_{KPC} in Latin America [5]. A sequence analysis of the genes encoding *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgtB* was aligned with the genomic DNA of the NTUH-K2044 strain. We identified an integrate 3,055 bp fragment truncating the *mgtB* gene. This truncation occurred between nucleotides +21 and +22 and the inserted fragment consisted of an ISEcp1-*bla*_{CTX-M-15} compound transposon (Fig. 1). In addition to this alteration in the *mgtB* gene, two other mutations were detected, in the *pmrB* gene (R256G) and the *phoQ* gene (D146G).

This study shows that insertion of the *bla*_{CTX-M-15} gene, along with the insertion element ISEcp1, into the chromosomally-located *mgtB* gene resulted in concomitantly acquired resistance to broad-spectrum cephalosporins and colistin, and was considered the main resistance mechanism to colistin in these isolates. This sequence has been observed previously in a study conducted by Jayol et al in France [6]. Although we detected two other mutations—R256G and D146G in the *pmrB* and *phoQ* genes, respectively—the most important finding was that *bla*_{CTX-M-15} was associated with an ISEcp1 insertion sequence in the *mgtB* gene.

Additionally, ~3000 bp fragments of the *mgtB* amplicons were observed in gel electrophoresis in the other isolates from the outbreak. These larger fragments are likely to correspond to the *mgtB* gene truncated by the *bla*_{CTX-M-15} gene, along with the insertion element ISEcp1. This observation reinforces the idea that the *mgtB* gene contains additional elements related to this gene.

Our study adds to the findings reported by de Jayol et al [6], and provides further evidence to indicate that resistance to broad-spectrum cephalosporins may be associated with resistance to colistin in *K. pneumoniae* in the hospital environment.

In conclusion, this study provides evidence that insertion of the *bla*_{CTX-M-15} gene along with the ISEcp1 element into the *mgtB* gene in a highly-resistant strain recovered from an environment with high use of antibiotics [2] led to colistin resistance.

Accession number. The sequence of the KPC40 strain was deposited in GenBank with accession number NRG000000000.

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Genome Note

Draft genome sequence of a KPC-2-producing *Klebsiella pneumoniae* ST340 carrying *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes: a rich genome of mobile genetic elements and genes encoding antibiotic resistance

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Urinary tract infection

ABSTRACT

Objectives: *Klebsiella pneumoniae* is considered an opportunistic pathogen and an important agent of nosocomial and community infections. It presents the ability to capture and harbour several antimicrobial resistance genes and, in this context, the extensive use of carbapenems to treat serious infections has been responsible for the selection of several resistance genes. This study reports the draft genome sequence of a KPC-2-producing *K. pneumoniae* strain (Kp10) simultaneously harbouring *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes isolated from urine culture of a patient with Parkinson's disease.

Methods: Classical microbiological methods were applied to isolate and identify the strain, and PCR and sequencing were used to identify and characterise the genes and the genetic environment. Whole-genome sequencing (WGS) was performed using a Nextera XT DNA library and a NextSeq platform.

Results: WGS analysis revealed the presence of 5915 coding genes, 46 RNA-encoding genes and 255 pseudogenes. Kp10 belonged to sequence type 340 (ST340) of clonal complex 258 (CC258) and carried 20 transferable genes associated with antimicrobial resistance, comprising seven drug classes. Although the simultaneous presence of different *bla*_{CTX-M} genes in the same strain is rarely reported, the *bla*_{SPC-2}, *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes were not associated with the same genetic mobile structure in Kp10.

Conclusions: These results confirm the capacity of *K. pneumoniae* to harbour several antimicrobial resistance genes. Thus, this draft genome could help in future epidemiological studies regarding the dissemination of clinically relevant resistance genes.

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For many years, cephalosporins, fluoroquinolones and trimethoprim/sulfamethoxazole were often used to treat *Klebsiella pneumoniae* infections [1]. However, owing to high rates of resistance to these agents, and specifically because of the emergence and dissemination of extended-spectrum β -lactamase

(ESBL)-producing strains, there was an increase in the use of carbapenems, which are the last line of effective therapy available for the treatment of infections by multidrug-resistant *K. pneumoniae* [1]. As a result, in the last decade carbapenem resistance has become a major public health threat [1].

The *K. pneumoniae* carbapenemase-2 (KPC-2) variant is the main mechanism of resistance to carbapenems and, so far, is the only one reported in Brazil, where it is endemic [2,3]. Moreover, ESBL-encoding genes are frequently identified in KPC-producing

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strains and this combination of resistance genes is not uncommon [1]. However, the simultaneous presence of different *bla*_{CTX-M} genes in the same strain is rarely reported. Thus, this study reports the first draft genome of a KPC-2-producing *K. pneumoniae* ST340/CC258 (strain Kp10) carrying both *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes.

Strain Kp10 was isolated in 2011 from a urine sample collected from a 78-year-old woman with Parkinson's disease admitted to a tertiary hospital located in the state of São Paulo, Brazil. Two months before this isolation, the patient was transferred to the referred hospital from another institution, presenting a hypogastric mass detected by abdominal ultrasound, haematuria, mental confusion and dysphasia, who was in use of peripheral venous and Foley catheters and bladder irrigation. A urine sample was collected and although the culture was negative, the patient received ceftriaxone (2g every 24h) for 3 days, ciprofloxacin (400mg every 12h) for 2 days and amoxicillin/clavulanic acid (500mg every 8h) for 9 days. During treatment the patient's condition deteriorated and 18 days after the admission she was transferred to the intensive care unit (ICU) with a diagnostic hypothesis of urosepsis. Urine and blood cultures collected upon admission to the ICU were negative. Cefepime (2g every 8h) was used for empirical treatment for 5 days and was then replaced by meropenem (1g every 12h) for 20 days. Urine culture performed during treatment with meropenem showed growth of a *K. pneumoniae* presenting susceptibility only to polymyxin B and tigecycline. The antibiotic prescription was changed and to polymyxin B [1 000 000 international units (IU)] was administered to the patient every 3 days for 2 weeks. After 39 days in the ICU, the patient was discharged to the clinical ward where she died 2 days later owing to cardiorespiratory arrest. The isolate was further identified as a KPC-2-producer by PCR and sequencing, and the location of the *bla*_{KPC-2} gene on a plasmid of ca. 50kb was determined by hybridisation of S1-digested DNA with a specific probe following previously reported protocols [4].

The total genomic DNA of Kp10 was sequenced using a NextSeq platform (Illumina Inc., San Diego, CA). A total of 15 718 312 paired-end reads (150 × 150bp) were generated with 471× coverage. In brief, the sequence reads were initially assembled de novo using Velvet v.1.2.10 (<https://www.ebi.ac.uk/~zerbino/velvet/>) and contigs were curated using Geneious software v.R10 (Biomatters Ltd., Auckland, New Zealand). Finally, the draft genome sequence was automatically annotated using PROKKA (<http://www.vicbioinformatics.com/software/prokka.shtml>).

Whole-genome sequencing analysis revealed the presence of 5915 coding sequences, 255 pseudogenes, 45 tRNAs, 1 rRNA (5S) and a G + C content of 56.6% in Kp10. Multilocus sequence typing (MLST), plasmid replicons and acquired antimicrobial resistance genes were identified using MLST 1.8 (<https://cge.cbs.dtu.dk/services/MLST/>), PlasmidFinder 1.3 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) and ResFinder 2.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>), respectively, available from the Center for Genomic Epidemiology.

Kp10 belonged to sequence type 340 (ST340) of clonal complex 258 (CC258), whereas resistome analysis revealed the presence of aminoglycoside resistance genes *aac(3)-IId*, *aac(6)-Ib*, *aadA1* and *aadA2*, β-lactam resistance genes *bla*_{KPC-2}, *bla*_{CTX-M-15}, *bla*_{CTX-M-59}, *bla*_{OXA-2}, *bla*_{OXA-9}, *bla*_{SHV-5} and *bla*_{TEM-1A}, fluoroquinolone resistance genes *aac(6)-Ib-cr*, *qnrB2*, *oqxA* and *oqxB*, the fosfomycin resistance gene *fosA*, the sulphonamide resistance gene *sul1*, the tetracycline resistance gene *tet(D)* and trimethoprim resistance genes *dfrA12* and *dfrA25*. Plasmids of incompatibility (Inc) groups R, H1B, FIB(Mar), FIB(K), FIA(H11), FII(K) and L/M(pMU407) were

identified. The genes *bla*_{KPC-2}, *bla*_{CTX-M-15} and *bla*_{CTX-M-59} were identified in different plasmids. We could not identify the incompatibility group of the plasmid harbouring *bla*_{KPC-2}, but the element responsible for mobilisation of this gene was transposon Tn4401b. The *bla*_{CTX-M-15} and *bla*_{CTX-M-59} genes were carried by IncR and IncFIA(H11) plasmids, respectively. In addition, *bla*_{CTX-M-59} was associated with a complex class 1 integron with *dfrA12* and *aadA2* genes in the variable region. *Klebsiella pneumoniae* Kp10 belonging to ST340 is a reflection of the Brazilian scenario, where CC258 is the most frequent KPC-2-producing lineage, especially ST11 and ST437 [2]. In addition, Kp10 is a model for 'genetic capitalism', in which constant accumulation of genetic platforms able to recruit and spread novel genes tends to occur in successful bacteria in specific environments [5], as in the case of genes encoding resistance to aminoglycosides and β-lactams, which are highly prescribed in our institution.

In our hospital, the first case of infection by a KPC-producing *K. pneumoniae* was identified in 2011. Before that, high rates of infection by CTX-M-2-type- and CTX-M-15-producing *K. pneumoniae* were observed [6]. However, coexistence of *bla*_{CTX-M-59} and *bla*_{CTX-M-15} in the same strain has never been identified. In summary, this study reports for the first time the isolation of a *bla*_{CTX-M-15}- and *bla*_{CTX-M-59}-harbouring *K. pneumoniae*, where these resistance determinants coexist with other important resistance genes, such as *bla*_{KPC-2} (among others), which culminate in a difficult-to-treat infection.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession no. **MUDD000000000**. The version described in this paper is version **MUDD010000000**.

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Competing interests

None declared.

Ethical approval

Not required.

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Short Communication

International high-risk clones of *Klebsiella pneumoniae* KPC-2/CC258 and *Escherichia coli* CTX-M-15/CC10 in urban lake waters

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HIGHLIGHTS

- Presence of MDR bacteria in public lakes and reservoirs has been investigated.
- Identification of high-risk clones of *K. pneumoniae* and *E. coli* is highlighted.
- The environmental dissemination of hospital-associated lineages has been discussed.

GRAPHICAL ABSTRACT

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ABSTRACT

The emergence of high-risk clones of multidrug-resistant (MDR) bacteria in aquatic environments has generated an important public health problem, creating an urgent need to strengthen surveillance. This study reports the occurrence of clinically significant MDR Enterobacteriaceae and non-fermentative bacteria carrying carbapenemases (KPC-2), extended-spectrum β -lactamases (CTX-M) and plasmid-mediated quinolone resistance (PMQR) genes in urban lakes and reservoirs, in Southeastern Brazil. In this regard, the detection of hospital-associated lineages of KPC-2-producing *Klebsiella pneumoniae* belonging to the international clonal complex CC258 (ST11) and CTX-M-15-producing *Escherichia coli* belonging to the international CC10 (ST617), in an urban lake, is reported for the first time. Whole genome sequencing (WGS) analysis of KPC-2-producing *K. pneumoniae* ST11 revealed that *bla*_{KPC-2} gene was carried by an IncN plasmid on a Tn4401b element. This study support that aquatic environments with public access can act as reservoirs of clinically important MDR bacteria, constituting a potential risk to human and animal health. On the other hand, the detection of high-risk clones highlights the extra-hospital spread of clinically significant bacteria into urban aquatic environments.

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1. Introduction

Aquatic environments have been directly affected by discharges of large quantities of urban wastewater, animal waste and hospital effluents (Bajaj et al., 2015; Finley et al., 2013), where the presence of multidrug-resistant (MDR) bacteria constitutes an important public

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health problem (Ben Said et al., 2016; Woodford et al., 2014). Consequently, in urban areas these environments can act as reservoirs of MDR and virulent bacteria, contributing to human and animal transmission (Montezzi et al., 2015; de Araujo et al., 2016; Rodrigues et al., 2016). Additionally, aquatic ecosystems are ideal for exchange of genetic material among bacterial species, facilitating the transfer and spread of resistance genes to clinically significant pathogens (Berglund, 2015; Tacão et al., 2014). In Brazil, the largest country in South America, reports about the identification of clinically significant β -lactamase-producing bacteria belonging to hospital-associated lineages, in wastewater, polluted rivers and coastal recreational waters, have been increasing steadily in recent years (Campana et al., 2017; de Araujo et al., 2016; Dropa et al., 2016; Montezzi et al., 2015; Oliveira et al., 2014; Picão et al., 2013; Turano et al., 2016). In the present study, we report additional information on the occurrence of clinically significant MDR bacteria carrying carbapenemases (*bla*_{KPC-2}), extended-spectrum β -lactamases (*bla*_{CTX-M}-type) and plasmid-mediated quinolone resistance (PMQR) genes, in urban lakes and reservoirs with public access.

2. Materials and methods

2.1. Sample collection and isolation of MDR bacteria

During the period from October/2012 to October/2013, surface water samples from aquatic environments with public access were collected from four different locations in the São Paulo state, Southeastern Brazil. Sampling included three urban lakes [RLI, 23°33'47.5"S 46°43'41.2"W; LDI, 22°31'10.2"S 46°38'05.8"W; IBL (2 samples), IBL1, 23°34'57.5"S 46°39'28.4"W and IBL2, 23°35'14.8"S 46°39'32.4"W] and one reservoir (GPR, 23°41'47.4"S 46°43'02.1"W). Bacteriological analysis was performed according to standard methods for the examination of water. In brief, volumes of 500 ml of surface water were collected in sterile bottles according to standard methods and transported to the laboratory under refrigeration (American Public Health Association, 2012). From each water sample, 100 ml was concentrated by filtration through sterile membrane filters with a pore size of 0.45 μ m. The filters were placed on MacConkey agar plates and incubated for 24 h at 37 °C. Next, the membrane filters were aseptically removed and placed separately into sterile tubes that had previously been filled with 10 ml of sterile Mueller–Hinton broth. After vortex treatment, an aliquot (100 μ l) of each culture was streaked onto MacConkey agar plates supplemented with cefotaxime (1 mg/l) or ciprofloxacin (1 mg/l). We have used cefotaxime in the selective media to select organisms that produce ESBLs and/or carbapenemases.

2.2. Identification of the isolates, antibiotic susceptibilities, ESBL phenotype and KPC production

Gram-negative isolates were identified by MALDI-TOF MS system (Bruker Daltonik). Resistance profiles were determined by Kirby-Bauer method, against 13 different antibiotics, and the results were interpreted as described in the CLSI (2015) guidelines or in EUCAST (2016). Additionally, minimum inhibitory concentrations (MICs) to ceftiofur, ceftazidime, enrofloxacin and ciprofloxacin were determined by agar dilution or Etest methods (CLSI, 2013, 2015). MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). ESBL production was screened by double-disc synergy test (DDST) and Etest ESBL strips containing ceftazidime/clavulanate (CLSI, 2015); whereas KPC producing isolates were identified using the modified Hodge test (MHT), the phenylboronic acid (PBA) disc test or by positive growth on CHROMagar™ KPC (Paris, France) (Nordmann et al., 2012).

2.3. Detection of ESBL-, pAmpC-, carbapenemase- and PMQR-encoding genes

Molecular characterization of resistance determinants was performed to identify specifically plasmid-mediated resistance. Plasmid-mediated genes conferring resistance to extended-spectrum cephalosporins (*bla*_{CTX-M}, *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, *bla*_{CTX-M-9} and *bla*_{CTX-M-15}), cephamycins (*bla*_{CMV-2}), carbapenems (*bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA}, *bla*_{IMP} and *bla*_{VM}), or encoding resistance to fluoroquinolones (PMQR: *aac*(6')-*lb-cr*, *qnr*, *oqx*A, *oqx*B, *qep*A) were identified by PCR and sequencing (Table A.1). In this regard, although it is not our focus it is important to record that the *aac*(6')-*lb-cr* is also an aminoglycoside resistance gene.

2.4. Clonal relatedness investigation and determination of phylogenetic groups of *E. coli*

For *E. coli* isolates, phylogenetic groups of virulence were determined by PCR, as previously described (Clermont et al., 2000), where virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal strains belong to low virulence phylogroups A and B1. On the other hand, the clonal relatedness of *E. coli* strains was investigated by ERIC-PCR (Aydin et al., 2007), where a cluster analysis of ERIC patterns was performed by using BioNumerics software (Applied Maths). Multilocus sequence type (MLST) was performed for *E. coli* (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>) and *K. pneumoniae* (<http://bigdb.pasteur.fr/klebsiella/klebsiella.html>) strains with positive production for CTX-M-15 and KPC-2 β -lactamases, respectively.

2.5. Whole genome sequence analysis

For KPC-2-producing *K. pneumoniae* and CTX-M-15-producing *E. coli*, the total genomic DNA was extracted, purified and used to construct a paired-end library (150 bp), which was sequenced using the NextSeq platform (Illumina). Genome assembly was carried out using Velvet v1.2.10 and contigs were annotated using Prokka (www.github.com/tseemann/prokka). Multilocus sequence type (MLST), plasmid replicons and antimicrobial resistance genes were identified using MLST 1.8, PlasmidFinder 1.3 and ResFinder 2.1 databases, respectively, available from the Center for Genomic Epidemiology (<http://genomicpidemiology.org/>) (Accessed March 15, 2017).

For detection of heavy metals (HM) and disinfectant resistance genes (DRG), we aligned sequence reads against our in-house database containing HM genes, conferring resistance to silver (*silE*, *silP*), copper (*pcoA*, *pcoD*, *pcoE*), arsenic (*arsB*, *arsH*), tellurite (*terD*, *terF*, *terX*, *terY3*) and mercury (*merA*, *merC*), and DRG genes conferring resistance to quaternary ammonium compounds (i.e., *qacA/B*, *qacC/qacD*, *qacE*, *qacF/H/I*, *qacE Δ 1*, *qacG*, *qacJ*, *qacH/qacZ*, *emrE*, *mdfA*, *sugE*, *ydgE* and *ydgF*) (Fang et al., 2016; Schwaiger et al., 2014). The criteria used was 90% similarity against our database.

3. Results

3.1. Occurrence and diversity of MDR gram-negative bacteria in urban lakes and reservoirs

A wide range of MDR Gram-negative bacteria, fermenting ($n = 14$) and non-fermenting ($n = 9$), were recovered from water samples collected from urban lakes and reservoirs in Southeastern Brazil (Table 1). In this regard, eleven different species were identified, most being *E. coli*, *P. aeruginosa* and *K. pneumoniae*. Antibiotic susceptibility studies revealed high levels of resistance to clinically important antibiotics, including amoxicillin/clavulanic acid (82.6%), cefotaxime (73.9%), ceftriaxone (69.6%), cefoxitin (60.9%), ceftazidime (43.5%), cefepime (47.8%), ertapenem (69.6%), imipenem (39.1%) and ciprofloxacin

Table 1

Antibiotic resistance profile of Gram-negative bacterial species isolated from water samples collected in urban lakes and reservoirs, selectively screened using MacConkey agar plates supplemented with cefotaxime (1 mg/l) or ciprofloxacin (1 mg/l).

Strain ^a	Specie	Water sample	Environmental location (latitude/longitude)	Antibiotic resistance profile ^b
GPR1	<i>Pseudomonas aeruginosa</i>	Reservoir	23°41'47.4"S 46°43'02.1"W	AMC, CTX, CRO, CFO, SUT, TIG
GPR2	<i>Escherichia coli</i>	Reservoir	23°41'47.4"S 46°43'02.1"W	GEN, SUT, CIP
GPR3	<i>Pseudomonas putida</i>	Reservoir	23°41'47.4"S 46°43'02.1"W	AMC, CTX, CRO, CFO, SUT, CIP, ETP, TIG
GPR4	<i>Klebsiella pneumoniae</i>	Reservoir	23°41'47.4"S 46°43'02.1"W	AMC, CTX, CRO, CAZ, CPM, GEN, SUT, CIP, TIG
RL1	<i>Enterobacter asburiae</i>	Lake	23°33'47.5"S 46°43'41.2"W	AMC, CFO, ETP, IMP
LDL1	<i>Pseudomonas aeruginosa</i>	Lake	22°31'10.2"S 46°38'05.8"W	AMC, CTX, CRO, CFO, SUT, ETP, TIG
LDL2	<i>Escherichia coli</i>	Lake	22°31'10.2"S 46°38'05.8"W	AMC, CTX, CRO, CAZ, CPM, CFO, AMI, SUT, CIP, ETP, IMP
IBL1.1	<i>Pseudomonas fulva</i>	Lake	23°34'57.5"S 46°39'28.4"W	AMC, CTX, CRO, CPM, CFO, SUT, ETP, TIG
IBL1.2	<i>Aeromonas hydrophila</i>	Lake	23°34'57.5"S 46°39'28.4"W	AMC, GEN, SUT, IMP
IBL2.1	<i>Providencia rettgeri</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, IMP, TIG
IBL2.2	<i>Pseudomonas aeruginosa</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CFO, SUT, ETP, TIG
IBL2.3	<i>Pseudomonas aeruginosa</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CFO, SUT, ETP, IMP, TIG
IBL2.4	<i>Klebsiella pneumoniae</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, SUT, CIP, ETP, IMP
IBL2.5	<i>Pseudomonas aeruginosa</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, GEN, SUT, CIP, ETP, IMP, TIG
IBL2.6	<i>Escherichia coli</i>	Lake	23°35'14.8"S 46°39'32.4"W	CTX, CRO, CAZ, CPM, GEN, SUT, CIP
IBL2.7	<i>Escherichia coli</i>	Lake	23°35'14.8"S 46°39'32.4"W	GEN, SUT, CIP
IBL2.8	<i>Enterobacter kobei</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, ETP, TIG
IBL2.9	<i>Stenotrophomonas maltophilia</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, ETP, IMP
IBL2.10	<i>Klebsiella pneumoniae</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, ETP, TIG
IBL2.11	<i>Pseudomonas putida</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, SUT, ETP, TIG
IBL2.12	<i>Citrobacter freundii</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CRO, CAZ, CPM, CFO, ETP, IMP, TIG
IBL2.13	<i>Escherichia coli</i>	Lake	23°35'14.8"S 46°39'32.4"W	SUT, ETP, TIG
IBL2.14	<i>Escherichia coli</i>	Lake	23°35'14.8"S 46°39'32.4"W	AMC, CTX, CIP, ETP, TIG

^a GPR, Guarapiranga Reservoir; RL1, Rua do Lago Lake; LDL, Lindóia Lake; IBL, Ibirapuera Lake.

^b AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; CPM, cefepime; CFO, cefoxitin; AMI, amikacin; GEN, gentamicin; SUT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; ETP, ertapenem; IMP, imipenem; TIG, tigecycline. Antimicrobial resistance profiles were determined by the Kirby-Bauer method and CLSI (2015) breakpoints were used to interpret results, except for CPM and TIG, for which the EUCAST criteria was applied (2016).

(43.5%). In this regard, it is important to consider that some bacterial species, identified in this study, present intrinsic resistance to beta-lactam antibiotics (i.e., *Pseudomonas* spp., *Aeromonas hydrophila*, *Stenotrophomonas maltophilia*) or tigecycline (i.e., *Pseudomonas* spp.) (Leclercq et al., 2013).

3.2. Molecular characterization of beta-lactamase genes

Isolates identified as *K. pneumoniae* ($n = 3$), *E. coli* ($n = 2$) and *Pseudomonas putida* ($n = 1$), displaying resistance to broad-spectrum cephalosporins, were screened by PCR and sequencing, confirming the presence of genes encoding CTX-M-type ($n = 1$), CTX-M-2 ($n = 2$), CTX-M-9 ($n = 1$) and CTX-M-15 ($n = 1$) ESBL variants. Additionally, *E. coli* strain LDL2 showed resistance to cefoxitin, related to the presence of the *bla*_{CMY-2} pAmpC gene, whereas the carbapenemase-resistant *K. pneumoniae* strain IBL2.4 carried the *bla*_{KPC-2} gene (Table 2). On the

other hand, although *E. coli* LDL2 and *K. pneumoniae* IBL2.10 strains exhibited resistance to carbapenems, the presence of carbapenemase-encoding genes (including *bla*_{NDM-1}, *bla*_{OXA-48}, *bla*_{IMP}, and *bla*_{VIM}-type genes) was not detected. Further identification of colistin-resistant Gram-negative bacteria carrying the *mcr-1* gene was unsuccessful (Liu et al., 2016).

3.3. Molecular characterization of PMQR

In five strains of *E. coli*, two *K. pneumoniae* and one *P. aeruginosa*, resistance to human (ciprofloxacin and levofloxacin) and veterinary (enrofloxacin) fluoroquinolones could be related to PMQR. In this regard, while the *aac*(6')-Ib-cr gene variant was confirmed in *E. coli* (LDL2, IBL2.6 and IBL2.7) and *P. aeruginosa* (IBL2.5) isolates, the *oqx*A was found in *E. coli* (GPR2) and *K. pneumoniae* (GPR4 and IBL2.4) strains; and the *oqx*B was identified in two *K. pneumoniae* strains

Table 2

Epidemiological and microbiological characteristics of beta-lactam-resistant and quinolone-resistant Gram-negative bacteria isolated from water samples collected in urban lakes and reservoirs, Brazil.

Strain ^a	Species ^b	Antibiotic resistance profiles ^c											β-lactamase genes	PMQR genes	Phylogenetic group ^d
		AMC	CTX	CRO	CTF	CAZ	CPM	CFO	ETP	IMP	CIP	ENO	LVX		
GPR2	<i>E. coli</i>	S	S	S	S	S	S	S	S	S	R	R	R	oqxA	A
GPR4	<i>K. pneumoniae</i>	R	R	R	R	R	R	S	S	S	R	R	R	oqxA, oqxB	–
LDL2	<i>E. coli</i>	R	R	R	R	R	S	R	R	R	R	R	R	CTX-M-2, CMY-2	B1
IBL2.4	<i>K. pneumoniae</i>	R	R	R	R	R	R	R	R	R	R	R	R	KPC-2	–
IBL2.5	<i>P. aeruginosa</i>	R	R	R	S	R	R	R	R	R	R	R	R	aac(6')-Ib-cr	–
IBL2.6	<i>E. coli</i>	S	R	R	R	S	S	S	S	S	R	R	R	CTX-M-15	A
IBL2.7	<i>E. coli</i>	S	S	S	S	S	S	S	S	S	R	R	R	aac(6')-Ib-cr	A
IBL2.10	<i>K. pneumoniae</i>	R	R	R	R	R	S	S	R	S	S	S	S	CTX-M	–
IBL2.11	<i>P. putida</i>	R	R	R	R	R	S	R	R	S	S	S	S	CTX-M-9	–
IBL2.14	<i>E. coli</i>	R	R	S	S	S	S	S	R	S	R	R	R	qnrB	D

^a GPR, Guarapiranga Reservoir; LDL, Lindóia Lake; IBL, Ibirapuera Lake.

^b *K. pneumoniae* strain IBL2.4 belonged to ST11 (CC258). *E. coli* strain IBL2.6 belonging to ST617 (CC10).

^c AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; CTF, ceftiofur; CAZ, ceftazidime; CPM, cefepime; CFO, cefoxitin; ETP, ertapenem; IMP, imipenem; CIP, ciprofloxacin; ENO, enrofloxacin; LVX, levofloxacin. Antimicrobial resistance profiles were determined by the Kirby-Bauer method and CLSI (2015) breakpoints were used to interpret results, except for CPM, for which the EUCAST (2016) criteria was applied and for ENO, for which the CLSI (2013) veterinary criteria was applied. S, susceptible; R, resistant. All resistant strains displayed CAZ and CTF MIC ≥ 16 mg/l and CIP and ENO MIC ≥ 32 mg/l, as determined by E-test or microdilution method (CLSI, 2015), respectively.

^d Phylogenetic group of *E. coli* determined by the Clermont method (Clermont et al., 2000).

(GPR4 and IBL2.4) (Table 2). The *qnrB* variant was carried by *E. coli* strain IBL2.14.

3.4. Clonal relatedness and phylogenetic groups of *E. coli*

Both ESBL- and PMQR-positive *E. coli* isolates were clonally unrelated by ERIC-PCR. The CTX-M-15-producing *E. coli* strain IBL2.6 was identified as belonging to the sequence type 617 (ST617), clonal complex 10 (CC10). On the other hand, the KPC-2-producing *K. pneumoniae* strain IBL2.4 was classified as belonging to the ST11, which is included into the worldwide disseminated CC258. Regarding the phylogenetic analysis of *E. coli* strains, CTX-M-producing *E. coli* belonged to the low-virulence phylogroup A and B1, whereas among PMQR-positive *E. coli* isolates, four strains belonged to low-virulence phylogroups A or B1 and, one *E. coli* strain carrying the *qnrB* gene belonged to the high-virulence phylogroup D (Table 2).

3.5. WGS analysis of KPC-2-producing *K. pneumoniae* ST11 (CC258) and CTX-M-15-producing *E. coli* ST617 (CC10)

WGS analysis of the *K. pneumoniae* strain IBL2.4 confirmed that this isolate belonged to high-risk hospital associated clone ST11/CC258 (*gapA*-3, *infB*-3, *mdh*-1, *pgi*-1, *phoE*-1, *rpoB*-1, *tonB*-4), further revealing that *bla*_{KPC-2} gene was carried on a conjugative IncN plasmid sizing ~40-kb, harbored on a Tn4401b element. Moreover, resistome analysis of IBL2.4 identified the β-lactam resistance gene *bla*_{SHV-11}, fluoroquinolone resistance genes *oqx*A and *oqx*B, the trimethoprim resistance gene *dfr*A30, the tetracycline resistance gene *tet*A, and the fosfomycin resistance gene *fos*A. Furthermore, IncFIB(pQII), IncFIB(K), ColRNAI, and IncFII(K) plasmids were also identified. On the other hand, *mer*A (mercury reductase), *pco*E, *pco*A, and *pco*D copper resistant genes, silver resistance genes *sil*P and *sil*E, tellurite resistance system genes (*ter*F, *ter*X, *ter*Y3) and disinfectant resistance genes *quac*E1, *quac*E, *quac*G, *yd*Gf, *yd*Gf and *sug*E(c), were detected by in silico analysis.

WGS analysis of the *E. coli* strain IBL2.6 confirmed that this isolate belonged to high-risk hospital associated clone ST617/CC10 (*adh*-10, *fum*C-11, *gyr*B-4, *icd*-8, *mdh*-8, *pur*A-13, *rec*A-73). Moreover, resistome analysis of IBL2.6 identified the β-lactam resistance gene *bla*_{CTX-M-15}, aminoglycoside resistance genes *aac*(3)-IIa and *aad*A5, the fluoroquinolone and aminoglycoside resistance gene *aac*(6')-Ib-cr, the trimethoprim resistance gene *dfr*A17, the tetracycline resistance gene *tet*B, the sulphonamide resistance gene *sul*I, the phenicol resistance gene *cat*B3 and the macrolide resistance gene *mph*A. Furthermore, three incompatibility plasmid groups were also identified, IncFIB, IncX4 and IncFIA. Heavy metals genes and disinfectant resistance genes were not detected by in silico analysis.

4. Discussion

Bacterial resistance is a global threat that directly affects public health, since the versatility of resistance mechanisms, currently expressed by clinically important species, has limited the use of commercially available antibiotics in human and veterinary medical practices. Concerning this issue, the increase in antimicrobial resistance among clinically significant bacteria, occasioned by the overuse and misuse of antimicrobial agents, have been affecting the aquatic environments, making this a natural reservoir (Conte et al., 2017; Marshall and Levy, 2011; Picão et al., 2013; Turano et al., 2016). Urban aquatic environments are susceptible to bacterial contamination, mainly from the community and even from health-care establishments. Therefore, in urbanized areas, rivers and lakes can act as reservoirs of high-risk pathogens (de Araujo et al., 2016; Montezzi et al., 2015; Rodrigues et al., 2016). In this regard, hospital-associated lineages of ESBL- and carbapenemase-producing Gram-negative bacteria have been identified in sewage, urban rivers and beaches (Campana et al., 2017; de

Araujo et al., 2016; Dropa et al., 2016; Oliveira et al., 2014; Picão et al., 2013; Turano et al., 2016; Yang et al., 2017).

In this study, we report the further identification of PMQR (mainly the *aac*(6')-Ib-cr gene variant, which confer further resistance to aminoglycosides), ESBL- and carbapenemase-producing bacteria in urban lakes and reservoirs with public access, in Southeastern Brazil. Of main concern is the identification of international high-risk clones of *E. coli* and *K. pneumoniae* producing CTX-M-15 and KPC-2, respectively, in an urban lake located in the major public park (Ibirapuera) of the São Paulo city, the largest and most populous metropolitan area in Latin America. This lake is inhabited by wildlife, where many flocks of birds (with inter-urban migratory flows) nest and roost near surface waters generating a lot of bird droppings, which can be considered as a point source of water pollution (Guenther et al., 2011; Tryjanowski et al., 2013).

The CTX-M-15-positive *E. coli* strain IBL2.6 was identified as belonging to ST617, which has been clustered into the international CC10, widely associated with *bla*_{CTX-M} (most *bla*_{CTX-M-15})-harboring human *E. coli* isolates in Belgium (Smet et al., 2010), Nigeria (Aibinu et al., 2012), Spain (Novais et al., 2012), China (Ho et al., 2013), Sweden (Brolund et al., 2014) and Mauritania (Ben Sallem et al., 2015); being further identified in companion animals in France (Dahmen et al., 2013) and Mexico (Rocha-Gracia et al., 2015); domestic farm animals in Tanzania (Seni et al., 2016); migratory Franklin's Gulls in Chile (Báez et al., 2015); urban wastewater in Algeria (Alouache et al., 2014); and wastewater treatment plant and municipal sewage in Tunisia (Ben Said et al., 2016). In Brazil, *E. coli* lineages belonging to the CC10 (ST570, ST571 and ST10) have been related to enteropathogenic pathotypes (Pitondo-Silva et al., 2009). More recently, in earlier studies by our research group, *E. coli* strains ST10/CC10, carrying the colistin-resistant *mcr*-I gene, have been identified in human and wildlife infections, and commercial chicken meat, in Natal and São Paulo cities (Fernandes et al., 2016; Monte et al., 2017; Sellera et al., 2017).

Regarding KPC-2 production, the plasmid-mediated *bla*_{KPC-2} gene has been widely spread among clinically significant members of the Enterobacteriaceae family, contributing with the establishment of predominant clones identified worldwide (Mathers et al., 2015). Pandemic KPC has occurred primarily by the dissemination of KPC-producing *K. pneumoniae* isolates belonging to the CC258, which include the sequence types ST258, ST11, ST340, ST437 and ST512 (Mathers et al., 2015; Woodford et al., 2011). In this study, the KPC-2-producing *K. pneumoniae* strain IBL2.4 belonged to the international high-risk clone ST11, which is endemic in Brazilian hospitals (Andrade et al., 2011, 2014; Pereira et al., 2013), as well as in other countries of Asia (particularly China) and Europe (Lee et al., 2016; Oteo et al., 2016; Qi et al., 2011). To our knowledge this is the first description of KPC-2-producing *K. pneumoniae* ST11 in an urban lake.

High-risk hospital-associated bacterial lineages have found several routes of entry into the environment, such as sewage from the community or hospitals (Roca et al., 2015). Specifically, in Brazil wastewater from treatment plants and hospitals have become a hot spot for horizontal transfer of antibiotic resistance genes (Conte et al., 2017; Dropa et al., 2016; Ferreira et al., 2011), and recent studies have report the occurrence of clinically relevant ESBL and/or carbapenemase producers in sewage and polluted rivers (Fontes et al., 2011; Oliveira et al., 2014; Picão et al., 2013; Turano et al., 2016). Of particular interest is the fact that the detection of KPC-2-producing *K. pneumoniae* belonging to ST340 and ST437 has also been reported in urban rivers in São Paulo city (Oliveira et al., 2014) demonstrating that high-risk clinical bacteria are not limited to nosocomial settings, which should exacerbate the public health concerns.

In the last 30 years, plasmid-mediated β-lactamases in Enterobacteriaceae has been widely reported in Brazil. In this regard, CTX-M-15-type ESBLs are disseminated among *E. coli* isolates in the hospital setting, whereas KPC-2-producing *K. pneumoniae* has become endemic during the last decade. Indeed, nowadays KPC-2 production is the

most frequent resistance mechanism (96.2%) in carbapenem-resistant *K. pneumoniae* (Sampaio and Gales, 2016), and include representatives of CC258 (i.e., ST11, ST340 and ST437) (Andrade et al., 2011; Pereira et al., 2013). Although, in this study, the direct source of hospital contamination could not be evidenced, hypothesis on the indirect contamination by nosocomial or domestic sewage through the Sapateiro stream could be raised, since this stream is the main suppliers of water for the lake that compose the landscape of the Ibirapuera Park. Most of the Sapateiro stream is channeled, however for decades it has received rainwater and urban runoff from the neighborhoods surrounding the Park, including homes, shopping malls and hospitals, resulting in poor water quality, from the point of view of abiotic and microbiological factors. At present, these waters are processed before they enter the lake, by a flotation treatment plant, in which an expressive part of suspended organic matter is withdrawn from the water through flocculation and micro-oxygenation; a process that focuses on obtaining water within the standard suitable for landscape use (Takahashi et al., 2012). Therefore, it is possible that this watercourse to continue to provide continuous flow of resistant bacteria and/or their resistance determinants for the urban lake.

In summary, the presence of MDR Gram-negative bacteria carrying genes previously identified in human and veterinary medicine supports the hypothesis that resistant bacteria can colonize different ecological niches beyond the hospital. Therefore, these findings support that aquatic environments with public access can act as reservoirs of clinically important MDR bacteria, constituting a potential risk to human and animal health. On the other hand, the detection of high-risk clones highlights the extra-hospital spread of clinically significant bacteria into urban aquatic environments.

5. Conclusions

Resistance to broad-spectrum cephalosporins, carbapenems and fluoroquinolones was detected in clinically relevant fermenting and non-fermenting Gram-negative bacteria isolated from urban lakes and reservoirs. Of main concern is the identification of CTX-M-15-producing *E. coli* and KPC-2-positive *K. pneumoniae* strains, belonging to international high-risk clones ST617 (CC10) and ST11 (CC258), respectively, from water samples collected in the main urban lake located in one of the major public parks of the São Paulo city, the largest and most populous metropolitan area in Brazil. On the other hand, this study support that aquatic environments, with public access, can be important sources for disseminating and/or transmitting a wide variety of MDR bacterial species and/or their genetic resistance determinants, for both humans and associated ecosystems, whereas the presence of successful clones of *E. coli* and *K. pneumoniae* suggests a contamination by domestic or hospital sewage. These results highlight the importance of environmental monitoring of MDR bacteria that can potentially serve as a source of resistance genes against clinically important antimicrobial agents.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this work.

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