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Review Article

REVIEW ARTICLE

Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance

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One sentence summary: This review highlights *Klebsiella pneumoniae* as a crucial pathogen in the burden of antibiotic resistance, encompassing multi and extremely drug resistant high-risk strains which cause worldwide infections. This poses the urgent need to identify new targeted strategies for prevention and treatment.

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ABSTRACT

Klebsiella pneumoniae is an important multidrug-resistant (MDR) pathogen affecting humans and a major source for hospital infections associated with high morbidity and mortality due to limited treatment options. We summarize the wide resistome of this pathogen, which encompasses plentiful chromosomal and plasmid-encoded antibiotic resistance genes (ARGs). Under antibiotic selective pressure, *K. pneumoniae* continuously accumulates ARGs, by *de novo* mutations, and via acquisition of plasmids and transferable genetic elements, leading to extremely drug resistant (XDR) strains harboring a 'super resistome'. In the last two decades, numerous high-risk (HiR) MDR and XDR *K. pneumoniae* sequence types have emerged showing superior ability to cause multicontinent outbreaks, and continuous global dissemination. The data highlight the complex evolution of MDR and XDR *K. pneumoniae*, involving transfer and spread of ARGs, and epidemic plasmids in highly disseminating successful clones. With the worldwide catastrophe of antibiotic resistance and the urgent need to identify the main pathogens that pose a threat on the future of infectious diseases, further studies are warranted to determine the epidemic traits and plasmid acquisition in *K. pneumoniae*. There is a need for future genomic and translational studies to decipher specific targets in HiR clones to design targeted prevention and treatment.

Keywords: antibiotic resistance; extremely drug resistant (XDR); resistome; epidemic plasmids; high-risk clones; mobile genetic elements (MGEs)

INTRODUCTION

After more than 70 years of extensive use of antibiotics to treat infectious diseases, antibiotic resistance is now being recognized as a worldwide crisis in modern medicine (The Review on Antimicrobial Resistance, O'Neill 2016). The dramatic increase in prevalence of infections caused by multidrug-resistant (MDR) and extremely drug resistant (XDR) pathogens belonging to the *Enterobacteriaceae* group poses a great concern since these pathogens are common natural inhabitants of our microbiome. Moreover, infections caused by these strains are often associated with high mortality rates, prolonged hospitalization and costs (Giske *et al.* 2008).

Antibiotic resistance is a multifactorial complex process (Watkins and Bonomo 2016). However, from the bacterial perspective it reflects evolution in action, concomitant to the continuous exposure to antibiotics, where selective pressure gives rise to the evolvement of multiple genetic mechanisms (Davies and Davies 2010). This constant evolution over the years has led to the emergence of MDR and XDR *Enterobacteriaceae* strains (Magiorakos *et al.* 2012) that exhibit resistance to nearly all antibiotics available, without possible treatment options

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(Hersh *et al.* 2012). The risk of global dissemination of these XDR pathogens has become a recognized global threat.

In the context of antibiotic resistance, a 'successful' bacterial strain should be an extremely effective vehicle for the dissemination of antibiotic resistance traits. This can be accomplished if it can transfer its antibiotic resistance traits vertically to its daughter cells, and also if it can act as a donor for mobile genetic elements (MGEs), like plasmids and transposons, that may be transferred horizontally to other strains, species or genera (Woodford, Turton and Livermore 2011).

In this era of antibiotic resistance, *Klebsiella pneumoniae* represents one of the most concerning pathogens involved in antibiotic resistance and as such, together with other highly important MDR pathogens, it has been classified as an ESKAPE organism (Boucher *et al.* 2009).

Klebsiella pneumoniae, belonging to the *Enterobacteriaceae* family, is a natural inhabitant of the gastrointestinal tract microbiome of healthy human and animals. It is a common opportunistic hospital-associated pathogen, accounting for about one third of all Gram-negative infections overall. It is involved in extra-intestinal infections including urinary tract infections, cystitis, pneumoniae, surgical wound infections and life-threatening infections, such as endocarditis and septicemia. It is also an important cause of serious community-onset infections such as necrotizing pneumonia, pyogenic liver abscesses and endogenous endophthalmitis (Podschun and Ullmann 1998).

Alongside with its high prevalence, *K. pneumoniae* is a major source for antibiotic resistance. Data retrieved from the European Antimicrobial Resistance Surveillance Network, (http://atlas.ecdc.europa.eu/public/index.aspx?Instance = GeneralAtlas) for the years 2005–2015, shows non-susceptible rates for *K. pneumoniae* and *Escherichia coli*, against the four major antibiotic classes: the third-generation cephalosporins, aminoglycosides, fluoroquinolones and carbapenems (Fig. 1). It is noticeable that resistance rates in *K. pneumoniae* steadily increase over the years. Resistances vary considerably between countries; Eastern and South-Western Europe, as well as Mediterranean countries, are endemic to MDR *K. pneumoniae*, due to ESBL production, exceeding 50%–60% non-susceptibility for third-generation cephalosporins, fluoroquinolones and aminoglycosides (Fig. 1A–C). As for carbapenems, while in 2005 almost all regions were free of carbapenem non-susceptible *Klebsiella*, in 2015 carbapenem-resistant *K. pneumoniae* (CRKP) has emerged in several countries, such as Romania, Italy and Greece, reaching non-susceptible rates of 40%–60% (Fig. 1D). Interestingly, despite the variations observed between countries, the data show the superiority of *K. pneumoniae*, compared to *E. coli*, in non-susceptible rates to all classes of antibiotics, especially to carbapenems, the last resort β-lactams. The percentage of carbapenem non-susceptible *K. pneumoniae* is extremely high in endemic countries compared to carbapenem non-susceptible *E. coli* (Fig. 1D). These differences in resistance rates between *E. coli* and *K. pneumoniae* are stable throughout the last decade, and highlight the major role of this later pathogen in the burden of antibiotic resistance.

The worldwide increase in occurrence of MDR and XDR *K. pneumoniae* reflects multifactorial dissemination processes that include (i) spread of High risk (HiR) global multiresistant genetic lineages (Woodford, Turton and Livermore 2011); (ii) acquisition of successful multiresistant plasmids and (iii) acquisition of resistance genes located on successful transposons.

In this review, we focus on the main features of *K. pneumoniae* that shape it as a highly important worldwide antibiotic-resistant pathogen. We provide an overview of all antibiotic resistance genes (ARGs) and mechanisms in this species, against the first and last line antibiotic treatments (Fig. 2). Using a time-line presentation, we demonstrate that under a continuous antibiotic selective pressure the resistome evolves constantly, and may accumulate various ARGs in a specific *K. pneumoniae* genetic background leading to the evolution of MDR and XDR carbapenem-resistant clones. Alongside the resistome, we present characteristics of 52 fully sequenced antibiotic resistance plasmids of *K. pneumoniae* that enable the inter- and intraspecies transfer of resistance via horizontal transfer (Table 1 and Fig. 3). These self-transferrable plasmids are referred to as *K. pneumoniae* 'mobilome' and they encompass plasmids that harbor ARGs and transposons. These diverse resistome and mobilome of *K. pneumoniae* contribute to the evolution of the XDR phenotype in both epidemic and sporadic sequence types (STs) or clones. When these ARGs and plasmids become associated with strains that have high epidemic potential, the clones become HiR (Woodford, Turton and Livermore 2011). We present the nine main epidemic *K. pneumoniae* clones associated with multinational outbreaks, and describe their ARGs repertoire and associated plasmids (Table 2). We discuss the unique traits described in the literature that may be involved in their epidemic success compared to non-outbreak clones. We highlight the importance of these clones to serve as HiR bacterial hosts and shuttles for antibiotic resistance, and summarize their possible dissemination routes including patient-to-patient spread, silent colonization via human and animals reservoirs, and across countries via medical tourism (Fig. 4).

KLEBSIELLA PNEUMONIAE RESISTOME

The resistome is defined as the collection of all the genes that confer antibiotic resistance (Wright 2007). We searched PubMed for all genes (ARGs) and mechanisms that confer resistance to five important classes of antibiotics used to treat *K. pneumoniae* infections, including β-lactams, aminoglycosides, quinolones, tigecycline and polymyxins. We summarized the *Klebsiella* resistome encompassing all ARGs reported in this species over the years since the first use of β-lactam antibiotics in the 1940s. We also marked the chromosomal or plasmid location of these genes (Fig. 2).

β-lactam resistance genes

β-lactam antibiotics, in clinical use since the 1940s, are a major class of antibacterial agents prescribed in human medicine. Since their introduction, hundreds of different β-lactamases have evolved among enteric pathogens including *K. pneumoniae*, reaching an astonishing number (>2000) and diversity (Bush and Jacoby 2010).

Broad-spectrum and extended-spectrum β-lactamases

Resistance to penicillin in *K. pneumoniae* was reported early in the 1960s, leading to the identification of the first β-lactamase genes, bla_{SHV-1} and bla_{TEM-1}. Two decades later, the first extended-spectrum β-lactamase (ESBL) gene, bla_{SHV-2}, was identified in *K. pneumoniae* recovered from an ICU patient in Germany (Kliebe *et al.* 1985). The gene exhibited an extended-spectrum activity against β-lactams, including third-generation cephalosporins and monobactams, and was defined as the first ESBL gene. Shortly after, another plasmid-mediated ESBL variant bla_{TEM-3} was reported from this pathogen in France (Sirot *et al.* 1987).

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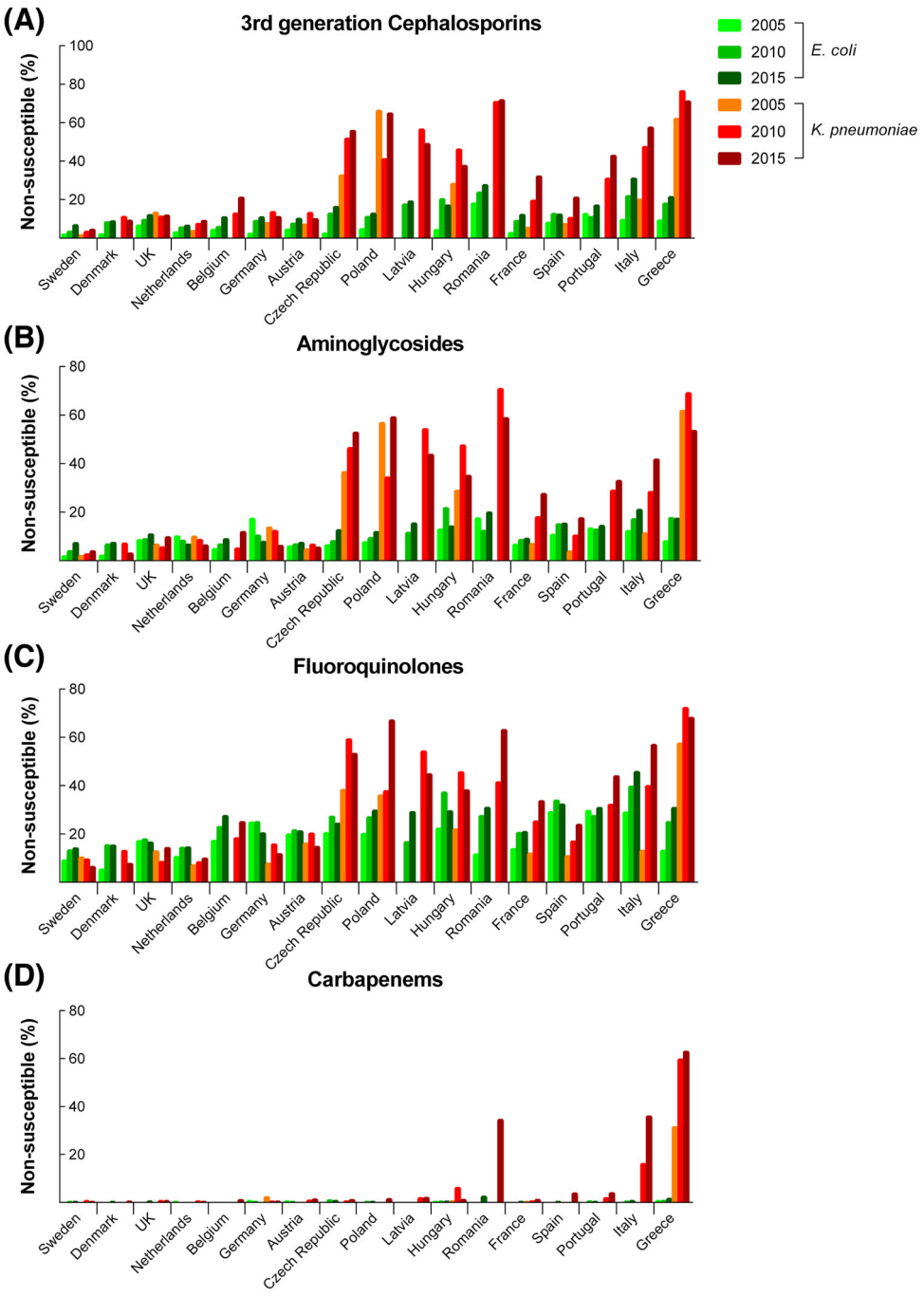


Figure 1. European data on non-susceptible rates of *E. coli* and *K. pneumoniae* to antibiotics (2005–2015). Data describing non-susceptible rates for *E. coli* and *K. pneumoniae* with respect to four classes of antibiotics, third-generation cephalosporins, aminoglycosides, fluoroquinolones and carbapenems. Percent non-susceptibility values were retrieved from the ECDC database http://atlas.ecdc.europa.eu/public/index.aspx?Instance=GeneralAtlas.

Since the emergence of ESBLs in *K. pneumoniae*, during the 1990–2000s, this pathogen has become the major ESBL-carrying pathogen associated in nosocomial outbreaks. In Israel and Spain, a prevalence of 40% of ESBL production among the total *K. pneumoniae* hospital isolates was reported (Navon-Venezia *et al.* 2003; Cantón *et al.* 2008). During that period, *K. pneumoniae* strains harbored mainly TEM and SHV β-lactamases (Chong, Ito and Kamimura 2011), with high occurrence and spread of various alleles in numerous countries (Fig. 2; Livermore 2012). In the 2000s, there was a shift in the type of ESBLs present in *K. pneumoniae*, causing hospital outbreaks, due to acquisition of plasmids and transposons encoding $bla_{\text{CTX-M}}$-type ESBLs, that led to the dominance of CTX-M-producing strains (Calbo and Garau 2015).

Additional groups of ESBL genes were transferred to *K. pneumoniae* by horizontal gene transfer (HGT) including bla_{OXA}-type ESBLs (Evans and Amyes 2014), and the rare genes bla_{GES} and bla_{SFO} (Bradford 2001), or bla_{PER}, bla_{TLA} and bla_{VEB} (Philippon *et al.* 2016). Moreover, inhibitor-resistant β-lactamase genes have emerged, encoding enzymes that are

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partially inhibited by clavulanic acid and tazobactam (Bush and Jacoby 2010).

After 30 years since their emergence, the occurrence of ESBL-producing *K. pneumonia* is increasing worldwide, reaching an epidemic proportion in many countries (Fig. 1; Calbo and Garau 2015), although prevalence and ESBL gene content varies between different geographical regions. According to World Health Organization (WHO), the occurrence of ESBL-producing *K. pneumoniae* has reached now endemic rates of up to 50% in many parts of the world, and up to 30% resistance rates in the community demonstrating the widespread nature of this resistance (Antimicrobial Resistance, Global Report on Surveillance. WHO 2014).

Plasmid-mediated AmpC genes

The remarkable versatility of *K. pneumoniae* to incorporate β-lactamase genes onto transferrable plasmids that enable their spread, gave rise to the emergence and spread of plasmid-mediated AmpC-like cephalosporins in this species (Jacoby 2009; Bush 2010). These genes emerged during the late 1980s and early 1990s, in parallel with the explosion of ESBL genes, and they are entirely plasmid-borne in *K. pneumoniae* (Jacoby 2009). The most abundant bla_{AmpC} gene families in this species belong to the CMY, DHA, FOX and MOX types, and their first years of occurrence in *K. pneumoniae* are shown in Fig. 2. Plasmid-encoded bla_{ACT}, bla_{MIR}, bla_{ACC} and bla_{LAT} seem to be highly rare AmpC genes in *K. pneumoniae*, and were not added to the time line. However, evolutionary tendency to incorporate resistant genes onto the chromosome occurred, and the first chromosomal AmpC $bla_{\text{CMY-2}}$ was identified in *K. pneumoniae* in 2009 (Fig. 2, Zamorano *et al.* 2015). *Klebsiella pneumoniae* strains showing enhanced resistance to β-lactams due to the existence of bla_{AmpC} combined with porin loss, or increased efflux, were also reported as with the case of $bla_{\text{ACT-1}}$. These genes can also be easily overexpressed on plasmids due to multiple copies, or increased promoter strength of plasmid genes, and even lead to carbapenem resistance (Jacoby 2009).

Carbapenem resistome

The endemic occurrence of ESBL-producing *K. pneumoniae* in various parts of the globe was reflected by ongoing exponential evolvement of new ESBL-types and alleles within this species, as well as in other *Enterobacteriaceae*, acquired by horizontal transfer of ESBL-encoding plasmids and transposons. The MDR phenotype characteristics of these ESBL-producing *K. pneumoniae* strains have led to a significant increase in carbapenem use, which became the last resort antibiotics to treat ESBL-producing *K. pneumoniae* (Livermore and Woodford 2006). The extensive use of carbapenems has resulted in the evolution of plasmid-mediated carbapenemases, i.e. enzymes that hydrolyze all β-lactams including the last-line carbapenems (Queenan and Bush 2007). Their appearance in *Enterobacteriaceae* led to carbapenem-resistant *Enterobacteriaceae* (CREs). Probably due to its hospital association, *Klebsiella* turned out to be the major CRE to have spread worldwide (Cantón *et al.* 2012), posing a significant public health threat (Chen *et al.* 2014c).

Imipenem was the first carbapenem antibiotic used to treat *K. pneumoniae* infections back in 1983 (Baumgartner and Glauser 1983), and imipenem-resistant strains were recognized already after 2 years (Knothe, Antal and Krcméry 1987). The earliest detected carbapenemase was IMP-1 metallo-enzyme, detected in *K. pneumoniae* from 1991 in Japan (Haruta *et al.* 2000). Several years later, in 1996, the first *K. pneumoniae* carbapenemase, named KPC, has emerged in the United States (Yigit *et al.* 2001). Although other carbapenemase genes occured in *K. pneumoniae* and were even reported in this species for the first time, such as $bla_{\text{OXA-48}}$ (Poirel *et al.* 2004) and $bla_{\text{NDM-1}}$ (Yong *et al.* 2009), it is undoubtable that bla_{KPC} became the most prevalent and highly impacting carbapenemase in *K. pneumoniae*. Strains carrying bla_{KPC} have been described in all continents, with $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$ being the most common genes, highly prevalent in strains involved in hospital outbreaks in many countries (Mathers, Peirano and Pitout 2015). The main driving force for spread of these genes was and is still clonal expansion of *K. pneumoniae* ST258, which, since its first report in the United States (Kitchel *et al.* 2009), has become endemic in many parts of the world (Munoz-Price *et al.* 2013), as will be discussed further. Along with clonal dissemination, bla_{KPC} genes reside in a unique Tn*4401* transposon variants (Naas *et al.* 2012), and are inserted into plasmids of various replicon types, which facilitates the dissemination of the gene to other bacterial species (Chmelnitsky *et al.* 2014). An interesting example of interspecies transfer of transposon-encoded $bla_{\text{KPC-3}}$ gene from *K. pneumoniae* to *E. coli* was reported in a patient from the United States. A *Serratia marcescens* isolate carrying *E. coli* plasmid harboring this transposon was sequentially recovered from the same patient, demonstrating *K. pneumoniae* as a source for KPC genes in multiple pathogens (Sidjabat *et al.* 2009).

Along with their activity against all β-lactam antibiotics and high transferability, KPCs often possess resistance to common β-lactamase inhibitors, posing a clinical challenge (Papp-Wallace *et al.* 2010). Increased mortality was shown for infections caused by KPC-producing *K. pneumoniae* (Munoz-Price *et al.* 2013).

With the increasing use of carbapenems to treat ESBL-producing *K. pneumoniae* infections, numerous processes occurred: (i) carbapenem resistance emerged without the actual carriage of a carbapenemase gene as a result of permeability alterations due to porin loss and overexpression of efflux pumps. The first description of this phenomenon was in 1988 (Van de Klundert *et al.* 1988), and was further demonstrated by a combination of high-level plasmid-mediated AmpC $bla_{\text{ACT-1}}$ together with porin loss (Bradford *et al.* 1997); decreased susceptibility to ertapenem by a combination of ESBL and loss of OmpK36 (Martínez-Martínez 2008; Leavitt *et al.* 2009), and the involvement of AcrAB efflux pump (Padilla *et al.* 2010). (ii) The emergence of unique ESBL genes with wider spectrum of activity, such as bla_{GES} (Queenan and Bush 2007). The first GES-type carbapenemase acquired in *K. pneumoniae* was $bla_{\text{GES-4}}$, identified in 2002 (Wachino *et al.* 2004). These enzymes are relatively rare in *K. pneumoniae*, and were mostly reported from Greece, Finland, South Korea and Brazil (Lee *et al.* 2016). (iii) New plasmid-encoded carbapenemase genes like $bla_{\text{OXA-48}}$ and bla_{VIM} arrived in *K. pneumoniae* (Fig. 2), now reported as endemic in different countries (Woodford, Turton and Livermore 2011). (iv) Translocation of carbapenemase-encoding genes, such as $bla_{\text{VIM-1}}$ and $bla_{\text{NDM-1}}$, from plasmids onto *K. pneumoniae* chromosome, by MGEs that were unique for these ARGs, making these resistances merely impossible to control (Fig. 2; Lee *et al.* 2016).

Multiple β-lactamase-encoding Klebsiella pneumoniae

Carriage of multiple β-lactamase genes in the same strain is a known ability of *K. pneumoniae* and may contribute to the selective success of this pathogen. Combinations of all types of *bla* genes were reported in this species (Lee *et al.* 2016). This may be due to either (i) carriage of an antibiotic-resistant plasmid encoding an array of ARGs due to acquisition of transposons containing different *bla* genes on the same plasmid or

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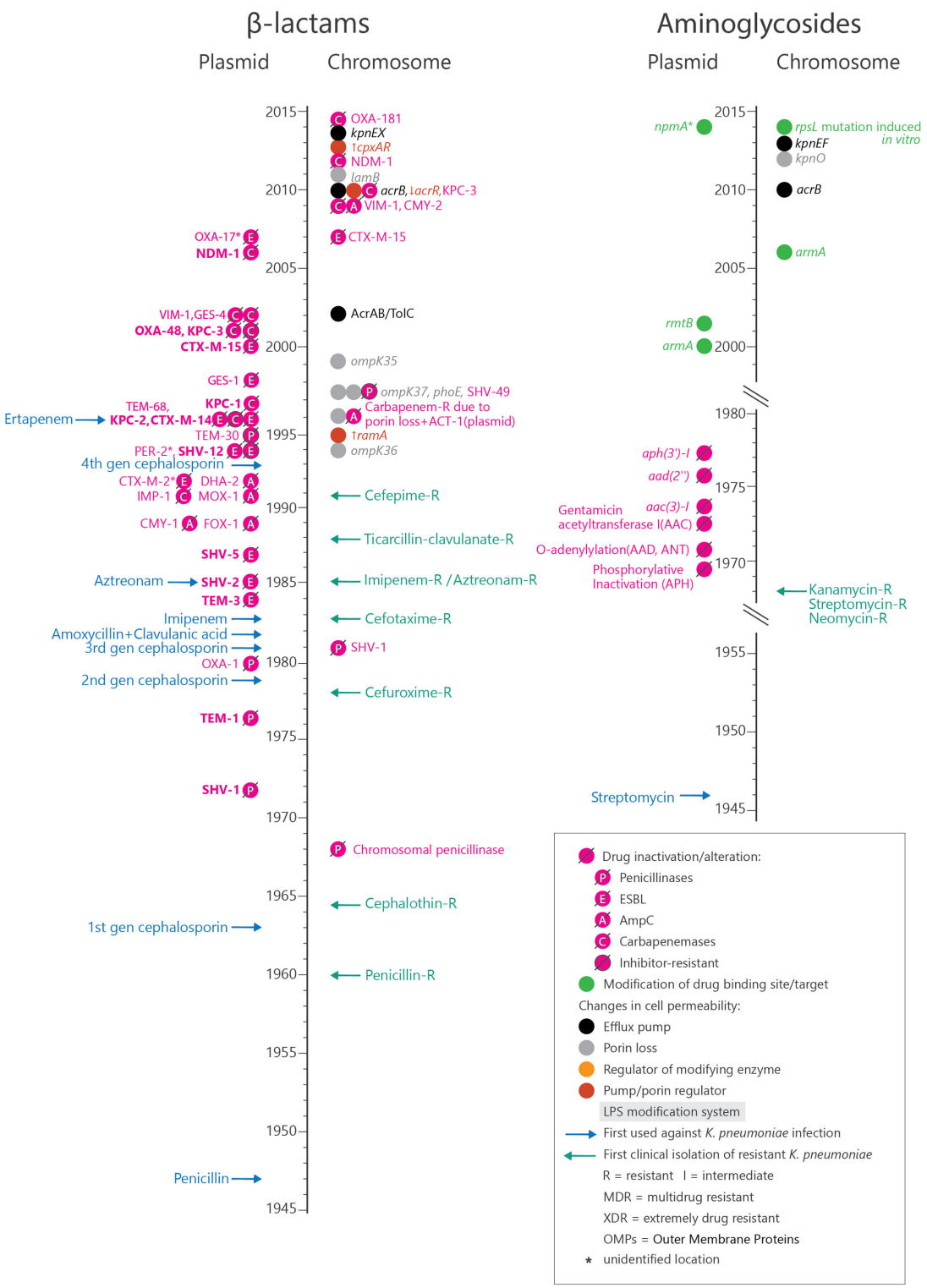


Figure 2. Time lines describing the evolvement of *K. pneumoniae* resistome. Time lines presenting resistance genes in *K. pneumoniae* against five important antibacterial classes. All ARGs presented were categorized into different resistance mechanisms, labeled with different symbols as indicated. The first year of isolation of *K. pneumoniae* carrying the indicated ARG (based on a PubMed search) is marked as well as its location (plasmid/chromosomal). Arrows on the left to the timeline indicate the first clinical use of the specified antibiotics. Arrows to the right of the timeline indicate the first appearance of resistance in a clinical *K. pneumoniae* isolate. Preference was given to the first members of a new resistance gene family, or for the first gene allele within a gene family with a new activity, or the first evidence of a family member presented in a new location. Highly common alleles of β-lactamase families are bolded. Rare β-lactamase gene families (appearing <20 times in PubMed search) were excluded from the figure.

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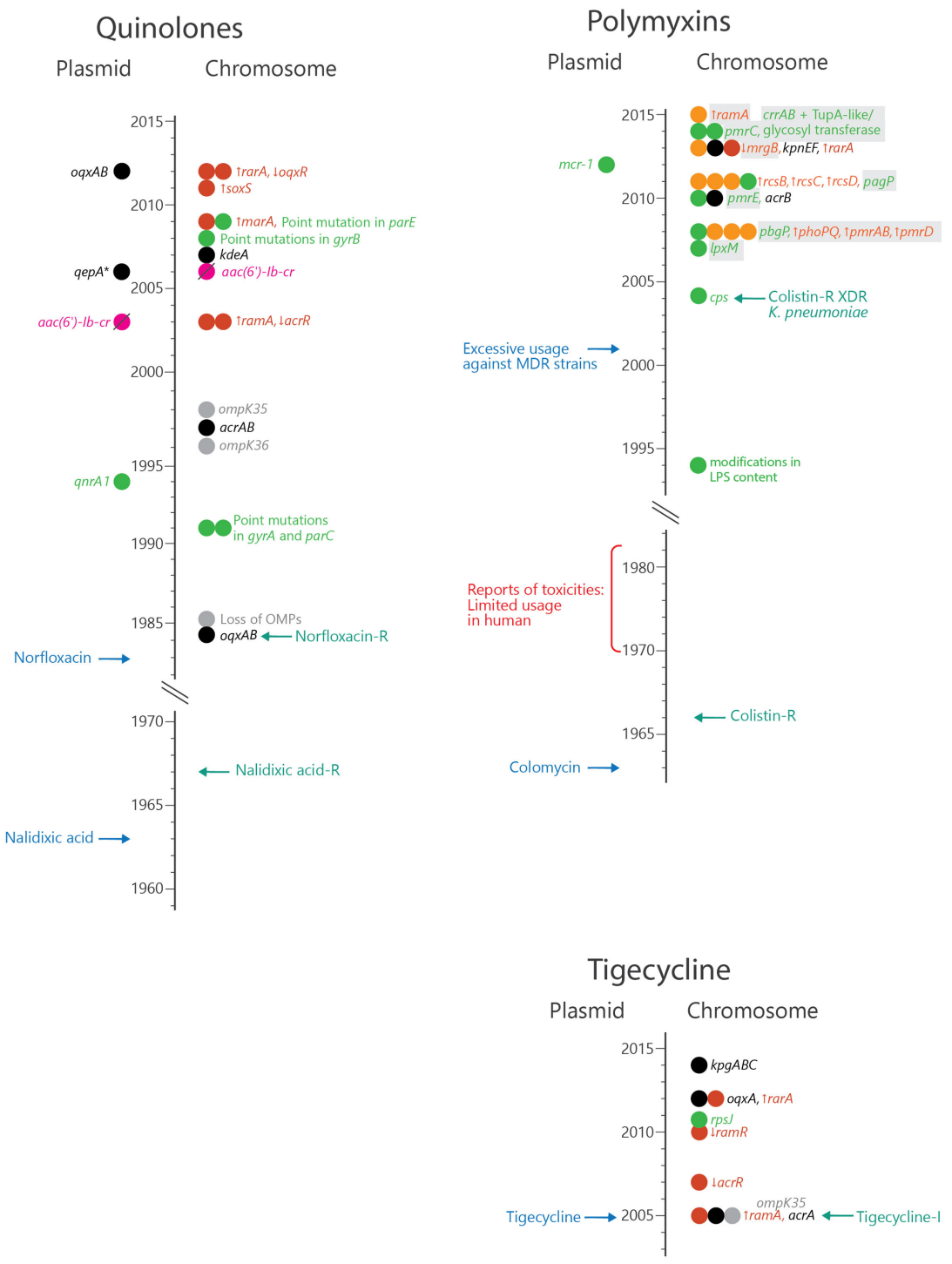


Figure 2. *Continued.*

(ii) co-carriage of more than one antibiotic resistance plasmid. Individual strains may carry multiple *bla* genes like the description of a strain isolated from a sputum of a hospitalized patient in New York City, carrying up to 10 different *bla* genes including a FOX-like plasmid-mediated AmpC, bla_{KPC}, bla_{SHV} and inhibitor-resistant β-lactamases (Moland *et al.* 2007), or a strain carrying bla_{NDM-1} and $bla_{OXA-232}$, on two different plasmids (Doi *et al.* 2014).

The evolutionary advantage for *K. pneumoniae* to harbor multiple β-lactamases with an overlapping catalytic activity, is an understudied area. There are indications that multiplicity of β-lactamases may be involved in extra reliable resistance. For example, IMP, VIM and NDM carbapenemases are susceptible only to aztreonam among β-lactam antibiotics, but they usually hide that gap with the presence of ESBL or AmpC β-lactamase (Molton *et al.* 2013). Extra copies of genes were also connected to

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higher resistance level (Chaves *et al.* 2001), or bacterial response towards increased antibiotic concentration (Huang *et al.* 2013). Further functional studies should be performed to elucidate the synergism of β-lactamases.

Aminoglycoside resistance genes

Aminoglycosides were actively used from mid-1940s to 1980s until they were replaced with third-generation cephalosporins, carbapenems and fluoroquinolones (Krause *et al.* 2016). During that period of use, *K. pneumoniae* gained the main resistance mechanisms against this group, involving drug modification enzymes with different activities, such as acetylation, adenylation or phosphorylation (Benveniste and Davies 1973). Within 10 years, plasmid-mediated resistance genes of all classes, *aac*, *ant* (grouped with *aad*), and *aph* gene families, were identified in *K. pneumoniae* (Fig. 2).

Decrease in aminoglycoside usage slowed down evolution of new resistance mechanisms until the discovery of 16S rRNA methylase, belonging to the *armA* gene family, that encodes enzymes which prevent aminoglycosides to bind to their 16S rRNA target (Doi, Wachino and Arakawa 2016). These genes are plasmid-encoded in *K. pneumoniae* (Galimand, Courvalin and Lambert 2003), and while drug-modifying enzymes have a narrow spectrum of activity, 16S rRNA methylases confer resistance to practically all aminoglycosides, including plazomicin, the most recent aminoglycoside compound developed (Poulikakos and Falagas 2013). Chromosomal location of *armA* was described in *K. pneumoniae* only once (Yu *et al.* 2009). Other known plasmid-mediated 16S rRNA methylases including Rmt family and NpmA were also found in *K. pneumoniae* (Krause *et al.* 2016), with no evidence of chromosomal location (Fig. 2).

Chromosomal resistance mechanisms against aminoglycosides in *K. pneumoniae* include modifications in cell permeability due to alterations in AcrAB-TolC and KpnEF efflux pump systems, and due to loss of putative porin, KpnO. Disruptions in AcrAB-TolC increased susceptibility to tobramycin and gentamicin (Padilla *et al.* 2010), whereas Δ*kpnEF* mutant showed strong change in resistance to tobramycin and spectinomycin (Srinivasan and Rajamohan 2013), but affected only slightly resistances to gentamicin and streptomycin. This may suggest different affinities of the permeability apparatus to different aminoglycosides. Direct involvement in aminoglycoside resistance was reported *in vitro* for KpnO porin, which upon loss caused resistances to tobramycin, streptomycin and spectinomycin (Fig. 2; Srinivasan *et al.* 2012).

Mutations which confer resistance via target modification, such *rrs* or *rpsL*, have not been found yet in clinical strains of *K. pneumoniae*. It is possible that *rpsL* mutations are connected with high fitness cost and reduced virulence, so they are less preferable. Multiple copies of *rrs* in *K. pneumoniae* chromosome may also complicate the development of resistance due to mutations in this gene (Beceiro, Tomás and Bou 2013; Tsai *et al.* 2014).

Quinolone resistance genes

Quinolones target bacterial topoisomerases blocking bacterial DNA replication. These drugs have been used in clinical practice since the 1960s, but their use increased extensively after the introduction of the first fluoroquinolones in the 1980s, which has led to development of bacterial quinolone resistance mechanisms (Naeem *et al.* 2016). *Klebsiella pneumoniae* resistome combines all the resistance mechanisms known for quinolone resistance in gram-negative bacteria (Redgrave *et al.* 2014), including target-site gene mutations, increased production of MDR efflux pumps, modifying enzymes and/or target protection proteins.

Cases of *Klebsiella* treated with nalidixic acid, the first clinically approved quinolone (Ward-McQuaid, Jichlinski and Macis 1963), and norfloxacin, the first fluoroquinolone used against *K. pneumoniae* (Guerra *et al.* 1983), were accompanied by the first evidences of chromosomal resistances to nalidixic acid (Davis, Lilly and Lowbury 1969) and to norfloxacin (Rydberg, Larsson and Miörner 1994) (Fig. 2). The first and major resistance mechanism is chromosomal mutations in the quinolone binding targets, DNA gyrase (*gyr*A-*gyr*B subunits) and topoisomerase IV (*par*C-*par*E subunits). Mutations in *gyrA* and *parC* in *K. pneumoniae* (Deguchi *et al.* 1997) were recognized earlier than mutations in *gyrB* (Nam *et al.* 2013) and *parE* (Guillard *et al.* 2015), and are probably more common (Fig. 2; Nam *et al.* 2013).

Changes in *K. pneumoniae* cell permeability have been reported to be involved in quinolone resistance, similar to their role in β-lactam resistance. These included OmpK36 deficiency (Martínez-Martínez *et al.* 1996), overexpression of *acrAB*, the multidrug efflux pump gene (Mazzariol *et al.* 2002) and unaltered production of *kdeA* (Ping *et al.* 2007) (Fig. 2). *In vitro* transferring of these genes into sensitive *E. coli* increased. Another important pump in *K. pneumoniae*, OqxAB, assumed to originate from the *K. pneumoniae* chromosome, has now been recognized to be involved in plasmid-mediated quinolone resistance (PMQR), and to mobilize and spread among other bacteria (Wong, Chan and Chen 2015). Efflux pump regulators were also reported to be involved in quinolone resistance in *K. pneumoniae*; the years in which the first resistance was identified are indicated, as well as the alteration in expression found in quinolone-resistant *K. pneumoniae* strains (Fig. 2).

Another group of quinolone resistance genes include PMQR determinants, which occur in *K. pneumoniae*, as in other *Enterobacteriaceae* (Jacoby, Strahilevitz and Hooper 2014). These genes encompass members of the *qnr* genes, which encode a family of proteins that physically protect DNA gyrase and topoisomerase IV from the inhibitory activity of quinolones. The first *qnr* gene was discovered on a plasmid of *K. pneumoniae* isolated in 1994 in the United States (Martínez-Martínez, Pascual and Jacoby 1998). There is no evidence of *qnr* genes on the *K. pneumoniae* chromosome, although these proteins are chromosomally encoded in other gram-negative bacteria including disease-causing species like *Shewanella algae, Citrobacter* spp., *Stenotrophomonas maltophilia, Vibrionaceae* and *Serratia marcescens* (Nordmann 2005; Ruiz *et al.* 2012).

Another PMQR gene, *aac(6′)-Ib-cr*, is the single gene responsible for quinolone modification in *K. pneumoniae*. It deactivates narrow spectrum quinolones such as ciprofloxacin and norfloxacin, which carry the unsubstituted piperazinyl group, a substrate of this enzyme (Ruiz *et al.* 2012). Initially, it was plasmid-encoded in *K. pneumoniae*, but was recently found on chromosome of this species (Fig. 2; Ruiz *et al.* 2012). Aminoglycoside resistance is often accompanied with co-resistance to β-lactams and fluoroquinolones; *aac(6′)-Ib-cr* conferred resistance to both aminoglycosides (tobramycin, amikacin and kanamycin) and fluoroquinolones (ciprofloxacin and norfloxacin) (Schultsz and Geerlings 2012). PMQR gene *qepA* encodes an efflux pump protein (Ma *et al.* 2009), and it has yet to be recognized on *K. pneumoniae* chromosome. Mechanisms provided by expression of PMQR genes produce low or moderate levels of resistance to quinolones, but they create favorable conditions for appearance of chromosomal gene mutants (Fàbrega *et al.* 2009).

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Interestingly, the presence of other resistance genes together with PMQR determinants provides cross-selection in spite of the antibiotic chosen for treatment. In Mexico, $bla_{CTX\text{-}M\text{-}15}$ was found on *K. pneumoniae* plasmids with *qnrB* (Silva-Sánchez *et al.* 2013). Cases of *K. pneumoniae* harboring $bla_{CTX\text{-}M\text{-}15}$ and *aac(6′)-Ib-cr* were reported from different world regions (Bado *et al.* 2016), and was suggested to possess an improved fitness due to the presence of $bla_{CTX\text{-}M\text{-}15}$ (Tóth *et al.* 2014). Thus, epidemic *K. pneumoniae* clones possess different modes of quinolone resistance which can be evolutionary important. Isolates belonging to the epidemic clones, discussed later herein, such as *K. pneumoniae* ST11, ST15 and ST147, often possess two or three resistance-causing substitutions in GyrA and ParC together with carriage of the PMQR gene *aac(6′)-Ib-cr* (Tóth *et al.* 2014). The presence of *oqxAB* and porin loss were described for *K. pneumoniae* ST258 (Mathers, Peirano and Pitout 2015).

Polymyxin resistance genes

Polymyxin disrupts membrane integrity through displacement of cations (Ca^{+2}/Mg^{+2}) in the outer membrane, by binding to the negatively charged lipopolysaccharides (LPS) and leading to cell lysis (Falagas and Kasiakou 2005). The history of polymyxin resistance in *K. pneumoniae* is shorter compared to other classes of antibiotics, owing to restricted use in human medicine between 1980s and 2000s, due to recognized toxicity (Fig. 2). The first clinical isolate of colistin-resistant *K. aerogenes* (now classified as *pneumoniae*) was isolated during the first period of usage (Davis, Lilly and Lowbury 1969). In the early 2000s, with the increasing occurrence of XDR carbapenemase-producing *K. pneumoniae* (CPKP) strains, therapy often relied on polymyxins, which became one of the last line of drugs (Falagas and Kasiakou 2005; Antoniadou *et al.* 2007). The first hospital outbreak of colistin non-susceptible MDR *K. pneumoniae* was reported in 2004 from Greece (Antoniadou *et al.* 2007), and since then an increasing number of reports on the recovery of colistin-resistant strains emerged from the clinical setting (Marchaim *et al.* 2011).

The major mechanism of polymyxin resistance in *K. pneumoniae* is target modification, achieved by chromosomal mechanisms, and is referred to as the 'LPS modification system'. Strains equipped with this multifaceted system alter the LPS structure, resulting in decreased anionic charge interfering with polymyxins binding. These changes in LPS are provided by mutations in several core genes, responsible for the maturation of lipid A (*lpxM* and its regulator *ramA*) (Clements *et al.* 2007; De Majumdar *et al.* 2015) and by neutralization of lipid A, by additional binding of amino arabinose (*pbgP*, *pmrE*), phosphoethanolamine (*pmrC*) or palmitate (*pagP*) (Mitrophanov *et al.* 2008; Llobet *et al.* 2011). Resistance also involves increased activity of numerous LPS-modifying gene regulators, such as *phoPQ*, *pmrA* and *pmrD* (Fig. 2, highlighted). Mutation in one of two other regulation genes, leading to *pmrB* overexpression (Jayol *et al.* 2014) or *mrgB* deactivation (Poirel *et al.* 2015), is already sufficient to cause resistance to polymyxins. Another potential pathway for LPS modification involves TupA-like/glycosyltransferase and CrrAB regulatory system described by Wright *et al.* These numerous pathways that lead to colistin resistance in *K. pneumoniae* in the clinical setting are complexed, may derive independently in a specific strain genetic background and are less common via patient-to-patient spread of resistant strains (Wright *et al.* 2015).

Additional mechanisms found to be involved in colistin resistance in *K. pneumoniae* are capsule polysaccharides (CPS) that may mask charged molecules on the outer membrane, and an increased expression of efflux pumps AcrAB-TolC and KpnEF (Srinivasan and Rajamohan 2013), due to positive regulation of their transcription by RarA (De Majumdar *et al.* 2013).

Plasmid-mediated polymyxin resistance was reported only recently, with the identification of the *mcr-1* gene in China (Liu *et al.* 2016). This gene encodes a phosphoethanolamine transferase enzyme family that modifies lipid A by connecting it with phosphoethanolamine, similarly to the activity of PmrC (Liu *et al.* 2016). The *mcr-1*-encoding plasmid, although originally found in *E. coli*, was conjugated and expressed *in vitro* in *K. pneumoniae*. Shortly after, widespread occurrence of this gene was reported in various countries across the globe. It was suggested to originate from *E. coli* with chicken origin, presumably due to extensive colistin use in the poultry (Schwarz and Johnson 2016; Zhi *et al.* 2016).

For CRKP, only tigecycline, colistin and some aminoglycosides still show favorable *in vitro* activities. Their XDR profile results in increased usage of colistin, which in turn leads to emergence of strains with induced resistance. This was demonstrated by widespread of colistin-resistance among KPC-producing *K. pneumoniae* (Lee *et al.* 2016). Colistin-resistant *K. pneumoniae* was found to be significantly higher in prevalence as compared to other *Enterobacteriaceae* species. In a recent global surveillance program, Bradford *et al.* (2016) reported that although resistance rates to colistin, among carbapenem-producing bacteria, were 12%, *K. pneumoniae* comprised 95% of all these isolates.

Co-selection of colistin resistance was observed also as a result of a broad-spectrum cephalosporin treatment in ESBL-producing *K. pneumoniae*, when resistance to colistin was achieved due to disruption of *mgrB* by $ISEcp1\text{-}bla_{CTX\text{-}M\text{-}15}$ (Jayol *et al.* 2016) or $ISEcp1\text{-}bla_{OXA\text{-}181}$ (Zowawi *et al.* 2015). High rates of colistin resistance (up to 36%) were found in carbapenem-resistant *K. pneumoniae* in endemic areas like Italy linked to higher mortality (Capone *et al.* 2013). Description of CRKP isolates carrying *mcr-1* has just been started, but spread of this gene is already considered to be a serious threat resulting in strains that are pan-resistant (Karaiskos *et al.* 2016).

Tigecycline resistance genes

Tigecycline is the first glycylcycline launched (Livermore 2005), and it has been in use against *K. pneumoniae* infections from 2005, after it was shown to escape from the main resistance mechanisms known against tetracyclines (Schedlbauer *et al.* 2015). Tigecycline was considered a promising drug, with a broad-spectrum activity even against ESBL-producing strains (Golan 2015). Shortly after its first use, an MDR *K. pneumoniae* strain possessing decreased tigecycline susceptibility (MIC 4 μg/mL) was isolated at a hospital (Fig. 2, Ruzin *et al.* 2005). According to the currently recommended tigecycline breakpoints, this strain was actually intermediately resistant to tigecycline (Osei Sekyere *et al.* 2016); however, since that report, tigecycline resistance among *K. pneumoniae* isolates has been increasingly reported. Known resistance mechanisms against this antibiotic are chromosomally encoded, and include modifications of both the 30S and the 16S ribosomal unit targets of the antibiotic, as well as changes in cell permeability (Osei Sekyere *et al.* 2016). Overexpression of the efflux pumps AcrAB-TolC and OqxAB and alterations in the expression levels of their regulators RarA, RamA, RamR and AcrR were also shown to contribute to resistance (Fig. 2; Osei Sekyere *et al.* 2016); however, their role in resistance is not definitive, and may be due to the increased expression of other pumps, such as KpgABC, a putative novel

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pump, that was offered as an alternative mechanism of tigecycline resistance (Nielsen *et al.* 2014). Resistance was also higher in *K. pneumoniae* strains with decreased transcription of porin gene, *ompK35* (Källman *et al.* 2008). The first mutation in ribosomal protein leading to susceptibility decrease was noticed in protein S10, encoded by *rpsJ* (Villa *et al.* 2014). *tetA* genes encoding efflux pump against tetracyclines were registered in tigecycline non-susceptible *K. pneumoniae* isolates, but their contribution to tigecycline resistance in this species is still unclear (Ahn *et al.* 2016).

XDR resistome

The emergence of multiple resistances towards different antibiotic groups is frequently found in hospital-adapted *K. pneumoniae* isolates due to the accumulation of arrays of ARGs that may be encoded on multiple plasmids forming a 'super resistome'. A super resistome may encompass combinations of ESBLs and/or carbapenemase genes with aminoglycoside modifying enzymes, or association of CTX-Ms or NDM carbapenemases with 16S rRNA methylases as described in a recent review (Krause *et al.* 2016). IncA/C plasmids, for example, encode NDM carbapenemase together with *rmt* methylases, quinolone resistance gene *qnrA* and AmpC-β-lactamase bla_{CMY} (Pitout, Nordmann and Poirel 2015). Another interesting example of a *K. pneumoniae* strain possessing a super resistome was shown carrying four different β-lactamases (bla_{NDM-1}, bla_{CMY-16}, *qnrA* and *armA* on one plasmid and bla_{OXA-48} and $bla_{CTX-M-15}$, each on separate plasmids), together with porin deficiency, quinolone resistance chromosomal mutations and additional ARGs (Seiffert *et al.* 2014).

These super strains may be practically resistant to all available classes of antibiotics, posing a great challenge to clinicians due to limited available treatment options (Munoz-Price *et al.* 2013; Nordmann and Poirel 2014), and are highlighted further in this review.

PLASMIDS ENCODING ANTIBIOTIC RESISTANCE IN *KLEBSIELLA PNEUMONIAE*

Antimicrobial resistance in *K. pneumoniae* has been attributed largely to the acquisition of large, self-conjugating plasmids. These extrachromosomal DNA molecules that encode factors promoting the initiation of replication, independently from the replication of the bacterial chromosome, maintain a constant copy number per bacterial cell and regulate their own replication rate (Nordström 2006). The minimal portion of a plasmid that controls the initiation of replication, maintaining the copy number of the parent plasmid, is called the basic replicon (Kollek, Oertel and Goebel 1980). In early studies performing bacterial typing of the emergent *K. pneumoniae* ST258 clone, plasmid replicon type was not determined, because most K. *pneumoniae* plasmids were not typable by the standard PCR-based replicon typing method (Carattoli *et al.* 2005). Thanks to genomics, the complete sequencing of *Klebsiella* plasmids was recently obtained, demonstrating that *K. pneumoniae* was endowed with new set of plasmids, carrying new replicons that were not previously described. However, most of these novel plasmid types were related to the so called IncF plasmid family. IncF plasmids have been described in all species of *Enterobacteriaceae*, often associated with virulence genes. IncF-related plasmids are in the range of 60–200 kb in size, of low copy number and carry FII-related replicons. The typical FII replicon is constituted by the *repA* gene whose transcription is controlled by antisense RNAs, acting *in trans* via sequence complementarity on target sense mRNAs (Blomberg *et al.* 1994; Nordström 2006). DNA sequences of FII replicons of plasmids from different species diverge, but may originate from a common ancestral replicon, showing overall >75% nucleotide identity. FII replicons characterize the large heterogeneous IncFII plasmid family, and can be subcategorized, based on PCR and sequencing, to the FII (*Escherichia coli*), FIIs (*Salmonella*), FIIy (*Yersinia*) and FIIk (*Klebsiella*) specific groups (Villa *et al.* 2010).

The IncFIIk plasmids

A total of 306 DNA sequences are currently available at the NCBI nucleotide database, corresponding to no redundant, complete sequences of plasmids, identified in *K. pneumoniae* (http://www.ncbi.nlm.nih.gov/nuccore/, December 2016). The majority of these DNA sequences were generated by high-throughput sequencing of entire genomes. Among them 27 are small (<15 kb) and 279 large plasmids. We identified the replicons on these plasmids using the PlasmidFinder database (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli *et al.* 2014). The most frequent replicon identified in this collection of *in silico* plasmids was the FIIk replicon (149/279 large plasmids, 53%). The FIIk was often found to be associated with replicons of the FIB-type (112/149 FIIk positives). The 'multireplicon' status allows the generation of plasmids that can replicate using alternate replicons, bypassing the incompatibility effect due to the simultaneous presence of two incompatible plasmids co-residing within the same cell (Osborn *et al.* 2000).

Two types of FIB replicons have been found more frequently in *K. pneumoniae* plasmids, named FIB_{pKPQIL} (48/149 FIIK positives) and FIB_{pKPN} (63/149 FIIK positives). These two FIB replicons distinguished plasmids pKPQIL and pKPN3, respectively (Fig. 3). These two plasmids are commonly found in fully sequenced *K. pneumoniae* genomes of the epidemic clonal group, CG258. Plasmids pKpQIL and pKPN3 very often co-reside within the same cell, show very similar transfer loci and FIIK replicons, and differ from the variable and the FIB-replicon regions (Fig. 3).

Plasmid pKPN3, initially identified in the genome of *K. pneumoniae* ST258 clone isolated from Israel and USA (Leavitt *et al.* 2010), encoded resistance to arsenic, copper and silver, by the *ars*, *cus* and *sil* loci, respectively (Fig. 3). In several pKPN3 variants, the *catA1*, *mphA*, *dfrA* and *aadA* genes, conferring chloramphenicol, macrolides, trimethoprim and streptomycin resistance, respectively, were also found. The most peculiar feature of pKPN3 derivatives is the presence of various virulence factors, such as the *lac* operon cluster, encoding a transcriptional regulator (LacI), β-galactosidase (LacZ) and lactose permease (LacY), that differ from the chromosomally encoded lactose operon, the Fec-like iron(III) dicitrate transport system and a glutathione ABC transport system (García-Fernández *et al.* 2012).

An interesting variant of pKPN3 is represented by the pKPN-CZ plasmid identified in a *K. pneumoniae* ST416, from the Czech Republic (Dolejska *et al.* 2013). This $bla_{CTX-M-15}$-encoding plasmid carried *mrkABCDF* gene cluster, showing >99% nucleotide identity with the *mrk* cluster encoding a type 3 fimbriae, identified in *K. pneumoniae* chromosome. The same cluster was previously identified on IncX plasmids from *E. coli*, and this genetic trait was demonstrated to profoundly enhance the ability of *E. coli* to form biofilm (Ong *et al.* 2009). The pKPN-CZ also encoded a thermo-resistance cluster, deriving from the mobilization of a similar cluster from the chromosome of *Cronobacter sakazakii* strain ATTC29544 (92% coverage, 99% nucleotide identity; Acc. No. FR714908).

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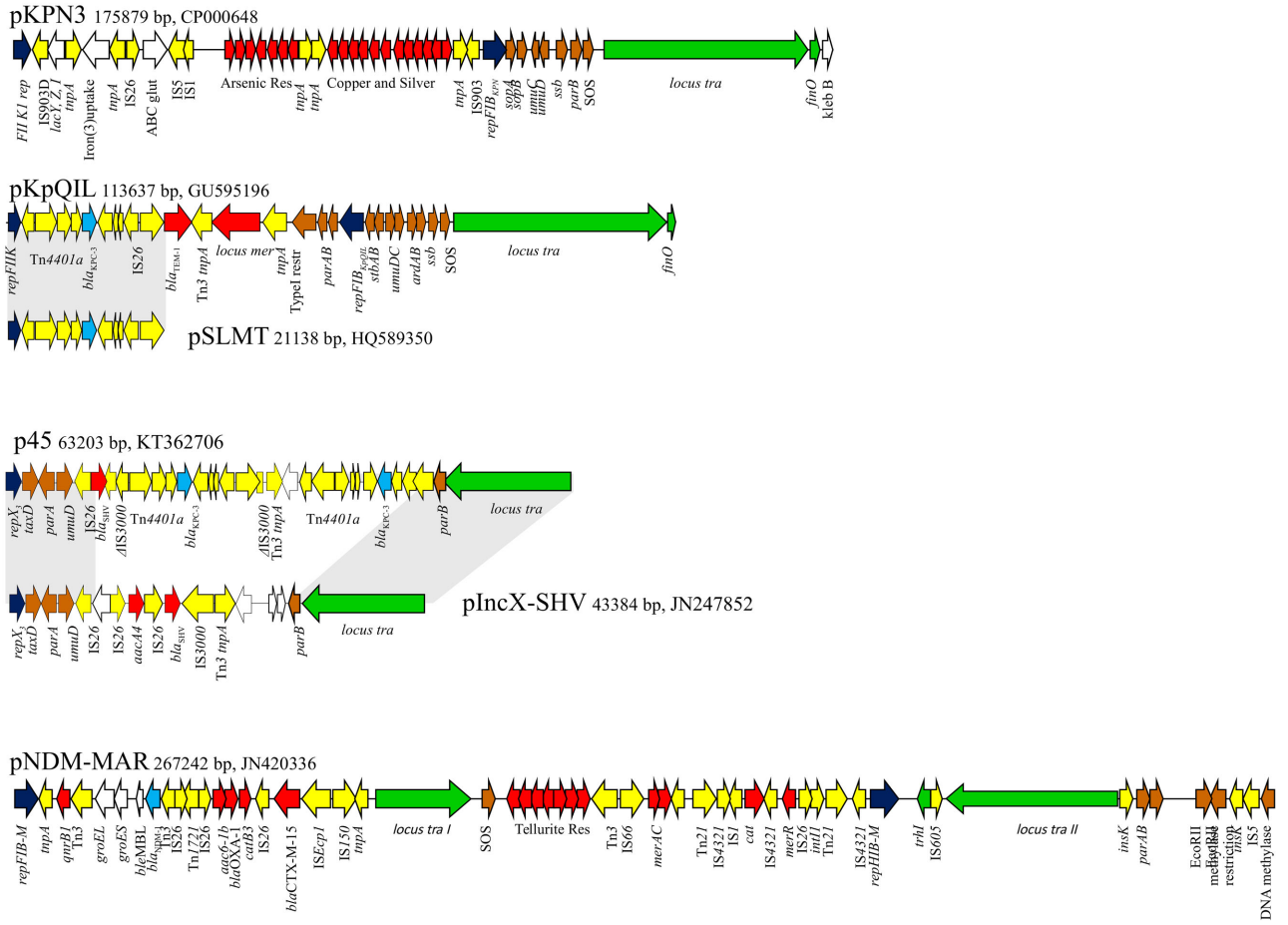


Figure 3. Schematic maps of six *K. pneumoniae* resistance plasmids. Schematic maps of six *K. pneumoniae* resistance plasmids discussed in the text. Colored arrows indicate open reading frames deduced from nucleotide sequences and represent: green, transfer loci genes (*traJ*, *D*: *traD*; *I*: *traI* etc.); red, resistance except carbapenemase genes; pale blu, bla_{kpc} and bla_{ndm} genes; yellow, transposon-related genes (*tnpA*, *tnpR*, *tnpM*), class 1 integrase genes, *tnpA* genes of insertion sequences; dark blue, replicase genes; brown, genes encoding restriction enzymes, DNA methylases, plasmid stability and partition functions.

The presence of these highly peculiar plasmid-mediated virulence traits assigns to pKPN3 and its derivatives, the role of *Klebsiella* specific virulence plasmids. These plasmids are very rarely reported in other enterobacterial species, presumably due to the narrow host range characteristic to IncFIIk plasmids that limits their dissemination to other species. The presence of these plasmids in *K. pneumoniae*, and their role in iron scavenging and biofilm formation, may contribute to *Klebsiella* persistence and survival within the hospital environment and in the human patient. Indications on the existence of pKPN3-derived plasmids exterritorial to a clinical setting were reported recently from a *K. pneumoniae* strain isolated from municipal wastewater treatment facilities in Israel that harbored a plasmid named pKPSH-11XI, encoding the quinolone resistance *qnrB* gene. These findings demonstrate the presence of these plasmids in the environment, suggesting the environment as a possible reservoir for antibiotic-resistant MGEs with significant clinical impact (Kaplan *et al.* 2015).

Plasmids associated with carbapenemase genes

bla_{KPC}-encoding plasmids

A retrospective study on *K. pneumoniae* isolates belonging to the epidemic ST258 clone carrying bla_{KPC}, isolated in early 2000s in the USA, demonstrated that both bla_{KPC-2} and bla_{KPC-3} genes were encoded on IncFIIk pKpQIL plasmids, which were identical to the first completely sequenced pKpQIL of ST258 from Israel (Fig. 3, Leavitt *et al.* 2010; Chen *et al.* 2014a). Since the mid-2000s, this plasmid was recognized in ST258 isolates from around the world, proving its epidemic nature. The most common resistance traits of pKpQIL plasmid are as follows: bla_{KPC-3} located in the Tn*4401a* transposon, *mer* operon, conferring resistance to mercuric ions, and the Tn3 transposon, carrying the bla_{TEM-1} gene (Fig. 3). The smallest bla_{KPC-3}-encoding pKpQIL derivative reported to date is pSLMT, identified in *K. pneumoniae* from the UK (HQ589358). This plasmid constituted only the portion of pKpQIL backbone consisting of the FIIk replicon and the bla_{KPC-3}:Tn*4401a* transposon. This configuration suggests the possible origin of multireplicon pKpQIL structure that resulted from a fusion between the minimal portion of pKpQIL together with another episome, carrying the FIB_{pKpQIL}, the transfer locus and other resistance determinants, generating the complete, frequently reported plasmid (Fig. 3).

There are also mosaic bla_{KPC}-encoding plasmids that seem to derive from IS26-mediated recombination between pKPN and pKpQIL, such as those generating pKPN3 derivatives carrying the bla_{KPC-2} identified in *K. pneumoniae* from the USA (Chen *et al.* 2013b). It is reasonable to assume that the coexistence of more than one IncFIIk plasmid within the same *K. pneumoniae* cell may cause frequent rearrangements among these plasmids, by

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recombination among homologous regions, represented by the FIIk replicons, the transfer loci and the numerous IS*26* elements scattered along the variable multidrug resistance regions.

Beside the IncFIIk, bla_{KPC-2} and bla_{KPC-3} were also identified on a wide variety of plasmids, including the IncN, IncX3, IncR, IncHI1 and IncI2 types (Table 1). Tn*4401b* carrying the bla_{KPC-3} on IncI2 has been extensively reported in the USA and UK. Interestingly, an association between the different Inc groups carrying bla_{KPC-3} and *K. pneumoniae* CG258 strain clades has been noted; IncI2 plasmids were found only in clade II, while pKpQIL were found in both clades I and II (Chen *et al.* 2013a; Chen *et al.* 2014c, Deleo *et al.* 2014).

IncR, IncN and IncX3 plasmids show broad host range and are often responsible for the horizontal transmission of bla_{KPC} by conjugation to other *Enterobacteriaceae*. Two tandem copies of the Tn3-like element Tn*4401a*-bla_{KPC-3} were identified on an IncX3 variant in *K. pneumoniae* ST512, with duplication due to the intramolecular transposition of the Tn*4401a* transposon within the IncX3 scaffold (Fig. 3). The plasmid conferred significantly increased MICs for carbapenems, cephalosporins and β-lactam/β-lactamase inhibitor combinations, compared to isogenic recipients carrying one copy of the Tn*4401a*-bla_{KPC-3} on pKpQIL. The MIC difference may be due not only to the double copy of the bla_{KPC} and their level of gene expression, but also to IncX3 and pKpQIL different copy numbers. On the same IncX3 plasmid, the bla_{SHV-11} was identified 4 Kb apart from the Tn*4401a*-bla_{KPC-3} integration site, followed by an IS*26* element. The same structure carrying IS*26*-bla_{SHV-11}-IS*3000* was previously detected on plasmid pIncX3-SHV, co-resident with pKpQIL carrying bla_{KPC-3} in ST258 strains (Fig. 3). It is therefore highly plausible that Tn*4401a*-bla_{KPC-3} transposed from pKpQIL into the pIncX3-SHV plasmid and the pKpQIL was then lost from the *K. pneumoniae* strain (Fortini *et al.* 2016).

Other plasmid rearrangements have been noted to involve portions of IncFIIk plasmids fused in a multireplicon status with IncR or IncN plasmids. These fusions had a significant evolutionary success. For instance, FIIK-R multireplicon plasmids carrying bla_{KPC-2} have been identified in Italy, USA, China, Russia and Taiwan, suggesting a successful international spread of these plasmids (Table 1; Frasson *et al.* 2012).

Other carbapenemase-encoding plasmids

VIM, IMP, NDM metallo-β-lactamases (MBL), GES and the carbapenem-hydrolyzing class D OXA β-lactamases (CHDL) have been described to be encoded on diverse plasmid types (Table 1), of which some types emerged and spread in unrelated, geographically distant *K. pneumoniae* strains.

In Greece, epidemic IncN1 bla_{VIM-1}-carrying plasmids were identified in unrelated *Klebsiella* strains from different hospitals. These plasmids encoded a variable region consisting of multiple integrons and transposons (Miriagou *et al.* 2010). Similar IncN1 plasmids were also described carrying the bla_{IMP4}-integron borne gene cassette (Lo *et al.* 2013).

The bla_{NDM-1} gene in *K. pneumoniae* was mostly acquired by broad host range IncA/C2, IncHI1, IncX3 and IncN2 plasmids (Table 1). The IncA/C2 group is particularly important since it carries multiple determinants, conferring resistance to aminoglycosides (encoded by *armA* or *rmtB* 16S RNA methylases), chloramphenicol, trimethoprim and sulphonamides, together with the AmpC β-lactamase CMY-2 (Carattoli *et al.* 2012). They show a very broad host range, being able to replicate in *Enterobacteriaceae*, but also in *Pseudomonas*. However, bla_{NDM-1} in *K. pneumoniae* also emerged on plasmids that were not previously identified in other *Enterobacteriaceae*. For example, it was identified on pKpQIL derivatives in Asia and America, but also on novel plasmids. Among them, the most innovative was represented by plasmid pNDM-MAR first identified in Morocco, but also detected in isolates from the USA and UK. Belonging to a novel group within the IncHI family, pNDM-MAR diverged from the previously described IncHI1 and IncHI2 groups. This plasmid was endowed by the HIB-M and FIB-M novel replicons and bla_{NDM-1}, as well as being characterized by the presence of the ESBL $bla_{CTX-M-15}$, and *qnrB1*, *aac6-1B*, bla_{OXA-1}, *catB3*, telluric and mercuric ions resistance genes (Fig. 3; Villa *et al.* 2012). The bla_{NDM-5} associated with the plasmid-mediated colistin resistance gene *mcr-1*, and bla_{NDM-7} and $bla_{OXA-181}$ carbapenemase gene variants were identified on IncX3 plasmids, suggesting that this plasmid type is highly frequent in *K. pneumoniae* (Table 1). The CHDL OXA-48 was identified in different *K. pneumoniae* STs (Table 2), but is always associated with the IncL plasmid, which is a highly conserved plasmid with epidemic occurrence in all continents (Poirel, Bonnin and Nordmann 2012). In contrast, bla_{IMP-8} and bla_{GES-5} genes were detected on novel plasmid types (Papagiannitsis *et al.* 2015).

Plasmids carrying ESBLs

ST11 and ST147 are HiR multiresistant *K. pneumoniae* clones, producing CTX-M-15 or CTX-M-14, that have spread globally (Table 2; D'Andrea *et al.* 2013). Plasmid typing performed on various collections of *Klebsiella* isolates demonstrated that bla_{CTX-M} genes were mostly located on IncFII plasmids that differ from those carrying $bla_{KPC,}$ and are rather highly similar to the IncFII of *E. coli*. However, despite the probable common origin, these plasmids in *Klebsiella* diverged. For example, pKF3-70 carrying the $bla_{CTX-M-14}$ gene from China had a conserved FII backbone but acquired an iron transport system, and several open reading frames of unknown function (Yi *et al.* 2010). Plasmid pKP12226 encoding $bla_{CTX-M-15}$ in *K. pneumoniae* ST11 identified from South Korea was a fusion of an IncFII plasmid carrying also the FIA replicon and the phage P1, which is capable of extrachromosomal replication by the IncY replicon (Shin and Ko 2015).

Several reports identified CTX-M-15 and CTX-M-1-producing *K. pneumoniae* in companion animals, associated with plasmids that were commonly reported in *E. coli* from animal reservoirs, such as the IncI1, IncR and IncN plasmids (Poirel *et al.* 2013; Donati *et al.* 2014). These evidences suggested that there was frequent horizontal exchange of ESBL-encoding plasmids among *Klebsiella* and other *Enterobacteriaceae*.

Interestingly, *Klebsiella*-specific $bla_{CTX-M-15}$-encoding plasmids were also recognized in *K. pneumoniae* ST416 clinical isolates from Czech Republic and Taiwan; pKDO1 (GenBank Acc. No. JX424423), pKF3-94 (FJ876826) and pK245 (NC˙010886) showed 52%–61% coverage, 84%–100% nucleotide identity with plasmid pKpQIL. Plasmid pKDO1 (Table 1, #37) carried the FII_{K7} replicon allele, associated with a novel and rare replicon encoding a Rep3 replicase. The plasmid pKDO1 contained a resistance region of 39760 bp, carrying *qnrB1* and the $bla_{CTX-M-15}$ gene. In addition, pKDO1 plasmid harbored the *Eco*RII restriction/antirestriction system flanked by an In4-type class 1 integron with the *dfrA14* gene cassette, in a configuration previously described for some IncN plasmids (Dolejska *et al.* 2013).

The $bla_{CTX-M-3}$ gene is disseminated among *Enterobacteriaceae* in Europe on pCTX-M-3, a conjugative plasmid of the IncM family, carrying the *armA* 16S RNA methylases (Gołebiewski *et al.* 2007). Plasmid pCTX-M-360, identified in *K. pneumoniae* in China, was highly related to the pCTX-M-3 plasmid, but lacked the region encoding *ArmA*, and had acquired multiple open reading frames encoding unknown functions (Zhu *et al.* 2009).

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Table 1. Prototypes of 52 fully sequenced antibiotic-resistance plasmids from *K. pneumoniae* carrying carbapenemases and ESBL genes.

No.	Plasmid reference	Accession no.	Resistance gene/s	Replicons	Countries of isolation
1	pKpQIL-234*	KJ146689	bla_{KPC-2}	FIIk, FIB_{pKpQIL}	USA, Greece, China
2	pKPN101-IT	JX283456	bla_{KPC-2}	R, FIIk	USA, Italy
3	Plasmid 9*	FJ223607	bla_{KPC-2}	N1 (A/C2, R)	Brazil, USA, China, Russia, Taiwan
4	pKpS90*	JX461340	bla_{KPC-2}	X3 (U)	Brazil, Hong Kong, France, Italy
5	pBK32179	JX430448	bla_{KPC-2}	FIIK, FIB_{pKpN3}	USA, China
6	pJM45-p1	CP006657	bla_{KPC-2}	R, FIIk, FIB_{pKpN3}	China
7	pHSO62105-3	KF623109	bla_{KPC-2}	other replicons	USA, UK, China, Greece
8	**pKpQIL***	GU595196	bla_{KPC-3}	FIIk, FIB_{pKpQIL}	USA, Israel, Italy, Taiwan
9	pNJST258N2	CP006926	bla_{KPC-3}	R, FIIk, FIB_{pKpN3}	USA
10	pBK30661	KF954759	bla_{KPC-3}	FIA-HI1 (X3)	USA
11	pBK15692	KC845573	bla_{KPC-3}	I2	USA, UK
12	Plasmid 12	FJ223605	bla_{KPC-3}	N1	USA
13	pNJST258C2	CP006919	bla_{KPC-3}	ColE	USA
14	**p45**	KT362706	bla_{KPC-3}, bla_{SHV-12}	X3	Italy, USA
15	pKPC-SMH	KT148595	bla_{KPC-3}	other replicons	UK, Canada
16	pBK31551	JX193301	bla_{KPC-4}	N1	South Korea, USA
17	pBK31567	JX193302	bla_{KPC-5}	X5	USA
18	**pNDM-KN***	JN157804	bla_{NDM-1}	A/C2 (R)	USA, Canada, Australia, Kenia, Taiwan
19	Plasmid 2	CP009115	bla_{NDM-1}, $bla_{CTX-M-15}$	FIIk, FIB_{pKpQIL}	Japan, Taiwan, USA, Canada
20	pTR1	JQ349085	bla_{NDM-1}	FIIK	Taiwan
21	**pNDM-MAR**	JN420336	bla_{NDM-1}, $bla_{CTX-M-15}$	HIB-FIB	Morocco, USA, UK
22	pJEG027*	KM400601	bla_{NDM-1}	X3	Canada, China, India, USA
23	pTR4	JQ349085	bla_{NDM-1}	N2	Taiwan
24	KP-plasmid 2	CP012755	bla_{NDM-1}	ColE	USA
25	KP96	KC958437	bla_{NDM-1}, $bla_{CTX-M-24}$	N1	China
26	Plasmid 3	CP006801	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{OXA232}	HI1, R	USA
27	pKPX-1	AP012055	bla_{NDM-1}	FIB_{pKpN3}, other replicons	China, Czech Republic, Oman, Taiwan, USA
28	pKpN01-NDM7	CP012990	bla_{NDM-7}	X3	Canada
29	pNDM5˙IncX3	KU761328	bla_{NDM-5}, *mcr-1*	X3	USA
30	Kpn-29144/15	KX523903	$bla_{OXA-181}$	X3	Czech Republic
31	pKpn-E1.Nr7*	KM406491	bla_{OXA-48}	L	Switzerland, Ireland, Australia, France, Netherlands
32	pNL194*	GU585907	bla_{VIM-1}	N1 (R)	Greece, Norway, Switzerland
33	pIMP-HZ1	JX457479	bla_{IMP}	N1	Hong Kong
34	p801-IMP	KT345947	bla_{IMP}	N3	China
35	pIMP-PH114	KF250428	bla_{IMP}	A/C2	China
36	pKP-M1144	KF745070	bla_{IMP-8}, bla_{GES-5}	ColE	Portugal
37	pGES-GZ	KT070138	bla_{GES-5}	N	China
38	pKF3-70	FJ494913	$bla_{CTX-M-14}$	FII	China
39	pKP09085*	KF719970	$bla_{CTX-M-15}$	FIIK, FIB_{pKpN3}	China, Sweden, Korea, Norway
40	pKDO1	JX424423	$bla_{CTX-M-15}$	FIIK, Rep3	Czech Republic
41	pKPX-2	AP012056	$bla_{CTX-M-15}$	HI1, FII	Taiwan, Germany
42	p628-CTXM	KP987217	$bla_{CTX-M-55}$	I1	China
43	pK29	EF382672	$bla_{CTX-M-3}$	HI2	Taiwan
44	pCTX-M-360	EU938349	$bla_{CTX-M-3}$	M	China
45	pKP12226	KP453775	$bla_{CTX-M-15}$	Y, FII, FIA	South Korea
46	pJIE137	EF219134	$bla_{CTX-M-62}$	N3	Australia
47	pIP843	AY033516	$bla_{CTX-M-17}$	ColE	Vietnam
48	pLJ1	EU880929	bla_{SHV-12}	N1	Australia
49	pKPS77	KF954150	bla_{SHV-12}	N1, R	France
50	pKPN-068	CP007733	bla_{SHV-12}	L	USA
51	pCAV1193-166	CP013324	bla_{SHV-7}	A/C2	UK, Japan
52	pH11	CP013215	bla_{SHV-12}	HI2	China, USA, Brazil, UK

Plasmids that are schematically presented in Fig. 3 are marked in bold.
Plasmids designated with an asterisk are considered as epidemic plasmids.

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Overall, taking into account the species-specific IncFIIk plasmids, the diverging IncHI, IncI2 and IncN2 plasmids, together with the completely new replicons identified in this species, *K. pneumoniae* resistance plasmids are very often unique and different from those described in other *Enterobacteriaceae*.

Interspecies spread of *Klebsiella* carbapenem-encoding plasmids

Transfer of *Klebsiella* plasmids to other species *in vitro* was described frequently in the literature, mostly to *E. coli*, demonstrating the conjugation ability of these plasmids, and their potential to operate as shuttles for antibiotic resistance. *Klebsiella* serves as a worldwide source for carbapenem resistance by dissemination of its plasmids facilitated by HGT to other species. Spread of these ESBL and carbapenemase-encoding plasmids poses a major threat as acquisition of these plasmids turns bacteria to MDR or XDR.

Originating from *Klebsiella* in the USA (Smith Moland 2003), bla_{KPC-2} has been shown to cause interspecies spreading to other *Enterobacteriaceae*, including *Salmonella* (Miriagou *et al.* 2003), *Enterobacter* sp. (Hossain *et al.* 2004), *E. coli* (Bratu *et al.* 2007) and *Proteus mirabilis* (Tibbetts *et al.* 2008). Further intercontinental dissemination of bla_{KPC-2} in various *Enterobacteriaceae* was reported in distant parts of the world, including *Enterobacter* and *E. coli* from Israel (Navon-Venezia *et al.* 2006; Marchaim *et al.* 2011), in the same species from Europe (Petrella *et al.* 2008), and in *Serratia marcescens* and *Citrobacter freundii* from Asia (Zhang *et al.* 2007; Cai *et al.* 2008; Zhang *et al.* 2008). Once bla_{KPC-2} is introduced in a certain location, especially in a hospital set-up, under antibiotic pressure, further dissemination of the gene may occur, as has been described in multiple clones of *E. coli* isolated during 2005–2008 in a tertiary hospital (Goren *et al.* 2010b). Dissemination of bla_{KPC-2} into other gram-negative non-fermenters pathogens was reported in *Pseudomonas aeruginosa* from South America (Villegas *et al.* 2007; Akpaka *et al.* 2009) and the USA (Poirel *et al.* 2010), in *P. putida* (Bennett *et al.* 2009) and in *Acinetobacter baumannii* (Robledo *et al.* 2010).

Transfer of *Klebsiella* plasmids was reported into clinically important strains, such as the worldwide epidemic *E. coli* ST131 clone (Nicolas-Chanoine *et al.* 2008). This ESBL-producing clone is a cause for multiple infections including bacteremia. Emergence of resistance to carbapenems in this clone by acquisition of bla_{KPC}-encoding IncFIIk plasmid with a scaffold similar to that of pKpQIL was reported in the USA (O'Hara *et al.* 2014), and resulted in a XDR hypervirulent strain.

Transfer of KPC from *K. pneumoniae* to a commensal *E. coli* strain in a patient gut was demonstrated by the *in situ* acquisition of bla_{KPC-3}-encoding pKpQIL plasmid from a CRKP ST258 strain colonizing the patient gut, leading to the emergence of a carbapenem-resistant *E. coli* strain (Goren *et al.* 2010a).

HIGH-RISK EPIDEMIC *KLEBSIELLA PNEUMONIAE* CLONES

The two main dissemination modes of an antibiotic-resistant pathogen in general and specifically *K. pneumoniae* are (i) clonal expansion of an antibiotic-resistant clone and (ii) HGT of antibiotic resistance elements from a resistant strain to a susceptible bacterium that turns resistant upon acquisition of the resistant element/s. Although both modes of spread act simultaneously throughout evolution, the main driving force for spread of *K. pneumoniae* over the years is the successful expansion of certain high-risk clones. The origin of a HiR clone in a certain vicinity can be either as a result of a local transmission of a specific clone or due to the import of a new clone from an endemic area.

The importance of HiR bacterial clones in the context of international dissemination of antibiotic resistance was first recognized by Woodford *et al.* The authors defined a 'high-risk clone' as isolates that belong to the same ST according to the multilocus sequence type (MLST) scheme, although isolates may have been isolated in a different geographic location, and time. Moreover, certain *K. pneumoniae* STs are considered more probable outbreak-causing agents as compared to others (Woodford, Turton and Livermore 2011; Chmelnitsky *et al.* 2013). These are the clones that should be identified, monitored and controlled.

High-risk XDR worldwide *Klebsiella pneumoniae* clones

The number of outbreaks caused by a bacterial clone represents its epidemic potential. We searched PubMed using the keywords (*K. pneumoniae*, outbreak/epidemic/pandemic/ST), and focused on papers published after 2005, when *K. pneumoniae* MLST scheme was established (Diancourt *et al.* 2005). Outbreaks of ESBL-producing *K. pneumoniae*, occurring prior to 2005, are well described elsewhere (Calbo and Garau 2015). The inclusion criteria for 'a HiR *K. pneumoniae* clone' were a clone that caused at least four recognized outbreaks and that was reported from ≥10 countries. All nine ST clones identified were known CPKP.

The following STs, ST16, ST20, ST48, ST307, and ST340, ST336 and ST395, although recognized as important international outbreaks clones, were excluded from our analysis because at the time of this review they did not fulfill our inclusion criteria. It is important to emphasize that these clones are continuously disseminating and may become HiR in the future.

Table 2 represents the distribution of *bla* gene alleles with highest enzymatic capacity within each HiR clone based on all reports connected to each ST including outbreaks, sporadic cases and surveillance reports. All HiR clones carry a diverse arsenal of *bla* genes including diverse carbapenemases and/or ESBLs (Table 2). Certain STs harbor a main *bla* carbapenemase gene such as ST258 and ST512 that are linked to bla_{KPC}, whereas isolates belonging to other STs may carry various carbapenemases. For example, ST15 is mostly a CTX-M-15 producer but also encodes all types of carbapenemases genes: bla_{KPC}, bla_{OXA-48}-like, bla_{NDM}, bla_{VIM} and bla_{IMP}. This high variation of resistant genes within and between the clones reflects HGT of circulating ESBL- and carbapenemase-encoding plasmids and transposons, which may depend on *bla* genes and allele distribution in bacterial population in different geographic locales.

Clonal group 258

Since the first emergence of CRKP, nearly 100 international outbreaks caused by high-risk clones were reported in the literature (Table 2). The most significant outbreak causing clonal group was CG258, responsible for 68% of all outbreaks and consisting of three STs: ST258, ST11 and ST512. The second most prevalent CG, responsible for ~20% of all outbreaks was CG15, consisting of ST14 and ST15. The remaining CG are epidemic but to a lower extent (Table 2).

CG258 is an intriguing clonal group due to its high spread and success. ST258, the most widespread clone of this CG, has emerged clinically in 2000. This hybrid clone emerged after recombination events between two *K. pneumoniae* clones. ST11 and ST442, and now is divided into two clades based on capsule type (Deleo *et al.* 2014). ST512 originated from ST258 clade II

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Table 2. High-risk XDR *K. pneumoniae* clones causing worldwide outbreaks in humans (2000–2016).

Clonal group	ST[a]	Relative occurrence (%) of *bla* gene alleles in *K. pneumoniae*[b]						Plasmid Types[c]	No. of Reports/ Countries/ continents[d]	No. of Outbreaks
		Carbapenemase-producing				Carbapenemase non-producing				
		KPC (%)	OXA (%)	NDM (%)	VIM/IMP (%)	CTX-M (%)	Other ESBLs			
258	258	2 (51.5) 3 (36.2) 7/11/9 (1.3) ND (4.4)	-	-	VIM 1/4 (0.7)	14 (1.8) 15 (1.1) 2/25 (0.7) ND (1.5)	SHV-5 (0.7)	R, FII, FIB, L/M[e], N, X, pKpQIL, pKPN3	137/35/5	32
	11	2 (40.2) 3 (1.9) 17 (1.9) 16/24 (0.8) ND (1.9)	48 (9.6) 162/163/ 245 (1.7)	1 (11) ND (0.4)	VIM 1 (2.8) VIM 4 (0.8) IMP 8/ND (0.8)	15 (7.4) 14 (2.7) 1/2/3/17/ 22 (2) ND (1)	SHV-11 (1.3) -5 (1.1) -1/12/22/31 (1.5) ND (1) DHA- 1 (5) ND (3) SFO-1 (0.4)	FII, FIB, L/M[e], N, R	133/35/5	25
	512	3 (85) 2 (13) ND (2.8)	-	-	-	-	-	F, FIB, X, pKpQIL	36/11/5	14
15	15	2 (12) 3 (5)	48 (12) 162 (1.4) 181 (0.7)	1 (10)	VIM 1 (4.2) 4/34 (1.4) IMP 8 (1.4) ND (1.4)	15 (28.2) 14 (4) 3 (2.1) 1/2/32/55 (1.8) ND (5)	SHV-28 (2.4) 1/2/5/55/12/ -106 (2.1) TEM-I (0.7) CMY-2 (1.4) DHA-1 (1.3)	R, A/C, L/M[e]	71/26/4	12
	14	2 (13.9) 3 (11)	181 (13.9) 48 (5.6) 232 (2.8)	1 (19.4) 5 (2.8) ND (2.8)	-	15 (4.7)	SHV-1 (2.8) -11 (2.5) -2/5 (1.4) ND (2.8) TEM-104 (1.4) OXA-1/9 (2.8) DHA: ND (0.8) CMY-2 (0.8) FOX-7 (5.6)	A/C, L/M[e]	36/22/4	7
147	147	2 (11.2) 3 (3.5)	48 (11.6) 181 (3.4) 204 (1.8)	1 (17.5) 5 (1.8) 7 (1.8) ND (1.8)	VIM-1 (5.3) 27 (2.8) 39 (2.3) 26 (1.2) 33 (1) ND (2.8) IMP-4 (1.9) 8 (1.9)	15 (12) 3 (1.9) ND (3.7)	SHV: 2 (1.9) ND (2.8) TEM-1 (1.9) DHA-1 (0.9) CMY-2 (0.9) LAP-2 (1.9)	FII, FIB, L/M[e], X	56/30/5	6
37	37	2 (28.6) 3 (3.6)	48 (10.7)	1 (10.7)	VIM-1 (3.6)	15 (10) 14 (2.8) 2 (1.8) 3 (1.8) 65 (1.8) ND (7.1)	SHV-12 (4.6) 11 (1.8) 2 (1.1) TEM-35 (1.1) DHA 1 (7.1) ND (1.8)	L/M[e]	28/15/4	6
101	101	2 (30.4) 3 (5.9)	48 (30) 181 (2.5) 204 (2)	1 (1.8)	VIM-1 (1)	15 (17.2) 14 (2.9) 3 (1.4)	SHV-12 (2) 11 (0.6) TEM-104 (0.8) DHA-1 (1.6)	L/M[e]	51/25/3	5
17	17	2 (17.3) ND (4.4)	181 (4.4)	1 (8.7)	VIM-1 (8.7)	15 (25) 14 (6.5) ND (4.4)	SHV-2 (4.4) 11 (3.9) 12 (2.2) ND (4.4) TEM-116 (1.3) DHA-1 (4.4)	FIIk, L/M[e]	23/11/3	5

[a]Determined by MLST scheme.

[b]The relative occurrence of each *bla* allele was determined by dividing the number of studies in which the indicated *bla* gene was reported in *K. pneumoniae* with the total number of studies retrieved from the search in PubMed for a specific ST (updated to October 2016). Each study was valued as one point. In the case of CPKP, only carbapenemases were taken into account. In presence of ESBLs or AmpC enzymes, penicillinases were neglected. When several *bla* genes were indicated in the same report, the one point was divided equally between all *bla* genes, i.e. if a strain carried bla_{OXA-48} and bla_{NDM-1}, each allele gained relative occurrence of 0.5.

[c]Plasmid types encoding *bla* genes only from reports described as outbreaks.

[d]Number of reports retrieved from PubMed using a search for the indicated *K. pneumoniae* ST. Number of countries where *K. pneumoniae* STXX was reported in (islands were considered as independent countries).

[e]L/M presently can be identified independently.

ND, not determined.

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via a single locus variation (Chen *et al.* 2014c; Bowers *et al.* 2015), harbors mostly bla_{KPC-3}. The majority of CG258 members are XDR due to their multiple ARGs. There are isolates of CG258 that show *ompK35* and *ompK36* porin alterations (Bowers *et al.* 2015) as well as mutated *mgrB* causing colistin resistance (Cannatelli *et al.* 2014). Comprehending the unique features of this epidemic CG and each of these epidemic STs will contribute to understanding their success, and will assist in designing improved diagnostic and prevention of their spread.

Linkage between KPC genes and successful clonal lineages

Whole genome sequencing (WGS) and molecular epidemiology data reveal that global dissemination of ST258 occurred after it had acquired bla_{KPC}-encoding plasmid. ST258 was reported with bla_{KPC} genes in more than >90% of all cases. Outbreaks associated with this clone were KPC carrying, mainly bla_{KPC-3} and bla_{KPC-2}. A tight connection was shown between this clone, bla_{KPC}/Tn*4401* and plasmids pKPN3 and pKpQIL (Table 2; Bowers *et al.* 2015). The remaining outbreaks are due to rare-VIM-producing isolates or non-producing carbapenemase derivatives, which are relatively rare. ST512 isolates are exclusively linked to bla_{KPC} (Table 2).

Interestingly, carbapenem-resistant bla_{KPC}-negative variants of ST258, described from Israel, lacking pKpQIL, were bla_{CTX-M} positive and *ompK35* deficient, and disappeared from the clinical setting two and half years since their recognition, reflecting their inferior fit to the hospital environment (Adler *et al.* 2012). It is speculated that acquisition of IncF plasmids significantly contributed to the evolutionary success not only of *K. pneumoniae* ST258, but also of *E. coli* ST131, a well-known pandemic *Enterobacteriaceae* clone (Mathers, Peirano and Pitout 2015). This suggests that *K. pneumoniae* STs linked to a specific IncF plasmid may possess an advantage over clones that may carry diverse plasmids.

Superiority of CG258

Antibiotic resistance is an important advantage among pathogens in clinical settings, but it is not unique for HiR clones. Several reports point out numerous superior features of CG258 that may be related to its worldwide success. Genomic comparison study revealed the presence of 50 unique genes present in ST258 clone and absent in a non-ST258 sporadic clone (ST376) found to be related to cell motility, secretion and DNA repair and modification (Chmelnitsky *et al.* 2013). Another study described the integrative conjugative element, ICEKp258.1, carried by ST258 and ST11, encoding a type IV secretion system, that possibly facilitates mobile elements propagation (Chen *et al.* 2014b). Another element, ICEKp258.2, found in ST258 and in its offspring ST512, encodes a type IV pilus gene cluster, and a type III restriction-modification system, which may improve adherence abilities and narrow range of compatible plasmids and other mobile elements, respectively (Pitout, Nordmann and Poirel 2015). This may explain the low diversity of *bla* carbapenemases in ST258 and ST512 compared to ST11 (Table 2). In addition to antibiotic resistance, *K. pneumoniae* ST258 showed reduced susceptibility to chlorhexidine, a disinfectant often used in hospitals, compared to other sporadic KPC-producing clones, suggesting its possible selective advantage in the hospital environment (Naparstek *et al.* 2012).

The capsule diversity was mentioned as another possible unique feature of CG258; Wyres *et al.* performed an *in silico* analysis based on 39 available WGS data (26-ST258, 9-ST11 and 4-other STs), identified 11 distinct capsule variants in this CG. *Klebsiella pneumoniae* ST11, the progenitor of CG258, was found to comprise seven *cps* locus types compared to one or two loci in other STs. It was suggested that this diverse capsule content may complicate the recognition of this CG by the immune system and explain the high occurrence of these STs (Wyres *et al.* 2015). Based on the evolutionary study that showed that two recombination events occurred in *cps* loci during the evolvement of ST258 (Chen *et al.* 2014c; Deleo *et al.* 2014), the *cps* region of *K. pneumoniae* was recognized as a 'hot spot' for genetic rearrangements and for chromosomal homologous recombination events. These may reflect the genetic flexibility of *K. pneumoniae* and possibly the route of emergence of HiR clones.

CG258 pitfalls

Contradictory data in the literature demonstrate weak physiological traits of CG258. A fitness study comparing ST512, a CG258 high-prevalent outbreak clone with ST321, a sporadic clone, showed, unexpectedly, the superiority of the later in growth competition experiments (Benenson *et al.* 2012). This *in vitro* study demonstrates the fitness enigma and presents the need of further understanding the relation between fitness of clones, epidemicity and host-related factors. Increased virulence of CG258 lineages is also far from being proved. ST258 isolates show variable virulence potential in several animal models (Pitout, Nordmann and Poirel 2015). In a study from Greece, ST258 isolates showed low virulence in a mouse model of septicemia, easily removable by the immune system *in vitro*, and shown to contain a non-virulent capsule type (Tzouvelekis *et al.* 2013). In another study, based on WGS of only three isolates, each belonging to a different ST, the numbers of virulence genes found in *K. pneumoniae* ST11 and ST512 were lower compared to those found in the high-risk clone ST101 (Oteo *et al.* 2016). With respect to biofilm formation, *K. pneumoniae* ST258 showed low biofilm production *in vitro* compared to non-ST258 sporadic clones (Naparstek *et al.* 2014), which was further supported by a transcriptome analysis (Bruchmann *et al.* 2015).

The success of non-CG258 clones

Understanding the successful dissemination of other (non-CG258) HiR clonal groups has yet to be studied. The importance of having a XDR phenotype together with the presence of certain MGEs was suggested. High-risk ST14, ST147, ST37 and ST101 tend to be armed with carbapenemases, whereas ST15 and ST17 are mostly ESBL-carrying clones; almost 40% of isolates belonging to each of these two high-risk clones are CTX-M producers (Table 2). A study from Taiwan on epidemic ESBL and AmpC-producing *K. pneumoniae* clones found that the high-risk clones ST11, ST15, ST147 and ST37 all share the same *ompK36* allele, group A, suggesting their convergent evolution, but the actual role of this porin allele in their success has not been further elucidated (Yan *et al.* 2015).

Contribution of plasmids to HiR clones success

The contribution of *K. pneumoniae* conjugable plasmids to the success of HiR clones was discussed in the context of co-resistance to multiple classes of antibiotics. The HiR ST258 emerged in early 2000s with the pKpQIL and pKpN3 plasmids, but these plasmids have been also identified in more recently isolated strains within CG258, suggesting that they are conserved and maintained stably within the clone (Leavitt *et al.* 2010; Chen *et al.* 2014a). pKpQIL contributed to the epidemiological success of the clone, conferring carbapenem resistance, but pKPN3 also could play a role in adaptation and fitness of CG258

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because it was conserved in the majority of the isolates. Along the years, pKPN3 evolved more than pKpQIL acquiring different determinants, including the carbapenem resistance and novel putative virulence genes. The discrimination in clades I and II of the CG258 corresponded to a discrimination of the plasmid content which resulted different in the two separated clades, thanks to the acquisition of an IncI2 by clade II (Chen *et al.* 2013a; Deleo et al., 2014). However, the virulence properties of these IncFIIk plasmids have not been formally demonstrated. A recent review by Lee *et al.* describes two examples of bacterial success related to plasmid carriage. The first is bla_{NDM}-encoding plasmids, especially IncA/C, which often harbor aminoglycoside and fluoroquinolone resistance genes that can contribute to their selection and spread among *K. pneumoniae*. A second example is IncL plasmids encoding bla_{OXA-48}, (Table 1); their high conjugation rate may lead to wide distribution with further selection under antibiotic pressure (Lee *et al.* 2016). Studies on the superiority of these HiR clones among other *K. pneumoniae* STs are lacking and should be further elucidated in order to eliminate or at least control their efficient spread.

Routes of global dissemination of HiR CRKP clones

XDR CRKP HiR clones have been spreading around us for nearly two decades, posing a great threat due to limited treatment options and increased mortality (Doi and Paterson 2015; Campos *et al.* 2016). All nine HiR clones show worldwide spread in three to five continents (Table 2). Spread of these clones is a complex process that includes local, national and international events. Although transmission routes may be enigmatic, the literature highlights numerous routes of transmission presented schematically in Fig. 4. Transmission modes may involve in-hospital patient-to-patient spread, acquisition following contacts with positive colonizers, as well as transmission via medical travel, and possibly through the food chain, companion animals and environmental sources (Fig. 4).

Medical-associated spread

Risk factors for acquisition of *K. pneumoniae* infection concern medical treatment and healthcare facilities, i.e. recent antibiotic use, catheter carriage and prolonged hospitalization (Boyle and Zembower 2015). Therefore, *K. pneumoniae* is recognized as a typical and even dominant nosocomial pathogen (Pitout, Nordmann and Poirel 2015). In hospitals, *K. pneumoniae* spreads from person to person. Interestingly, the ability to spread CRKP varies between different carriers: only few of them, probably those with higher rectal *K. pneumoniae* concentration, are significantly responsible for environmental contamination (Lerner *et al.* 2015). Colonization may last up to 2–4 years (Conlan *et al.* 2016). Carriers of *K. pneumoniae* do not always develop disease.

The ability of *K. pneumoniae* to survive on hospital equipment surfaces facilitates *K. pneumoniae* distribution. A ventilator-associated outbreak in China is a dramatic example of successful transmission of KPC-2 ST11 *K. pneumoniae* bypassing patient-to-patient contact (Hu *et al.* 2016). Dissemination of *K. pneumoniae* ST278 isolated from different patients in the same hospital unit in Calgary demonstrated rapid changes within resistance regions pointing out the high genetic plasticity of this bacterium (Lynch *et al.* 2016). Thus, the most sufficient measures during ongoing outbreak are active surveillance and strict *K. pneumoniae* carrier isolation, including spatial segregation and division of staff into one group caring for infected or colonized patients and the other working with non-carriers only (Schwaber and Carmeli 2014). In Italy, an outbreak in a neonatal intensive care unit was successfully prevented by an active surveillance program when all carriers were detected and treated with antibiotic combination before development of acute infection (Giuffrè *et al.* 2013). Early recognition of positive colonization by active surveillance was found as the most effective measure against KPC-producing *K. pneumoniae* (Campos *et al.* 2016).

Long-term care facilities (LTCFs) are hypothesized to be a connecting link in the spread of MDR *Enterobacteriaceae* between the hospital and the community settings (Endimiani *et al.* 2009). Additional reports supported the importance of LTCF as a reservoir for CRKP (Adler, Katz and Marchaim 2016). LTCFs can even be sources of reintroduction of bacteria into hospitals and implementation of prevention measures is much more complicated in these wards (Schwaber and Carmeli 2014).

Spread of CRKP in the community setting is still scarce. The first report described transfer of a hospital-acquired CRKP after discharge to a family member (Gottesman *et al.* 2008). Additional cases included complicated cystitis in an old woman with NDM-producing *K. pneumoniae* (Nordmann *et al.* 2012), and a case of urinary tract infection caused by KPC-producing *K. pneumoniae* ST258 in a young pregnant woman without recent health care exposures or travel history (Khatri *et al.* 2015). These few reports highlight the potential emergence of CRKP in community-associated infections and echoes the uncontrolled ESBL-producing *K. pneumoniae* situation in the community.

Animal reservoir of CRKP

Possible reservoirs for circulating CRKP clones are animals that can exchange pathogens with humans via the food chain, close contact or occupational transmission. High-risk human CR *K. pneumoniae* STs were reported in various companion animals in the community: for example, *K. pneumoniae* ST15 carrying bla_{OXA-48} in dogs from a veterinary clinic in Germany (Stolle *et al.* 2013), and different animals from Japan, France, Germany and other European countries infected and colonized with $bla_{CTX-M-15}$ carrying clones (Poirel *et al.* 2013; Ewers *et al.* 2014; Harada *et al.* 2016). *Klebsiella pneumoniae* ST11-producing DHA-1 was associated with morbidity of wild animals in Europe (Pilo *et al.* 2015; Loncaric *et al.* 2016). The role of animals as a reservoir for HiR CRKP clones and its impact on human health still remains unclear and should be further explored.

International spread

Global spread of HiR clones can occur due to international travel and tourism (Van der Bij and Pitout 2012). This may include medical travel (Rogers *et al.* 2011), during trips in which people are occasionally in contact with healthcare facilities, or visiting an endemic country. The first case of bla_{KPC-3} encoding ST258 outside the USA was reported in Israel (Navon-Venezia *et al.* 2009). Patients can be transferred from epidemic regions and spread a novel pathogen as it was shown for bla_{OXA-48}-carrying *K. pneumoniae* ST101 isolated from foreign patients in Israel (Adler *et al.* 2011); or a patient can be subjected to infection during hospitalization in an endemic country, as with VIM-27-producing *K. pneumoniae* ST147 which moved from Greece to Luxembourg (Bogaerts *et al.* 2011). Acquisition of various HiR clones bla_{NDM-1}- and $bla_{KPC-2/3}$-producing CRKP was reported in Canadian travelers who returned from different countries, and needed further hospitalization (Peirano *et al.* 2014). Active surveillance of returning patients hospitalized in endemic areas and infection control actions are recommended to eliminate further spread to avoid new acquisitions.

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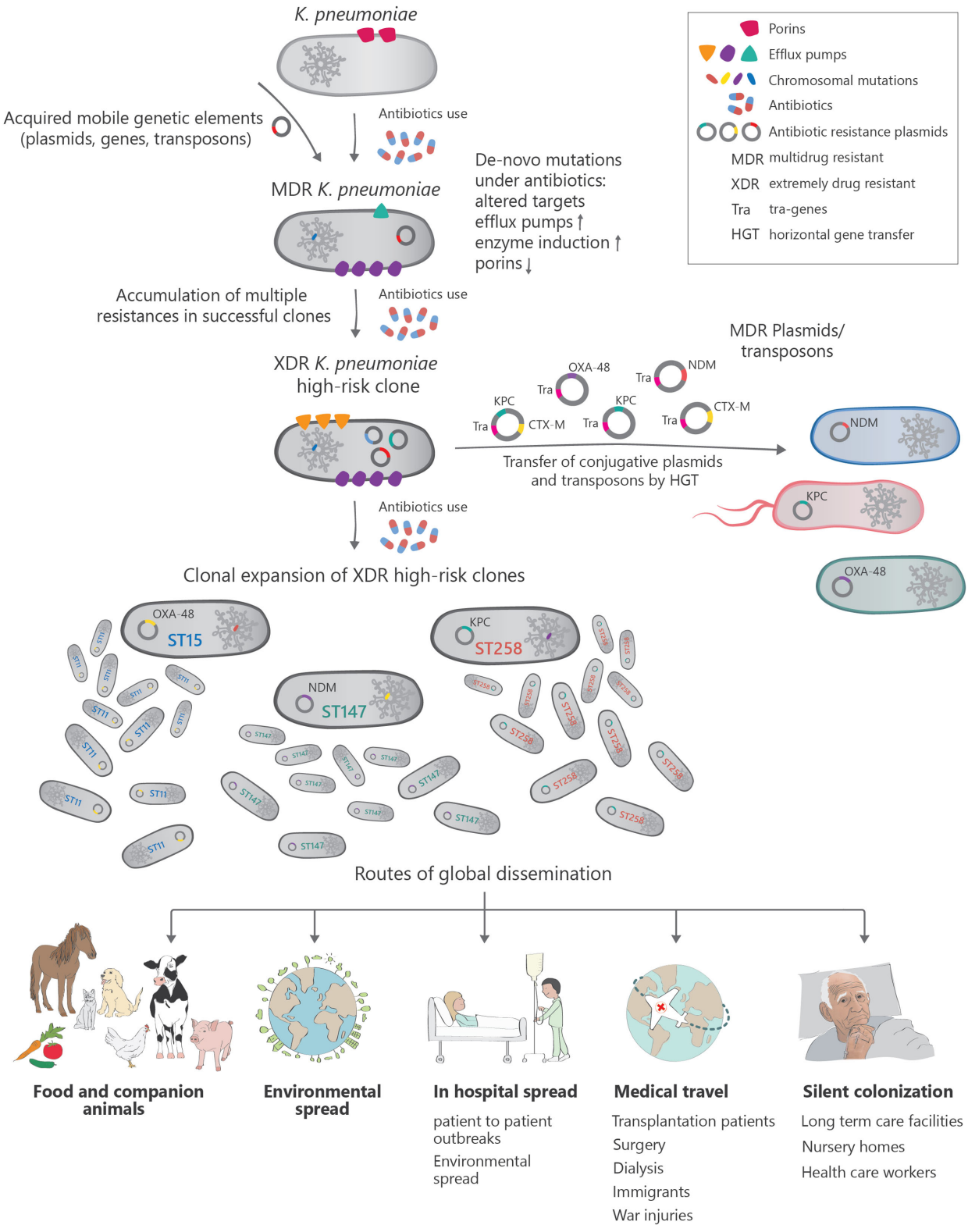


Figure 4. Hallmarks of emergence and spread of MDR and XDR *K. pneumoniae* and ARGs.

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Transfer of ESBL-producing HiR clones may be linked to the food chain and international trade relations like consumption of various vegetables in Switzerland carrying bla_{CTX-M}-encoding ST15 and ST37, and bla_{SHV}-encoding ST17 and ST147, acquired from endemic countries (Zurfluh *et al.* 2015). The global spread of KPC-carrying *K. pneumoniae* and NDM-1-producing *Enterobacteriaceae* was summarized by Molton *et al.* (2013), without indicating the STs involved.

Thus, fast dissemination of *K. pneumoniae* clones and resistance genes is a serious threat requiring immediate actions. Active surveillance must be introduced in all healthcare facilities; measures for arresting environmental contamination should be developed and become routinely used. Further studies are warranted for understanding the secret of success of high-risk clones and their transmission. This could be further elucidated using RNA-seq approach to identify differentially expressed genes with clinically relevant phenotypes for specific high-risk clones, and to understand the conditions that facilitate their spread. Identification of unique markers for specific high-risk clones will assist in effective screening of carriers, control transmission by rational cohorting and improve diagnostics.

CONCLUSIONS

Klebsiella pneumoniae plays a major role in the worldwide burden of antibiotic resistance. This burden is mainly hospital-associated, and involves HiR epidemic strains that possess a super resistome and cause infections with limited treatment options, leading to increased complications, higher mortality rates and costs.

Being a hospital-associated pathogen, *Klebsiella* is continuously exposed to multiple antibiotics resulting in constant selective pressure, which in turn leads to additional mutations that are positively selected. We showed that the resistome of *K. pneumoniae* encompasses a wide array of ARGs that continuously evolve and diversify (Fig. 2). Although various *K. pneumoniae* isolates may differ in their resistome repertoire, depending on geographic location, cultural population exchange, antibiotic stewardship and possible resistant reservoirs, this species shows a flexible ability to accumulate and switch resistances. Interestingly, ARGs that encompass the resistome can be located on the bacterial chromosome or on plasmids. Within a single genome, chromosomally encoded AR elements can move onto plasmids and vice versa. Plasmid-mediated resistome and transposons can also transfer and disseminate horizontally to other *K. pneumoniae* strains and occasionally to other bacterial species.

Plasmids are present in almost all *K. pneumoniae* strains, and the replicon content in this species very often diverges from other *Enterobacteriaceae* species replicons. Plasmid analysis highlights the impressive diversity of replicon types associated with the same carbapenemase or ESBL gene. Among them, broad host range, successful plasmids of the IncX3, IncA/C, IncN and IncL groups often associated with the bla_{NDM}, bla_{OXA-48} and bla_{CTX-M} genes. *Klebsiella pneumoniae* acquired these epidemic plasmids and then often acts as the harbinger of resistance determinants among other enterobacteria.

However, the most frequent plasmids in *K. pneumoniae* are the narrow host range IncFIIk plasmids, associated with the KPC carbapenemase. These plasmids and bla_{KPC} are highly specific for *Klebsiella* genomes and are characteristics of the *K. pneumoniae* species. Numerous open reading frames encoding proteins with unknown function have been annotated on the *K. pneumoniae* plasmids and are very often unique, not being present in other bacterial genomes, and likely deriving from HGT from undefined species. These gaps of knowledge in the attribution of functions to plasmids have left open the possibility that they contribute significantly to the success of this species not only as vehicles of resistance genes.

We described nine HiR worldwide MDR and XDR *K. pneumoniae* STs associated with multicontinent outbreaks. These clones possess a wide diversity of *bla* gene content, diverse resistomes and multiple plasmid types, supporting the complex epidemiology involved in multiresistant *Klebsiella*; this indicates an ability to acquire locally prevalent ARGs, and ARG-encoding plasmids, together with direct dissemination of a particular clone combined with a specific resistome, as well as a continuous influx of strains due to globalization and silent reservoirs. Taking into account these dynamic processes and the severe outcome related to infections caused by these clones, there is a need for an accurate molecular typing of both clones and genes. With the reduction in cost, and availability of MLST and WGS, we presume these methods will become more and more available for diagnosis of infectious diseases, both in patients and carriers. There are numerous examples in the literature showing that active surveillance to identify positive CREs colonizers is highly effective to control spread. We highlight the importance of molecular identification of specific HiR clones which may be crucial for applying the suitable infection control actions to avoid spread.

Figure 4 summarizes the hallmarks of the emergence and spread of MDR and XDR *Klebsiella* clones and mentions the routes of global dissemination of this pathogen. We propose that the key for the success of *K. pneumoniae* as a pioneer and spreader of antibiotic resistance is directly related to the fact that this pathogen is sited in, and highly fitted to hospitals. It is interesting to note that it is enteric counterpart, carbapenem-resistant *E. coli*, which has been known to acquire plasmid-mediated carbapenemases, has not become epidemic. The prevalence of carbapenem-resistant *E. coli* is still very low compared to CRKP (Fig. 1), even in countries in which it has been introduced, carbapenem-resistant *E. coli* clones are not spreading successfully. We can speculate that the epidemic traits of HiR *Klebsiella* clones involve plasmid–strain combination and need further understanding.

In conclusion, while past reports described that the type of CRE depends on the country, and might be associated with historical and or cultural relations and exchange of populations with high-prevalent countries (Cantón *et al.* 2012), it is now clear that globalization plays an important role in the dissemination of CRKP. Countries that were considered as endemic for only one type of carbapenemase such as KPC-producing *K. pneumoniae*/ST258 in Israel and VIM-producing *K. pneumoniae* in Greece, now deal with numerous carbapenemases and even multiple clones (Mathers, Peirano and Pitout 2015; Pitout, Nordmann and Poirel 2015).

The vast information and knowledge retrieved from whole genome DNA sequencing data of HiR clones and MGEs drives the need to deepen our understanding of functional resistance, including conditions that affect the expression of the resistome, as well as to define the mechanisms involved in these expression patterns. It is therefore crucial to understand the role of antibiotics on regulation of ARG expression and on their rate of dissemination. Future genomic and translational studies should be designed to decipher the secrets of success and adaptation of *Klebsiella* HiR clones.

Multiple efforts for tracking XDR *Klebsiella* using active surveillance for identifying gastrointestinal carriage in local (Forcina *et al.* 2016) and nation-wide levels have been undertaken, and are reported as highly successful in limiting the

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spread and decreasing the number of new acquisitions (Metan and Akova 2016). In spite of these efforts, resistance rates have increased both in *Klebsiella* and in other human-affecting pathogens, giving rise to concerns about the importance of additional reservoirs.

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