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Chapter 4

MOLECULAR EPIDEMIOLOGY OF *KLEBSIELLA PNEUMONIAE* CARBAPENEMASE

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ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* have emerged as a class of pathogens that pose a significant threat to patients admitted to healthcare facilities. This phenotype is mostly due to the production of carbapenemases, which constitute the group of β -lactamases capable of hydrolysing all β -lactam antibiotics, including carbapenems.

However, the successful worldwide dissemination of carbapenem resistant *K. pneumoniae* has been linked to the emergence of a specific type of carbapenemase: KPC (*K. pneumoniae* carbapenemase). Although this carbapenemase has been identified in several sequence-types (STs), the pandemic seems to be mainly driven by the spread of KPC-producing *K. pneumoniae* from ST258.

Apart from the triumph of the clonal spread, there is a considerable variability in the number of mobile genetic elements that KPC-producing

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K. pneumoniae might harbour, and that contribute to the mobilization and transference of the KPC-encoding gene (*bla_{KPC}*). This transmission can be mediated by different molecular mechanisms that include the mobilization of minor genetic elements and the horizontal transfer of different conjugative plasmids.

Tn4401 transposon is highly involved in the horizontal dissemination of *bla_{KPC}*. This transposon can even assume different isoforms that, in turn, may become associated with multiple *bla_{KPC}*-bearing plasmids. Although many plasmids have been linked to the dissemination of *bla_{KPC}* gene, the incompatibility groups enclosing Tn4401 seem to be predominant.

In *K. pneumoniae*, other carbapenem resistance determinants have been identified throughout the years but none has disseminated to the extent of KPC. Its spread and success seems to be multifactorial with virulence factors, antibiotic resistance determinants and mobile genetic elements playing major roles. Only the early detection of these factors may ease their establishment worldwide and prevent their emergence in non endemic countries.

INTRODUCTION

Antibiotic resistance has been critically increasing over time and constitutes now one of the major priorities in human medicine. Instead of bacteria resistant to single classes of antibiotics, pathogens frequently harbor their multidrug resistance, which constitutes a serious therapeutic challenge that many times cannot be overcome, leading to higher morbidity and mortality (Giske et al. 2008).

First line antibiotics are becoming outdated, resulting in an increase of untreated infections that are forcing the clinicians to administer stronger formulas more often (Giske et al. 2008; Laxminarayan et al. 2013). Carbapenems constitute a group of antibiotics that are often considered last resort agents, reserved for the treatment of infections caused by multidrug resistant bacteria. However, carbapenem-resistant *Enterobacteriaceae* (including microorganisms such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* spp) have emerged as the major family of pathogens that pose a significant threat to patients admitted to healthcare facilities (Akova et al. 2012).

Apart from the success of the clonal spread, there is a considerable variability in the number of mobile genetic elements (such as plasmids, transposons, prophages, integrative and conjugative elements and insertion

sequences) that carbapenemase-producing *K. pneumoniae* might harbour, and that contribute to the mobilization and transference of the carbapenemase-encoding genes (Chen et al. 2012). This transmission can be mediated by different molecular mechanisms that include the mobilization of small genetic elements and the horizontal transfer of different conjugative plasmids (Chen et al. 2014a; Manageiro et al. 2015).

The evolution of *K. pneumoniae* towards a successful pathogen enables the escape from the host immune response by a mechanism of capsule switching. The *cps* (capsule polysaccharide) locus, which is one of the major determinants of antigenicity, seems to be evolving through a recombination process that allows DNA exchange between different strains (Shu et al. 2009).

It is known that factors beyond antibiotic resistance in *K. pneumoniae* may favour the strain's fitness, providing a benefit that underlies its prevalence. In fact, in *K. pneumoniae*, other carbapenemases have been identified along the time, but none has disseminated to the extent of KPC (*K. pneumoniae* Carbapenemase) (Chen et al. 2014a; Mathers et al. 2015). Its spread and success seems to be multifactorial, with pathogenicity determinants, virulence factors, and mobile genetic elements playing major roles (Chmelnitsky et al. 2013).

CARBAPENEM RESISTANCE MECHANISMS IN *KLEBSIELLA PNEUMONIAE*

Resistance to carbapenems in *K. pneumoniae* can be associated to a diversity of molecular events (Hawkey, 2015). The production of β -lactamases such as Ambler class A Extended-spectrum β -lactamases (ESBLs) or Ambler class C AmpC β -lactamases (e.g., CTX-M-15 and CMY-2, respectively) are likely to contribute to carbapenem nonsusceptibility when combined with a significant decrease in the cell permeability (Goessens et al. 2013; Nordmann and Mammeri, 2007). However, it is the carbapenem-hydrolyzing enzymes belonging to classes A, B and D of Ambler that create the greatest public health risk (Grundmann et al. 2010).

In what concerns class A carbapenem hydrolyzing enzymes, although there have been scattered reports of GES-5 (Guyana Extended-Spectrum β -lactamase) worldwide, KPC β -lactamases constitute the most extensively described carbapenemase in *K. pneumoniae* (Manageiro et al. 2013; Pitout et al. 2015).

KPC enzymes provide resistance to penicillins, cephalosporins, cephamycins, monobactams and carbapenems, being weakly inhibited by common β -lactamase inhibitors (such as clavulanic acid and tazobactam) and strongly inhibited by boronic acid, as shown in Figure 1 (Queenan and Bush, 2007). KPC-2 and KPC-3 constitute the most common variants of these enzymes and have already been described in other members of the *Enterobacteriaceae* family, particularly *Enterobacter* spp (Mathers et al. 2015). Nevertheless, the most severe nosocomial outbreaks of KPC-producing bacteria are most often linked to *K. pneumoniae*. The primary source of KPC remains unknown, but it is likely that it was acquired from the chromosome of an environmental microorganism, as it happened for other clinically relevant antibiotic resistance genes. Since its first emergence in United States of America, KPC is currently the most clinically significant carbapenemase (Grundmann et al. 2010). Its rapid international spread has become a well-known public health threat. Nowadays, KPC-producing *K. pneumoniae* is considered to be endemic in USA, Brazil, Israel, China, Poland, Greece, Italy, among other countries, and have been linked to a major clone – the ST258; however, the factors contributing to the epidemiological success of this clone remain largely unknown (Tzouveleki et al. 2013). In many hospitals worldwide, the reports of sporadic infections caused by non-clonal KPC-producing *K. pneumoniae* are eventually substituted by outbreaks due to ST258 or other high risk clones, leading to its endemicity (Won et al. 2011).

The class B β -lactamases (metallo- β -lactamases) that have been identified in *K. pneumoniae* have also been detected in other *Enterobacteriaceae* species. Microorganisms carrying these types of β -lactamases are often nonsusceptible to penicillins, cephalosporins, cephamycins and carbapenems, remaining susceptible to monobactams. Moreover, their action is inhibited by metal chelating agents (e.g., EDTA, dipicolinic acid) (Queenan and Bush, 2007). This group includes enzymes from IMP (Imipenemase), VIM (Verona Imipenemase) and NDM (New Delhi Metallo- β -lactamase) families, among which NDM is currently predominant worldwide (Bonomo, 2011; Queenan and Bush, 2007; Struelens et al. 2010). Although IMP producers are more common in Asia-Pacific area (such as China, Japan and Australia), VIM-harboring *K. pneumoniae* are mostly detected in the Mediterranean region (Walsh et al. 2005). The NDM is the most recently discovered family of mobile class B carbapenemase; the first reports worldwide involved foreign individuals who had received medical care in India and then travelled back to their country of origin (Yong et al. 2009). It is estimated that there are reservoirs for NDM-1 in the countries of the Balkan region, while there is also

an unknown burden in the Middle East, where people often travel to and from India (Dash et al. 2014; Livermore et al. 2011).



Figure 1. Antibiotic susceptibility testing of a KPC-producing *K. pneumoniae*. a, amoxicillin; b, ampicillin; c, ceftazidime with clavulanic acid; d, aztreonam; e, amoxicillin with clavulanic acid; f, temocillin; g, cefotaxime with clavulanic acid; h, cefotaxime; i, cefpodoxime; j, cefepime; k, doripenem; l, boronic acid; m, imipenem; n, amoxicillin with clavulanic acid plus cloxacillin; o, ceftazidime; p, piperacillin with tazobactam; q, meropenem; r, imipenem plus dipicolinic acid; s, ertapenem; t, cloxacillin; u, ceftoxitin. Disks with indicator antibiotics and/or inhibitors are used to detect: f, class D carbapenemases; c, e, g, ESBL; n, ESBL in the presence of an AmpC β -lactamase; l, class A carbapenemases; r, class B carbapenemases; t, AmpC β -lactamases.

The only class D carbapenem-hydrolyzing β -lactamases (CHDL) ever detected in *K. pneumoniae* isolates were OXA-48-type enzymes (Oxacillinase) (Poirel et al. 2012). Unlike other carbapenemases, which efficiently hydrolyze

all known β -lactams including carbapenems, this β -lactamase degrades penicillins, weakly hydrolyses carbapenems, and saves third generation cephalosporins (Poirel et al. 2012). For this reason, OXA-48-like enzymes can be difficult to detect and can represent a challenge for infection control. The use of temocillin has been reported as an indicator of the presence of this class D carbapenemases. OXA-48-producing *K. pneumoniae* is currently widespread in several Middle Eastern, North African and European countries, showing a wide range of antimicrobial susceptibility profiles to β -lactam antibiotics (Cantón et al. 2012).

There is also a growing number of reports of the majority of these carbapenemases found either in bacteria isolated from zoonotic species and in other non-human sources (Guerra et al. 2014). To the best of their knowledge, there are still no reports of KPC-producing *K. pneumoniae* in non-human sources. However, KPC have already been reported in other *Enterobacteriaceae* species from hospital sewages, effluents and rivers. Moreover, VIM, IMP and OXA-48-producing *K. pneumoniae* have been reported in rivers from Tunisia and Switzerland and in companion animals from Germany (Woodford et al. 2014). The ability to limit the emergence and spread of carbapenemase producers in non-human sources is essential to keep the circle of transmission interrupted.

HIGH RISK CLONES OF KPC-PRODUCING *KLEBSIELLA PNEUMONIAE*

The rate at which carbapenem resistant *K. pneumoniae* has disseminated motivated cause for concern among the scientific community (Grundmann et al. 2010). To date, KPC has been detected in more than 100 distinct sequence types (STs). However, this pandemic is mainly driven by the spread of a unique KPC-producing *K. pneumoniae* clone that is included in clonal complex 258 (CC258). This group consists of one predominant ST (ST258), and other STs with minor epidemiological expression, such as ST11, ST340, and ST512. Overall, *K. pneumoniae* ST258 represents a model of a high-risk clone with regard to its epidemiology, genetic features and antimicrobial resistance (Chen et al. 2014a; Munoz-Price et al. 2013).

The *bla*_{KPC}-harbouring *K. pneumoniae* was first identified in a non-ST258 isolate in the South of the United States of America during the 1990s, after which sporadic reports across the country were followed (Yigit et al. 2001).

But it was only in 2009 that became clear that this clone represented 70% of *bla*_{KPC}-harbouring *K. pneumoniae* from different parts of the country (Kitchel et al. 2009). The endemicity of ST258 KPC-producing *K. pneumoniae* in USA was followed by reports of this ST from many countries of South America, Europe and Asia, suggesting a dispersion pattern similar to other previously reported international multidrug resistant high risk clones (Mathers et al. 2015). Recently, the complete genome sequencing of *K. pneumoniae* ST258 urinary isolates from New Jersey suggested a clustering system that grouped the isolates in two different lineages based on the single nucleotide polymorphism (SNP) analysis of the core genomes. The two different groups, denominated clade I and clade II, were associated with two distinct KPC gene variants, known as KPC-2 and KPC-3, respectively, and mainly differed in one 215-kb genome region associated to the biosynthesis of the *cps*, which represents an essential virulence factor in this species (Deleo et al. 2014).

When isolates from ST258 (clades I and II) were compared with isolates from other STs (ST11, ST442, and ST42) with regard to the *cps* coding region and the core genome SNPs, it was found that a 1.1-Mbp area in ST258 clade II was identical to the same area of ST442, while the remaining part of the ST258 genome was homologous to ST11. This indicated that ST258 clade II was a hybrid clone produced by a recombination event between ST11 and ST442. Further investigations indicated that ST258 clade I evolved from ST258 clade II due to the replacement of the *cps* region from ST42 (Chen et al. 2014b).

Other STs have also played a role in the dissemination of KPC-producing *K. pneumoniae*. ST11, which belongs to CC258, constitutes one of major STs in the Asian continent and in South America (Munoz-Price et al. 2013; Yang et al. 2013). ST11 has also been associated with nosocomial outbreaks in Greece and United Arab Emirates caused by NDM-producing *K. pneumoniae* isolates (Pitout et al. 2015). In turn, *K. pneumoniae* ST147 is becoming an emergent high risk clone. It was first identified in Greece and has been associated not only with *bla*_{KPC} but also with *bla*_{VIM} in that country (Giakkoupi et al. 2011). This ST has also been associated with other class B and class D carbapenemases from other countries, including in North America (Lascols et al. 2013; Peirano et al. 2011).

KPC-HARBOURING MOBILE GENETIC ELEMENTS

The present success of *bla*_{KPC} is the result of its capacity for horizontal gene transfer, which has been greatly mediated by a Tn3-based transposon - Tn4401 (Chen et al. 2014a). This mobile genetic element has 10kb in length and is enclosed by two 39bp inverted repeat (IR) sequences. Its formation originated from the integration of Tn3 transposase and resolvase genes upstream of *bla*_{KPC}, followed by the insertion of two specific sequences, *ISKpn6* and *ISKpn7*, downstream and upstream of *bla*_{KPC}, respectively. Because the two pairs of IRs are still contiguous to these insertion sequences, its integration in the backbone of Tn4401 appears to be recent (Cuzon et al. 2011). Moreover, several active Tn4401 isoforms have been identified so far, differing by the existence of different genetic events upstream of the *bla*_{KPC} gene (Figure 2) (Pitout et al. 2015).

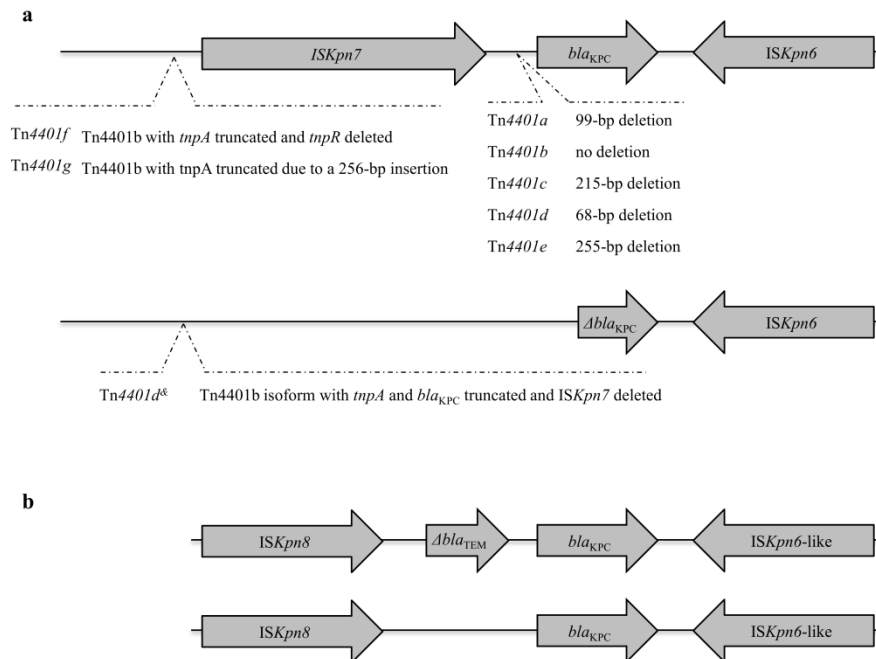


Figure 2. Schematic representation of the genetic structures enclosing the *bla*_{KPC} gene. a, eight isoforms of Tn4401, differing by polymorphisms located upstream of the *bla*_{KPC} and by other genetic re-arrangements. b, examples of other mobile genetic elements non-Tn4401 flanking *bla*_{KPC}.

Recently, a hybrid transposon structure resulting from the recombination of Tn4401 and Tn1331 has been observed in plasmids from different incompatibility groups (Inc groups). Tn1331 is typically known to enclose genes that confer resistance to aminoglycosides (*aac(6')-Ib* and *aadA1*), and β -lactams (*bla_{OXA-9}* and *bla_{TEM-1}*), contributing to the establishment of multidrug resistance (Gootz et al. 2009). The presence of the KPC-encoding gene has also been associated with other non-Tn4401 mobile genetic elements but usually in non-ST258 *K. pneumoniae* or in other bacterial species (Shen et al. 2009).

KPC is frequently plasmid encoded, and is often carried on conjugative elements of different incompatibility groups, such as IncF (with FIIk1, FIIk2, and FIA replicons), IncI2, IncX, IncA/C, IncR, and ColE1. Nevertheless, the most predominant *bla_{KPC}* plasmid types associated with *K. pneumoniae* ST258 are IncF with FIIk replicons (Chen et al. 2014a).

In fact, seems that the presence of plasmids carrying *bla_{KPC}* is crucial to the epidemiological success of ST258. The loss of *bla_{KPC}*-bearing plasmids by this high risk clone has diminished the capability of these isolates to successfully spread, which suggests that the *bla_{KPC}* in association with other plasmid-mediated virulence factors promoted an increase in the fitness of ST258 (Chmelnitsky et al. 2013). This is further sustained by the finding that non-ST258 *K. pneumoniae* with *bla_{KPC}* did not exhibit the same worldwide success as ST258 with *bla_{KPC}*. Hence, it appears that the thriving dissemination of *K. pneumoniae* ST258 is greatly influenced by the factors contained on *bla_{KPC}*-bearing IncF plasmids and on its own chromosome (Adler et al. 2012).

To date, more than 50 *bla_{KPC}*-harbouring plasmids have been completely sequenced, showing the presence of genes encoding added resistance to aminoglycosides, quinolones, trimethoprim, sulphonamides, and tetracyclines. This evidence constitutes cause for concern since co-selection frequently leads to the transmission of multidrug resistance (Gootz et al. 2009; Pitout et al. 2015). In fact, the association of the *bla_{KPC}* with other resistance determinants may facilitate the occurrence of hitchhiking events even in the absence of carbapenem selection pressure (Chen et al. 2014a).

Globally, these findings indicate that the existence of a vast genetic background with regards to genes, mobile genetic elements and clones has given rise to predominant clones.

CONCLUSION

K. pneumoniae is one of the most common pathogenic agents responsible to healthcare-associated infections worldwide. The concerning rates of high level multidrug resistant *K. pneumoniae* that have been proved to be associated with the dissemination of high risk clones and spread of epidemic plasmids by horizontal gene transfer have motivated the need to comprehend the genetic architecture of this pathogen. In fact, understanding the molecular evolution of carbapenemase-producing *K. pneumoniae*, and specifically of KPC producers, may help us to track the spread of specific mobile genetic elements and clones, and thus control of the spread of such microorganisms. Trough concerted actions of prevention and detection of KPC-producing *K. pneumoniae* we may avoid not only the infections but also the economic and health burden that derive from it.

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