Chapter 4

Review - Understanding β-lactamase Producing *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae is a nosocomial pathogen commonly implicated in hospital outbreaks with a propensity for antimicrobial resistance towards mainstay β-lactam antibiotics and multiple other antibiotic classes. The successful proliferation, transmission and infection of the Gram-negative bacterium can be attributed to a myriad of factors including host factors, environmental factors, virulence factors and a large repertoire of antibiotic resistance mechanisms. The poor treatment outcomes and limited treatment options are consequences of the successful pathogenesis and spread of antibiotic resistance in the increasingly common β-lactamase producing *K. pneumoniae* bacterium. The review briefly explores the biology, successful pathogenesis and antibiotic resistance of *K. pneumoniae* as well as the detection and characterisation techniques of important strains.

Keywords: *Klebsiella pneumoniae*, β-lactamases, Antibiotic resistance

1. Introduction

The evolution of the Gram-negative bacillus in an era of antibiotic use has resulted in a changed epidemiology, wherein *K. pneumoniae* now commonly occurs in healthcare facilities, such as hospitals, and is responsible for a range of serious infections involving the urinary tract, lungs, abdominal cavity, intra-vascular devices, soft tissues surgical sites and causing bacteraemia [1]. Treatment of *K. pneumoniae* infections has been complicated by the rapid and easy acquisition of antimicrobial resistance along with the unmatched development of novel antimicrobials to combat them [1–5]. Resistance determinants in *Enterobacteriaceae* are typically encoded on the chromosome, plasmids, integrons and transposons [6]. *Klebsiella pneumoniae* is an *Enterobacteriaceae* member which often displays resistance towards β-lactam antibiotics,

particularly through β-lactamase expression of which the most important are cephalosporinases, such as extended-spectrum β-lactamases (ESBLs) and carbapenemases [6].

Several mechanisms contribute towards antimicrobial resistance and virulence in Gram-negative bacteria and may even work in concert to achieve multidrug resistance profiles [7–8]. Resistance determinants usually mediate resistance by inactivating the antimicrobial agent, modifying the antibiotic or its target and decreasing antimicrobial drug concentrations within the cell [9–11]. A common form of enzymatic inactivation of antibiotics is the acquisition and expression of β-lactamase genes within bacterial species, such as *K. pneumoniae*, which can be classified into Ambler classes A to D [12]. Extended-spectrum β-lactamases typically confer resistance towards penicillins, first-, second- and third-generation cephalosporins as well as aztreonam, but remain mostly inhibited by clavulanic acid, an inhibitor [13]. Extended-spectrum β-lactamase-producers can additionally express resistance towards other antibiotics, such as aminoglycosides and fluoroquinolones, and are typically treated with carbapenems [2,3,5,14]. The use of antibiotics, such as aminoglycosides, carbapenems, cephalosporins, fluoroquinolones as well as β-lactam/β-lactamase inhibitors, has been identified as one of the several risk factors associated with carbapenem-resistant *Enterobacteriaceae* (CRE) infection [15,16]. Treatment of CREs are often reliant on last resort antimicrobials, such as colistin, fosfomycin and/or tigecycline, which can be rendered ineffective due to antimicrobial resistance evolving or emerging [4,16–18]. The rise in carbapenemase-producers both locally and internationally poses a treatment dilemma as fewer efficacious antibiotics are available and all are threatened in light of the emergence of extensively drug-resistant (XDR) and pan-drug-resistant (PDR) Gram-negative bacteria [16]. Carbapenem resistance in *Enterobacteriaceae* has been detected worldwide at alarming frequencies, including in Africa, Asia, Europe, North America and South America [19–22]. The prevalence and geographical distribution of various *K. pneumoniae* strains differ, but a particularly important strain involved in national and international epidemics is the sequence type (ST) 258 harbouring the *K. pneumoniae* carbapenemase (KPC) [19,23–25].

Characterisation of clinically relevant *K. pneumoniae* isolates has elucidated strains implicated in both community-associated and healthcare-associated infections, of which the former has displayed a metastatic spread uncommon for enteric Gram-negative bacilli [1,15,26]. Clinical manifestations of infection and even geographical restriction of particular infections can be attributed to a myriad of factors, inclusively virulence factors and host-associated factors [26, 27]. The hypervirulent *K. pneumoniae* (hvKP) strains, variants of the "classical" *K. pneumoniae*, typically cause pyogenic liver abscesses, pneumonia, meningitis and endophthalmitis in otherwise healthy individuals [1]. "Classical" *K. pneumoniae* strains have typically exhibited a propensity for multidrug-resistance acquisition, whereas hvKP has remained largely susceptible with only a few reports of multidrug-resistant (MDR)-hvKP [1].

Once established in the hospital setting, the proliferation and spread of MDR strains can occur within and between hospitals [28]. The molecular characterisation of β-lactamases and the molecular typing of *K. pneumoniae* MDR isolates thus provide insight into current resistance profiles and possible routes of transmission. Whether by evolution of local clones through genetic determinant acquisition or introduction of successful international clones and their β-

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lactamases, the increase in multidrug-resistant *K. pneumoniae* isolates can be associated with poor treatment outcome [1,29,30]. The diversity and high number of resistance genes found in *K. pneumoniae* are indicative of an ever-growing resistance gene pool [31]. Future research should thus encompass deeper analysis of virulence factors implicated in the successful pathogenesis of *K. pneumoniae* working in concert with the existing β-lactamases, which attribute to its survival and proliferation within and outside its host. In addition, whole genome sequencing of important *K. pneumoniae* strains with multidrug resistance and the use of computational tools is an important next-step for elucidating gene characteristics, such as the virulence genes, through comparative genomics [32,33].

2. Epidemiology of multidrug-resistant *Klebsiella pneumoniae*

Klebsiella pneumoniae is a nosocomial pathogen commonly isolated from the intensive care unit (ICU) and implicated in hospital outbreaks, which is increasingly displaying high drug-resistant profiles through β-lactamase production, such as ESBL production and globally emerging carbapenem resistance [3,21,27,34]. The existence of β-lactamase enzymatic activity was first observed in 1940, which is prior to the implementation of penicillin for treatment [12]. The existence of the β-lactamases was therefore naturally present within environmental isolates [12,35,36].

Broad-spectrum β-lactamases initially emerged in *E. coli* during the 1960s and 1970s but rapidly spread to other bacterial species, including within the *Enterobacteriaceae* family, which led to treatment using second- and third-generation cephalosporins [11,37,38]. The first β-lactamase enzyme described in 1965 was the Temoneria (TEM)-1 enzyme and soon thereafter the sulphydryl variable (SHV)-1 β-lactamase, which can typically confer resistance to penicillins but not to cephalosporins [39]. Temoneria- and SHV-type β-lactamase derivatives described as ESBLs were soon thereafter detected and found to have activity against oxyimino-β-lactam antibiotics through minor active site modifications [14,39,40]. Resistance to oxyimino-β-lactam antibiotics was recorded briefly after (year 1982) the introduction of third-generation cephalosporins in *K. pneumoniae* and *Serratia marcescens* [41].

Hospital outbreaks of ESBL-producing bacteria, particularly *K. pneumoniae* and *E. coli*, are a threat that has existed for several years, since its first recorded outbreak in French hospitals in the 1980s [42–44]. Historically, the predominating β-lactamases encoded were of the TEM- or SHV-type, for example, in the United States of America, but a shift has occurred with the Cefotaximase-Munich (CTX-M)-type being the most commonly detected ESBL [44,45]. Worldwide distribution of ESBL-producing *Enterobacteriaceae*, especially *K. pneumoniae* and *E. coli* encoding CTX-M, has been recorded with an increase in prevalence over the years [46]. The predominating ESBL enzyme within clinical isolates mediating resistance can be geographically variable [44]. The lack of consistent studies or few studies reporting on the ESBL prevalence and genes detected in some African countries, particularly within Eastern and Western Africa, makes it difficult to determine trends in antimicrobial resistance patterns [22]. Non-ESBL-resistant phenotypes are also still present in clinical isolates and are attributed to

the production of broad-spectrum β-lactamases, such as TEM-1, TEM-2 and SHV-1 [47]. Alternately, high-level resistance can be attributed to inhibitor-resistant β-lactamases, which are TEM derivatives or due to cephalosporinase production [47]. Several other ESBL variants exist [48]. The only Ambler class D ESBLs are of the OXA-type enzymes of which OXA-1 has been frequently associated with other ESBL encoding genes and OXA-2 with PER-1 ESBLs [48–50]. The result of the former OXA-1 association with other ESBLs, particularly with $bla_{\text{CTX-M}}$ genes, could be β-lactam-β-lactamase inhibitor combination resistance [49]. Infections by ESBL-producing *K. pneumoniae* ranging from urinary tract infections to complicated sepsis are preferentially treated with the carbapenem antibiotic [3–14].

Carbapenem resistance in *Enterobacteriaceae* has been detected worldwide at alarming frequencies, including in Africa, Asia, Europe, North America and South America [19–22]. The prevalence and geographical distribution of various *K. pneumoniae* strains differ, but a particularly important strain involved in national and international epidemics is the sequence type (ST) 258 harbouring the *K. pneumoniae* carbapenemases (KPC) [19,23–25]. The most important carbapenemases belong to the Ambler Class A [*K. pneumoniae* carbapenemase (KPC)], Class B [metallo-β-lactamases (MBL), such as New Delhi metallo-β-lactamases (NDM-1)] and Class D [Oxacillinases, particularly OXA-48-type carbapenemases] [6,20,25, 51,52].

3. Classification of *K. pneumoniae* isolates

Klebsiella belongs to the Phylum *Proteobacteria*, the Class *Gammaproteobacteria* and the Order *Enterobacteriales*. The genus *Klebsiella* further belongs to the *Enterobacteriaceae* family and can be subdivided into a range of species, including *Klebsiella granulomatis*, *K. mobilis*, *K. ornithinolytica*, *K. oxytoca*, *K. planticola*, *K. pneumoniae*, *K. singaporensis*, *K. terrigena*, *K. trevisanii* and *K. variicola* [53–55]. The bacterium *K. pneumoniae* can be further subdivided into *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* (Table 2.1) [53,55,56]. *Klebsiella pneumoniae* is closely related to several other genera within the *Enterobacteriaceae* family, such as *Citrobacter*, *Escherichia*, *Enterobacter* and *Salmonella* [11,57].

A study conducted by Drancourt and collegues (2001) aimed at re-establishing and confirming the taxonomy of the genus *Klebsiella* determined the carbon assimilation patterns, 16S rDNA and β-subunit of RNA polymerase B (*rpo*B) sequences for eight *Klebsiella* species [54]. Seven of the *Klebsiella* species, namely *K. ornithinolytica*, *K. oxytoca*, *K. planticola*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. terrigena*, could be distinguished by the inability of the *K. pneumoniae* subspecies to grow at 10°C or utilise L-sorbose as the sole carbon source [54]. The 16S rDNA and *rpo*B sequence analyses furthermore indicated a 98.2% to 99.7% and 99.4% to 100% similarity, respectively, between the three *K. pneumoniae* subspecies and *K. granulomatis* [54]. Sequence analysis of the *rpo*B gene is confirmatory for *K. pneumoniae* but is typically used for characterisation utilising MLST [28, 58]. *Klebsiella pneumoniae* is the most relevant and common species isolated from clinical specimens [59].

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4. General characteristics of *K. pneumoniae* bacteria

Klebsiella species are ubiquitous and can occur within two broadly defined habitats, namely the environment and mucosal surfaces of mammals, including humans [59]. In the environment it can be found to exist in surface water, sewage, soil and even on plants whilst on their human host the saprophyte can be located in the nasopharynx and the intestinal tract [59]. The human skin is not conducive for the growth of *Klebsiella* species and so is merely considered to be transiently colonised [59].

Klebsiella pneumoniae presents typically as Gram-negative straight rods between 0.3 and 1.8 μm in size [60]. The non-motile bacteria are lactose-fermenting, facultative anaerobes that proliferate at 37°C and produce characteristically mucoid colonies on carbohydrate- rich media, attributed to the presence of a capsule [54,60]. Biochemical reactions can be utilised for the identification and differentiation of *Klebsiella* species [59].

4.1. Culture and metabolic characteristics

Klebsiella species are easily cultured on media suitable for *Enterobacteriaceae* bacteria, including: nutrient agar, tryptic casein soy agar, bromocresol purple lactose agar, Drigalski agar, MacConkey agar, eosin-methylene blue (EMB) agar and bromothymol blue (BTB) agar [61]. No additional growth factors are required by *K. pneumoniae,* which is capable of both fermentative and respiratory metabolism [54]. The facultative anaerobe can have a variable mucoid appearance, which may vary between different strains and be influenced by the composition of the medium used [54,61].

Useful tests in determining enterobacterial taxonomy include carbon source utilisation tests, glucose oxidation test in the presence or absence of pyrroloquinoline quinone, gluconate- and 2-ketogluconate dehydrogenase tests and tetathionate reductase and β-xylosidase tests [62]. All *Klebsiella* strains are capable of utilising L-arabinose, D-arabitol, D-cellobiose, citrate D-fructose, D-galactose, D-glucose, 2-ketogluconate, maltose, D-mannitol, D-melibiose, D-raffinose, D-trehalose and D-xylose, whilst lactose and D-sorbitol can be used as a carbon source by all strains, except *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *Ornithinolytica* [61]. A unique characteristic to both *K. pneumoniae* subsp. *pneumoniae* and *K. mobilis* is the ability to oxidise glucose to gluconate using glucose dehydrogenase in the absence of pyrroloquinoline quinone [61]. *Klebsiella pneumoniae* subsp. *pneumoniae* in addition possesses enzymes involved in the glycerol dissimilation pathway, namely glycerol dehydrogenase type I and 1,3-propanediol dehydrogenase, which permits fermentative growth on glycerol [32,61,63,64].

Klebsiella species are oxidase negative, catalase positive and often Voges-Proskauer test positive, with the exception of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *Rhinoscleromatis* [54,61]. Most strains can hydrolyse urea, reduce nitrates without the production of H_2S gas, as well as utilise glucose and citrate as carbon sources [54]. In the case of fermentation of glucose, a gas and an acid are produced [54]. Glucose fermentation also results in the formation of acetoin and 2,3-butanediol [61].

4.2. Genomic characteristics

Klebsiella pneumoniae isolates' genome size ranges from 5.1 to 5.6 Mb with extensive genetic variation being reported among intraspecific strains due to genomic rearrangements (often as a result of chromosomal inversions, plasmids and mobile genetic elements) as well as strain-specific genes [11,65]. The nine *K. pneumoniae* subsp. *pneumoniae* whole genomes currently available on public databases [NCBI GenBank Entrez Genome database (http://www.ncbi.nlm.nih.gov/genome)] include: *K. pneumoniae* strain MGH78578, NTUH-K2044, 342, HS11286, KCTC2242, CG43, JM45, KP13 and 1084 [32,65]. In a study conducted by Kumar and colleagues (2011) where two *K. pneumoniae* strains were sequenced and compared with previously sequenced strains, 3 631 common proteins were identified and considered to be the core set of orthologous genes [11]. A comparison with the information on known biological functions of 342 *K. pneumoniae* proteins revealed that 52.11% of the protein-encoding genes were dedicated to transport and binding proteins, energy metabolism, regulatory function and cell envelope, respectively [11]. A five-gene cluster involved in anaerobic sugar metabolism that was also identified in two of the strains, namely strain 1162281 and JH1, was found to be similar to Gram-positive genera homologs [11]. In a separate study by Ramos and colleagues (2014), the Kp13 chromosome was compared to strains MGH78578, NTUH-K2044 and 342 and it was found to harbour a similar G+C content (57.5%, 57.5%, 57.7% and 57.3%, respectively) [65]. The G+C content for Kp13 was, on the other hand, lower for the six plasmids, suggestive of DNA acquired through horizontal gene transfer (HGT) [65]. At least 32 *K. pneumoniae* plasmids have been sequenced, which range in size from 3 to 270 kb [66].

Microbial pathogens are capable of modifying inherent virulence or patterns of spread through evolutionary processes, which can often be mediated by HGT [1]. The acquisition of pathogenicity islands and virulence plasmids are mechanisms by which *K. pneumoniae* may laterally transfer genes [1]. Resistance genes could also be acquired by Gram-negative bacteria through recombination, integron-mediated mobilisation of gene cassettes or transposition [67]. An example of a lateral plasmid transfer mechanism is the acquisition of a large 180 to 220 kb virulence plasmid by hypervirulent *K. pneumoniae* (hvKP) strains that are not typical in "classical" *K. pneumoniae* strains and encode virulence factors, such as the RmpA (regulator of the mucoid phenotype) and iron acquisition factors [1].

Klebsiella pneumoniae has acquired multiple resistance genes over time [67]. The common statement that antibiotic use is solely to blame for increased antibiotic resistance over time is challenged by Projan (2007), who hypothesised that the ability of a bacterium to develop resistance could be a function of genome size because larger genomes have more genetic information to draw from [68]. In support of this school of thought, smaller genomes of some bacteria appear more specialised, such as *Treponema pallidum*, whereas those with larger genomes are more environmentally adaptable and versatile, such as *K. pneumoniae* and *Acinetobacter baumannii*, thus developing multidrug resistance more easily [68]. Resistance genes acquired, particularly ESBL genes, are widely disseminated even between species, such as strains of *Escherichia coli*, *Enterobacter aerogenes*, *Proteus mirabilis* and *Pseudomonas aeruginosa* [48].

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5. Virulence factors and the role in pathogenesis of *K. pneumoniae*

The significant impact of *K pneumoniae* in the clinical setting as a healthcare-associated pathogen has prompted investigation into the factors implicated in its pathogenesis [7]. The factors aiding in basic pathogenesis of *K. pneumoniae* are the fimbrial and non-fimbrial adhesins, a capsule, siderophores (particularly enterobactin), urease, lipopolysaccharide (LPS), serum resistance as well as biofilm formation [7,8,46,69,70,71]. On the other hand, enhancing factors aiding invasion include other siderophores (aerobactin and yersiniabactin), catechols receptor, mucoid factor and hypermucoviscosity [8,72].

The prerequisite to an infection is often the mucosal pathogen's ability to adhere [7,73]. *Klebsiella pneumoniae* expresses numerous fimbrial and non-fimbrial adhesins capable of recognising varied receptors, which in turn can facilitate the adherence to several target cells [7]. Fimbrial adhesins include mannose-sensitive type 1 fimbriae, type 3 fimbriae and plasmid-encoded fimbriae designated as KPF-28, while a non-fimbrial adhesin includes the CF29K factor [7,59,61,70,74,75]. The above mentioned type 1 and type 3 fimbriae are frequently detected in *K. pneumoniae* isolates, particularly mediating urinary tract infections (UTIs) and biofilm formation, respectively [8,65,75]. The expression of the various fimbriae can be both beneficial in that it may facilitate attachment or disadvantage the bacterium due to the heightened host immune response that may be triggered, thus outlying the opportunistic nature of *K. pneumoniae* [7].

Surface saccharides that have been associated with *K. pneumoniae* virulence in a human host include an LPS and capsule [69]. Capsules can play an important role outside the human host by offering some protection against desiccation in the environment or in the host by resisting complement-mediated lysis or phagocytosis and possibly having a neutralising effect against antibodies through the release of excessive capsular material [69,76,77]. At least 78 antigenically varied capsular types have been identified in *K. pneumoniae* [1,78–81] Resistance to phagocytosis was found to be higher in K1 and K2 capsular serotypes [1,82]. Particular types may also play a more significant role in virulence, such as the K2 capsule, which has frequently been isolated from clinical isolates implicated in urinary tract infections, pneumonia and bacteraemia [7,46,61,79]. The LPS is, on the other hand, a component situated in the outer membrane of bacteria and part of it forms the O-antigen of which there are only 12 differing antigens [59,61].

Finally, the growth of *K. pneumoniae in vivo* necessitates essential elemental iron for which it competes with the host by producing high-affinity extracellular ferric chelators (iron-binding molecules) [7,72]. A hypervirulent strain of *K. pneumoniae* was found to possess greater quantities of biologically active siderophores [1,72]. The genes encoding siderophores include *ent*B (enterobactin), *iut*A (aerobactin), *irp*1-*irp*2-*ybt*S-*fyu*A (yersiniabactin) and *iro*N (ferric-catecholates receptor) [72].

Virulence genes typically researched include *uge* (encoding uridine diphosphate galacturonate 4-epimerase), *wab*G (involved in the biosynthesis of the outer core lipopolysaccharide), *ure*A (related to the urease operon), *mag*A (mucoviscosity-associated gene A), *mrk*D (type 3 fimbriae adhesion), *all*S (activator of the allantoin regulon), *kfu*BC (iron-uptake system), *rpm*A (regulator of mucoid phenotype) and *fim*H (fimbrial gene encoding type 1 fimbrial adhesion) due to their

role in bacterial pathogenesis [32,80,83]. The virulence of *K. pneumoniae* is further exacerbated by the additional, easy acquisition of β-lactamase encoding genes; however, successful infection is ultimately also reliant on a number of host-dependent factors [8].

6. Clinical manifestations of *K. pneumoniae* infections

Klebsiella pneumoniae is both known as a commensal bacterium found in the environment and as an important healthcare-associated pathogen involved in a myriad of infections, ranging from blood, respiratory, urinary and intra-abdominal infections, in predominantly incapacitated patients [62,73,75,80,84]. Clinical presentations of disease caused by *K. pneumoniae* are affected by the quantity and type of virulence factors expressed, whereas the resulting infections can be divided into community-associated and healthcare-associated infections [26,75].

Klebsiella pneumoniae mostly affects patients in the ICU and is an important contributor to in-hospital mortality [3]. In the clinical setting, *K. pneumoniae* is second only to *E. coli* in causing catheter-associated urinary tract infections and is an important blood stream pathogen [27,75]. On the other hand, *K. pneumoniae* is also responsible for diseases, such as community-associated pneumonia, pyogenic liver abscess, rhinoscleroma, atrophic rhinitis and less frequently meningitis, necrotising fasciitis and prostatic abscess [75,80,85–87]. Rhinoscleroma and atrophic rhinitis are specifically caused by *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *Ozaenae*, respectively [80]. Community-associated infections, such as pneumonia and liver abscesses, meningitis or endophthalmitis have been identified in Taiwan and South Africa [26]. *Klebsiella pneumoniae* implicated in community-associated meningitis led to mortality rates ranging from 30% to 83% in adult cases with added severe neurologic sequelae in survivors in South Africa [72]. It was also noted that bacteraemic community-associated pneumonia mediated by *K. pneumoniae* had a poorer prognosis than *Streptococcus pneumoniae* mediated bactereamia [88].

Unlike their Gram-positive counterparts, invasive infections and metastatic spread are rare for extra-intestinal Gram-negative pathogens, such as *K. pneumoniae* [72,73]. Hypervirulent strains of *K. pneumoniae* have, on the other hand, been identified and associated with community-associated liver abscesses as well as spread to bone, eyes, joints, kidneys, lungs, muscle/fascia, pleura, prostate, spleen, soft-tissue, skin and the central nervous system (CNS) [72,73]. *Klebsiella pneumoniae* is largely thought of as an opportunistic pathogen, but the emergence of hypervirulent strains over the past decade have demonstrated the capacity to infect otherwise healthy individuals [72,73].

The virulence factors expressed could contribute to the range of clinical manifestations of infections, but the geographical restriction of certain manifestations could alternately be dependent on host factors typical to that region [26,27,89]. Host factors could include the frequency of diabetes mellitus, genetic predilections, underlying prevalent diseases, alcoholism, socioeconomic determinants and the availability of quality healthcare [26,27,89,90].

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7. Treatment of *K. pneumoniae* infections

Appropriate therapeutic options are often determined based on the antibacterial spectrum, convenience of use and tolerability of antimicrobials, such as third- and fourth-generation cephalosporins.[91] The factors influencing appropriate antimicrobial treatment are also dependent on local bacterial susceptibility patterns and patient risk profiles, which may ultimately determine the risk of infection with opportunistic and potentially antibiotic-resistant pathogens [92]. Multidrug-resistant bacterial strains, such as *K. pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, present a therapeutic conundrum due to their ability to undermine treatment, whilst also reducing appropriate antibiotic options available and causing a delay in appropriate treatment due to inefficient empirical treatment [29,93].

7.1. Treatment of multidrug-resistant *K. pneumoniae* infections

The global emergence of multidrug-resistant Gram-negative bacilli is an unprecedented problem, which is exacerbated by the focus on improving existing classes of drugs instead of developing new classes of drugs with alternate targets over the past 50 years [4,5]. The rise in the rate of multidrug-resistant bacteria and the increasingly limited treatment options is exemplified by ever-prevalent ESBL-producing *K. pneumoniae* for which carbapenems were the mainstay treatment but are increasingly rendered ineffective by the sporadic emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) [3,5,18].

Typical characteristics of ESBL-producing members of the *Enterobacteriaceae* family include resistance to amino- and carboxy-penicillins, second-generation cephalosporins and several third- and fourth-generation cephalosporins as well as monobactams (such as aztreonam) though some may remain susceptible to cephamycins [45,47,48,94,95]. Extended-spectrum β-lactamase producers additionally exhibit synergy between the former-mentioned antibiotics and clavulanate, a β-lactamase inhibitor, and may exhibit additional resistance towards other antibiotics, such as fluoroquinolones, aminoglycosides, trimethoprim and sulphamethoxazoles [2,11,14,47]. Treatment failure could be attributed to a drug's inability to reach therapeutic concentrations at the site of infection, particularly when the minimum inhibitory concentrations of the bacterium are close to the susceptibility breakpoint of drugs, such as ciprofloxacin sometimes used against temoneria (TEM)-52 ESBLs [91]. Empirical treatment should match information on pathogens distributed in the clinical setting and their respective susceptibility patterns so as to better ensure correct initial antimicrobial therapy [39]. Delayed appropriate treatment can increase the likelihood of death [39].

In a retrospective study conducted by Micek and colleagues (2010), a better outcome was believed to be associated with correct initial combination antimicrobial therapy when empirically treating Gram-negative bacteria-mediated sepsis as compared to monotherapy [92]. In the aforementioned study, a combination of a antipseudomonal fluoroquinolone, such as ciprofloxacin, or an aminoglycoside with a carbapenem (imipenem and meropenem), piperacillin-tazobactam or cefepime as initial treatment for severe Gram-negative bacterial infections offered a broader spectrum of activity [92]. Additional retrospective studies further favour combination therapy in CRE infections for which treatment options have been reduced

mainly to colistin, tigecycline, some aminoglycosides and fosfomycin [4,18]. Although fosfomycin appears active *in vitro*, there is little clinical experience with the drug as well as knowledge of adequate combinations for treatment without encouraging antimicrobial resistance [4,96,97]. Tigecycline, on the other hand, has demonstrated effectiveness against MDR *Enterobacteriaceae* and despite requiring dosage adjustments, due to low blood levels, has good clinical experience [2,4,98]. An unfortunate drawback to tigecycline could include the selection of Gram-negative bacteria with efflux pump mutations [2,5]. Colistin has been recommended for use only in cases of known colistin-sensitive MDR strains or nosocomial and ICU late sepsis shock where MDR strains are suspected [4]. The use of colistin for a prolonged period (>13 days) of time has been suggested as responsible for the emergence of colistin-resistant or pandrug-resistant bacterial strains in some instances [17,99,100]. The emergence of MBL and KPC strains of *K. pneumoniae* has rendered them resistant to all but one antibiotic, namely colistin [101,102].

8. Antibiotic-resistance mechanisms in *K. pneumoniae* isolates

Innate antimicrobial susceptibility could be impacted by adaptive responses, resulting in alterations to gene expression and cell physiology, which are induced in response to the pathogen's natural environmental stresses or within a host [10,103–105] Three modes of antibiotic resistance existing in bacteria, such as *K. pneumoniae*, include drug modification or enzymatic inactivation, antibiotic target modification or decreased concentrations of antimicrobial drugs within cells (possible by reduced permeability) and increased efflux activity [9–11,106,107]. These modes of action are encoded either intrinsically or acquired through mutation and resistance gene acquisition [10,106]. The adaptive responses are not only triggered by antibiotics but can occur as a response to environmental stresses and include: (i) cessation of growth, (ii) stress-induced acquisition of resistance determinants, (iii) changes to target sites, (iv) altered membrane barrier functions, (v) induction of resistance-conferring mutations and (vi) promotion of biofilm formation [10,11,103]. Ironically, some protective responses activated as a result of the stress caused by antimicrobial drugs can lead to resistance towards these very same antimicrobial drugs [103,105].

Changes in membrane permeability and drug flux can be influenced by variable expression and regulation of the efflux pumps [11]. Within the *Enterobacteriaceae* Gram-negative bacteria, a significant bacterial efflux pump family is the resistance nodulation division (RND) [6,65,106,108]. The active expression of the chromosomal native AcrAB-TolC efflux pump of the RND family contributes to fluoroquinolone resistance in *E. coli, Enterobacter* spp. and *Klebsiella* spp [6,108,109]. Alternately, alterations in outer membrane proteins of both *K. pneumoniae* and *E. coli*, either due to mutations or deletion of porins, may limit influx of antimicrobial agents or alternately increase efflux [107]. Besides the major OmpK35 and OmpK36 porins, the alternative OmpK37, PhoE and LamB porins may be expressed by *K. pneumoniae* [110]. The latter three porins' role in antimicrobial resistance has not been thoroughly investigated but is suspected to be important in the absence of OmpK35 and OmpK36 [110]. Modification or loss of the OmpK35 and OmpK36 porin proteins can affect resistance in various

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ways either leading to elevated minimum inhibitory concentrations (MICs) or resistance towards carbapenems and expanded-spectrum cephalosporins, reduced fluoroquinolone susceptibility, or it may occasionally confer additional cross-resistance to quinolones, aminoglycosides and co-trimoxazole within broad-spectrum β-lactamase- or ESBL-producers [36,48,110–113]. An additional modification to the outer membrane aiding in resistance, other than porin loss, is the upregulation of capsule polysaccharide (CPS) production in *K. pneumoniae* [6,114].

Bacterial cells can exist as single cells, the planktonic form, or within communities drawn together by a self-produced biopolymer matrix and attached to a surface [46,105,115,116]. The latter is referred to as a biofilm and confers survival advantages in the form of improved resistance to host immune defences, resistance to biocides, increased resistance to antimicrobial compounds and higher plasmid transfer rates within that environment, which could include antibiotic resistance genes [10,46,75,115,116]. Genetic elements conferring potential resistance genes are easily transferred horizontally both intra- and interspecies due to the close genetic resemblance between bacteria of the *Enterobacteriaceae* family [10,11]. The reduced antimicrobial drug effect against bacterial populations within a biofilm is largely unclear but could be as a result of several mechanisms acting in conjunction, such as: (i) poor compound diffusion, (ii) the slower growth and uptake of antibiotics by the bacteria in mature biofilm (>24 hours old), (iii) the production of antimicrobial inactivating enzymes, (iv) general stress responses, (v) the expression of efflux pumps and (vi) the presence of persister cells (Figure 2.2) [10,46,105,115–117]. Biofilm formation in *K. pneumoniae* is influenced by cell density-dependent quorum sensing signalling *via* the non-specific bacterial type-2 QS regulatory molecules, AI-2 autoinducers [118]. The mannose-resistant *Klebsiella*-like (MR/K) haemagglutinins or "Mrk proteins" are encoded by the genes *mrk*ABCDF within an operon and form part of type 3 fimbriae, which is important in mediating biofilm formation in *K. pneumoniae* [119]. Antimicrobial drug resistance can increase up to 1000-fold for bacterial cells existing within the biofilm [115,120].

Finally, resistance towards β-lactam antibiotics are mainly mediated by β-lactamase enzyme production, which is capable of hydrolysing third-generation cephalosporins and monobactams [48,58,107,121,122]. Other factors at play besides ESBL production include cases of ESBL hyperproduction due to promoter upregulation after direct mutation, inserted transposable elements in close proximity to the promoter and the capacity of a strain to coproduce more than one ESBL [48].

9. Classification of β-lactamases

Enzyme-mediated resistance to β-lactam antibiotics was initially discovered in *E. coli* but has since spread to a large number of bacterial species in the form of over 890 unique β-lactamases [12]. Both the chromosomal and the plasmid encoded β-lactamases can be classified into either Bush-Jacoby-Medeiros functional groups based on hydrolysis and inhibition characteristics or four Ambler molecular classes based on the proteins' amino acid sequences, as illustrated in Table 1 [12,123]. The former Bush-Jacoby-Medeiros classifies the β-lactamases into three

groups and 16 subgroups [6,12,123,124]. Some resistance genes exist through natural selection of resistant clonal lineages or have been acquired through mobile genetic elements, such as plasmids, transposons and insertion sequence elements (ISs) [23,24,125].

Functional group	Molecular class	Common name	Resistance to β-lactams
1	C	Cephalosporinase	Penicillins, cephalosporins, carbapenems*, monobactams*
2b	A	Penicillinase	Penicillins, early cephalosporins, β-lactamase inhibitor combinations*
2be	A	Extended-spectrum β-lactamase	Penicillins, cephalosporins, monobactams, β-lactamase inhibitor combinations
2d	D	Cloxacillinase	Penicillins (including oxacillin and cloxacillin)
2df	D	Carbapenemase	Carbapenems and other β-lactams
2f	A	Carbapenemase	All current β-lactams
3	B	Metallo-β-lactamase	All β-lactams, except monobactams

*β-lactams that are resistant as a function of high β-lactamase production in combination with efflux and porin modifications

Table 1. Major groups of β-lactamases in Gram-negative bacteria [12]

Ambler molecular classes A, C and D enzymes typically possess serine within the active site, while class B enzymes contain zinc [6,37,38,51]. Nine structural/evolutionary families have been described during the classification of ESBL variants [48]. The variants include Belgium extended-spectrum β-lactamase (BEL), Brazilian extended-spectrum β-lactamase (BES), CTX-M, Guyana extended-spectrum β-lactamase (GES), oxacillinase (OXA), *Pseudomonas* extended resistance (PER), *Serratia fonticola* (SFO), SHV, TEM, Tlahuicas (TLA) and Vietnam extended-spectrum β-lactamase (VEB) [6,14,48,126]. Other β-lactamases of importance are carbapenemases detected in *Enterobacteriaceae*, which typically include the OXA-48-type, KPC-type and MBL-type enzymes, Imipenem (IMP), Verona integron-encoded metallo-β-lactamases (VIM) and New Delhi metallo-β-lactamase (NDM) [20,37,127].

Three definitions of ESBLs have been proposed, which include a classical definition, a broadened definition and an all-inclusive definition [94]. The classical definition originally defined an ESBL as derivatives of broad-spectrum TEM and SHV enzymes and later more functionally defined as β-lactamases of the Ambler class A or functional group 2be capable of hydrolysing extended-spectrum cephalosporins and monobactams, while still being inhibited by β-lactamase inhibitors and poorly hydrolysing cephamycins and carbapenems [94]. The classical definition did not, on the other hand, account for the β-lactamases with similar hydrolysis profiles and dissimilar evolutionary backgrounds, such as CTX-M, GES and VEB enzymes [94]. A broader definition by Livermore (2008), included TEM and SHV variants with weaker ESBL activity, the enzymes with similar hydrolysis but dissimilar sources, as well as

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β-lactamases possessing wider resistance to the parent types that do not fall within the 2be functional group (e.g. OXA variants and AmpC type mutants). The wider resistance observed is to oxyimino-cephalosporins [94]. Lee and colleagues (2012) have independently extended the broadened definition of ESBLs to include AmpC ESBLs from the Ambler class C; thus designating ESBLs as: aESBLs, cESBLs and dESBLs [94]. The broadened definition is limited in that ESBLs with concurrent carbapenem and oxyimino-cephalosporin resistance are excluded [94].

Finally, the all-inclusive definition classifies ESBLs into three classes: $ESBL_A$ (class A ESBLs), $ESBLS_M$ (miscellaneous ESBLs including as AmpC and OXA-type ESBLs) and $ESBL_{CARBA}$ (β-lactamases encompassing ESBLs with carbapenem hydrolysing activity) [94,128]. The GES-1 β-lactamase, for example, has hydrolysis profiles resembling that of other ESBLs, but six GES β-lactamases have illustrated carbapenemase activity, being GES-2, -4, -5, -6, -11 and-14 [129]. Bush and colleagues (2009), on the other hand, felt the term $ESBL_{CARBA}$ as clinically confusing as ESBLs should be treatable with carbapenems and should thus remain more accurately classified as carbapenemases [130]. Bush and colleagues (2009) further disputes the definitions set by Giske (2009) by stating that AmpC-producers although treatable with carbapenems may develop resistance easily and should thus not be classified together with ESBLs [128,130]. The all-inclusive definition thus further excludes the clinical criteria in which ESBLs should have sensitivity to available β-lactamase inhibitors and current definitions of ESBLs, AmpC β-lactamases and carbapenemases should be kept independent [130]. The most common ESBL-encoding genes detected include SHV-, TEM- and CTX-M-type enzymes [6].

10. Risk factors for ESBL-producing *K. pneumoniae* infections

The clinical outcomes of inadequate empirical treatment with broad-spectrum antibiotics with no activity against the isolated causative bacterium (*in vitro*) or a bacterium with additional antibiotic resistance can lead to: (i) treatment failure, (ii) adverse patient outcomes, (iii) perpetuation of the increase in antimicrobial resistance and (iv) a financial burden to society [92,131]. The colonisation pattern in a patient after admission into hospital is largely influenced by the local antibiotic policy with increases in colonisation observed after 2 weeks, especially after treatment with broad-spectrum antibiotics, which lead to higher attack rates by nosocomial *K. pneumoniae* [59].

Generalised factors in at-risk patients commonly include severe illness, underlying medical conditions, recent surgery, haemodialysis, multiple or excessive antibiotic use, the use of medical devices, such as lines and tubes, prolonged hospitalisation, ICU admittance, admittance at long-term health facilities or nursing homes and international travel to endemic areas [132]. An important risk factor in modern society is the risk of acquiring ESBL-producing *K. pneumoniae* or *E. coli* when travelling to high-risk countries, particularly when travel is directed to endemic areas, such as to Asian countries or Greece, Turkey and the United States of America (USA), which have ESBLs and carbapenemases (such as KPC, VIM, OXA-48 and NDM) [45]. The risk of infection with ESBL-producing *E. coli* and *K. pneumoniae* is particularly higher if antibiotics were consumed during travel, often for traveller's diarrhoea [45,133].

The clinical manifestation of disease can be attributed to numerous host-dependent factors, which may range geographically but it is also influenced by socioeconomic determinants and the quality of healthcare at hand [26,27,89,90]. Underlying complications or illness that may result in an increased risk of *K. pneumoniae* infection include malignancy, cirrhosis, biliary tract disorders, diabetes mellitus and alcoholism [134].

11. Spread, prevention and control

The rise in antimicrobial-resistance among bacteria, such as those described as 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp.), has highlighted the need for new antibiotics due to the 'escape' from currently marketed antimicrobial drugs [135]. The impact of infections with β-lactamase producing bacteria include increased mortality rates, particularly in blood stream infections (BSI), as well as increase in length of hospitalisation and hospital costs [2]. Principle reservoirs typically implicated in healthcare-associated outbreaks or spread includes the patients, the healthcare staff and the environment (such as sink drains) [21].

Factors impacting the spread and control of MDR bacteria include spread of plasmids and are impacted by the food chain or international travel [136]. During the travels, acquisition can occur in the absence of healthcare contact or along with leisure and medical tourism [45,137]. In healthcare settings, overcrowding is a key factor in exacerbating the faecal–oral route of transmission by either direct or indirect contact by healthcare workers [132]. The contact that staff have with patients during unassuming social interactions, such as taking a patient's blood pressure and the touching of inanimate objects in the patient's environment, could contribute to horizontal spread of pathogens, especially when elective hand hygiene practices are neglected [4,138]. The implementation of alcohol-based hand rubs and regular educational programmes are thus important steps in control measures undertaken [138]. The role of post-acute care facilities in dissemination of MDR bacteria is also stressed by Perez and colleagues (2010) [139].

Infection control measures undertaken can include: (i) increased barrier precautions, (ii) isolation of infected patients, (iii) appropriate antibiotic treatment duration and (iv) epidemiological standards for the handling of equipment as well as patient wounds [4,14,59]. A method investigated for its potential to reduce cross-contamination and infection rates in clinical settings, such as the ICU, is the effect of selective digestive tract decontamination (SDD) for the elimination of cephalosporin-resistant *Enterobacteriaceae* [140–143].

Several key shortcomings have, however, been identified by the World Health Organization (WHO) in the combat against antimicrobial resistance [144]. The issues are discussed under four topics which include: (i) lack of commitment and data, (ii) unconfirmed drug quality and irrational use, (iii) poor prevention and control of infections and (iv) languishing research into new antimicrobial agents and tools, including diagnostic tests and antimicrobials [144]. The resulting policy package recommended by the WHO thus firstly suggests that governments adopt and finance comprehensive national plans with accountability and engaging civil society

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by creating public awareness [144]. The second recommendation is based on improving surveillance and laboratory capacities, whilst the third advises local governments to guarantee an uninterrupted supply of essential, quality-assured medication [144]. The regulation and promotion of the correct use of former-mentioned medication is also emphasised along with good patient care [144]. Finally, the last two recommendations involve improvement of infection prevention and control while encouraging research and development of new tools, including diagnostic tests and antimicrobials [144].

12. Laboratory diagnosis of β-lactamase producing *K. pneumoniae* isolates

In light of increasing antibiotic resistance among bacteria, surveillance of drug-resistance patterns within clinical settings and clinically relevant pathogens is significant particularly when deciding on appropriate treatment for complicated infections [27]. The detection of ESBL-producing bacteria requires tests that can accurately discern between ESBL producers and bacteria possessing alternative resistance mechanisms, such as inhibitor-resistant-β-lactamases, cephalosporinase overproduction and SHV-1 hyperproduction [47].

12.1. Biochemical and phenotypic detection techniques

Characteristics associated with ESBL-producing *Enterobacteriaceae* include the synergy observable between the antibiotics amino- and carboxy-penicillins, second-generation cephalosporins and up to several third- and fourth-generation cephalosporins when combined with β-lactamase inhibitors, such as clavulanate [14,47]. *Klebsiella pneumoniae* can encode all three ESBL-encoding genes whilst simultaneously encoding carbapenemases [145]. The characteristics associated with KPC and MBL carbapenemase production differ to ESBLs in that the KPC enzyme is capable of hydrolysing all β-lactams, whereas the MBL enzymes are capable of hydrolysing all β-lactams but not aztreonam [127]. The former KPC β-lactamase is partially inhibited by inhibitors, such as boronic acid, clavulanic acid and tazobactam, whereas the latter MBL enzymes are inhibited by ethylene diamine tetra-acetate (EDTA) [127,146]. Detection of these MDR *K. pneumoniae* can be manually screened for utilising several techniques, which include culturing on chromogenic agar (such as ChromID® ESBL agar medium) (bioMérieux, France), Etest MBL (AB BioDisk Company, USA), MicroScan panels (MicroScan, USA), modified Hodge test, disk diffusion techniques on Mueller-Hinton agar and enriched medium (such as tryptic-soy broth containing 2 $mg.L^{-1}$ cefpodoxime) [14,19,21,27,58,84]. Phenotypic techniques are often reliant on observable results, such as with the double disk synergy test (DDST), ESBL Etests (bioMérieux, France) and the combination disk method [47].

Initially, the DDST following methodology specified by the Clinical and Laboratory Standards Institute (CLSI) guidelines was intended for the differentiation between ESBL-producing *Enterobacteriaceae* strains and strains overproducing cephalosporinase, but the combination of cefotaxime or ceftazidime with clavulanic acid can also be predictive of a CTX-M-producer, particularly in *E. coli* [44,47]. The test makes use of a 30 μg disk of, e.g. cefotaxime and a disk of amoxicillin-clavulanate (10 μg clavulanate) approximately 30 mm apart, or at 20 mm for greater sensitivity [14,47]. The resistance breakpoints towards all third- and fourth-generation

cephalosporins are not always apparent, regardless of whether disk diffusions in agar or automated systems are used [47]. False-negative results can occur when testing isolates encoding SHV-2, SHV-3 or TEM-12.[14] Alternatively, the ESBL Etests are capable of quantifying synergy with one end of the strip containing gradients of cefotaxime, or ceftazidime, or cefepime and the other end a combination of the same former-mentioned antibiotic with 4 $mg.L^{-1}$ clavulanate [47]. A limitation may include failure to detect ESBLs when ranges fall outside MIC ranges on the strip or misinterpretation of the inhibition ellipse [47,147,148]. Another phenotypic testing method that can be utilised is based on broth microdilution assays, which includes the commercially available MicroScan panels (Dade Behring MicroScan, Sacramento, USA) that make use of dehydrated panels for microdilution antibiotic susceptibility [14].

Cloxacillin has been added to agar media for the inactivation of cephalosporinases, an AmpC β-lactamase, whereas both clavulanate and EDTA have been added when MBLs are produced concurrently with ESBLs for the latter's identification and confirmation [47]. The detection of extended spectrum Ambler class D OXAs is, on the other hand, complicated due to weak inhibition and no inhibition observed towards clavulanate and EDTA, respectively [47,149]. A unique characteristic attributed to most class D β-lactamases, including OXA-48-type enzymes, is the inhibition of activity by sodium chloride (NaCl) *in vitro* at a concentration of 100 mM [49].

Carbapenemases can, on the other hand, also be screened for in at-risk patients using selective media, such as CHROMagar KPC medium (CHROMagar Ltd, France), Brilliance™ CRE medium (Thermo scientific, UK) and SUPERCARBA medium [127]. Typically, methods of detecting carbapenemases make use of inhibition tests utilising boronic acid, clavulanic acid, EDTA and tazobactam [112,127]. Carbapenemase resistance in *Enterobacteriaceae* can be confirmed phenotypically using the modified Hodge test (MHT) according to CLSI guidelines, although several limitations have been recorded [112]. Limitations include variable sensitivity and specificity recorded in the detection of carbapenemases other than KPC (>90% respectively) and the occurrence of false positive MHTs in the absence of carbapenemase production due to reduced susceptibility or resistance to carbapenems [112,150,151]. The latter limitation could be as a result of isolates expressing alternative mechanisms of carbapenem resistance, such as ESBL production coupled with loss of porin proteins [112,151]. The MHT test demonstrated good sensitivity in the detection of OXA-48-producers [152]. Inhibition-based carbapenemase detection is limited due to variable specificity and sensitivity [127].

12.2. Automated detection of ESBLs

Automated systems used for the detection of ESBLs are the VITEK®2 ESBL test (bioMérieux, France) and the Phoenix ESBL test (Becton Dickinson, USA), both of which monitor the bacterial growth response to expanded-spectrum cephalosporins [14,47]. The VITEK®2 ESBL test (bioMérieux, France) consists of cards with wells, whereas the automated Phoenix ESBL test (Becton Dickinson Biosciences, USA) consists of five wells containing a cephalosporin with or without clavulanic acid [47]. Another method that could be used for the detection of β-lactamase and carbapenemase activity is the matrix-assisted laser desorption ionization-time

of flight (MALDI-TOF) mass spectrometry (MS), which analyse carbapenem molecule hydrolysis, although its efficiency in detecting OXA-48 producers remains uncertain [127,152–154].

12.3. Newer detection methods

Molecular investigations of outbreaks can be complicated when spurred by the spread of highly mobile plasmids [21]. Antimicrobial resistance genes are often carried on varied plasmids, which have been implicated in MDR Gram-negative bacteria outbreaks, as illustrated in a study by Tofteland and colleagues (2013), wherein the bla_{KPC} encoding plasmid was transmitted among varied strains and even species [21]. Non-phenotypic tests, including molecular techniques, that are available for antibiotic gene detection and typing include: polymerase chain reaction (PCR) assays, real-time PCR assays, next-generation sequencing (NGS) methodologies, microarrays, MALDI-TOF MS and PCR/electrospray ionization mass spectrometry (PCR/ESI MS) [127,152,155–158]. Molecular techniques, particularly PCR are the standard for detecting genes encoding ESBL, OXA-48-Like, VIM, KPC and NDM enzymes [50,152,159]. The detection of carbapenemases also includes the novel biochemical Carba NP test and a UV spectrophotometer-based technique [127,160,161]. The UV spectrophotometer-based method relies on the analysis of imipenem hydrolysis by extracted proteins from the isolate tested and demonstrates less variability in sensitivity (100%) and specificity (98.5%) as compared to inhibition-based methods [127,160,161]. Imipenem has also been used to detect carbapenemases using novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays [162].

13. Typing of *K. pneumoniae* isolates

Genetic typing of *K. pneumoniae* isolates is important for outbreak investigations, investigating sources or reservoirs, understanding transmission, managing hospital infections and for epidemiological referencing [163–165]. Several typing methods exist for the characterisation of *K. pneumoniae* isolates, which can be subdivided into phenotypic and molecular methods, but the appropriate method used is dependent on the question that needs answering[28,29]. Originally, typing methods for *K. pneumoniae* included phenotypic typing methods, such as biotyping, serotyping, phage typing and bacteriocin typing [28,57,59,163,166,167]. The most popular serotyping method implemented in the past that gave the most reproducible results was capsule typing [59]. The technique was not, however, without its shortcomings as considerable serological cross-reactions could occur between the 77 capsule types [59]. Methods developed since then include molecular typing methods, such as amplified fragment length polymorphisms (AFLP), MALDI-TOF MS, MLST, multilocus variable-number tandem-repeat analysis (MLVA), NGS, pulsed field gel electrophoresis (PFGE), plasmid profiling, sodium dodecyl sulphate polyacryamide gel electrophoresis (SDS-PAGE), restriction fragment length polymorphism (RFLP), ribotyping and random PCR methods [28,29,57,62,163,165–168]. Random PCR methods include random amplified polymorphic DNA (RAPD) and repeat-based PCR (rep-PCR) [62].

The PFGE molecular method is highly discriminatory and is the gold standard typing method in the characterisation of *K. pneumoniae* isolates [163,164,169]. Pulsed-field gel electrophoresis discrimination is based on genomic DNA restriction utilising a rare-cutting restriction enzyme, such as *Xba*I for *K. pneumoniae* [29,170]. The disadvantage of PFGE lies in that intra-laboratory reproducibility of results requires substantial managing and it is technically demanding [164]. Multilocus sequence typing, on the other hand, is a useful technique utilised for determining the clonal relatedness between *K. pneumoniae* isolates and provides unambiguous, portable data [28,163,167]. The MLST and MLVA methods are both described numerically and much like MLST, the MLVA data are portable [29]. In MLST the internal segments of seven house-keeping genes in *K. pneumoniae* are amplified and the variations in each sequence described as unique alleles, which comprise the allelic profile of the isolate, otherwise known as a sequence type (ST) [29,167]. The disadvantage lies in that the discrimination may not be defining enough for outbreak analysis but it is useful to compare to global epidemiology [28, 29]. The MLVA, on the other hand, determines the number of repeat units at multiple loci and can be modified to the desired resolution depending on the loci chosen, thus allowing for a higher resolution than PFGE [29].

14. Commonly characterised *K. pneumoniae* strains

Sequence typing has allowed for the characterisation of *K. pneumoniae* strains and led to the recognition of widespread MDR clones [1,29]. Although a vast number of sequence types have been recorded globally, which can be accessed on public databases (such as www.pasteur.fr/mlst and http://pubmlst.org), a few important STs are frequently reported and discussed. Typing has elucidated widespread multidrug-resistant clones, such as *K. pneumoniae* ST 258, which can often produce KPC carbapenemases and the virulent *K. pneumoniae* clonal complex (CC) 23 (including ST 23 and ST 57) [21,23,29,30,171,172]. Besides the "classical" *K. pneumoniae* STs, a few STs associated with hvKP strains include ST 23 and ST 57, which are associated with the K1 capsular serotype, as well as the ST 86, ST 375 and ST 380, which are associated with the K2 capsular serotype [1,172]. It has been suggested that particular clones acquire resistance genes easily and may have evolutionarily changed similar genes acquired so as to maintain or improve bacterial fitness [173].

15. Conclusion

Enterobacteriaceae in the clinical setting have adapted to a harsh environment created by the use of antibiotics through several mechanisms, which include the expression of β-lactamases capable of hydrolysing penicillins as well as other β-lactam antimicrobials [6,12,54]. The β-lactamases commonly implicated in a range of serious infections by *K. pneumoniae* include cephalosporinases (particularly ESBLs) and carbapenemases [6]. Extended-spectrum-producing *K. pneumoniae* forms part of the ESBL-producing *Enterobacteriaceae*, which is collectively listed as one of six dangerous pathogens by the Infectious Disease Society of America together

with *A. baumannii*, *P. aeruginosa*, vancomycin-resistant *E. faecium*, methicillin–resistant *S. aureus* and *Aspergillus* species. Other mechanisms of resistance and co-expression of several β-lactamases could work in concert to further extend the range of antimicrobial resistance by *K. pneumoniae*, often spurred on by excessive antimicrobial use in the clinical setting [10,11,103]. The consequences of the broadening resistance among Gram-negative bacilli, particularly towards the commonly implemented carbapenem antimicrobials, are often increased mortality rates and hospital costs, thus giving importance to tests with the capacity to discern between ESBLs, carbapenemases and other mechanisms of resistance being expressed [3,47,92]. The typing of bacterial isolates is also a paramount step in determining infection sources and possible dissemination routes [163,164,165].

Antibiotic resistance is often discussed in terms of selection and subsequent proliferation of MDR strains or the horizontal transfer of genetic elements encoding resistance, such as plasmids [30]. A combination of proteomics and molecular techniques could thus be used for the characterisation of plasmids within outbreak *K. pneumoniae* isolates [174]. Comparative studies of MDR bacterial proteomic information under specific *in vitro* conditions can also be used for the identification of proteins associated with antibiotic resistance [175]. Proteomic techniques could additionally be used for the investigation of possible immunogenic K. pneumoniae antigens, such as FepA (ferrienterobactin outer membrane receptor), OmpA (outer membrane protein A), OmpK36 (outer membrane porin) and the Colicin I receptor, for vaccine development [176,177]. Improving the understanding of the progression of drug resistance and mechanisms involved could aid attempts to improve the efficacy of current antimicrobials, an alternative solution in light of the lack of new drugs under development in recent years [3–5,175].

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