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Klebsiella pneumoniae: Morphological characteristics, Growth, cultural characters, Antigenic Structures, Virulence Factors and Vaccines Strategies

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Abstract

Members of the genus Klebsiella have rapidly evolved within the past decade, generating organisms that simultaneously exhibit both multidrug resistance and hypervirulence (MDR-hv) phenotypes; such organisms are associated with severe hospital- and community-acquired infections. Carbapenem-resistant infections with unknown optimal treatment regime were of particular concern among the MDR-hv Klebsiella strains. Recent studies have revealed the molecular features and the mobile resistance elements they harbour, allowing identification of genetic loci responsible for transmission, stable inheritance, and expression of mobile resistance or virulence-encoding elements that confer the new phenotypic characteristics of MDR-hv Klebsiella spp.

Keywords: Klebsiella, MDR, virulence.

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1. Introduction

The Klebsiella genus comprises a wide diversity of species, including the *Klebsiella pneumoniae* species complex (KpSC) and several more genetically distant species [1]. Strains of this genus were first isolated in the late 19th century and named by Trevisan (1885) to honor the German microbiologist Edwin Klebs [2]. Klebsiella is classified under the Enterobacteriaceae family which contained a large array of biochemically distinct genus, including the model organism Escherichia coli and the notorious human pathogens Salmonella, Yersinia, Serratia, Enterobacter, Citrobacter, Kluyvera, Leclercia, Raoultella, Cronobacter, etc [1]. The Klebsiella genus currently comprises a wide diversity of species, including species belonging to the *K. pneumoniae* species complex (KpSC) and other Klebsiella species (K. indica, K. terrigena, K. spallanzanii, K. huaxiensis, K. oxytoca, K. grimontii, K. pasteurii and K. michiganensis) that share an average of only 90% nucleotide identity with KpSC [1]. Seven phylogroups that belong to KpSC have been classified, including K.pneumoniae (Kp1), K.quasipneumoniae subspecies quasipneumoniae (Kp2), K.variicola subsp. variicola (Kp3), K.quasipneum oniae subsp. similipneumoniae (Kp4), K.variicola subsp. tropica (Kp5), K. quasivariicola (Kp6) and K. africana (Kp7) [3]. Two subspecies of *K. pneumoniae*, namely *K. pneumoniae* subsp. ozaenae and *K. pneumoniae* subsp. rhinoscleromatis, which are associated with a specific disease syndrome (atrophic rhinitis and rhinoscleroma, respectively), have been reported [4].

2. Morphological characteristics:

Klebsiella spp. is non-motile, straight rod shape, 0.3-1µm in diameter and 0.6-6µm in length, when these bacteria are stained with a Gram stain, they give pink color (negative bacteria) with a prominent polysaccharide capsule. This capsule encases the entire cell surface and provides resistance against many host defense mechanisms [5].

3. Habitat:

Klebsiella pneumoniae has a strong association with human as resident flora and colonises almost every part of the human body with most preferential in the respiratory, gastrointestinal and urinary tract. The other predominant habitats include soil, plants, surface water, sewage and industrial effluent [6]. Despite the source of origin, clinical isolates cause nosocomial and community-acquired infections. This leads to the serious therapeutic threats given the increase in drug resistant phenotypes and decrease in effective antibiotics [7].

4. Growth and cultural characters:

Based on the presence or absence of capsular (K) somatic(O) and slime(M) antigens ,the Klebsiella strains have beem divided into 4 smooth and 4 rough forms

Smooth forms	Rough forms
1. MKO	MKR mucoid capsulated
2. KO	KR non mucoid capsulated
3.	MO MR mucoid non capsulated 4. OR non mucoid non capsulated [8].

When kept at room temperature, cultures remain viable for weeks or months. They are facultative anaerobic bacteria. There is no haemolysis of horse or sheep red cells. Lactose fermenting large moist glistening colonies due to polysaccharide capsule (K-antigen) in MacConkey agar. The optimum temperature for growth is 37º the limits are 12º and 43º [9]. When much capsular material is produced, the growth on agar is luxuriant, greyish white, mucoid and almost diffluent .This is due to high proportion of water(92%)in the capsular material [10]. Moist heat at 55º kill The organisms in 30 min. They are survive drying for months [11].

5. Biochemical activities:

The key biochemical tests used to differentiate Klebsiella species are listed in table (1).

6. Antigenic structures and virulence factors (Fig.2):

All K.pneumoniae isolates carry a set of core genes responsible for their pathogenicity and required to establish opportunistic infections in humans and other hosts [13].

A) K-Antigens :

The surface of Klebsiella species is shielded by a thick layer of capsular polysaccharide, historically known as K-antigen, that protects the bacteria from phagocytosis and prevents killing of the bacteria by bactericidal serum factors [14]. Hyper virulent K.pneumoniae (Hv-Kp) strains are known to produce a hypercapsule, resulting in a hypermucoviscous phenotype which further contribute to increased resistance to complement-mediated or phagocyte-mediated killing [15]. Traditionally, 77 K-antigens have been identified among Klebsiella spp. based on the diversity in their sugar composition, type of glycosidic linkages, and the nature of enantiomeric and epimeric forms [16]. Recently, additional K-types have been reported based on the arrangement of the capsule polysaccharide synthesis (cps) locus or K locus (KL), known as the KL series [17]. Among the different K-antigens, K1, K2, K5, K16, K23, K27, K28, K54, K62 and K64 are some of the most commonly isolated serotypes globally [18]. K1 and K2 strains are generally more virulent than strains of other serotypes based on the most frequently isolated serotypes collected from patients and results from mouse experiments. In addition, K2 strains are the most prevalent type of *K. pneumoniae* strain isolated clinically, followed by K1 strains,and they are more resistant to phagocytosis and intracellular killing by alveolar macrophages and neutrophils than other strains, and this phenotype is independent of whether they are hypercapsule producers [19]. Plasmid located virulence gene(regulator of mucoid phenotype A (rmpA)) is responsible for Synthesis of capsular polysaccharides; highmucus phenotype of (hvKP),while, rmpA2 is responsible for capsule upregulation [20].

B) Lipopolysaccharide(Endotoxin) :

Polysaccharide represents an important and essential factor in bacterial pathogenicity, especially *K. pneumoniae*, as it is one of the superficial compositions of bacteria that helps it to resist phagocytosis and participates in protecting bacteria against the host's complement system.(Fig.1) [21]. LPS is both a benefit and a hindrance for *K. pneumoniae* during infection, as it is an important virulence factor that protects against humoral defenses but also can be a strong immune activator [22]. LPS consists of three parts: O-antigen, a core polysaccharide, and lipid A antigen which are encoded by genes in the wb, waa, and lpx gene clusters, respectively [23].

i. O-Antigens:

The O-antigen moiety of the LPS has a limited range of structures, resulting from different sugar composition, glycosidic linkages and epimeric or enantiomeric forms of the sugars [24]. The most recent classification includes 11 O-serotypes: O1, O2a, O2ac, O2afg, O2aeh (previously known as O9), O3 (divided in subserotypes O3, O3a and O3b), O4, O5, O7, O8 and O12 [17]. Among clinical *K. pneumoniae* isolates O1 is the most common serotype [25].

ii. Lipid A :

Lipid A is formed in the cytoplasm by conserved constituent enzymes and transmitted by the ATP-binding cassette(ABC) transporter MsbA and is concentrated in the outer membrane [26]. Lipid A, is a potent ligand of Toll like receptor-4 (TLR4) which stimulation leads to the production of cytokines and chemokines that help recruit and activate cellular responses, including neutrophils and macrophages, which combate *K. pneumoniae* infection and control spread to other tissues [22]. *K. pneumoniae* may use another method to prevent recognition by the immune response which is modification of the LPS to a form that is no longer recognizable by certain immune receptors [27]. The lipid A portion of *K. pneumoniae* LPS plays a beneficial role in virulence as both in vitro and in vivo experiments have shown that lipid A protects against some cationic antimicrobial peptides [19].

iii. Core polysaccharide :

Two types of core polysaccharide (Type I and Type II) have been identified to be produced by these bacteria,they are encoded by two different groups of wa gene cluster [28].

C) Fimbriae

Fimbriae are organelles appointed to facilitate attachment and adherence to eukaryotic cells, but also involved in other functions, such as interaction with macrophages, biofilm formation, intestinal persistence, and bacterial aggregation [29]. Most clinical *K. pneumoniae* isolates express two types of fimbrial adhesins, type 1 fimbriae and type 3 fimbriae [30]. Besides type 1 and type 3 fimbriae, a third type named *K. pneumoniae* complex (KPC) fimbria was identified and its heterologous expression in E. coli demonstrated an active role of this protein in biofilm formation [13]. Several genes encoding Fimbriae in *K. pneumoniae* have been described such as fimH, mrkD and cf29A that encode type 1 fimbriae, type 3 fimbriae and non-fimbrial adhesion factor ,respectively [31].

Table 1: Differentiation of Klebsiella species.

Properties	K.pneumoniae	K. ozaenae	K.rhinoscleromatis	K. oxytoca
Indole	-	-	-	+
Vogesproskauer	+	-	-	+
Urea hydrolysis	+	-	-	+
Lysine decarboxylase	+	V	-	+
ONPG	+	+	-	+
Growth at 4°C	-	-	-	-
Growth at 10°C	-	-	-	+
Growth at 44°C	+	V	UK	+

+ most strains positive; - most strains negative; V some strains positive, others negative; UK unknown; ONPG (O -nitrophenyl-B - galactopyranoside test) [12]

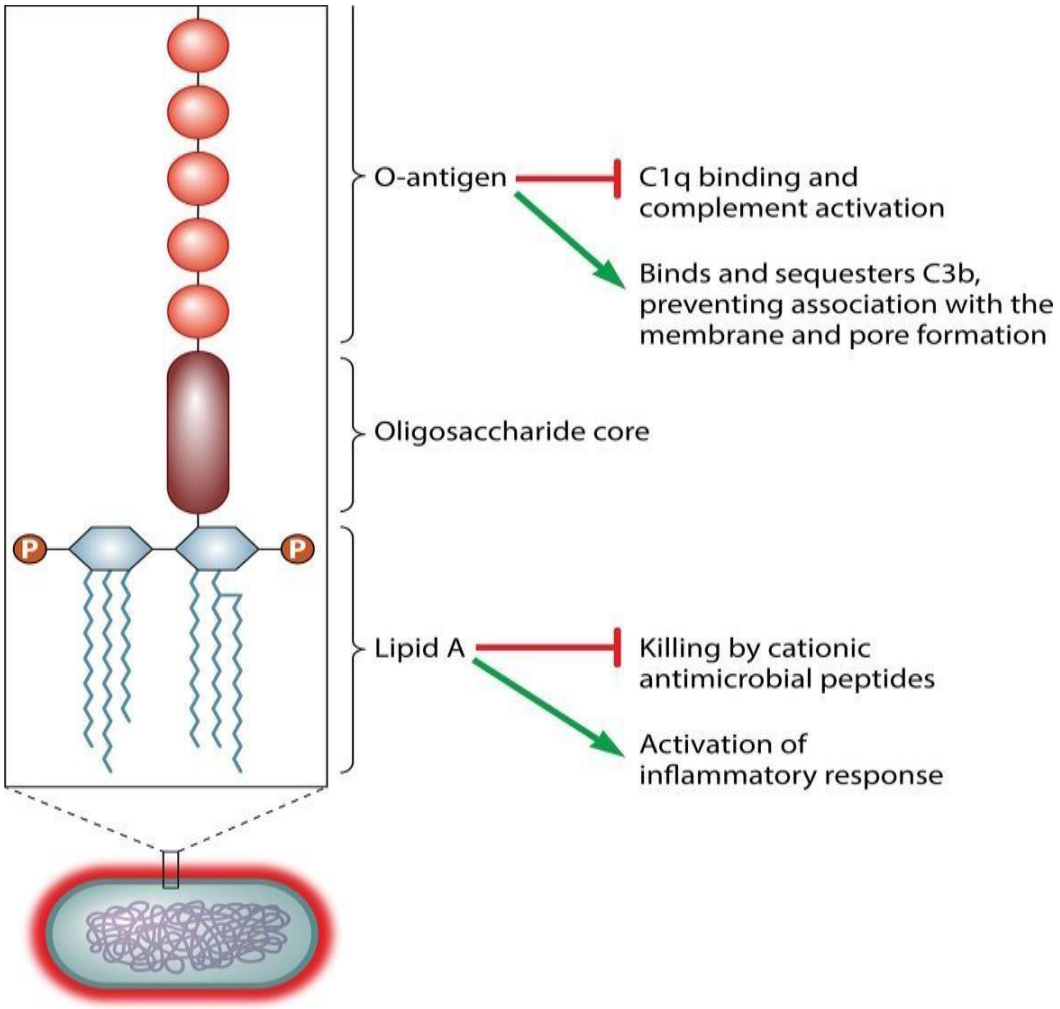

Figure 1: Role of lipopolysaccharide in *K. pneumoniae* virulence, [19].

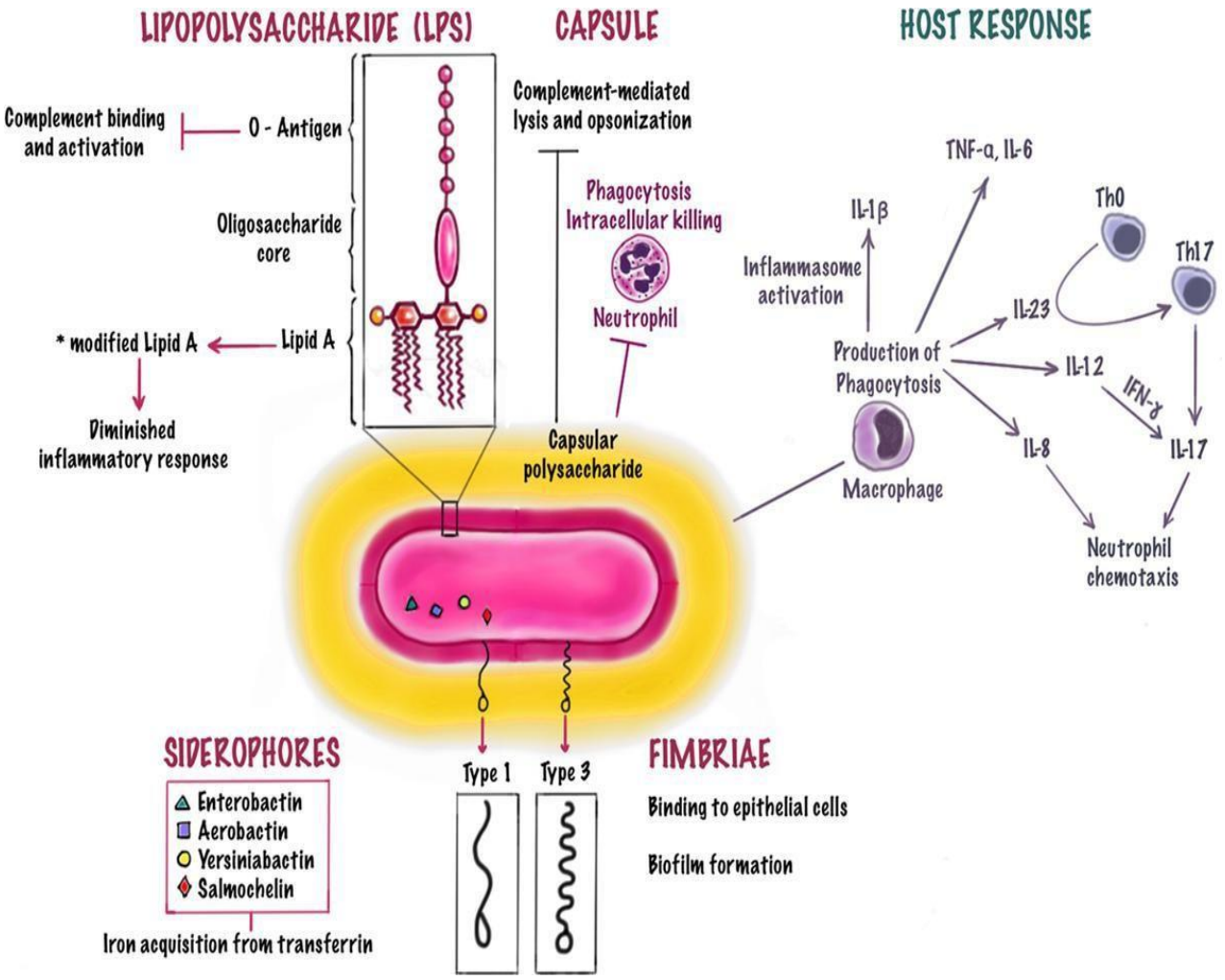

Figure 2: Schematic presentation of *K. pneumoniae* virulence factors and host innate immune response, [6].

Another *K. pneumoniae* fimbrial antigen named KPF-28 has been reported to be expressed by several *K. pneumoniae* circulating strains and its role in colonization has been demonstrated by using KPF-28 antisera to inhibit bacterial adhesion to intestinal cells. However, besides a correlation of the expression of this adhesin with an antibiotic resistance phenotype, no further information has been collected about KPF-28 over the last two decades [13]. Type 1 fimbriae are made by a polymer of the major building element FimA. FimH, together with the minor subunits FimF and FimG, forms a flexible tip fibrillum that is connected to the distal end of the pilus rod and that is responsible for the adhesion to the host [32]. Type 1 fimbriae were described to be phase-variable with a different role in lungs and urinary or intestinal tract infections. Indeed, fimbrial expression was found to be highly upregulated in the bacterial population in urine and infected bladders and downregulated in the lungs. In this organ, expression of type 1 fimbriae may be a disadvantage for the bacteria because of their ability to adhere to phagocytic cells in the lungs and therefore to be rapidly eliminated [33]. Type 3 fimbriae have been extensively proven to mediate binding to extracellular matrix (ECM) proteins such as collagen molecules and to strongly promote biofilm formation [34].

D) Siderophores :

Siderophores are secreted small molecules that can bind the iron present externally and re-enter to the bacterial cells through specific receptors [35]. Since iron is essential for the survival of bacteria, it is also necessary to obtain iron source through siderophores during the colonization stage of *K. pneumoniae*. Nearly all bacteria, including all opportunistic pathogens of the airway, require an iron source to survive [36]. *K. pneumoniae* production of siderophores such as enterobactin, yersiniabactin, salmochelin and aerobactin has been shown to strongly correlate with in vivo virulence and differentiate Hv-Kp from classical *K. pneumoniae* strains. For this reason, siderophores have been suggested as potential biomarkers to be further implemented in screening laboratory tests [37]. Each of these molecules presents a specific receptor on the bacterial surface able to bind the siderophore and to mediate the iron uptake, which is essential for the pathogen survival [38]. Salmochelin is associated with invasive diseases, and is common in highly virulent *K. pneumoniae* strains that cause severe community related infections, such as liver abscess and pneumonia [39].

7. Pathogenic strains and infections caused by *K. pneumoniae*:

Historically, *K. pneumoniae* infections have been caused by "classic" *K. pneumoniae* (cKp) strains which were found widely in hospital environs in Asia, causing infections in immunocompromised patients with debilitating conditions . On the other hand , Hypervirulent *K. pneumoniae* (Hv-Kp) variant strains first isolated in the Asian Pacific Rim [40]. The HvKp strains cause life threatening community-acquired metastatic and invasive infections such as liver abscess, meningitis, endophthalmitis and septic arthritis in immunocompetent individuals [41]. HvKp infection requires minimal number of bacteria to occur. As studies in mice showed that an inoculum dose of as little as 50 bacteria was lethal compared to the inoculum size of 107 needed for classical *K. pneumoniae* (cKp) strains [42]. The recent emergence and widespread dissemination of the new cKp strains of multidrug-resistant (MDR) *K. pneumoniae* and the global spread of hvKp strains put *K. pneumoniae* on the WHO and the US centers for disease control list of priority MDR pathogens [43]. With the acquisition of an extended-spectrum beta-lactamase, many strains have additional resistance to amoxicillin, ceftazidime, ceftriaxone, and carbenicillin [6]. In 2009, a novel gene called New Delhi metallobeta- lactamase (NDM-1) was reported in strains of *K. pneumoniae* in India and Pakistan and gives *K. pneumoniae* resistance even to intravenous antibiotic carbapenem. To treat these infections, Physicians have resorted to use older previously discarded antibiotics which include colistin and tigecycline with few novel antibiotics under development [6]. Recent reports have indicated the presence of colistin-resistant strains of KP in ICUs. Consequently, superbugs with pan resistance to all known classes of antibiotics, including the polymyxins, have emerged [6]. *K. pneumoniae* is the causative agent of a range of infections, including:

A) Respiratory infections:

K. pneumoniae pneumonias can be split into two broad categories:

1) Hospital-acquired pneumonias (HAPs).

2) Community-acquired pneumonias (HAPs).

1) Hospital -acquired pneumonias (HAPs):

K.pneumoniae HAPs are far more prevalent than *K. pneumoniae* CAPs .HAP is generally defined as pneumonia that presents at least 48 h after admission to a hospital in individuals with no symptoms of pneumonia prior to admission and about 11.8% of HAPs are caused by *K. pneumoniae* [44]. HAPs occur in both ventilated and nonventilated patients, and *K. pneumoniae* is the causative agent in 8 to 12% and 7% of these cases, respectively [45].

There is a significantly higher risk of *K. pneumoniae* being multidrug resistant in nosocomial infections than in community-acquired infections because many patients have been treated with antibiotics and are carrying antibiotic-resistant flora [46].

2) Community-acquired pneumonias (CAPs):

CAPs are potentially serious infections that can progress rapidly and lead to hospitalization, intensive care unit (ICU) stays and high rates of morbidity and mortality [47]. Both classical and HV strains can cause CAPs, the increased prevalence of *K. pneumoniae* as the etiological agent of CAPs in Asia and Africa is likely due to the increased prevalence of hypervirulent strains in these areas [19]. Reports estimate that *K. pneumoniae* CAPs comprise 22 to 32% of cases requiring admission to the ICU, with mortality rates in these ICU patients ranging from 45 to 72% [19-48]. Cases usually present with cough, fever, leukocytosis and chest pain which are similar to symptoms typical of acute pneumonias and can also display the trademark *K. pneumoniae* characteristic of “currant jelly sputum” which is the production of thick blood-tinged mucous resulting from high levels of inflammation and necrosis in the lungs [49].

B) Urinary tract infections:

K. pneumoniae strains are the second or third most frequent cause of UTIs behind Escherichia coli, which causes the vast majority of UTIs [50]. *K. pneumoniae* accounts for 2 to 6% of nosocomial UTIs and 4.3 to 7% of community-acquired UTIs [51]. *K. pneumoniae* UTIs resulting from seeding of *K. pneumoniae* from the GI tract [52]. The symptoms include dysuria, increased frequency, urgency of voiding and hematuria [53]. C) Bacteremia: *K. pneumoniae* is second only to E. coli among gram-negative pathogens as the causative agent of both community-associated and nosocomial bacteremias [54]. Mortality rates following *K. pneumoniae* bacteremia ranged from 27.4 to 37% [55]. The increase in antibiotic resistance makes treatment of lung and bladder *K. pneumoniae* infections more difficult and prolonges time that patients carry *K. pneumoniae* at these sites, allowing *K. pneumoniae* to spread to the bloodstream and brain [56].

D) Gastrointestinal tract infections:

HV strains cause primary liver abscesses in patient populations that do not appear to have any underlying liver disease but are likely initiated from a breach in host defenses in the GI tract that permits intestinal microbiota to seed tissue sites unlike other pyogenic liver abscesses caused by polymicrobial sources [57]. The intestinal flora has an effect on *K. pneumoniae* colonization. For instance, a healthy gut microbiome provides an extra layer of defense and helps eliminate exogenous bacteria. However, changes in intestinal flora after antibacterial treatment lead to increase the level of available monosaccharides in the intestinal tract and promot the growth of pathogenic or opportunistic bacteria *K. pneumoniae* [58].

E) Central nervous system infections:

In Taiwan, several epidemiologic studies of adult bacterial meningitis (ABM) have also revealed that *K. pneumoniae* is the most commonly implicated pathogen of community-acquired infection .Presence of DM and liver disease especially cirrhosis are the most common underlying medical conditions of this specific group of CNS infection [59].

Brain abscess, which may occur alone or in combination with ABM, is the most common form of

intracranial focal suppuration of K. pneumonia infection [59].

F) Other infections:

HV *K. pneumoniae* infections can also lead to severe skin and soft tissue infections (e.g., cellulitis, necrotizing fasciitis, and myositis), endophthalmitis and abscesses in a number of other tissues (e.g., neck, lungs and kidneys) [60]. *K. pneumoniae* has been implicated in the development of ankylosing spondylitis because of the high incidence of Klebsiella in the bowel flora of patients whose disease is in an active state [61].

8. Vaccines and Monoclonal Antibodies (mAb) Strategies

With little hope of new drugs and an increase in antimicrobialresistant and virulent strains, vaccines offer the best solution against the rapidly increasing global threat of *K. pneumoniae* infections, as recognized by the WHO [62]. No vaccines are currently licensed against *K. pneumoniae*, but several vaccine targets have been described in the past few decades .Moreover, a therapeutic approach based on the use of monoclonal antibodies (mAbs) against antimicrobial resistant (AMR) pathogens has been taking place over the last years [63].

A) K-Antigen Based Approaches

Bacterial capsule polysaccharides have been used historically as vaccine target antigens and several formulations have been developed against different pathogens [64]. The ideal capsule-based vaccine should be multivalent in order to cover the majority of all bacteraemic isolates. Subsequently tested a polyvalent Klebsiella vaccine, composed of six K-serotypes (K2, K3, K10, K21, K30, and K55), which resulted to be safe and immunogenic in humans. This study raised the awareness that a Kp vaccine able to cover around 70% of clinically relevant *K. pneumoniae* strains should include at least 25 capsular polysaccharides [65]. An interesting study was performed in 2019 by Feldman et al. [42] who tested for the first time a bioconjugate vaccine encompassing capsules from the K1 and K2 serotypes, causing around 70% of all Hv-Kp infections worldwide.

B) O-Antigen Based Approaches

K. pneumoniae O-antigens are alternative targets for the vaccine development, due to their limited range of structures. Indeed, four serotypes (O1, O2, O3 and O5) were predicted to cover over 80% of clinically relevant Kp strains [18]. Immunization with purified O1 LPS vaccine could protect mice against K2:O1 challenges.Additionally, it was reported that O1 LPS, incorporated either into liposomes or sodium alginate microparticles, could protect rodents against lobar pneumonia and resulted to be less toxic than free LPS in mice [66]. Hegerle et al. [67] reported the development of combined *K. pneumoniae* and Pseudomonas aeruginosa glycoconjugate vaccine comprising O1, O2, O3, O5 Kp O-serotypes, chemically linked to the two Pseudomonas flagellin types (FlaA, FlaB). The quadrivalent conjugate vaccine generated antibody titers to the four *K. pneumoniae* O-antigens and both Fla antigens in rabbits. Innovative strategies have been recently proposed for the generation of O2a glycoconjugate vaccines including a semi-synthetic approach and a bioconjugation approach [68]. Anti *K. pneumoniae* O-antigen mAbs also proved to be able to reduce bacterial burden, enhance survival, and show synergy with current standard of care therapy [69]. Serotype specific anti-O1 (KPE33) and anti-O2 ((KPN42) human mAbs are able to protect mice against *K. pneumoniae* infection via opsonophagocytic killing [70]. Anti-O3 mAb 2F8 able to cross-react with all three O3 sub-serogroups (O3, O3a and O3b) [71].

C) Protein Based Approaches

Protein virulence factors have also been proposed as targets for the development of vaccines and therapeutic mAb against *K. pneumoniae*. These antigens are characterized by low variability, especially when compared to the K-antigens and O-antigens polysaccharides. In particular, type 3 fimbriae structural protein MrkA has gained a strong interest as a putative target for a vaccine as, besides being expressed by most of *K. pneumoniae* strains, including Hv-Kp, it shows a structural position on the pilus filament that may ensure easy access to antibodies. There are already data showing that immunization with purified fimbriae was able to protect mice against a lethal challenge in a model of acute pneumonia [72]. Type 1 and 3 fimbriae were used as carrier proteins conjugated to E. coli core oligosaccharide, proving to be immunogenic also in rabbits [73]. Recently, using bioinformatic prediction tools, Zargaran et al., [74] found four B cell epitopes (each for one Fim antigen) that were proposed as suitable vaccine candidates for *K. pneumoniae* as these epitopes may be recognized by the B cell receptor, triggering humoral responses. Indeed, in silico analysis suggested that these epitopes are immunogenic and antigenic, not similar to human peptides, not allergenic and not toxic. However, these results still need to be supported by in vitro and in vivo testing. A reverse vaccinology approach is applied on a total of 222 available complete genomes of *K. pneumoniae* and four outer membrane proteins were shortlisted for vaccine designing: the outer membrane protein OmpA, the copper/silver efflux RND transporter, the phosphoporin PhoE and the peptidoglycan-associated lipoprotein Pal. Of these antigens, a total of four epitopes were joined together using flexible linkers resulting in a potential multi-epitope vaccine against *K. pneumoniae* [75]. Another virulence factor recently investigated as a potential vaccine target is the highly conserved YidR, an ATP/GTP-binding protein that mediates the hyperadherence phenotype and is involved in biofilm formation .Recombinant YidR has been proven to protect mice from challenge with a low dose of *K. pneumoniae* [76].

9. Conclusions

The molecular features and the mobile resistance elements they harbour, allowing identification of genetic loci responsible for transmission, stable inheritance, and expression of mobile resistance or virulence-encoding elements that confer the new phenotypic characteristics of MDR-hv Klebsiella spp.

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