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A Review of the Innate Immune Evasion Mechanisms and Status of Vaccine Development of *Klebsiella pneumonia*

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Authors' contributions

This work was carried out in collaboration between both authors. Author FT conducted and synthesized the literature searches and wrote the first draft of the manuscript. Author LM made edits to the first draft and contributed to the content. Both authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Klebsiella pneumoniae (KP) is a human pathogen causing a broad spectrum of diseases such as urinary tract infections (UTI), pneumonia, pyogenic liver abscess, bloodstream infections, and sepsis. Neonate, geriatric and immunocompromised individuals are the most vulnerable to KP infections. The success of KP as an infectious agent is due to the evolution of various mechanisms to evade the host's immune system. These diverse mechanisms have led to the dominance of KP infections in community settings where hypervirulent strains predominate and in hospital-acquired infections where multidrug-resistant strains predominate. KP infections in the past decades have been increasingly associated with high morbidity and mortality due to the emergence of multidrug-resistant and hypervirulent strains capable of evading both the internal immune defense mechanisms and external antimicrobial agents. The pharmaceutical industries have very few and often expensive new antibiotics in the pipeline, offering little hope for antibiotic therapy. The development of new therapeutic strategies such as polyvalent, biconjugate vaccines that can provide protective immunity, especially against vulnerable populations, can mitigate the effects of

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KP infections. In this review, we discuss the virulence mechanisms of KP and how it evades the innate host immunity, and the interplay between the virulence and immune evasion strategies. The progress in the search for a vaccine to protect against KP infections will also be highlighted.

Keywords: Klebsiella pneumoniae; antimicrobial peptides; biofilm; hypervirulence; innate immunity; complement system, biconjugate vaccines.

1. INTRODUCTION

The bacterial genus *Klebsiella* was named after a renowned German bacteriologist Edwin Klebs (1834-1913), and is also known as Friedlander's bacillus in honor of Carl Friedlander, a German pathologist. Friedlander proposed that the bacterium was the causal organism of pneumonia seen primarily in people suffering from debilitating conditions such as alcoholism and people with chronic diseases that include: diabetes, malignancy, liver disease, glucocorticoid therapy, renal failure [1,2].

Klebsiella pneumoniae (KP) is a gram-negative, rod-shaped, encapsulated, non-motile, facultative anaerobe, lactose-fermenting bacterium. It appears mucoid when grown in the laboratory on agar media like MacConkey and sheep blood agar [1]. Traditionally, KP isolates were classified based on capsular serotyping (K-typing) using specific antisera. There are 79 KP capsular serotypes, but most human infections are caused by a limited number of serotypes that include K1, K2, K4, and K5 [1,3]. KP strains belonging to the K1 and K2 serotypes are hypervirulent, causing community-acquired infections, especially pyogenic liver abscesses [4]. Apart from capsular typing, KP isolates are also typed based on the polysaccharide O-antigen (O-typing). The O-antigen is the outermost domain of lipopolysaccharide (LPS) on the outer membrane, forming a vital cell wall component. Among clinical isolates, the O1 serotype appears to be the most prevalent [5,6].

Some KP strains have quickly achieved superbug status, resistant to almost all clinically essential antibiotics in recent years. Few new classes of antimicrobials are in the pharmaceutical pipeline to use against pan drug-resistant gram-negative bacterial infections [3,7,8]. Consequently, developing strategies to prevent infections and mitigate antibiotic resistance through vaccine development has become an urgent priority [9,10].

1.1 *K. pneumoniae* Human Carriage and Clinical Significance

Humans are the main reservoir for KP as healthy individuals are asymptomatic KP carriers in the nose, stool, and throat [8,11,12]. Carriage of KP in the community ranges from 5 to 38% and 1 to 6% in stool samples and the nasopharynx, respectively [1,3]. Ambulatory alcoholic patients have been shown to have a higher nasopharyngeal carriage. Increased carriage rates have been noted in hospitalized patients, with rates of 77%, 19%, and 42% indicated in stool, pharynx, and hands, respectively [13]. The higher rates of colonization among hospitalized patients are due to increased transmission and greater antibiotic use [1,8,14]. This increase in carriage rates is clinically significant because, as one report indicates, *Klebsiella* hospital-acquired infection was four times higher in stool carriers than non-carriers [15,16].

KP is a significant cause of life-threatening infections. It is the most commonly isolated gram-negative bacterium from the lower respiratory tract and the second most common cause of bacteremia [16,17]. Widespread use of broad-spectrum antibiotics in the past three decades has contributed to the rise in the frequency of infections caused by gram-negative aerobic bacilli, such as KP [14,18]. Of these infections, those categorized as pneumonia or bacteremia carry a relatively high attendant fatality ratio ranging from 10-50%. Before the introduction of antibiotics, KP was a common cause of community-acquired pneumonia, especially among diabetic patients and alcoholics [10,19]. In the antibiotic era, KP has become a predominantly nosocomial infection [4,20,21] causing opportunistic, and hospital acquired infections (HAI) which disproportionally affect neonates, the elderly, and the immunocompromised [14,22]. The main risk factors for hospital-acquired KP infection are indwelling venous or urinary catheters, mechanical ventilation, and prolonged stay in high dependence units [10,14]. Individuals with weakened immunity due to immunosuppressive therapy or an underlying medical condition (such

as diabetes mellitus, cancers, and human immunodeficiency virus) and low birth weight in preterm infants are more prone to KP infections 20. These infections include urogenital infections, necrotizing pneumonia, surgical wound infections, endogenous endophthalmitis, septicemia, pyogenic liver abscesses, and endocarditis [23].

1.2 Evolution and Challenges of Pathogenic KP Strains

KP infections have historically been caused by "classic" *K. pneumoniae* (cKp) strains. These strains were found to be ubiquitous in hospital environs in Asia, causing infections in immunocompromised patients with debilitating conditions [24]. These cKp strains are different from the hypervirulent *K. pneumoniae* (hvKp) variant strains initially isolated in the Asian Pacific Rim [24,25]. The hvKp strains cause life-threatening community-acquired, metastatic, and invasive infections such as liver abscess, meningitis, endophthalmitis, and septic arthritis in immunocompetent individuals [3,26]. hvKp presents a unique challenge in managing KP infections due to the minimal number of bacteria required to cause an infection. Studies in mice showed that an inoculum dose of as little as 50 bacteria was lethal compared to the inoculum size of 10^7 needed for classical *K. pneumoniae* (cKp) strains 9. The recent emergence and widespread dissemination of the new cKp strains of multidrug-resistant (MDR) KP and the global spread of hvKp strains put KP on the WHO and the US centers for disease control list of priority MDR pathogens [7,17]. The common antibiotics used in treating KP include fluoroquinolones, aminoglycosides, parenteral or oral cephalosporins. However, the emergence of KP strains resistant to the previously mentioned antibiotics requires last-line antibiotics to treat the infections. These infections are a big challenge for clinicians as not all healthcare facilities can afford to stock the expensive last-line antibiotics. MDR infections may therefore result in a higher mortality rate. With the acquisition of an extended-spectrum beta-lactamase, many strains have additional resistance to amoxicillin, ceftazidime, ceftriaxone, and carbenicillin [12]. Reports have indicated varying degrees of beta-lactamase inhibition using clavulanic acid. In 2009, a novel gene called New Delhi metallo-beta-lactamase (NDM-1) was reported in strains of KP in India and Pakistan. This gene gives KP resistance even to intravenous antibiotic carbapenem. With few novel antibiotics under development, physicians have resorted to using older previously discarded antibiotics which include colistin and tigecycline to treat these infections [12]. However, recent reports have indicated the presence of colistin-resistant strains of KP in ICUs12. Consequently, superbugs with pan resistance to all known classes of antibiotics, including the polymyxins, have emerged [12]. The rise of multidrug-resistant and hypervirulent KP isolates warrants a deeper understanding of both resistance and virulence mechanisms and the development of alternative treatment and prevention strategies.

1.3 Innate Immune Response to *Klebsiella pneumoniae*

The human body is continuously exposed to microorganisms in the environment, living symbiotically with the host or shed from other individuals and animals [13]. Usually, the presence of a competent physical barrier (epithelial cell layers, cilia, etc.) and chemicals (stomach acid, sebum, saliva, cerumen, etc.) is enough to protect the body from bacterial infections [10,13]. However, upon breach of this physical barrier and invasion of the human body by the bacteria, the innate immune system provides the first line of response capable of eliminating the bacteria within minutes to hours upon infection [27]. This rapid elimination of invading bacteria is made possible by innate immune cells, including sentinel macrophages, a plethora of complement proteins, and other non-specific antimicrobial peptides present in blood [3,10,28]. Immune cells in the body recognize invading bacteria using pattern recognition receptors (PRRs), which trigger immune mediators [29,30]. The PRRs recognize conserved motifs called pathogen-associated molecular patterns (PAMPs) on bacterial cells [31,32].

The monocyte/macrophage system is central to the innate immune response. The cells possess phagocytic capabilities and orchestrate other immune response components via the production of cytokines and chemokines [12]. The humoral innate immune response consists of numerous components, including the naturally occurring antibodies (NAb), pentraxins, and the complement and contact cascades. These innate proteins and soluble plasma components provide crucial elements in preventing and controlling disease [12]. Cytokines, like the tumor necrosis factor-alpha (TNF-α), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 4 (IL-4), and interferon-gamma (IFN-γ) have been isolated in patients with sepsis

caused by gram-negative bacteria (GNB) [5,33]. These cytokines are produced by various cell types, predominantly leukocytes, and serve as mediators of inflammatory cascades, immune regulation, and hematopoietic processes [33]. These cytokines determine how the innate immune cells will proliferate, mature, detect and kill (phagocytose) invading pathogens and present the microbial antigens to lymphocytes such as T cells and engage the adaptive immune response [10,12].

1.4 Complement Activation for Clearance of *K. pneumoniae*

The complement system is part of the innate immune defense. It constitutes an extensive network of plasma proteins that trigger a proteolytic cascade upon recognizing the PAMP microbial patterns often found on the bacterial cell wall [31]. The complement cascade can be activated via the classical, alternative, or lectin pathways [10,34].

1.4.1 Classical pathway

The complement system's classical pathway is activated by the LPS and outer membrane proteins (OMPs) of KP [31,35,36]. The copiously expressed outer membrane protein K36 (OmpK36) of KP interacts directly with the rounded domains of the heterotrimer complement component (C)1q (C1q) in an antibody-independent way and activates the classical pathway (Fig. 1). The binding of C1q activates C1s leading to the formation of a C3 convertase that cleaves C3 into C3b, exposing a previously hidden thioester that covalently links C3b to bacterial surfaces [31,37]. OMPs and LPS located on the KP bacterial surface are crucial targets for C3b and C5b-9 deposition [36,38]. Deposition of C3b complement proteins on the bacterial surface tags bacteria for uptake and destruction by phagocytic cells [31] (Fig. 1). The phagocytic cells recognize the C3b deposited on bacterial cells via complement receptors expressed on their surfaces [31,34]. This recognition triggers phagocytosis of the tagged or opsonized bacteria. Following phagocytosis, the bacteria are engulfed in a phagosome where they are chemically attacked by reactive oxygen species (ROS) and antimicrobial peptides [32]. C3b deposition also initiates the formation of a C5 convertase that cleaves C5 into C5b, triggering the formation of the membrane attack complex (MAC). MAC is a multi-protein complex consisting of the proteins C5b, C6, C7, C8, and numerous copies of C9 (C5b-9) that can indiscriminately kill gram-negative bacteria but not gram-positive bacteria through forming pores in the outer membrane [31,39]. The exact mechanism by which the MAC causes cell lysis is unclear [31]. C5a is also produced during the proteolytic cascade of the complement system and recruits other immune cells to the site of infection [33,36]. Furthermore, complement activation enhances the adaptive immune response by, for example, inducing cross-talk between antigen-presenting cells (APCs) or through detection of complement cleavage products by complement receptors expressed on T and B cells [35,40] (Fig. 1).

1.4.2 Alternate pathway

The alternative complement pathway is activated by the KP lipopolysaccharide (LPS). The LPS structure differs between KP strains and influences the potency of the complement pathway [33,38,41]. Activation of the alternate complement pathway amplifies the production of the C3 convertase and the ensuing complement cascade.

1.4.3 Lectin complement pathway

Capsular polysaccharide moieties of some KP strains can activate the lectin complement pathway through the interaction with the mannose-binding lectin (MBL) [31,42]. The MBL and ficolins bind to carbohydrates, consequently activating C4b2b, which activates C3 to its active fragments C3a and C3b. The deposition of C3b on bacterial surfaces leads to the binding of factor B and conversion into C3 convertase (C3bBb), which cleaves more C3 into C3b, thereby amplifying the complement response [42]. Additionally, complement factors such as C5a and C3a are strong chemoattractants guiding macrophages, monocytes, and neutrophils to the sites of infection [43]. Complement activation occurs by identifying capsular polysaccharides containing mannobiose or rhamnobiose [42]. Although KP capsular polysaccharide activates the lectin complement pathway, this component is crucial in protecting KP against' 'host's immunity. As seen experimentally, poorly encapsulated strains display a relatively high-level of C3b on their surface and are easily phagocytosed. Consequently, loss of capsular polysaccharides is associated with loss of serum resistance and diminished virulence [32,44] Fig. 1.

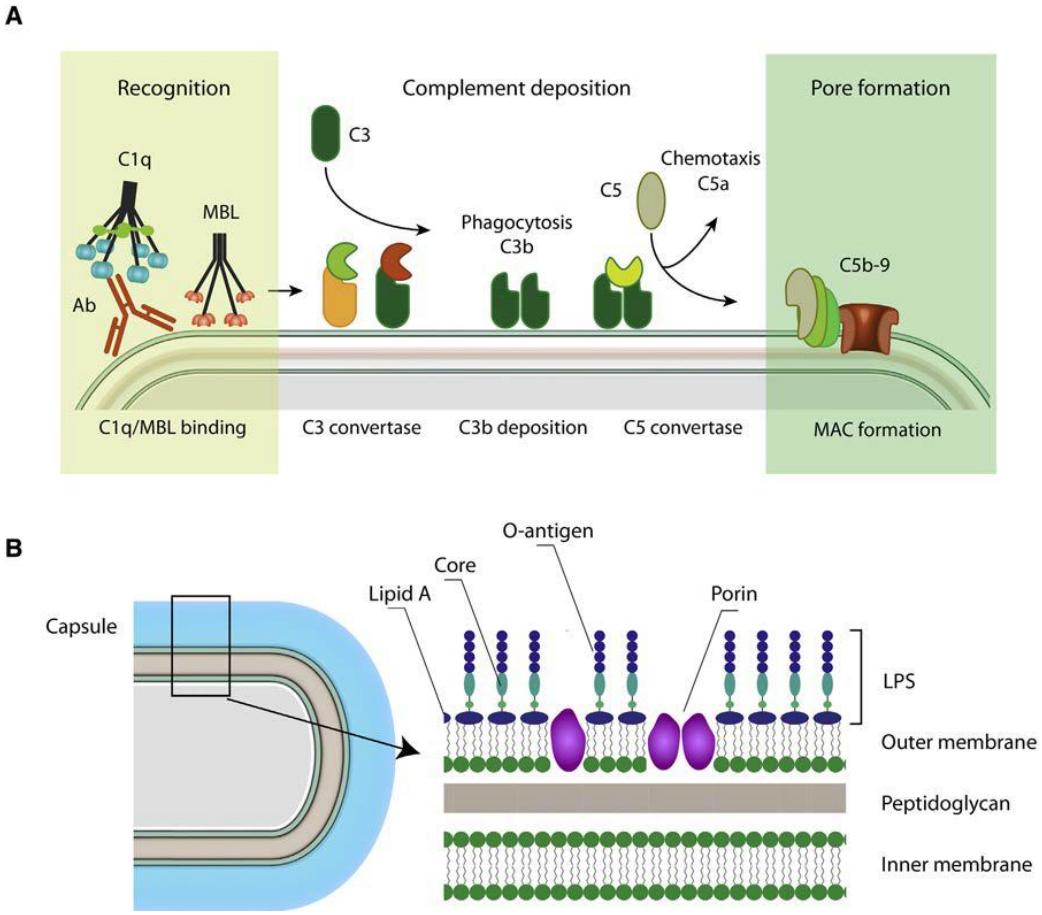

Fig. 1. Complement activation and the gram-negative cell structure. (A) Schematic overview of the complement system. Activation occurs via recognition of antibodies by C1q in the classical pathway or via recognition of sugar motifs via MBL or ficolins. This recognition leads to activation and cleavage of several complement proteins leading to the deposition of C3 convertases that cleave C3 into C3b. The alternative pathway amplifies C3 cleavage by generating additional C3 convertases leading to massive C3b labeling of the bacterial surface. The deposition of C3b triggers phagocytosis and the formation of C5 convertases that cleave C5 into the chemoattractant C5a and C5b, causing the formation of the membrane attack complex. (B) Gram-negative bacteria have a thin peptidoglycan layer located in the periplasmic space between the outer and inner membrane. The capsule consists of a polysaccharide layer that protects the bacterium from the outside environment

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1.5 *K. pneumoniae* Innate Immune Evasion Strategies

KP possesses the ability to circumvent mechanical and chemical barriers and escape the host's humoral and cellular innate defense mechanisms to cause an infection [29,31]. A review of KP infection biology by Bengoechea and co. in 2019 indicated that the bacterium's specific strategies to circumvent the immune defense mechanisms are yet to be fully elucidated. However, some factors are known to play a role. These inhibitory mechanisms are illustrated in Fig. 2 [12].

Components of the capsule serve primarily to protect KP against elimination by the host immune response. Excess production of the thick capsular material resulting in a hypermucoviscous phenotype and resistance to killing by the complement proteins are a defining feature of the highly virulent hvKp strains [45]. In contrast, other strains use the thick capsule to mask the LPS from detection by toll-like receptors (TLRs), mainly TLR4, which

recognizes gram-negative bacteria. TLRs are single-pass membrane-spanning receptors commonly expressed on sentinel immune cells such as dendritic cells and macrophages, which recognize structurally conserved microbial molecules 12. Certain KP strains can modify their lipopolysaccharide (LPS) to the extent that they are unrecognizable by the host immune cells. These bacterial modifications effectively dampen the inflammatory response's cascade, decreasing bacterial clearance from a host [12,37]. Studies have also shown that the versatility of KP lipopolysaccharide is critical in ensuring its survival by resisting antimicrobial peptides produced by the body 12. Each of these strategies will be discussed in more detail.

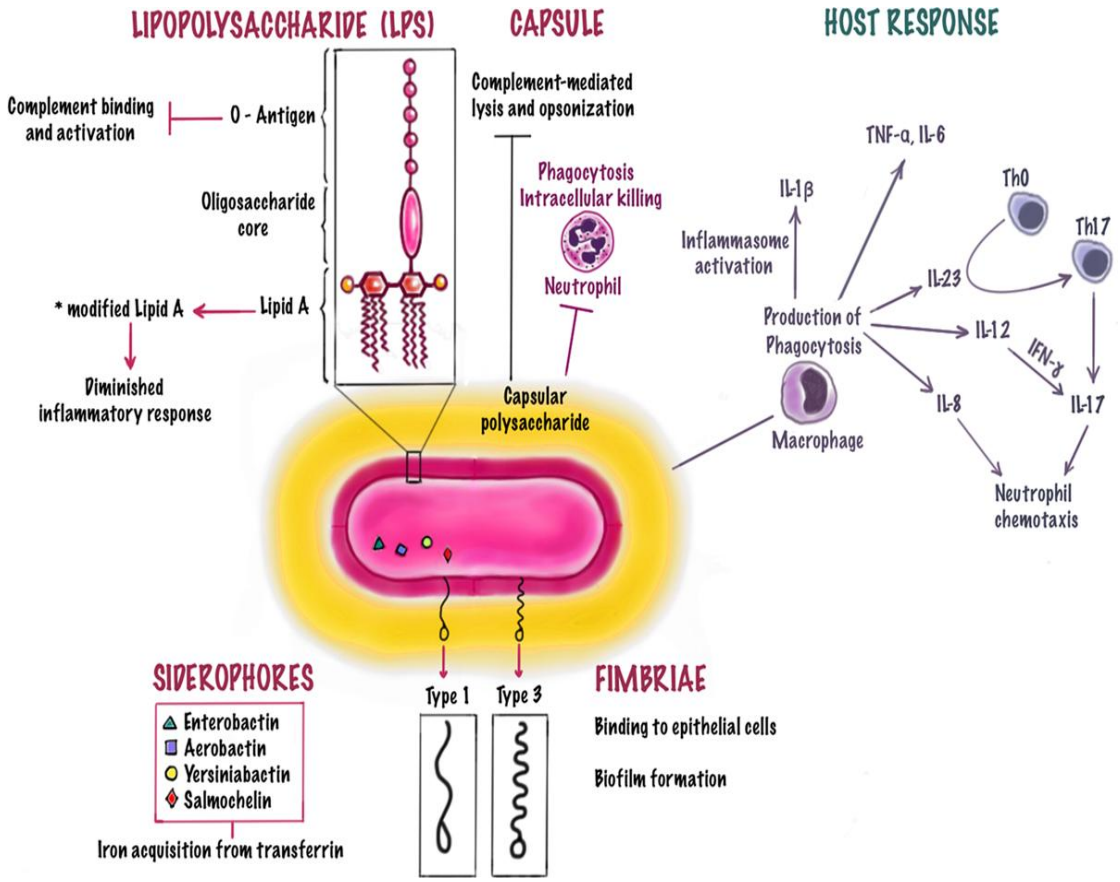

Fig. 2. Schematic presentation of *K. pneumoniae* virulence factors and host innate immune response. Capsular polysaccharides prevent phagocytosis and block complement-mediated lysis and opsonization. The intact LPS elicit a robust inflammatory response and prevent the binding of C1q to bacteria and the subsequent activation of the complement pathway. Certain strains may modify the LPS making it unrecognizable to immune cells, whereas others may use the capsule to prevent LPS detection by toll-like receptor (TLR4). K. pneumoniae is equipped with types 1 and 3 fimbriae, mediating adhesion to biotic and abiotic surfaces facilitating epithelial cell invasion and biofilm formation. It also synthesizes siderophores (enterobactin, aerobactin, yersiniabactin, and salmochelin) to acquire iron from the host. The monocyte/macrophage system plays a central role in the innate immune response through phagocytosis and the production of immune mediators such as cytokines and chemokines. An important mediator in this process is IL-23, which induces IL-17 production, which, along with IL-8, promotes neutrophil recruitment. IL-12 also amplifies IL-17 expression through IFN-γ. Other important cytokines are IL-1β, produced via activation of the NOD-like receptor pyrin domain-containing (NLRP3) inflammasome pathway, and other pro-inflammatory cytokines such as tumor necrosis factor- α (TNF-α) and IL-6. Perpendicular line (——|) indicates inhibition

1.5.1 Protection by the capsule

KP has a capsule ~160 nm thick comprised of polysaccharide fibers, which effectively shields the bacterium from hostile environments [22]. Deleting genes essential for capsule formation in clinical strains renders them non-pathogenic [22]. Capsular polysaccharides are high molecular weight structures made up of linear or branched repetitive units of 2 to 7 monosaccharides. The capsular switching observed in the hospital-enriched KP CC258 strain demonstrated that the capsular polysaccharide composition could change rapidly and is crucial for virulence [22,31,45]. Numerous studies indicate that the KP capsule provides an efficient barrier against bacterial killing via the MAC. In a study of clinical isolates, the capsule protected against serum killing and C3b deposition via the three different complement activation pathways [5,32,46].

The capsular polysaccharide motifs differ between KP strains and are detected to varying degrees by the innate immune system. Some KP strains can modify their capsular constituent to prevent identification by the lectin pathway, as was reported in strains of the K2 serotypes with varied glycan composition [6,26,37,39]. These strains are deficient in mannobiose and rhamnobiose that are recognized via the lectin pathway [42]. Sialylation of the terminal end of capsular polysaccharide hampers complement-mediated phagocytosis of hypermucoid KP strains [28,43]. The enzymatic removal of sialic acid reinstated complement-mediated phagocytosis [43]. The KP capsule prevents complement activation and protects against antimicrobial peptides [42].

1.5.2 Lipopolysaccharide (LPS)

The capsule constitutes the primary physical barrier for the MAC, but the outer membrane of KP is the main target for the MAC [47]. As a central component of the gram-negative cell wall, LPS plays a crucial role in outer membrane stability and protection against the outside environment. LPS is essential for gram-negative bacteria's fitness, but modifications in LPS are common and crucial for adapting to different environments [43,48]. LPS constitutes the O-antigen, a glycan polymer linked to a polysaccharide core attached to lipid A, which is hydrophobic and anchors LPS into the bacterial outer membrane [32]. Cationic antimicrobial peptides can damage the outer membrane by displacing cations such as Mg^{2+} that primarily stabilize the outer membrane through electrostatic interactions between lipid A moieties [32].

Furthermore, the innate immune system identifies the lipid A part of LPS via Toll-like receptor 4 (TLR4). Upon identification of the LPS, the immune cells react via cellular activation and cytokine production [5,46]. Structural modifications of lipid A greatly affect the capacity of lipid A to stimulate TLR4. Lipid A variations could influence the interaction with the host complement negative regulator factor H, as previously seen in *Neisseria gonorrhea* 49*.* KP has also evolved various mechanisms by which it can vary its lipid A during colonization and infection, indicating how crucial lipid A modifications are to survival within the host [49,50].

KP modifications of LPS, such as elongation and changes in the polysaccharide composition of the O-antigen side chain, have been shown to result in serum resistance changes [32]. The O-antigen is highly variable and exposed, so it is easily detected by antibodies 32. KP strains with a long O-antigen make a high molecular weight (smooth phenotype) LPS. In contrast, strains lacking O-antigen side chains have low molecular weight (rough phenotype) LPS. Typically, strains with rough LPS are more vulnerable to serum killing than smooth LPS strains [32,35].

Interestingly, isolates with rough LPS activate the classical pathway, while those with smooth LPS activate the alternative pathway [32]. The elongated O-antigen in smooth LPS probably protects against direct C1q binding or binding to antibodies aimed against KP surface molecules. Consequently, long O-antigen side chains result in C3b deposition further away from the bacterial surface [37], inhibiting the MAC's proper deposition on the bacterial surface, leading to reduced pore formation and bacterial killing. Opsonization studies of different KP strains showed slightly less C3b and no C5b9 deposition on serum resistant strains [38]. Adding purified KP LPS to strains that are serum sensitive effectively hampered serum-mediated killing. Finally, KP and other gram-negative bacteria secrete LPS-rich outer membrane vesicles that can absorb complement proteins, leading to inhibition of complement deposition on the bacterial membrane [32,42,46].

1.5.3 Outer Membrane Proteins (OMPs)

KP also use the outer membrane proteins (OMPs) to avoid detection by the complement

system [32]. Deleting the genes encoding the OMPs peptidoglycan-associated lipoprotein (Pal) and murein lipoprotein (LppA) decreased serum survival of KP 6. The deletion of *lppA* slightly reduced the ability of KP to survive in serum, whereas serum vulnerability increased significantly in the *pal* deletion mutant [5,51]. On top of increased serum susceptibility, phagocytosis of the *pal* and *lppA* mutants was considerably increased. Similar studies by Phan and co. in 2013 showed that the murein lipoprotein was a crucial protein for the serum survival of uropathogenic *E. coli*. Both the *pal* and *lppA* mutants have an elevated outer membrane permeability, leading to increased susceptibility to serum [52].

Furthermore, loss of the outer membrane protein OmpK36 enhances antibiotic resistance and is frequently observed in multi-drug resistance clinical isolates. This resistance is because the outer membrane protein (Omp) acts as a channel to regulate the exchange of extra- and intracellular substances, such as antibiotics, nutrients, and iron, in gram-negative bacteria. Therefore, deletion of these access channels renders the bacterium antibiotic resistance [53]. The deletion of the gene encoding the outer membrane protein OmpK36 increases serum resistance slightly. In contrast, the deletion of OmpK36 promotes neutrophils uptake, and therefore, the mutant is less virulent in an animal model [22,46].

1.5.4 Resistance against Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are a particular class of non-specific cell-derived mediators with broad-spectrum bactericidal properties. Some AMPs are continuously produced by the body, whereas others are only produced or upregulated in response to pathogen invasion [10,49]. AMPs may trigger cell damage in microorganisms in many ways, such as damaging their membranes, destroying DNA and RNA, or hampering cell-wall synthesis [3,54]. Depending on the specific antimicrobial strategy, a particular AMP may counteract only particular bacteria, e.g., gram-negative.

A majority of the AMPs are ubiquitous on the skin, but they are also found in other body regions [10]. Defensins, a family of AMP, can be produced by epithelial cells throughout the body together with other cellular defenses such as neutrophils and macrophages. Defensins may be secreted or act intracellularly within host cells [6,44]. AMPs known as bacteriocins are secreted exogenously by some members of the resident microbiota within the gut to destroy pathogenic bacteria [31,55]. Genes coding for these types of AMPs are typically plasmid-encoded and can be passed via lateral or horizontal gene transfer between different species within the resident microbiota [3]. Other AMPs include cathelicidin, dermicidin, and histatins and achieve their antimicrobial activity by either disrupting the bacterial cell membrane or disrupting the intracellular function [31].

KP has evolved strategies to circumvent host cationic antimicrobial peptides (CAMPs), key among them defensins. CAMPs and antibiotics such as polymyxins and quinolones use the outer membrane of gram-negative bacteria (GNB) as their common initial target [10,42]. Research has shown a relationship between resistance to CAMPs and polymyxins. KP uses the versatility of its LPS and capsular polysaccharides (CPSs) to impede the bactericidal action of polymyxins and CAMPs [46]. CPS dampens the interaction of polymyxins and CAMPs with the KP bacterial surface. A direct correlation between polymyxin B resistance and the CPS levels expressed by a given strain has been shown experimentally [10,20]. Furthermore, unbound CPS released from the bacterial surface binds CAMPs canceling out their bactericidal effect. In this case, the CPS acts as a bacterial decoy for CAMPs [10]. This feature is shared by anionic CPS expressed by *Streptococcus pneumoniae* and *Pseudomonas aeruginosa,* strongly suggesting that trapping CAMPs is a universal trait of anionic CPS [23,31].

KP also rearranges its LPS lipid A domain to counteract polymyxins and CAMPs. KP lipid A can be remodeled with palmitate, phosphoethanolamine, 4-amino-4-deoxy-L-arabinose, and 2-hydroxymyristate [7,26,52]. These decorations make KP resistant to CAMPs. Mutant KP strains deficient in these lipid A modifications are attenuated for virulence in in-vivo mouse pneumonia models 6. Experimental reports also indicate that acylation of lipid A mediates resistance to CAMPs [10,46]. A notable feature exhibited by KP lipid A is its plasticity. Following just a brief incubation with CAMPs, the production of lipid A species bearing such modifications are upregulated [10].

1.6 Interplay between Virulence and Immune Evasion

1.6.1 Hypermucoviscosity phenotype

KP virulence and immune evasion have been correlated with the hypermucoviscosity (HMV) phenotype [56], characterized by the formation of a mucoviscous string of 5 mm diameter when you pass a loop through a colony [25,45]. The presence of the regulator of the mucoid phenotype A *(rmpA)* gene has been associated with KP metastatic infections and biofilm formation, especially in immunocompetent patients with community-acquired infections (CAIs) [57]. The *rmpA* gene acts as an enhancer of the hypermucoviscosity (HMV) phenotype since it activates extra-capsular polysaccharide synthesis [25,56]. HMV-KP strains are more resistant to innate immunity mechanisms, such as antimicrobial peptides, complement-mediated lysis, and phagocytosis 57. This resistance occurs by blocking pathogen detection mechanisms, including toll-like receptors (TLR), mainly TLR4, which consequently inhibits the expression of interleukin-8 28. The inhibition of the TLRs pathogen recognition mechanism conceals the bacteria from the immune system, enabling them to survive and replicate within the host, leading to infection [1,45].

Traditionally, hypermucoviscuous KP strains have been categorized as hypervirulent due to their ability to resist innate immune clearance and, therefore, persist in a healthy host to cause an invasive infection [1,58]. Even though the *rmpA* gene is categorized as a critical gene in KP hypermucoviscosity, other genes such as*magA* have also been associated with hypervirulence [3,45].

1.6.2 Capsular antigens

There are 77 types of capsular antigens, but K1 and K2 serotypes have been classified as the most important ones. Amongst these two capsular serotypes, K2 is associated more with patients with pneumoniae, UTI, and bacteremia [3,56]. KP genetic analysis indicates that the region encoding the capsule contains the following gene clusters: 1. cps (capsular polysaccharide synthesis), 2. wb (the wb gene cluster directs O-specific polysaccharide), 3. *rmpA*, *rmpA1,* and *rmpA2* (regulators of the mucoid phenotypes A, A1, and A2, respectively), 4. *magA* (mucoviscosity associated gene A) [21,45]. These four genes are conserved in the majority of KP isolates and have very distinct roles. The cps gene is involved in capsular polysaccharide synthesis, whereas *RmpA* and *rmpA2* regulate the synthesis of the extracellular polysaccharide capsule [56]. The *wb* gene is responsible for synthesizing lipopolysaccharide, and the 35-Kb, *magA* gene is in a genomic locus with genes for biosynthesis, transfer, and glycosylation of lipopolysaccharide [45]. *MagA* plays a crucial role in metastatic *Klebsiella* infections such as bacteremia, septicemia, pneumonia, liver and lung abscesses [45], likely due to the hyperviscous phenotype, which causes increased resistance to phagocytosis (innate immune clearance) [1]. The genetic locus containing *magA* is a novel pathogenicity island responsible for the increased virulence of KP strains [21,45]. Mutations (frameshift, deletion, insertion mutations) in the genes regulating the KP hypermucoviscosity, such as *rmpA* and *magA* genes, correspond to diminished or negative hypermucoviscosity phenotype of KP isolates. These mutations result in variable serum resistance assays or mouse lethality in experiments using live mouse models [1,10].

1.6.3 Biofilm formation

The hypermucoviscous component of KP facilitates biofilm-associated infections primarily on indwelling medical devices [20,59]. Biofilms are aggregates made up of cells that remain embedded within a self-made matrix of extracellular polymeric substance. Biofilms cause bacterial cells to adhere to each other [53]. The extracellular polymeric substance is a multiplex structure made up of proteins, polysaccharides, and DNA [20]. KP biofilms formed on catheters' inner surfaces and other indwelling devices are the most clinically significant [18,60]. KP biofilms are a leading cause of gastrointestinal, respiratory, and urinary tract colonization and the subsequent development of metastatic infections, especially among immunocompromised patients [53,60,61].

The development of KP biofilms on solid surfaces is a process that proceeds from cell-to-cell adherence leading to the formation of microcolonies, maturation, and ultimately dispersal of the free-living cells to colonize new surfaces [21]. Type 3 fimbriae and the capsular polysaccharide are the key surface structures involved in the biofilm formation process [60,61]. Fimbriae mediate strong adherence, whereas capsular polysaccharides affect cell-to-cell communication and biofilm architecture [7,8,60]. KP cells forming a biofilm are moderately

protected from host immune defenses. The biofilm matrix conceals the bacteria from host-specific immunoglobulins and other antibacterial peptides and reduces the efficiency of complement proteins and phagocytosis [53]. It is also possible that biofilm formation skews the immunity toward a diminished inflammatory response, leading to the establishment of a chronic infection within the host [10,28].

Besides aiding resistance to immune clearance, the KP biofilm matrix also forms a critical antibiotic resistance mechanism [53]. Biofilms lead to antimicrobial resistance due to reduced bacterial growth. Within the biofilm's "inner core," the bacteria are acclimatized to starvation and low oxygen, leading to growth arrest that consequently lessens antibiotics' efficiency targeting metabolically active and dividing cells [53]. The biofilm matrix consisting of a dense matrix of proteins, polysaccharides, and DNA may also prevent the efficient diffusion of antibiotics, thus decreasing antibiotic exposure to the bacteria.

1.7 *Klebsiella pneumoniae* Vaccine Development Milestones

With little hope of new drugs and an increase in antimicrobial-resistant and virulent strains, vaccines offer the best solution against the rapidly increasing global threat of KP infections, as recognized by the WHO [17]. Understanding the pathogen biology and identifying conserved antigens that can be used as immunogens in a vaccine is key to the success of KP vaccine research and development [3,62]. Vaccines based on KP surface polysaccharides are promising candidates. Still, their success will be realized once researchers address the diversity of surface-exposed polysaccharides, synthesized as O-antigens (lipopolysaccharide, LPS) and K-antigens (CPS) [9,63,64].

KP possesses a unique, well-defined capsule surrounding the bacterial cellular components in a dense layer [9,64,65]. The capsule is comprised of repetitive units of monosaccharides collectively forming a high molecular weight polymer. These capsular polysaccharides (CPS) confer the K (or capsular) antigenic specificity upon their bacterial cells [20,50]. In experimental animal models, ribosomal vaccines and killed whole-cell vaccines have been shown to provide a certain degree of protection against *Klebsiella* infections [51]. From immunological correlate analysis, conferred immunity resulted from the induction of a serotype-specific anti-CPS immune response 51. However, attempts to stimulate a protective immune response in mice models via vaccination with purified non-pyrogenic, immunogenic CPS have been unsuccessful. This failure has been attributed to CPS's low immunogenicity in mice [51].

This same outcome has been observed in clinical practice. CPS-derived vaccines have been demonstrated to be poor immunogens in children <2 years of age since they lack an established T-helper response [35]. Also, CPS-derived vaccines fail to induce immunologic memory and have been shown to provide active protection for a limited period of only 3-5 years [51,64]. Although pure polysaccharide vaccines are immunogenically inferior compared to their protein subunit vaccine counterparts, such as recombinant hepatitis B vaccine [1,9,41], the vaccines are relatively safe to use with minimal chances of evoking adverse events. Protein subunit vaccines are manufactured using living organisms that include bacteria and yeast, that require substrates on which to grow them, and thorough hygiene to avoid contamination with other microorganisms [63]. The specific manufacturing process depends on the type of subunit vaccine being made. Protein subunit vaccines, such as the recombinant hepatitis B vaccine, are manufactured by inserting the genetic code for the immunogenic antigen into yeast cells, which are relatively easier to manipulate and grow [63]. These yeast cells are capable of synthesizing huge volumes of the protein. The yeast cells are grown in large fermentation tanks and then split open to allow harvesting of the synthesized protein [9]. Following purification of the protein, other vaccine components such as stabilizing preservatives and immune boosters, adjuvants, such as alum, are added [1,9]. Protein vaccines often elicit strong immune reactions that at times provide lifetime immunity without the need to administer subsequent vaccine booster dose 1,9. Major drawbacks in manufacturing protein vaccines are that their production process is expensive, and sometimes the immune reaction elicited following administration of vaccine might be too strong, causing serious side effects such as fever, chills, tiredness, headache, swollen lymph nodes, muscle, and joint aches, etc. [9].

Current vaccine strategies are geared towards polyvalent immunizing preparations comprised of non-toxigenic serotype-specific polysaccharide preparations [9]. Numerous vaccine preparation methods have been used to produce CPS as a

KP vaccine. Many trials have been conducted using preparations obtained by treating purified KP capsular polysaccharides (CPS) with a deacylation agent such as sodium hydroxide (NaOH) to come up with a non-pyrogenic, immunogenic CPS preparation [35,63]. One study demonstrated that ultra-purified CPS antigen immunogenic preparations could be obtained from the culture supernatant of *Klebsiella* spp. cultivated in a medium designed to support the capsule's proliferation. The highly purified antigens were immunogenic and non-pyrogenic in animal models [51]. For one polyvalent immunogenic vaccine derived from capsular polysaccharides of at least two different serotypes of *Klebsiella* spp., the CPS antigens were treated with a deacetylating agent like sodium hydroxide. Non-pyrogenic, immunogenic CPS of each serotype was generated and later combined to make the polyvalent immunogenic vaccine [66]. Antibodies produced following human trials of this polyvalent vaccine had a short half-life and rapidly disappeared in immunized subjects [66]. Other strategies utilize immunogenic CPS from different *Klebsiella* species to provide immunity against all pathogenic *Klebsiella* spp. infections. In the 1980s, a 24-valent, entirely capsular polysaccharide vaccine (Klebgen Bema) was developed by the Swiss Serum and Vaccine Institute [9]. The human trials demonstrated the vaccine's ability to elicit an exponential rise in serotype-specific IgG responses in adult cohorts [9,11]. As with many polysaccharide-only vaccine preparations, the total serotype-specific IgG titer dropped rapidly in just 18 months, close to baseline levels [9]. A vaccine prepared from such antigenic components can be used to protect individuals at a higher risk of contracting KP infections, such as ICU patients and individuals with debilitating conditions that cause deterioration of the immune system [9,63].

Other studies have found that KP O-polysaccharide (OPS) vaccine concentrates are immunogenic and experimental mice passively transferred with the OPS vaccine produce antibodies protective against systemic classic *K. pneumoniae* (cKp) infection [35,63]. Even though these preclinical studies were promising, OPS-based vaccines were ineffective against hvKp isolates since molecular studies have demonstrated that the capsular polysaccharide in hypervirulent strains can mask the OPS antigens [35].

Polyvalent protein-conjugate CPS vaccines are highly effective against bacterial pathogens such as *Streptococcus pneumoniae* [11,59]. In KP, multivalent vaccines based on the K-antigen have been developed and reached phase I trials in humans [63]. However, the high diversity of KP and the perplexing seroepidemiology complicate the process and increase the expense of developing a vaccine with broad coverage. Compared with other Enterobacteriaceae such as *E. coli* with 161 defined O serotypes and Shigella flexneri with around 47 O serotypes, KP has an astonishingly low number of reported O serotypes. These few O-serotypes offer promise for a more viable alternative for vaccine design and development compared to K-antigen-based vaccines [29,64].

Efforts to engineer a biconjugate vaccine against the most common hypervirulent serotypes of KP, K1, and K2, demonstrated that K1 and K2 glycoengineered bioconjugates are both immunogenic and efficacious in protecting mice from fatal infection against two different hypervirulent strains of KP [9]. These two serotypes (K1 and K2) account for over 70% of the hypervirulent KP cases [10,26,28]. From the existing literature, the K1/K2 biconjugate vaccine presented by Fieldman and co. is the first case of a vaccine protective against hvKp isolates that phenotypically overproduce capsular polysaccharides and cause extremely lethal infections [9]. In vivo studies have proved the 'vaccine's efficacy against hvKp lung infections and should be tested to determine its effectiveness against hvKp infections of the liver, blood, and meninges [9]. In future studies, developing a vaccine concentrate that expands the serotype coverage to target both the classical and hypervirulent KP pathotypes is recommended.

Despite the efforts to produce a KP vaccine, there isn't yet any vaccine available and effective for the immunological control of *Klebsiella* infections [9]. New therapeutic strategies should be explored, including bacteriophage therapy that has proved a success in treating bacterial infections. The first documented application of bacteriophage to treat human infections came in 1921 from Richard Bruynoghe and Joseph Maisin, who used bacteriophage to treat staphylococcal skin disease [67]. With bacterial pathogens rapidly becoming immune to mainstream antibiotics, it's essential to exploit the 'bacterial killers' as an alternative to treat resistant bacterium. Bacteriophages are viruses that adhere to bacterial cells and insert a viral genome into the cell. The viral genome replicates and forms numerous phage particles that lyse

the bacteria. Phage therapy avoids the adverse effects of antibiotics: the risk of developing antibiotic resistance, destroying of the host microbiome, and drug side effects. The downside to phage therapy is the challenge of finding a phage specific for a particular bacterial infection. A focus on viral phage cocktails targeting a wide range of KP strains would be the most effective strategy. With continued research on antibiotics, vaccines and bacteriophages hopes are high that the fight against the KP menace will soon be won.

2. CONCLUSION

The emergence of virulent, multi-drug-resistant KP strains spawning a new generation of hypervirulent 'superbugs' presents a clear global health threat that requires more proactive strategies to curb their spread. The few new antibiotics in the pipeline for curing pan-drug resistant KP infections offer little hope of an antimicrobial "magic bullet". As the prospect of new novel antibiotics dwindles, more resources should be devoted to developing vaccines against bacterial pathogens using the new bioconjugate, multivalent antigen, and immune-boosting strategies to elicit long-lasting, safe immunologic responses and memory. Such potent vaccines would be targeted for use by the most vulnerable population (immunocompromised, geriatric, organ recipients, hospitalized patients, etc.) to produce immune protection to mitigate the impacts of KP infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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