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BY
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AALBORG UNIVERSITY
DENMARK

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by

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"Complement mediated *Klebsiella pneumoniae* capsule changes". Trine S. Jensen, Katharina V. Opstrup, Gunna Christiansen, Pernille V. Rasmussen, Mikkel E. Thomsen, Daniel L. Justesen, Henrik C. Schønheyder, Mads Lausen, Svend Birkelund. *Microbes and Infection* 22 (2019) 19-30

"The apparent prevalence of skin lesions suspected to be human inflicted in Danish finishing pigs at slaughter". Søren Saxmose Nielsen, Anne Marie Michelsen, Henrik Elvang Jensen, Kristiane Barrington, Katharina Vester Opstrup, Jens Frederik Agger. *Preventive Veterinary Medicine* 117 vol. 1 (2014) 200-206

SUMMARY

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen, which causes severe infections such as urinary tract infections, pneumonia, bacteremia and sepsis. *K. pneumoniae* is an emerging pathogen in nosocomial settings and community-acquired infections are common. The number of invasive *K. pneumoniae* cases is rising in Denmark, and the treatment of *K. pneumoniae* infections is reliant on efficient antibiotics such as the beta-lactam class, an important and safe class of antibiotics. However, the antibiotic resistance of *K. pneumoniae* and the *Enterobacteriaceae*, especially the worldwide emergence of extended spectrum beta-lactamases (ESBL) and carbapenemases, render many broad-spectrum beta-lactams useless. The pathogenesis of *K. pneumoniae* infections is not fully elucidated, but *K. pneumoniae* seems to adapt a defensive strategy rather than an offensive. Important virulence factors include a large polysaccharide capsule, lipopolysaccharides and outer membrane proteins, which can be recognized by the innate immune system as pathogen associated molecular patterns.

In blood and tissue, the complement system is a pivotal part of the innate immune system, and consists of both soluble and membrane-bound proteins. Once activated, the proteolytic cascade can kill Gram-negative bacteria directly via the membrane attack complex, opsonize the pathogen for phagocytosis or release inflammatory mediators. Complement is functional in serum, and in vitro, serum is used to examine the complement mediated killing, and serum resistance is an important bacterial virulence trait. The *K. pneumoniae* polysaccharide capsule and lipopolysaccharides mediate serum resistance, thus circumventing the complement mediated killing.

In this dissertation, deposition of complement components, complement activating pathways, and complement killing of clinical *K. pneumoniae* blood isolates were examined. Furthermore, the interactions of beta-lactam antibiotics with ESBL-producing *K. pneumoniae* in serum were investigated. These studies have expanded the knowledge of the complex *K. pneumoniae* pathogenesis by exploring the interaction of *K. pneumoniae* and the complement system, thereby providing new insight in the *K. pneumoniae* escape of the humoral innate immune system.

The three studies showed that serum resistance varied between *K. pneumoniae* isolates, with the majority exhibiting a serum resistant phenotype. Whole genome sequencing of 30 clinical *Klebsiella spp*. showed many different capsular types and O-types, supporting the heterogeneity of *K. pneumoniae*, and no particular type was linked to serum susceptibility. Regardless of serum resistance, the isolates activated the complement system, as serum resistant isolates deposited iC3b, and serum sensitive isolates deposited iC3b and C3b. C4 components were deposited on serum

sensitive isolates together with C3b and iC3b, supporting deposition of classical convertases. Serum resistant isolates did not deposit C4 components, hence these isolates only activated the alternative pathway. Testing of serum susceptibility in different serum concentrations showed three principal groups of serum susceptibility amongst the *K. pneumoniae* isolates. One group was serum resistant regardless of serum concentration. One group was killed in concentrations between 30-75% normal human serum requiring activation of two or more pathways. The last group, the most serum sensitive isolates were killed in 5% normal human serum, and it was demonstrated that the alternative pathway was impaired in this concentration. Thus, for the most serum sensitive isolates, deposition of a classical C5-convertase was sufficient for the subsequent generation of the membrane attack complex and bacterial lysis. The membrane attack complex was present on the surface for the serum sensitive isolates in serum concentrations corresponding to C4 activation and bacterial killing. Therefore, C4 activation is necessary for complement-mediated killing of *K. pneumoniae*, and the location of the membrane attack complex pivotal. Complement mediated extensive capsular changes regardless of serum resistance, and capsular shedding may be a defense mechanism for complement evasion. Immuno-electron microscopy showed presence of C3 components and the terminal proteins C5b-9 in the capsule of serum resistant isolates, thus evading the insertion of the membrane attack complex into the outer membrane.

Furthermore, it was demonstrated the complement could enhance the effects of beta-lactam antibiotics on a partly serum susceptible ESBL-producing isolate, as the presence of the outer membrane attack complex increased the susceptibility to ceftazidime, a third generation cephalosporin, and meropenem, a carbapenem. However, regardless of being killed by meropenem, a serum resistant ESBL-producing isolate did not activate C4, thus not revealing any classical or lectin pathway activating surface structures. Ceftazidime induced similar morphological changes of both isolates with elongation and filamentation in serum. Thus, this underlines the importance of serum resistance as a virulence trait, as synergy of the antibiotics and normal human serum depended on the serum susceptibility of the isolates.

RESUME

Klebsiella pneumoniae er et Gram-negativt opportunistisk patogen, der forårsager alvorlige infektioner så som urinvejsinfektioner, penumoni, bakteræmi og sepsis. *K. pneumoniae* er et hyppigt patogen i hospitalsmiljøet, og samfundserhvervede infektioner er almindelige. Antallet af *K. pneumoniae* sygdomstilfælde i Danmark er stigende, og for at sikre en effektiv behandling af *K. pneumoniae* infektioner, er man afhængig af antibiotika som f.x de vigtige og sikre beta-lactamer. Udviklingen af antibiotikaresistens hos *K. pneumoniae* og *Enterobacteriaceae*, især extended spectrum beta-lactamaser (ESBL) og carbapenemaser, betyder imidlertid, at mange af de bredspektrede beta-lactamer ikke kan anvendes til behandling. *K. pneumoniae*s patogenese er der ikke fuldstændigt redegjort for, men noget tyder på, at bakterien hellere undgår immunforsvaret via en defensiv strategi i stedet for at angribe. Den store polysakkarid kapsel, lipopolysakkarider og proteiner i ydermembraner er vigtige virulensfaktorer, som det innate immune system genkender som patogen-associerede molekylære mønstre.

Komplementsystemet er en afgørende del af det innate immunsystem i blod og væv, og består af både opløselige og membranbundne proteiner. Komplementsystemet er en proteolytisk kaskade, som kan dræbe Gram-negative bakterier direkte ved hjælp af membranangrebskomplekset, opsonisere patogener for fagocytose, eller udskille inflammatoriske mediatorer. In vitro bruges serum for at undersøge komplementmedieret drab, og serumresistens er en vigtig bakteriel virulensfaktor. Serumresistens hos *K. pneumoniae* kan blandt andet forårsages af polysakkaridkapslen eller lipopolysakkarider, så bakterien derved undgår at blive dræbt af komplement.

I denne afhandling blev deponering af komplementproteiner, komplement-aktiverende veje og komplementdrab af kliniske *K. pneumoniae* blodisolater undersøgt. Derudover blev interaktioner mellem beta-lactam antibiotika og ESBL-producerende *K. pneumoniae* isolater i serum afdækket. Disse studier har bidraget til en øget forståelse af den komplekse *K. pneumoniae* patogenese ved at undersøge interaktionen mellem *K. pneumoniae* og komplementsystemet. Dette har givet ny viden om *K. pneumoniaes* undvigelse af det humorale innate immunsystem.

De 3 studier viste, at graden af serumresistens varierede hos *K. pneumoniae*, og at langt de fleste isolater var serumresistente. Helgenomsekventering af 30 kliniske *Klebsiella* spp. isolater viste, at de havde mange forskellige kapseltyper og O-typer, hvilket understøtter mangfoldigheden i *K. pneumoniae*. Der kunne ikke associeres en bestemt kapsel- eller O-type med serumfølsomhed. Isolaterne aktiverede komplementsystemet uanset om de var serumresistente eller ej, da serumresistente isolater deponerede iC3b, og serumfølsomme isolater deponerede både iC3b og

C3b. På serumfølsomme isolater blev C4 komponenter deponeret sammen med C3b og iC3b, hvilket understøtter, at serumfølsomme isolater havde klassiske konvertaser deponeret. C4 komponenter var ikke deponeret på serumresistente isolater, hvilket viste, at disse isolater kun aktiverede den alternative aktiveringsvej. Efter test af serumfølsomhed med forskellige serumkoncentrationer, blev det vist, *K. pneumoniae* isolaterne kunne inddeles i 3 principielle grupper indenfor serumfølsomhed. En gruppe var serumresistent uanset serumkoncentration. En gruppe blev dræbt af serumkoncentrationer mellem 30-75% normal humant serum, og krævede aktivering af to eller flere aktiveringsveje. Den sidste gruppe var de mest serumfølsomme isolater, der blev dræbt i 5% normalt humant serum, og det blev vist at den alternative aktiveringsvej ikke var funktionel i denne koncentration. Det var derfor tilstrækkeligt med deponering af en klassisk C5-konvertase på de mest serumfølsomme isolater for dannelsen af membranangrebskomplekset og efterfølgende drab af bakterien. Membranangrebskomplekset kunne findes på overfladen af de serumfølsomme isolater i serumkoncentrationer, der svarede til, hvornår C4 blev aktiveret og drabet af bakterien. C4-aktivering er derfor nødvendig for at komplement kan dræbe *K. pneumoniae*, og deponering af membranangrebskomplekset på membranen er afgørende. Komplement forårsagede omfattende kapselændringer, og afkastning af kapslen kan være en forsvarsmekanisme til at undgå komplement. Immuno-elektronmikroskopi viste, at C3 komponenter og de terminale C5-9 proteiner var lokaliseret i serumresistente isolaters kapsel, hvorved de kunne undgå at membranangrebskomplekset blev indsat i ydermembranen.

Det blev yderligere vist, at komplement kunne forstærke effekten af beta-lactam antibiotika på et delvist serumfølsomt ESBL-producerende isolat. Deponeringen af membranangrebskomplekset i ydermembranen gav øget følsomhed overfor ceftazidime, et tredje generations cephalosporin, og meropenem, et carbapenem. Selvom et serumresistent ESBL-producerende isolat blev dræbt af meropenem, aktiverede dette isolat ikke C4, og overfladestrukturer der kunne aktivere den klassiske eller lektin-medierede vej blev derved ikke afdækket. Ceftazidime inducerede ensartede morfologiske forandringer i både det serumresistente og serumfølsomme isolat med elongering og filamentering i serum. Dette understreger vigtigheden af serumresistens som en virulensfaktor for *K. pneumoniae*, da synergi mellem de inkluderede antibiotika og normal humant serum var afhængig af isolaternes serumfølsomhed.

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Lastly, but importantly, thank you to my wonderful family. I could not have done this without you.

TABLE OF CONTENTS

Preface

This dissertation is based on three experimental studies, which were conducted between 2017-2021 under the Doctoral Program Clinical Science and Biomedicine, Aalborg University, Denmark. The research was done in the Group of Medical Microbiology and Immunology, Department of Health Science and Technology, Aalborg University and the Department of Biomedicine, Aarhus University.

The three experimental studies present current aspects of complement-mediated *Klebsiella pneumoniae* killing. The two first studies focus on complement activation and subsequent killing of *K. pneumoniae* clinical isolates, and the third study investigates the interactions of beta-lactam antibiotics with ESBL-producing clinical *K. pneumoniae* isolates.

The introduction consists of two chapters. In chapter 1, the epidemiology, infections, virulence factors and antibiotic resistance of *K. pneumoniae* are introduced. In chapter 2, the complement system is presented with focus on complement activation pathways and complement mediated bacterial lysis.

The objectives and the main outcomes of the three included studies are described in chapter 3 and 4.

In chapter 5, the activation of the complement system and serum resistance of *K. pneumoniae* are discussed in relation to the literature and the main findings of the included studies.

Citations of the three studies will be marked in **bold** to underline the contribution of the studies to the field.

List of studies

Studies included in this dissertation:

Study I: “Complement mediated *Klebsiella pneumoniae* capsule changes”. Trine S. Jensen, Katharina V. Opstrup, Gunna Christiansen, Pernille V. Rasmussen, Mikkel E. Thomsen, Daniel L. Justesen, Henrik C. Schønheyder, Mads Lausen, Svend Birkelund. Microbes and Infection 22 (2019) 19-30

Study II: “Complement killing of clinical *Klebsiella pneumoniae* isolates is serum concentration dependent”. Katharina V. Opstrup, Gunna Christiansen, Svend Birkelund. Submitted to *Microbes and Infection*, 2021

Study III: “Beta-lactam induced morphological changes of ESBL-producing *Klebsiella pneumoniae* blood isolates”. Katharina V. Opstrup, Gunna Christiansen, Svend Birkelund. Manuscript.

List of abbreviations

AP	Alternative pathway
C4bp	C4-binding protein
CAZ	Ceftazidime
CP	Classsical pathway
CR	Complement receptor
DAF	Decay accelerating factor
ESBL	Extended-spectrum beta-lactamase
FB	Factor B
FH	Factor H
FI	Factor I
HIHS	Heat-inactivated human serum
IEM	Immuno-electron microscopy
IMB	Immunoblotting
KL	Capsular locus type
KPC	*K. pneumoniae* carbapenemase
LP	Lectin-mediated pathway
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein
mDAP	Meso-diaminopimelic acid
MLST	Multi-locus sequence-typing
MRP	Meropenem
NHS	Normal human serum
OMP	Outer membrane protein
pAb	Polyclonal antibody
PAMP	Pathogen associated molecular patterns
PBP	Penicillin binding proteins
PRM	Pattern recognition molecule
PRR	Pattern recognition receptor
ST	Sequence type

TEM	Transmission electron microscopy
TLR	Toll-like receptor
UTI	Urinary tract infection
WGS	Whole genome sequencing

Introduction

1 *Klebsiella pneumoniae*

In 1882, Carl Friedländer described a pathogen that caused acute pneumonia, which was later named *Klebsiella pneumoniae* after the German microbiologist, Edward Klebs, who had discovered it in 1875 [1,2]. *K. pneumoniae* is a Gram-negative, facultative anaerobe, rod-shaped, non-motile member of the family *Enterobacteriaceae* measuring approximately 1 x 1,5-3 μm. It exists ubiquitously in nature [3–5]. It is a common inhabitant of the gastrointestinal tract of humans and animals, and colonizes most mucosal surfaces [2,5].
As many early discovered microbes, the genus of *Klebsiella* has been reclassified over the years, and with whole genome sequencing becoming the method of choice, the genus today holds several species with *K. pneumoniae* being the type species. In the past decade, genome analysis has divided the *K. pneumoniae* complex into six different phylogroups (KpI-VI) [6–8]. These closely related phylogroups share common features, cause human disease, and routine typing by MALDI-TOF has been shown to fail in distinguishing between *K. pneumoniae sensu strictu (KpI)*, *K. variicola (KpIII)* and *K. quasipneumoniae (KpII and Kp IV)* [**7**,8]. In the following, the type species *K. pneumoniae* is described.
The genome size of *K. pneumoniae* is approximately 5.5 Mb, and the pan genome is comprised of 5000-6000 genes [6,**9**]. The core genome of *K. pneumoniae* consists of approximately 2000 conserved genes, which are present in more than 95% of the *K. pneumoniae* population [6]. The remaining genes, the accessory genome, vary amongst isolates, and can be located on plasmids or the chromosome, and the diversity is great, as more than 30.000 genes have been described. These genes include virulence factors and antibiotic resistance genes, and underline the diversity of *K. pneumoniae* [2].

1.1 *K. pneumoniae* epidemiology

Originally described as an opportunistic agent of community-acquired pneumonia, *K. pneumoniae* has been a leading cause of nosocomial infections including sepsis, bacteremia, pneumonia and urinary tract infections (UTI) over the last decades [5,10]. In Denmark, the number of invasive cases of *K. pneumoniae* has been increasing steadily since 2010 with an increase from 14.4 cases pr. 100.000 inhabitants to 23.4 cases pr. 100.000 inhabitants in 2019 [11]. Generally reported as the second leading cause of Gram-negative blood stream infections, *K. pneumoniae* is also a common cause of nosocomial pneumonia [2].
The transmission of *K. pneumoniae* in the hospital setting happens primarily from

colonized patients or from contaminated hands of health care personnel [5]. In a study conducted in an Australian intensive care unit, up to 63% of the patients were colonized with *K. pneumoniae* upon admission, and 50% of the subsequent infections with *K. pneumoniae* were caused by the strain carried by the patient, underlining the importance of colonization [12]. Risk factors for *K. pneumoniae* bacteremia include cancer, chronic liver disease, dialysis and diabetes [13].

Despite most infections occurring in immunocompromised individuals, the first reports of so-called hyper-virulent variants as opposed to the classical *K. pneumoniae* infections emerged in the 1980's in Asia [14]. These strains appear to cause community-acquired infections in healthy younger individuals, causing liver abscesses, pneumonia, meningitis and endophthalmitis, and today, cases have been observed worldwide [15,16].

Multi-locus sequence typing (MLST) of seven house–holding genes have been widely used to track *K. pneumoniae* infections. Many different sequence types (ST) are reported as causes of multidrug-resistant *K. pneumoniae* infections, including the extended-spectrum beta-lactamase (ESBL) and carbapenemase producing isolates [**7**,17,18].

1.2 *K. pneumoniae* infections and the innate immune system

To infect the host, *K. pneumoniae* must overcome the first line of defense; either the skin or mucosal surfaces in order to reach deeper tissues. Medical devices, such as catheters, present a possibility for biofilm production and breaking of mucosal surfaces, giving access of multiplying rapidly in situ, and establishing a urinary tract infection (UTI). Furthermore, *K. pneumoniae* UTIs are reported to have a higher association with catheters than *E. coli* [19]. In pneumonia, aerosol transmission has been demonstrated, but *K. pneumoniae* infections are linked to direct transmission, aspiration pneumonia and ventilator-associated pneumonia [20–22]. Since *K. pneumoniae* does not produce exotoxins, the inoculum plays an important role in the severity of infection, and the ability of the bacteria to quickly multiply within the host is essential [20].

K. pneumoniae is primarily an extracellular pathogen, but it can survive in macrophages and in lung epithelial cells [23,24]. Before and after crossing the physical barriers, pathogens are met by antimicrobial peptides such as defensins, produced primarily by epithelial and neutrophil cells and collectins, e.g. lung surfactant proteins [25,26]. Once having crossed the first line of defense, an intruding pathogen is confronted by cellular mechanisms such as phagocytic cells, dendritic cells and the humoral defenses, where complement has a pivotal role in opsonophagocytosis and direct killing of Gram-negative bacteria [27–29]. The complement system will be presented in detail in section 2.

The pattern recognition receptors (PRR), which recognize the pathogen-associated molecular patterns (PAMPs) on the surface of the bacterium inducing a cytokine

response, are an important part of the innate immune system [30]. PRRs are present in immune cells such as phagocytic cells and lymphocytes, but also endothelial and epithelial cells, and can be located on the cell membrane or in the cytosol depending on function. Examples of important PRRs involved in recognition of bacteria are the toll-like receptors (TLR) and the nod-like receptors [30,31].
The recognition induces activation of macrophages and dendritic cells, which can secrete pro-inflammatory mediators. Chemokines such as IL-8, induced by IL-1β, and cytokines e.g. TNFα and IL-6, initiate inflammation and attract neutrophils and monocytes to the site of infection, and are essential for the defense against *K. pneumoniae* [32,33]. From the primary site of infection, such as UTIs or pneumonias, *K. pneumoniae* can spread to the blood stream, causing bacteremia, and in some cases, sepsis [**7**,**9**,34]. Sepsis is a complicated pathogenesis mechanism defined by severe organ malfunction caused by a dysregulated host immune response to infection with high mortality [35].

1.3 *K. pneumoniae* surface morphology

The surface of *K. pneumoniae* is highly diverse, with prominent virulence factors such as capsule, lipopolysaccharides (LPS), fimbriae and outer membrane proteins (OMP). In the following, the well-described surface virulence factors, the capsule, the LPS and the OMPs will be covered in more detail.

1.3.1 The capsule and capsular typing

K. pneumoniae produces a polysaccharide capsule protecting the outer membrane surface (Fig. 1). The acidic, negatively charged capsule consists of repeat sugar units, also known as K-antigen, and vary in composition, depending on the combination of polysaccharides, such as mannose or rhamnose [10]. Colonies present with a mucoid appearance on agar, and the capsule is visible when examined by negative staining (Fig. 1) [**7**,36].
The capsule is essential for virulence as it protects the bacterium from phagocytosis and in some instances, complement-mediated killing [**9**,37,38]. Strains lacking capsule adheres better to cell surfaces, but appear to be avirulent in infection models [36]. Studies have shown that the capsule is pivotal for causing pneumonia in murine models [39,40].

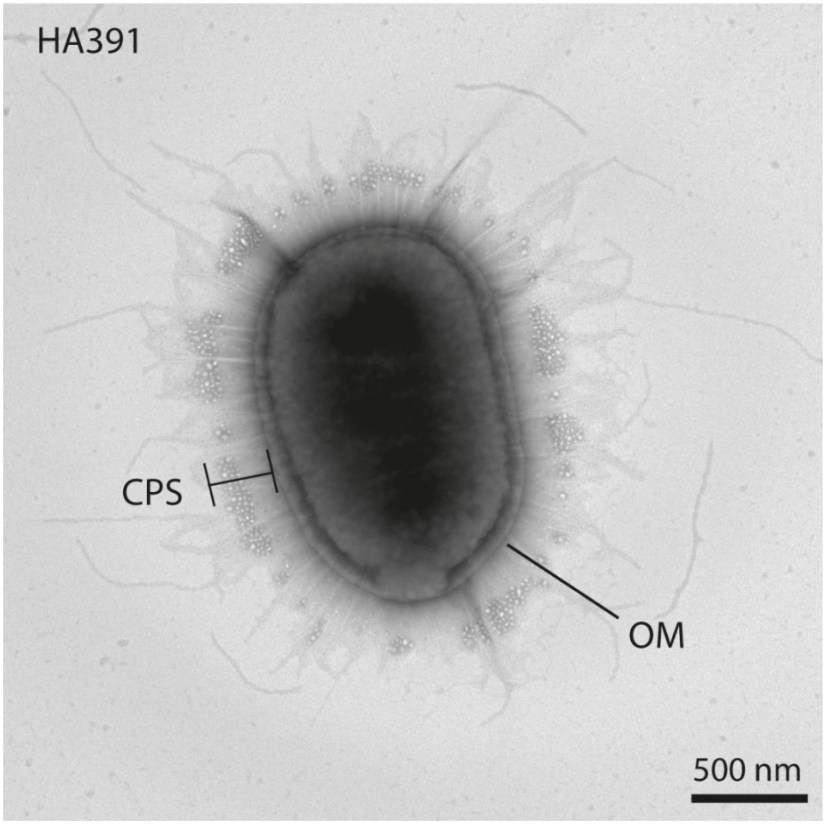

Figure 1. Electron micrograph of K. pneumoniae isolate HA391, grown on Müller-Hinton agar, visualized by negative staining*. Capsule, LPS, fimbriae and outer membrane vesicles are visible together with the distinct outer membrane.*

Serotyping of the *K. pneumoniae* capsule has been performed since the beginning of the 20th century, and 77 serotypes have been described, and named K1-K77 [41,42]. Capsular typing based on the wzi-protein, an essential capsule assembly enzyme, has been proposed as an alternative to serotyping. This approach is based on polymerase chain reaction followed by sequencing of the highly conserved *wzi*-gene of the capsule synthesis locus (cps or K-locus) [**9**,43]. The use of whole genome sequencing (WGS) has made in silico capsular typing of the capsule synthesis loci (K-loci) possible, and revealed great diversity of distinct capsular types. This approach has so far identified more than 130 K-locus (KL) types, correlating the serotypes K1-K77 with the KL-types, and typing isolates not typed by serology [**7**,**9**,44,45].
The genes encoding the capsule split into two principal groups. One group consists of shared conserved genes such as *wzi, wza,* and *wzb* encoding translocation and

assembly, and the other group of specific genes which encode biosynthesis, thus conveying the diversity of capsules [41]. Some capsular types have been linked to serum resistance, namely K1 and K2 [45]. These capsular types are associated with hyper-virulent strains of *K. pneumoniae*, causing liver abscesses in healthy individuals, but also to a highly successful carbapenemase-producing clone, ST258 [15,46].
However, the number of capsule types found in blood stream infections, pneumonia and urinary tract infections is vast, and infection with *K. pneumoniae* is not limited to certain capsular types, thus complicating the development of vaccines targeting capsule antigen [**7**,10,47].

1.3.2 Structure of the *K. pneumoniae* cell envelope

Covered by the polysaccharide capsule, the *K. pneumoniae* cell envelope is a complex construction consisting of an outer membrane, the periplasm containing the peptidoglycan layer and the inner cytoplasmic membrane (Fig. 2) [48].
The outer membrane is an asymmetrical bilayer of phospholipids in the inner leaflet, and mainly LPS in the outer leaflet together with beta-barrel transmembrane proteins and lipoproteins, which secures integrity of the membrane by anchoring the outer membrane to the peptidoglycan layer [49,50]. This construction allows the outer membrane to function as a superior permeability barrier, but at the same time allowing passive influx of hydrophilic nutrients via the transmembrane proteins [51].
The periplasm is an aqueous space surrounding the inner membrane, which amongst other features offers protection of the cytoplasm of toxic compounds, assists in cell division machinery, protein folding and transportation, secretion of LPS and outer membrane proteins to the outer membrane [52].
The peptidoglycan layer, made of glycan strands based on repeats of disaccharide N-acetyl glucosamine-N-acetylmuramic acid, and cross-linked by short peptides, protects the bacterium from bursting, and maintains the structure of the cell (Fig. 2) [53].

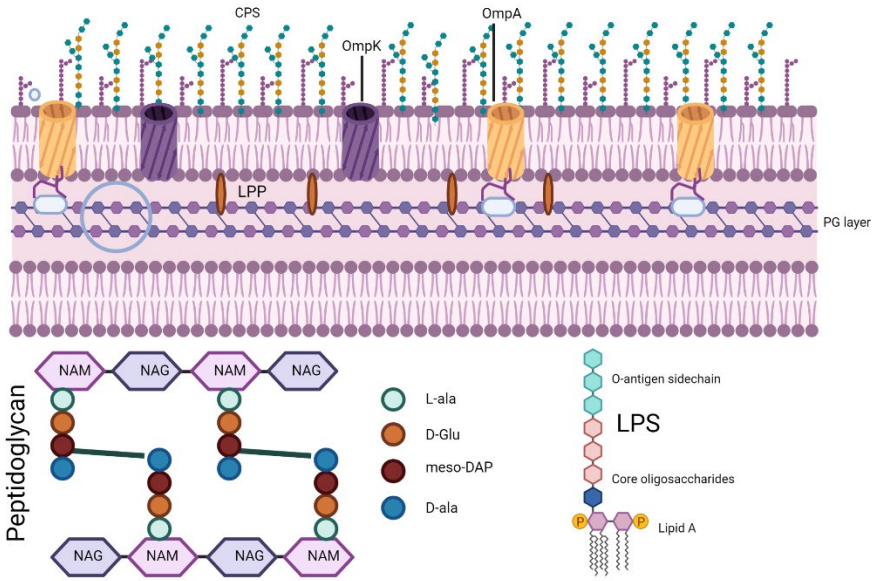

Figure 2. Cell envelope and surface structures of K. pneumoniae. *The cell envelope of K. pneumoniae is covered by the polysaccharide capsule. The asymmetrical bilayer of the K. pneumoniae outer membrane consists of an inner leaflet of phospholipids, and an outer leaflet of mainly lipopolysaccharides (LPS), beta-barrel proteins such as OmpK and OmpA, and lipoproteins (LPP). The LPS is composed of three subunits, the Lipid A, the core oligosaccharide and the O-antigen. The periplasmic domain of OmpA, an abundant OMP in K. pneumoniae, is linked to the peptidoglycan layer. The LPP secures the outer membrane by anchoring it to the peptidoglycan layer, which is located in the periplasmic space protecting the inner cytoplasmic membrane. The peptidoglycan layer is made of repeat glycan strands of NAG (N-acetyl glucosamine) and NAM (N-acetyl muramic acid), cross-linked by short peptides. D-ala: D-alanine, L-ala: L-alanine, D-Glu: D-glucosamine, meso-DAP: meso-diaminopimelic acid. Created with BioRender.com*

1.3.2.1 Lipopolysaccharides and O-typing

K. pneumoniae produces, like other Gram-negative bacteria, LPS, also known as endotoxin, which stabilizes the outer membrane by forming the outer leaflet of the outer membrane, acts as a permeability barrier and protects against host recognition [54]. The LPS consists of three parts; the lipid A, the core oligosaccharide and the O-antigen sidechain (Fig. 2). The hydrophobic lipid A anchors the LPS in the outer membrane, and the core oligosaccharide links the hydrophilic O-side chain, also known as O-antigen.

Lipid A is recognized by TLR4, and is considered to be a PAMP, whereas the O-antigen

can be recognized by antibodies [55]. The polysaccharide part of LPS protects the bacterium, but the O-side chain is not necessary for the growth of the bacterium [55]. The O-side chain is a highly variable region, which makes the basis for serotyping of the O-antigen. Nine O-types based on the O-antigen are described for *K. pneumoniae*, with serotypes O1, O2, O3 and O5 accounting for the majority of infections worldwide [**7**,41,47].
WGS has made in silico O-typing based on the serotypes accessible in recent years [**7**,45]. The repeating patterns of sugars generate the variety of the LPS, but Lipid A modifications can protect the bacterium from innate host recognition and killing by antibiotics targeting Lipid A, such as colistin, a peptide antibiotic used as a last resort drug for carbapenemase producing isolates [56,57].

1.3.2.2 Outer membrane proteins

The vast majority of integral transmembrane outer membrane proteins, referred to as OMPs, is composed of the beta-barrel proteins, which are made of beta-sheets, bundled into a cylinder, and vary in number of strands [48]. Together with the lipoproteins, which secure the outer membrane to the peptidoglycan layer, the transmembrane proteins constitute about 50% of the outer membrane mass [50]. Porins are strictly speaking non-specific β-barrel protein channels, allowing influx of nutrients. However, the use of porin is also used to address specific protein channels such as LamB and PhoE [51,58].
Two major OMPs in *K. pneumoniae*, OmpK35 and OmpK36, are co-regulated porins responsible of nonspecific influx of nutrients, but also other hydrophilic molecules such as beta-lactam antibiotics [59]. Loss of OmpK porins has been correlated to an increase in resistance against beta-lactam antibiotics, and in some cases, a decrease in virulence [**4**,60,61]. Complement activation by OmpK has been demonstrated via C1q binding [62].
OmpA, a highly conserved OMP in *Enterobacteriaecae,* is massively represented in the Gram-negative outer membrane with a copy number of approximately 100.000 pr. cell, and stabilizes the outer membrane by linking to the peptidoglycan layer [50,63,64]. It is involved in adhesion and invasion of eukaryotic cells, induces inflammatory responses, works as bacteriophage receptor, protects against collectins, and is associated with resistance against antimicrobial peptides of the innate immune system [65–67]. Host defenses targeting OmpA include serum amyloid A binding to OmpA, which promotes phagocytosis [68]. Furthermore, OmpA has been shown to mediate serum resistance by binding of complement regulator C4b-binding protein (C4bp) [69,70].

1.4 Antimicrobial drug resistance

In 2017, third-generation cephalosporin- and carbapenem-resistant *Enterobacteriaceae* were named critical pathogens by the World Health Organization [71]. *K. pneumoniae* infections are becoming increasingly difficult to treat, as resistance against the most used and effective antibiotics steadily rises [72]. The selective pressure resulting from the extensive use of antibiotics has generated multi-drug and pan-resistant isolates, and horizontal gene transfer trough mobile genetic elements has been a pivotal factor for the rapid dissemination of resistance genes within the *Enterobacteriaceae* [**4**,73,74]. Multidrug-resistant isolates are resistant to at least one agent in three different classes of antibiotics, limiting the treatment options to very few drugs [**4**,75].

1.4.1 The beta-lactam antibiotics and penicillin binding proteins

The beta-lactam class of antibiotics has been developed through the years and include the penicillins, the cephalosporins, the monobactams , and the carbapenems [76]. It remains a very important and widely used class of antibiotics due to the broad-spectrum activity and the safety of use [76]. The first penicillins were natural products of fungus, notably *Penicillinum notatum*, described by Alexander Fleming. The first large scale production of penicillin during World War II revealed that different fungi under varying conditions could produce different active compounds. The beta-lactam ring was recognized as a common structure for the penicillins in the mid-1940's [76]. The introduction of methicillin in 1960 by chemically adding a side-chain to the beta-lactam ring became the breakthrough for semi-synthetic production of beta-lactams. However, natural compounds were still explored, leading to the discovery of cephalosporin in 1961, and semi-synthetic derivatives from this group presented a new group with broad-spectrum activity [77]. The important broad-spectrum carbapenem group was discovered in the search for beta-lactamase inhibitors in the 1970's as antibiotic resistance towards the beta-lactams increased. Thienamycin, produced by Streptomyces, became the parent compound of the carbapenems, and since then, several semi-synthetic compounds have been produced [78].

The beta-lactam class of drugs works by inhibiting the bacterial cell wall in the final steps of peptidoglycan synthesis by binding to enzymes known as penicillin binding proteins, the transpeptidases (PBP), which are attached to the cytoplasmic membrane [53,79]. The peptidoglycan layer is composed of alternating N-acetylmuramic acid and N-acetylglucosamine chains cross-linked by peptides (Fig. 2) [53]. Biosynthesis of the peptidoglycan layer happens in three main stages. In the cytoplasm, the monomer precursor uridine diphosphate *N*-acetylmuramic acid pentapeptide (UDP-Mur*N*Ac pentapeptide) is synthesized. The conversion of the

UDP-MurNAc pentapeptide to lipid II, the last monomeric precursor, happens at the cytoplasmic membrane. Next, lipid II is flipped to the periplasmic side of the cytoplasmic membrane. Here, the glycosyltransferase PBPs polymerize the glycan strands from lipid II, and the transpeptidase PBPs cross-link the peptides of adjacent glycan strands by binding to the C-terminal D-Alanine D-Alanine moiety of the N-acetylmuramic acid pentapeptide to meso-diaminopimelic acid (meso-DAP) side chain amino group [53,80]. The beta-lactam antibiotics are sterically similar to the D-Ala D-Ala terminal, and covalently acylate the serine residues of PBPs, thus preventing the cross-linking of peptides [81,82].

Two main peptidoglycan synthesis complexes are suggested to be responsible for the cell elongation and division, named the elongasome and the divisome [83,84]. Although not completely elucidated, the active complexes, which can move along the cell membrane, are comprised of different proteins such as cytoskeletal proteins, membrane proteins, lipid II synthesis proteins, the synthases (PBPs), regulators and hydrolases. The elongasome mediates side wall cell envelope growth, and the divisome is responsible for growth and separation at the cell division site [85]. In *E. coli*, 12 different PBPs are described, but it was only recently that six PBP sequences for *K. pneumoniae* were confirmed [86].

The three most important PBPs, the high molecular mass PBPs, are PBP-1, -2 and 3. Low molecular mass PBPs include PBP-4, -5 and -6 for *K. pneumoniae* [86]. PBP-1a/b is essential for cell growth and division, has both transpeptidase and glycosyltransferase effect, and blocking leads to rapid cell death [81]. PBP-2, which only possess transpeptidase properties, is part of the elongasome, which maintains the rod-shape of the bacterium [83]. PBP-3 is involved in cell division as an essential protein of the divisome [81].

The beta-lactams have affinity for different PBPs, and this affinity can vary between bacterial species [87]. Ceftazidime (CAZ) is a third-generation cephalosporin, which is widely used in the clinic for serious infections such as bacteremia [**4**]. In *K. pneumoniae*, CAZ has an affinity for PBP-1 in high concentrations, and PBP-3 in lower concentrations [86]. Meropenem (MRP), which is a carbapenem, and used for treating ESBL-producing *K. pneumoniae*, was shown to bind all known PBPs in *K. pneumoniae* with a high affinity for PBP-2 and PBP-4 [86].

1.4.2 Resistance against beta-lactam antibiotics

Antimicrobial drug resistance has been recognized ever since the discovery of antibiotics, and every time a new drug has been introduced; resistance has been reported shortly after. The beta-lactamases, which hydrolyze beta–lactams depending on spectrum, are the most important mechanism of beta–lactam resistance. Other mechanisms of beta-lactam resistance in *K. pneumoniae* include but are not limited to efflux pump systems and alterations in outer membrane

permeability by loss or modification of OMPs [78,88]. The RND (resistance-cell-nodulation-division) family that include the AcrAB and OqxAB pumps are often expressed by clinical *K. pneumoniae* isolates [89,90]. Efflux pumps work by actively pumping toxic compounds out of the bacterial cell, and up-regulation under antibiotic pressure can contribute to increased resistance [90].

The beta-lactamases constitute more than 2000 unique enzymes [91]. They have been classified by two different schemes, based either on substrate hydrolysis (Bush-Jacoby group 1-4) or amino acid homology (Ambler classes A-D) [92]. The class A TEM (TEMoinera) and SHV (SulfHydryl reagent Variable) family and the class D OXA (OXAcillin) family is abundant in *K. pneumoniae*. The beta-lactamase (*bla*) encoding genes *bla*TEM, *bla*SHV and *bla*OXA are found on plasmids and in the chromosome of *K. pneumoniae* [**4**,91,93,94].

In 1983, the first extended-spectrum beta-lactamases (ESBL) were described [95]. Originally derived from point mutations from classic plasmid borne SHV-1, TEM-1 and 2 and OXA-10- β-lactamases, the ESBLs hydrolyze the oxy-imino cephalosporins (e.g. the 3rd generation cephalosporin CAZ) and monobactams, but cannot hydrolyze carbapenems or cephamycins [92]. They are largely susceptible to beta-lactamase inhibitors e.g. clavulanic acid, and this trait is utilized when phenotypically screening for the presence of ESBLs by testing for synergy between a third generation cephalosporin and clavulanic acid. However, the class D OXA family is not inhibited by beta-lactamase inhibitors in general.

Today, the CTX-M (CefoTaXime-Munich) family, a class A ESBL, has outcompeted the SHV, TEM and OXA ESBLs in general [93,94]. The CTX-M is believed to be derived from *Kluyvera* spp., an intestinal commensal and spread via mobile genetic elements to the *Enterobacteriaceae*. Initially, the CTX-M enzymes strongly hydrolyzed cefotaxime, but not CAZ. Nonetheless, some CTX-Ms such as the dominant CTX-M-15 also hydrolyze CAZ [96,97]. More than 170 variants of CTX-M have been identified. The evolvement of the CTX-M family may have happened via point mutations and horizontal gene transfer after the introduction into the *Enterobacteriaceae* [98].

Co-selection of resistance has facilitated the dissemination of ESBLs, as selective pressure of one antibiotic agent selects for resistance towards another agent by carriage of multiple resistance genes on one plasmid, such as genes encoding the ESBLs and aminoglycosides resistance genes [**4**,99]. *K. pneumoniae*, showing a high permeability of plasmids, has been involved in the spread of resistance plasmids within the species and to other pathogens, such as *E. coli* [100,101]. In Denmark, the most widespread ESBL is CTX-M-15, but decreasing rates of ESBL-producing *K. pneumoniae* isolates have been observed the past decade probably due to the antimicrobial stewardship [**4**,11].

However, the spread of multidrug-resistant isolates combined with an increase of invasive isolates, and the outbreak of carbapenemase–producing Enterobacteriaceae in other parts of the world give rise to concern [**4**,91]. The carbapenems have been a critical first choice treatment option for ESBL-producing *K. pneumoniae* isolates [102]. The carbapenemases hydrolyze most beta-lactams

including the carbapenems. The widespread class A carbapenem, KPC (*K. pneumoniae* carbapenemase) was first identified in *K. pneumoniae*, and plasmid-mediated transfer to *E. coli* has led to KPC being the leading cause of carbapenem resistance in the United States. It is noteworthy that KPC not only hydrolyze the carbapenems, but in addition most beta-lactamase inhibitors such as tazobactam, sulbactam and clavulanic acid [91,94].

In the European Union, the emergence of carbapenem-resistant *K. pneumoniae* varies between countries. The percentage of invasive carbapenem-resistant *K. pneumoniae* in the Northern countries is below 1% in 2017, but in Italy between 25-50% and in Greece > 50%. However, as this only includes invasive isolates from blood an cerebrospinal fluid, a self-assessment survey by national experts of carriage and other infections have shown an evolving pattern of carbapenem-resistance within several countries, including Denmark, listed at the inter-regional stage just below an endemic stage [103]. This calls for attention, as mortality rates for carbapenem-resistant *Enterobacteriaceae* in bloodstream infections have been reported as high as over 50% [104].

2 The complement system

Acting as one of the key players in the humoral innate immune system, the complement system protects the host from intruding pathogens as a first line of defense, and acts as a link between innate and adaptive immunity. However, research in recent years has revealed a more complex role of complement in the body, with negative effects, such as involvement in autoimmune diseases and cardiovascular diseases [105].

2.1 A proteolytic cascade

In the late 19th century, immunology pioneers at the Pasteur institute found a heat-labile substance in serum, which could kill the anthrax bacilli. Originally named "alexin" by Jules Bordet, when it was discovered in the late 19th century, it was later renamed complement by Paul Ehrlich, as the functions were believed to complement antibodies, the heat-stable component. It is now clear that activation of complement also occurs independently of antibodies and is an ancient protection, well preserved throughout evolution [105–107].

The complement system is a cascade system, consisting of both fluid phase proteins, produced primarily by hepatocytes, and membrane-bound regulators and receptors. However, complement proteins can also be produced locally by other cell types, including immune cells [108]. Several of the proteins of the complement system are zymogens, also named proenzymes [109]. Complement proteins are present in considerable concentrations in plasma but are also present in other body fluids and tissue under normal physiological conditions, with tissue concentrations increasing under inflammatory conditions [108,110].

The proteins, from C1 and onwards, are named in the sequence of their discovery, and not their place in the activation cascade. For example, C2 was discovered before C4, and even though C4 precedes C2 in the activation cascade, the nomenclature has been maintained to avoid confusion [109]. However, recent updates concerning the naming of the C2 fragments, have changed the designation of the large fragment to C4b and the smaller fragment to C2a, in consistency with the other fragments [111].

The complement system works as a proteolytic cascade of zymogens, enzymes and their substrates, which are activated after recognition of foreign surfaces such as bacteria.

The activation of the complement cascade can generate direct killing of Gram-negative bacteria via the membrane attack complex (MAC). Surface bound cleavage products e.g. iC3b and C3dg opsonize the pathogen for phagocytosis. Furthermore, other non-surface bound cleavage products, the anaphylatoxins C3a and C5a work as important inflammatory mediators [**9**,112,113]. Three activating pathways are currently known; the classical (CP), the alternative (AP) and the lectin-mediated

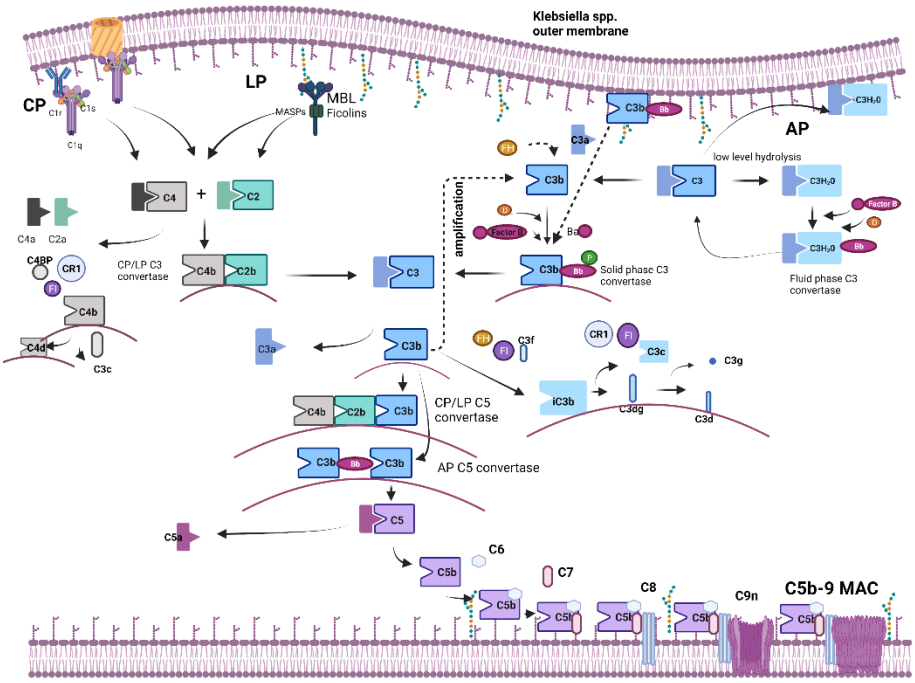

Figure 3. Complement activating pathways. *The classical pathway (CP) is activated, when C1q recognizes antibody bound to the bacterial surface or pathogen associated molecular patterns (PAMP) e.g. outer membrane proteins (OMP). The lectin pathway (LP) is activated collectins or ficolins recognizing carbohydrate moieties. CP and LP activation results in generation of a classical C3 convertase, C4bC2b, by cleavage of C4 and C2. If not engaging in a CP/LP C3 convertase, C4b can be cleaved into C4d, mediated by complement receptor 1 (CR1) and Factor I (FI). The alternative pathway (AP) is constantly low-level self-activating due to spontaneous hydrolysis of the C3 internal thioester domain, and C3(H_2O) can bind Factor B (FB). Upon cleavage of FB by Factor D (FD), an unstable fluid phase C3 convertase, C3(H_2O)Bb is formed. The C3 convertases cleave C3 into C3a and C3b. There are three known outcomes of C3b: 1. It can bind FB to C3bBb, which stabilized by properdin generate a solid phase C3 convertase, thus initiating the amplification loop. 2. It can be cleaved into iC3b and C3dg, which function as opsonins. The C3b cleavage is mediated by Factor H (FH), FI, and CR1. 3. C3b can generate C5 convertases by binding to the C3 convertases. The C5-convertases cleave C5 into C5a and C5b. Together with C6-9, C5b generate the membrane attack complex (C5b-9), which, when in close proximity to a membrane, can form pores of approximately 10 nm leading to rapid lysis. Created with BioRender.com*

pathway (LP), which lead to cleavage of the key component of complement, namely C3 via the CP/LP C3 convertase or the.C3 convertase (Fig. 3) [**7**,**9**]**.** In normal human serum (NHS) the complement system is active, which can be utilized in testing complement killing of bacteria in vitro, as the complement system can be inactivated by heating serum to 56° C, named heat-inactivated human serum (HIHS) [**7**,**9**].

2.1.1 Classical and lectin mediated pathways

The CP and LP are initiated by different pattern recognition molecules (PRM), however structural similarities of the involved PRMs exist. The CP was in the beginning the only recognized complement activation pathway and it was initially believed to be only antibody-dependent. Despite the name, the CP is evolutionary younger than the AP, as the C1 complex has only been identified in higher vertebrates [114]. C1 is not synthesized in the liver, but by monocytes, macrophages and immature dendritic cells. Activation occurs when C1q binds to antibodies bound to foreign surfaces (antibody-dependent activation). Additionally, C1q can recognize and bind to PAMPs such as OMPs or LPS, or to other mostly charged targets (antibody-independent activation) [**9**,110].
The complex of C1 exists in an inactive form, consisting of six molecules of the PRM C1q and two molecules of the serine proteases C1r and C1s, and the activation is Ca^{2+} dependent [114]. The C1q molecules are heterotrimers, each composed of C1qA, C1qB and C1qC chains [115] The binding of C1q to e.g. surface bound antibody mediates the C1r dependent activation of C1s, which cleaves C4 into C4a and C4b, and C2 into C2a and C2b, generating the classical C3 convertase, C4b2b [**7**,111]. C4b contains an active thioester bond, which covalently can bind to adjacent surfaces (Fig. 3) [**9**].
The LP was discovered in the late 1980's, when low levels of mannose-binding lectin (MBL) were linked to immune deficiencies in children and reduced levels of opsonophagocytosis [116,117]. Two main types of PRMs exist for the LP, the collectins and the ficolins. The collectins, such as MBL, consist of a collagenous region with a C-type lectin recognition domain, and primarily recognize carhohydrate structures on pathogen surfaces. The ficolins, comprised of a collagen-like and fibrinogen-like region, are in humans represented by H-ficolin, L-ficolin and M-ficolin [118]. The ficolins primarily recognize acetyl groups.
After binding of e.g. MBL to a carbohydrate group on the pathogen surface, the co-circulating MBL-associated serine proteases -1,-2, and 3 (MASPs) are activated and the MBL/MASP complex subsequently generates the common classical C3 convertase, C4bC2b. MASPs are structurally similar to C1r and C1s, and the structure of MBL reminisces C1q [119]. MASP-1 activates MASP-2, and while MASP-1 and MASP-2 can cleave C2, MASP-2 activation is necessary for C4 cleavage [109]. Although MASP-2 can auto-activate, MASP-1 activation seems to be pivotal for LP

activation [120]. The function of MASP-3 is not completely elucidated, but together with MAp44 (MBL-associated protein) it has been proposed that they bind MBL competitively of MASP-2, acting as negative regulators [121]. However, recent studies have demonstrated a role of MASP-3 as an activator of complement pro-factor D, thus linking the LP and AP [122].

2.1.2 The alternative pathway

The AP is evolutionary the oldest complement activating pathway, but it was not until the 1950's that Pillemer and coworkers suggested an alternative activation of complement independent of antibody. Initially described as the properdin pathway, some controversy was present regarding the theory and it was not widely accepted. The pathway was named the alternative pathway 20 years later, and properdin was recognized as a stabilizer of the alternative pathway [123].
The Mg^{2+} dependent activation of the AP is in contrast to the LP/CP not dependent on PRMs, but on spontaneous low-level hydrolysis of C3. C3 contains a labile internal thioester bond, and therefore C3 is continuously hydrolyzed to an unstable C3 convertase, C3(H_20). During this hydrolysis, the conformational change of the thioester reveals a binding site for factor B, which upon binding is cleaved by Factor D, a serine protease, to Bb and Ba, forming an unstable fluid phase C3 convertase, C3(H_20)Bb. This C3 convertase cleaves C3 to C3a, a potent pro-inflammatory mediator, and C3b, which via its thioester domain can covalently bind to nearby surfaces containing amino or hydroxyl groups [**9**,112,124].
After C3b binding to a surface, factor B binds, which is then cleaved by Factor D to Bb and Ba, in the same manner as the fluid phase convertase [**9**,125]. This yields a surface bound C3 convertase, C3bBb, which has a short half-life of 90s [110]. Properdin, secreted by monocytes, macrophages and neutrophils can stabilize the membrane-bound C3 convertase, aiding the amplification loop as the surface bound C3bBb can yield even more C3b.
Hence, the AP works as an amplifier of the CP/LP as the CP/LP generated surface bound C3b can participate in the feedback amplification loop [126]. Properdin might have an activator role in the AP as it possibly binds directly to microbial surfaces, initiating C3 convertase assembly [127]. However, this suggestion has been challenged by Harboe et al. who showed that binding of properdin to surfaces was C3b-dependent [128].

2.2 The C5 convertases and the terminal pathway

Upon C3 cleavage by the C3 convertases, C3b can bind to the surface bound C3

convertases, yielding the C5 convertase, C4b2aC3b and C3bBbC3b respectively, which cleaves C5 into C5a, a potent pro-inflammatory mediator, and C5b, which initiates the common terminal pathway, leading to the formation of MAC [**7**,**9**]. The conformational change of C5b reveals a labile binding site, which can bind C6. This conformation is kept throughout the assembly of the rest of the complex [129]. C5bC6 binds reversely to the surface, and C7 then binds to C5bC6, making the complex lipophilic. The C5b-7 complex is anchored in the outer membrane of the Gram-negative bacteria, and subsequently, C8, a heterotrimeric consisting of three subunits, is essential for insertion of the complex into the membrane, rendering unstable pores [**9**,129]. The MAC is completed by C9 binding to the C8α-subunit, yielding several stable pores via polymerization (Fig.3) [112]. The pores are approximately 10 nm, and rapid lysis follows [**9**,112].

2.3 Regulators of complement

The proteolytic fragments of C3, C4 and C5 are essential for the subsequent immunological response. In addition to continuing the cascade by C3b, C4b and C5b, the fragments are involved in facilitating phagocytosis e.g iC3b, mediating an inflammatory response (the anaphylatoxins C3a and C5a), and stimulating B-cells (iC3b and C3d(g)). To protect the host from self-damage, the complement cascade must be tightly regulated.

Complement regulators are present in fluid phase as soluble plasma proteins, and on host cells. CR1, decay accelerating factor (DAF) and membrane cofactor protein (MCP) protect the host cell by inhibiting convertases on the membrane [110]. If convertases are deposited on host cells, these membrane-bound receptors rapidly dissociate the convertases, preventing further activation. Intruding pathogens do not have these membrane-bound receptors, and thus, activation can proceed.

CR1 and MCP also work as cofactors for Factor I (FI)-mediated proteolysis of C3b and C4b [110]. The soluble factor H (FH) inhibits the alternative convertase in fluid phase by binding to C3b, and additionally functions as a specific co-factor for FI-mediated C3b inactivation on the cell surface [130]. C4bp is a specific co-factor for FI-mediated C4b inactivation, can prevent convertase formation by binding to C4b and accelerate deterioration of the convertase [131]. FI is a soluble plasma protein, and essential for the cleavage of C3b and C4b, respectively, in the presence of the mentioned co-factors (Fig. 3) [109,110].

If products of the final lytic complex (C5b-7, C5b-8 and C5b-9) are generated in soluble phase, the regulators clusterin and vitronectin can bind to the terminal fragments preventing further binding to membranes. Furthermore, the binding of C8 to non-membrane bound C5b-7 changes the complex conformation and the pore-forming capacity is lost [110,132].

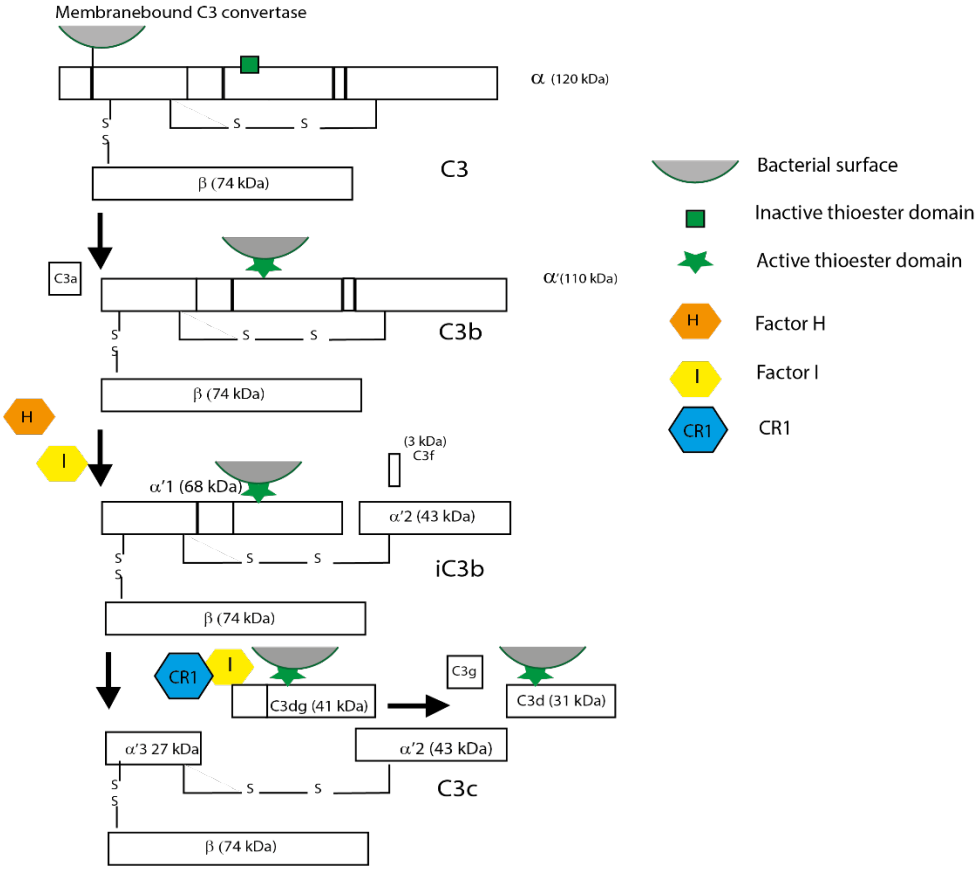

Figure 4. Cleavage of C3. *C3 consists of the α-chain (120 kDa) connected by disulfide bridges to the β-chain (74 kDa). The α-chain contains an inactive thioester domain. Upon cleavage of C3 into C3b, the small anaphylatoxins C3a is released, and the thioester domain on the α'-chain (110 kDa) is exposed. The presence of Factor H facilitates the Factor I mediated cleavage of C3b into iC3b, which contains the α'1 (68 kDa), the α'2 (43 kDa) and the uncleaved β-chain. Complement receptor 1 (CR1) mediates FI cleavage of iC3b into C3dg and finally C3d. C3dg and iC3b function as opsonins, as they remain membrane bound due to the location of the active thioester domain, and are recognized by major CRs.*

2.4 Proteolysis of C3 and C4

C3, the essential protein of the complement cascade, has a molecular weight of 195 kDa, and is composed of two chains, the α-chain (120 kDa) linked by disulfide bridges to the β-chain (74 kDa) (Fig. 4) [**7**,9,133]. After cleavage by the C3-convertase, C3b

consists of the α'chain (110 kDa) where C3a (9 kDa) has been cleaved off, and the intact β-chain of 74 kDa [**7**,**9**,133].
C3b can bind to a wide range of cellular surfaces via the thioester domain, but the larger bulk (approx. 90%) of generated C3b remain in fluid phase and undergo rapid degradation. C3b and the generated fragments upon processing, iC3b, C3dg and C3d contain the thioester domain, and if C3b is surface bound, these C3 fragments remain surface bound after cleavage [124].
C3b is cleaved by FI to iC3b depending on the presence of co-factor H, and the generation of additional convertases is prevented. iC3b (183 kDa) is comprised of the α'1- (68 kDa), the α'2-(43 kDa) and the intact β-chain. Thus, the C3f (3 kDa) has been cleaved off the α'-chain [**9**,124]. FI can cleave iC3b further to C3dg (41 kDa) in a CR1-dependent manner. The final product C3d (31 kDa) is present when C3g is cleaved off C3dg (Fig. 4) [**7**,**9**,134].
C4b (191 kDa) is comprised of the α'-chain (86 kDa) and intact β-(75 kDa) and γ-chains (30 kDa). Upon cleavage of C4, C4a is released, leaving an active component C4b (Fig. 3), which can bind to surfaces via the thioester domain on the α'-chain (191 kDa) [**9**,124]. C4b is cleaved by FI and an intermediate C4b (iC4b) is generated before cleavage to C4d (45 kDa) and subsequently C4c (146 kDa) is released. If C4b is surface bound, C4d remains bound (Fig. 3). The function of C4d is not known [135]. iC3b and C3dg are important opsonins, which are recognized by CRs such as the major phagocytic receptor CR3, thus facilitating phagocytosis of opsonized pathogens [110].
.

3 Objectives

The complex pathogenesis of *K. pneumoniae* seems to be based on evasion of the immune system and it is not completely elucidated. An important *K. pneumoniae* virulence factor is the large heterogenic polysaccharide capsule, which facilitates resistance against complement-mediated killing. The complement system is a pivotal part of the innate immune system, which can be activated by intruding pathogens. Upon activation, the products of the proteolytic cascades lead to direct killing via MAC, opsonophagocytosis and generation of inflammatory modulators. However, the mechanisms of serum resistance in *K. pneumoniae* are incompletely understood. Furthermore, the occurrence of MDR *K. pneumoniae* has raised steadily in recent years. More knowledge of the complex interactions between complement mediated host defenses, pathogen and antibiotics is needed, as the treatment of these infections are increasingly difficult.

Therefore, the objective of this project was to examine how clinical *K. pneumoniae* isolates activate the complement system, if serum killing of *K. pneumoniae* was complement mediated, the possible differences in the complement activating pathways and to investigate the interactions of complement and *K. pneumoniae* with beta-lactam antibiotics. Three experimental studies were carried out with the described purposes:

Study I: To determine if and how clinical *K. pneumoniae* isolates activate complement, and to visualize complement interaction with the capsule.

Study II: To analyze how serum concentration affect complement activation of clinical serum sensitive *Klebsiella spp*. and the complement activating pathways of *Klebsiella spp*.

Study III: To study if and how serum influenced beta-lactam treatment of ESBL-producing *K. pneumoniae*, and to characterize the antimicrobial resistance of 30 clinical *Klebsiella spp.* isolates from Northern Jutland.

4 Main outcomes

4.1 Study I

Complement mediated *Klebsiella pneumoniae* capsule changes

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Main outcomes

This study showed that 10 *K. pneumoniae* isolates of different capsular types activated the complement system via the alternative pathway regardless of serum resistance, as deposition of C3-components were demonstrated on all isolates. A serum sensitive isolate activated the classical/lectin mediated pathway as C4 components were deposited on the surface, suggesting that two or more activating pathways were needed for serum killing. Deposition of complement components mediated capsular and morphological changes in serum resistant and serum sensitive isolates. Lysis and membrane attack complexes were shown on the serum sensitive isolate. Thus, this indicated that capsular changes mediated protection against complement killing of *K. pneumoniae*.

4.2 Study II

Complement killing of clinical *Klebsiella pneumoniae* isolates is serum concentration dependent

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Main outcomes

In this study, 30 clinical *Klebsiella spp.* bacteremia isolates were analyzed for serum resistance, complement activation, MLST, O and KL-types. Serum resistance was not linked to a specific MLST, KL- or O-type, and all isolates activated complement in 50% NHS. Complement mediated killing of serum sensitive isolates varied with serum concentration; two isolates were killed in 5% NHS, while six were killed in 30-75% NHS and the presence of MAC was demonstrated.
We showed that complement killing of serum sensitive isolates depended on C4-activation and the deposition of a classical C5-convertase, as serum resistant isolates had C3 deposition, but no C4 deposition. Furthermore, it was shown that the AP was inactive in 5% NHS, thus indicating that deposition of a classical convertase was sufficient for the subsequent killing of the most serum sensitive isolates. Lastly, the classical C5 convertase needed acceleration by the AP to generate MAC on less serum sensitive isolates.

4.3 Study III

Beta-lactam induced morphological changes of ESBL-producing *Klebsiella pneumoniae* blood isolates

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Main outcomes

In this study, we analyzed 30 clinical *Klebsiella spp.* isolates for antimicrobial resistance by phenotypic testing and WGS. Several ESBL-isolates harbored CTX-M-15, and based on serum resistance, MLST and the carriage of CTX-M-15 two *K. pneumoniae* isolates were selected for investigation of the interaction between serum and CAZ/MRP. We showed that morphological changes by CAZ/MRP were induced equally in NHS, HIHS and growth medium. Outer membrane damage by the formation of MAC contributed to killing of an ESBL-producing isolate with CAZ despite phenotypic CAZ resistance.
By immunoblotting, we demonstrated that complement activation was not altered by addition of the antibiotics, and that the serum resistant ESBL-producing isolate did not activate C4 despite being killed by MRP. Thus, two complement activating pathways were needed to generate MAC, and the beta-lactam induced morphological changes did not cause the serum resistant isolate to activate C4. Synergy between complement and CAZ/MRP depended on the serum susceptibility of the isolates.

5 Complement killing of *Klebsiella pneumoniae* blood isolates

K. pneumoniae frequently causes bacteremia in immunocompromised individuals, and the complement system acts as an essential participant in the immediate defense against this opportunistic pathogen. However, the vast majority of clinical *K. pneumoniae* isolates are serum resistant when tested in vitro, thus circumventing the direct complement-mediated killing by MAC [**7**].
The importance of the complement system against intruding pathogens is stressed by the recurring infections in complement-deficient individuals [136]. Uncovering details of the complement activation by *K. pneumoniae* and the possible subsequent generation of MAC is needed to understand more of how *K. pneumoniae* has become an established health-care associated pathogen causing an increasing number of serious infections such as bacteremia. In the following, the complement activation and subsequent complement-mediated killing of *K. pneumoniae* blood isolates will be discussed.

5.1 Component C3 is central for serum killing of *K. pneumoniae*

Complement is considered a first line of defense that can kill to kill susceptible *K. pneumoniae* via MAC within minutes [**9**,136]. Complement component C3 is essential for the complement activation and subsequently, the possible generation of MAC [**9**,15]. Direct killing via MAC of Gram-negative bacteria is an important host protective factor, underlined by the fact that many clinical *K. pneumoniae* isolates are serum resistant, thus evading lysis [**7**,**9**,137].
Serum killing is inactivated when serum is heated to 56°C, as serum sensitive isolates grow unhindered in HIHS (Fig. 5B) [**7**,**9**]. Several studies have focused on whether *K. pneumoniae* isolates are killed in NHS, but not if this is complement mediated [29]. When studying the effect of a C5-specific inhibitor on serum killing of Gram-negative bacteria, it was shown that killing of a serum sensitive *K. pneumoniae* isolate was not only attributed to complement killing and the authors proposed an unknown heat-labile mechanism [138].
However, we demonstrated that serum killing was dependent on active complement with C3, as serum sensitive isolates were unaffected when incubated in C3-depleted serum, thus in this case eliminating the possibility that other heat labile serum proteins may mediate serum killing (Fig. 5A) [**9**,29]. Recently, we showed a slight inhibition of growth in NHS and HIHS compared to growth medium for a serum resistant isolate, indicating that other factors interfere with growth in NHS and HIHS [**4**].

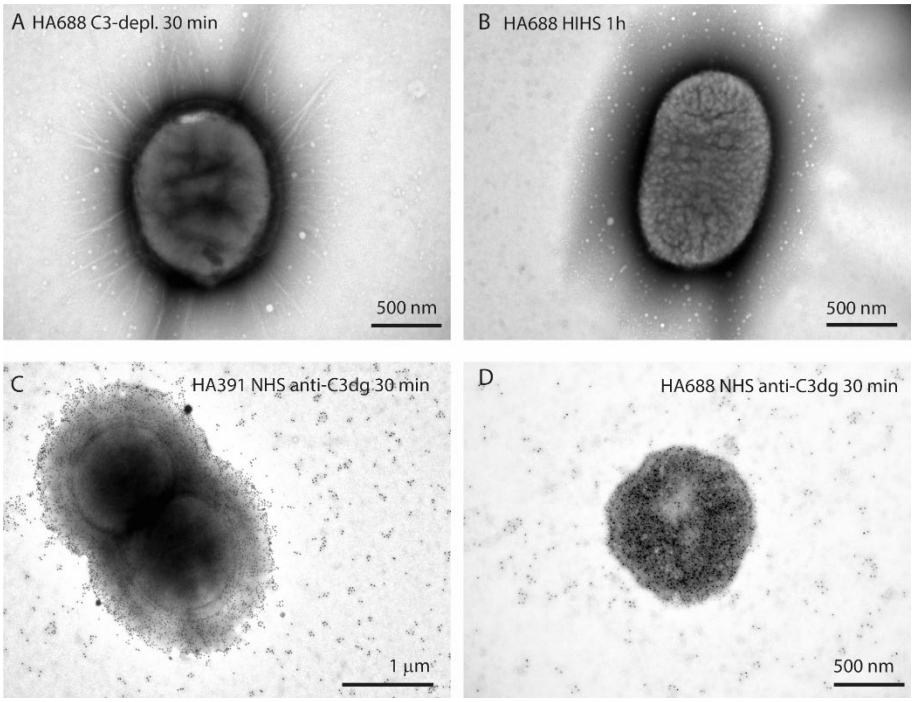

Figure 5. C3-dependent complement activation. *K. pneumoniae isolates were incubated in normal human serum (NHS), heat-inactivated serum (HIHS) or C3-depleted (C3-depl.) serum at 37°C, and processed for transmission electron microscopy (TEM) or immuno-electron microscopy (IEM). A: Serum sensitive isolate HA688 in C3-depl. B: Serum sensitive isolate HA688 in HIHS. C-D: Immuno-stain of serum resistant HA391 and serum sensitive HA688 with anti-C3dg in 50% NHS. Anti-rabbit Ig conjugated with 10 nm immune gold was used as secondary antibody for visualization.*

Components of C3 are deposited on encapsulated *K. pneumoniae* isolates regardless of serum resistance (Fig. 5C, 5D, 6A, 6B) [**7**,**9**,139]. Isolates producing extra capsular materials such as K1 and K2, linked to hyper-virulence, did not deposit C3 [140]. However, this has also been observed for other K-types not linked to hyper-virulence, where serum resistant strains avoided C3 deposition [141,142].

By immuno-electron microscopy (IEM), complement activation of a serum resistant and a serum sensitive *K. pneumoniae* isolate in NHS was visualized using a polyclonal antibody (pAb) against C3-components and gold conjugated secondary antibodies. Both isolates reacted with the pAb C3dg, but the serum resistant isolate primarily had C3 components deposited in the capsule together with extensive capsular changes (Fig 5C and 5D) [**9**].

Serum killing of *K. pneumoniae* varies depending on serum concentration, with some isolates being sensitive to concentrations as low as 5% NHS [**7**,143,144].

Furthermore, complement induced morphological capsular changes in a C3-dependent manner in both serum resistant and serum sensitive isolates (Fig. 5C, 5D, 6E and 6F) [**7**,**9**].

5.2 Complement activating pathways of *K. pneumoniae*

Complement activation by the CP, LP and AP is described for *K. pneumoniae* [**7**,**9**,62,145,146]. OMP and LPS activate the classical pathway when recognized by antibodies, and C1q recognizes OMP in an antibody-independent manner [29,62]. In consistency with this, we demonstrated C4 activation in non-immune serum [**7**]. Recognition of sugar patterns in the capsule can activate the LP [145]. LPS of *K. pneumoniae* has also been shown to activate the AP, and C3b binds readily to carbohydrates [62,147]. Two or more activating pathways are necessary for complement mediated killing of *K. pneumoniae* as serum resistant isolates solely activate the AP [**7**,**9**,62,139].

By IMB, we showed that only the serum sensitive isolates activated C4 in 50% NHS with high molecular weight bands present, and had reactions with bands corresponding to the C4α' and C4bβ, whereas serum resistant isolates activated the AP, demonstrated by binding of C3 components, and no C4 components (Fig 6A-C) [**7**,**9**]. In agreement with this, it was demonstrated that serum *resistant K. pneumoniae* strains avoided C1q binding and subsequent CP activation, and that smooth LPS activated the AP [139] When examining complement activation of a serum sensitive isolate, no C1q binding was present, indicating a possible C4 activation via the LP instead of the CP [**9**].

In another study, the authors suggested that the AP was pivotal in killing of serum sensitive isolates by using FB-depleted serum [148]. However, we showed that killing of serum sensitive isolates was dependent on CP/LP activation, as serum sensitive isolates were killed only upon activation of C4 [**7**,**9**]. A possible explanation for the differences in these observations could be the variability in the degree of serum sensitivity in *K. pneumoniae* isolates.

Some *K. pneumoniae* isolates are, as mentioned in 5.1, very serum sensitive and readily killed in 5% NHS [**7**]. Serum sensitive *E. coli* isolates are killed in concentrations as low as 0.3% NHS, underlining the differences in serum susceptibility [149]. Therefore, AP activation might be sufficient for killing of very susceptible isolates in 50% NHS, whereas less serum susceptible isolates require amplification of the AP together with deposition of classical C3 convertases to provide sufficient surface bound C3b for generation of the C5 convertases [**7**,136]. C3 deposition could be detected after 5 min on a serum sensitive isolate, with only weak C4 deposition. After 20 min considerable binding of C4 was observed together with MAC, implying AP activity preceding CP/LP activity and supporting the subsequent necessity of AP amplification of the CP/LP [**7**,**9**,62,139]

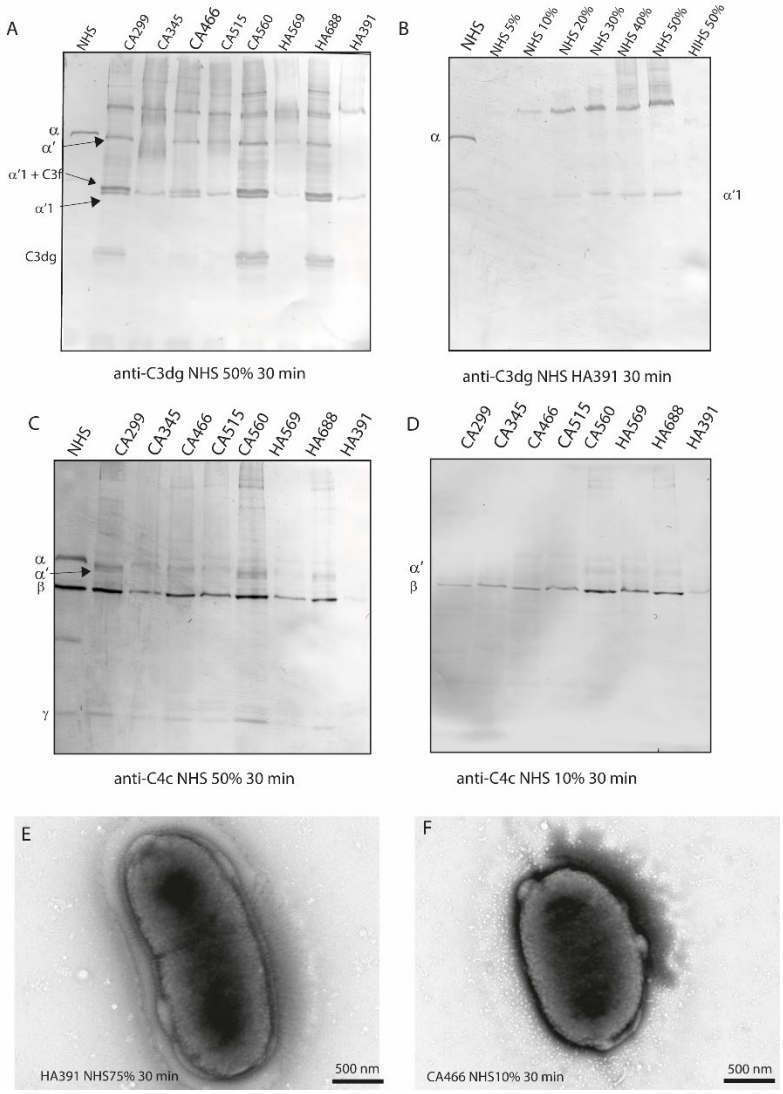

Figure 6. Complement activation is dependent on serum concentration. *K. pneumoniae isolates were incubated in normal human serum (NHS) and heat-inactivated human serum (HIHS) in concentrations from 5-75% NHS 37 °C for 30 min, and processed for immunoblotting (IMB) or transmission electron microscopy (TEM). Lane NHS is IMB of serum prior to addition of bacteria. A: IMB with anti-C3dg of seven serum sensitive isolates, and one serum resistant (HA391) in 50% NHS. B: IMB of serum resistant HA391 from 5-50% NHS and 50% HIHS with anti-C3dg. C: IMB with anti-C4c of seven serum sensitive isolates, and one serum resistant (HA391) in 50% NHS. D: IMB with anti-C4c of seven serum sensitive isolates, and one serum resistant (HA391) in 10% NHS. E: TEM of serum resistant HA391 in 75% NHS. F: TEM of serum sensitive CA466 in 10% NHS. Modified from* [**7**].

5.2.1 The classical C3/C5 convertase is essential for serum killing of *K. pneumoniae* isolates

As described in section 5.2, we demonstrated that complement-mediated killing of serum sensitive isolates of *K. pneumoniae* was C4-dependent, as serum resistant isolates only activated the AP [**7**,**9**]. Serum sensitive *K. pneumoniae* isolates can activate complement differently depending on serum concentrations, with a group of isolates being serum resistant in 10% NHS, but serum sensitive in 50% NHS. Furthermore, these isolates only activated the AP in 10% NHS, stressing the importance of deposition of classical convertases, as C4 components were deposited corresponding to the killing concentration of serum (Fig. 6D) [**7**]. The generation of MAC on the serum sensitive isolates was linked to C4 activation, as MAC was found in serum concentrations where the isolates had C4 components deposited, confirming the complement mediated killing. When no C4 activation occurred in 10% NHS (Fig. 6D), the serum sensitives isolates (Fig. 6F) showed similar changes with capsular shedding, as observed for a serum resistant isolate (Fig. 6E) in higher serum concentrations (Fig. 6D-F) [**7**].

We showed that the difference between a serum sensitive isolate and serum resistant isolate was the presence of an active classical C3/C5 convertase [**9**]. In concordance with this, serum killing of *Salmonella minnesota* was shown to be dependent on C1q and C4 presence, and C1q binding was demonstrated by electron microscopy, supporting that classical activation plays a pivotal role in complement mediated serum killing [150–152]. Several studies have shown that complement-mediated killing of *K. pneumoniae* is enhanced when antibodies against *K. pneumoniae* are present, thus eliciting an antibody-dependent classical pathway activation of complement [144,153–155].

In serum depleted of *K. pneumoniae* specific IgG, a carbapenem resistant *K. pneumoniae* strain showed significantly better survival [144]. Therefore, C4 activation is essential for serum killing both via antibody recognition by C1q or by antibody-independent activation [**7**,62]. However, the antibody subclass is important as high titers IgG_2 blocked antibody-dependent complement-mediated killing by binding to the O-antigen of LPS in *Pseudomonas aeruginosa*. IgG_2 has poor binding properties of C1q [156]. In a study of mouse antibodies against *K. pneumoniae*, the authors demonstrated that IgG_3 mediated better complement killing than IgG_1 [154].

5.2.2 Alternative pathway activation by *K. pneumoniae* may be impaired in low serum concentrations

The C4-dependent killing of serum sensitive *K. pneumoniae* as described above, is seemingly dependent on AP amplification of the CP/LP [**7**,**9**,62]. However, when we

examined complement activation in 5% NHS by immunoblotting (IMB), no C3 components were deposited on a serum resistant isolate, thus confirming no AP activation, as serum resistant isolates solely activated AP in higher serum concentrations. C3-component deposition intensified with increasing serum concentrations, and in 10% NHS weak binding was observed (Fig. 6B) [**7**]. Serum sensitive isolates killed in 5% NHS had C3 and C4 components deposited in 5% NHS. In agreement with this, Vogel et *al*. showed that C3b deposition on *Neisseria meningitidis* in 10% NHS was solely CP dependent, whereas both AP and CP were activated in 40% NHS [157].
When examining the deposition of C3b on an *E. coli* isolate, Gordon et al. showed that C3 components were readily deposited in 50% NHS, but no deposition was observed in 10% NHS [158]. Thus, this supports that sole AP activation is functionally impaired in low serum concentrations [**7**]. The deposition of C4 components in 5% NHS together with C3 components on very serum sensitive *K. pneumoniae* isolates indicate that the deposition of a classical convertase is sufficient for subsequent generation of MAC and killing of very serum sensitive isolates, stressing the significance of CP/LP activation [**7**]. The differences in complement activation depending on serum concentration could be of relevance when considering complement concentrations in extravascular tissue [158].

5.3 Generation of functional MAC is dependent on surface proximity and binding of complement components

Complement mediated killing of *K. pneumoniae* is facilitated by generation of the final pathway when MAC (C5b-9) is assembled after cleavage of C5 by the C5 convertases. By electron microscopy, we demonstrated that only isolates activating C4 had pores in the membrane corresponding to MAC (Fig. 7C) [**7**,**9**]. Using a monoclonal antibody (mAb) against C5b-9, we confirmed that MAC was present on the surface of a serum sensitive isolate in significantly higher levels than on a serum resistant isolate, and the C5b-9 was located in the capsule of the serum resistant isolate (Fig. 7A-C) [**9**]. This agrees with DeLeo et al., who showed that both serum sensitive strains and serum resistant isolates deposited more C5b-9 with increasing serum concentrations corresponding to serum killing [144].
Likewise, generation of MAC is dependent on outer membrane surface bound C5 convertases, and thereby requiring closeness to the surface for MAC to be assembled properly (Fig. 7A) [159]. As described in 5.1, some serum resistant isolates avoid deposition of C3 components completely, thereby evading the generation of MAC, and some serum resistant isolates deposit C3 components away from the outer membrane [**9**,141], where it can be quickly degraded as described in the following section.

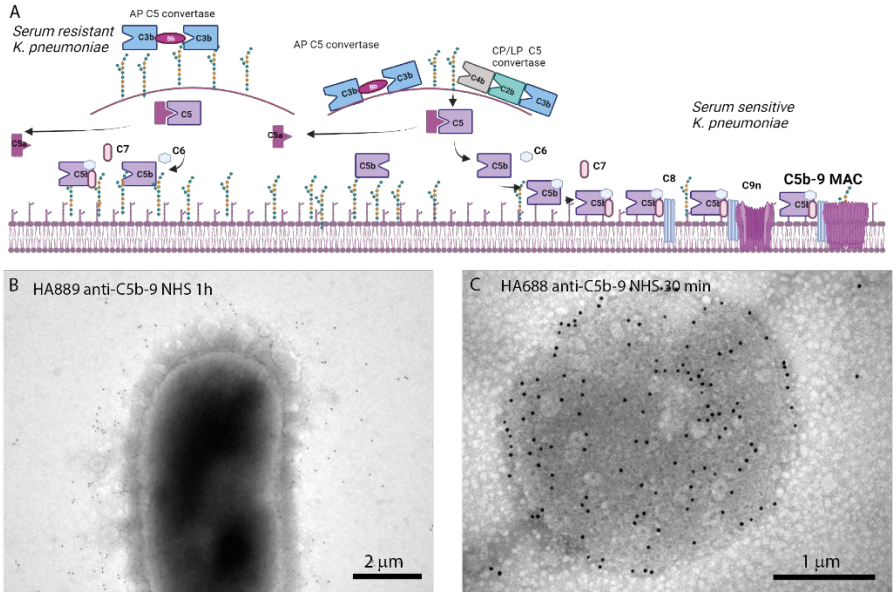

Figure 7. Generation of the membrane attack complex. A*: Location of C5b in close proximity to the bacterial membrane is essential for generation of a functional membrane attack complex (MAC, C5b-9). The serum resistant isolate only activates the alternative pathway (AP), and deposits C3b and C5b in the capsule thus circumventing insertion of a fully assembled MAC into the outer membrane. The serum sensitive isolate activates the classical (CP) and/or the lectin (LP) pathway together with the AP, and has C5b membrane bound. Next, the C5b-7 complex anchors to the outer membrane, C8 inserts the complex into the outer membrane and the finally, the complex is stabilized by C9 polymerization, generating MAC and rapid lysis. Created with BioRender.com* ***B-C****: K. pneumoniae isolates were incubated in 50% normal human serum (NHS) at 37 °C, and processed for immuno-electron microscopy with anti-C5b-9. Anti-mouse Ig conjugated with 10 nm immuno-gold was used as a secondary antibody.* ***B****: IEM of serum resistant HA889 NHS for 1 h. C: IEM of serum sensitive HA688 NHS for 30 min.*

5.4 Deposition and processing of complement C3 components on the surface of *K. pneumoniae*

After binding of C3b to a bacterial surface, C3b has the possibility to engage in C3 or C5 convertases ultimately resulting in generation of MAC if C5b-7 is anchored in the membrane (Fig. 7A) [160]. However, serum resistant clinical *K. pneumoniae* isolates activate complement by the AP in NHS without subsequent generation of MAC anchored in the membrane (Fig. 7B) [**7**,**9**,139]. After cleavage of C3 by an active C3

convertase, an active thioester domain located on the α-chain is revealed on the C3b fragment (Fig. 4) allowing covalent binding (Fig. 5).
Analysis of the degradation of C3b on the bacterial surface of *K. pneumoniae* by IMB using an antibody against C3c and C3dg (Fig. 6A), respectively, showed variance in the processing of C3b on serum sensitive and serum resistant isolates [**7**,**9**]. We showed that clinical *K. pneumoniae* isolates deposited iC3b independent of serum resistance [**7**,**9**]. For the serum sensitive isolates, pAb C3dg recognized the C3α' in 50% NHS, supporting that serum sensitive isolates had C3b deposition in addition to iC3b (Fig. 6A) [**7**,**9**]. High molecular weight bands confirmed binding of C3 components to the bacterial surface [**7**,**9**]. In agreement with this, Albertí et al. demonstrated that C3b was rapidly degraded to iC3b on *K. pneumoniae* serum resistant isolates and showed presence of both C3b and iC3b on serum sensitive isolates. Even though rapid degradation of C3b to iC3b also occurred on serum sensitive isolates, the greater number of C3b on serum sensitive isolates than serum resistant isolates led to subsequent MAC generation and killing [139].
However, binding of iC3b can facilitate opsonophagocytosis of serum resistant isolates, as iC3b is recognized by CR3, expressed on neutrophils and monocytes [40,139,161,162].

5.5 The role of the capsule and LPS in serum resistance of *K. pneumoniae*

The diversity of *Klebsiella* capsule types is vast, and the presence of capsule essential for virulence [**7**,41]. However, the role of the capsule in complement activation and evasion is not completely elucidated. The heterogeneity within clinical isolates is abundant when regarding serum resistance and capsular types [**7**,137]. Recent research has been focused on specific lineages of *K. pneumoniae* either carrying carbapenemases (ST258) or hyper-virulent isolates often associated with K1 and K2 capsules [144,163,164]. However, *K. pneumoniae* infections caused by less known STs or K-types are frequently observed [**7**,165,166]. Capsular serotype has not been directly linked to serum resistance, although the sugar composition, thickness and *rmpA* induced hyper-mucoidity have been reported as influencing serum resistance [**7**,**9**,137,167,168].
Serum sensitivity of the *K. pneumoniae* ST392 clone, an international ESBL-clone, varied within the ST from 5-75 % despite close genetic relatedness with similar KL, ST , O-types and PFGE [7]. This supports that other factors apart from capsule and LPS play a role in serum resistance. This clone presented with a large intact capsule when incubated in HIHS, showing that having capsule does not necessarily protect against serum killing [**7**,**9**,62,141]. In addition to this, LPS O antigen has been demonstrated to be essential for serum resistance [62,169].
Rough or smooth phenotype of Gram-negative LPS have been shown in several

studies to be essential for serum sensitivity, as rough phenotype LPS isolates are readily killed in NHS [141,169–171]. The smooth phenotype consists of all three regions of LPS, whereas the rough phenotype lacks the O-antigen side chain. The O-antigen may mask lipid A or porins, which can activate the CP in an antibody-independent manner [37,141,172].

Adding to the complexity, capsular polysaccharides mediated complement resistance in O-side-chain deficient isolates, thus showing that deficiency of the O-side chain does not necessarily render serum sensitivity for *K. pneumoniae* isolates, and that the capsule can hide complement activating surface structures [37].

When examined by IEM, C3 components were primarily deposited in the capsule of serum resistant isolates and shedding of the capsule without damage to the bacterial membrane were observed, supporting the role of the capsule as a barrier to avoid deposition of C3b in close proximity to the outer membrane. The shedding of capsule did not promote C4 binding near the outer membrane, suggesting that no CP/LP activating structures were revealed (Fig. 5C, 5D and 6C-F) [**9**,144].

The importance of the capsule was backed by Cortés et al. who demonstrated that only mice challenged with encapsulated *K. pneumoniae* strains developed pneumonia and bacteremia and that a non-encapsulated mutant was readily phagocytosed [39]. Furthermore, Merino et al. showed that *K. pneumoniae* exhibiting smooth type LPS not masked by capsule activated complement without being killed, whereas rough type LPS isolates had C3b binding close to the membrane regardless of capsule, and were subsequently killed [141]. Loraine et al. did not find correlation between specific KL- or O-types and serum resistance when screening more than 150 *K. pneumoniae* isolates [137] indicating a complex interplay of surface structures in the context of serum resistance [**7**,140].

O1, O2 and O3 serotypes cause the vast majority of infections worldwide and in consistency with this, we observed these O-types as the majority amongst 30 clinical *K. pneumoniae* isolates from Northern Jutland, Denmark [**7**,41,47]. Yet, no specific O- or KL-types were related to the serum susceptibility of these isolates in non-immune serum, and no K1/K2 serotypes were present. Amongst these 30 isolates, eight were serum susceptible in varying serum concentrations from a healthy seronegative donor, despite causing HA and CA bacteremia [**7**]. However, low C4 levels have been associated with an increased risk of Gram-negative sepsis, which may partly clarify the pathogenic course of these isolates [173]. Furthermore, deficiency of MBL, an LP PRM, is associated with severe and recurrent infections such as pneumonia [174,175].

5.6 In vitro interactions of complement and beta-lactam antibiotics in the killing of ESBL-producing *K. pneumoniae*

As described in 5.5, in vitro highly serum susceptible and serum resistant *K. pneumoniae* isolates can cause bacteremia. Beta-lactam antibiotics are widely used for treatment of *K. pneumoniae* infections, and the continual use for decades has prompted the emergence of MDR *K. pneumoniae*, and complicating treatment [72]. The beta-lactam antibiotics have been known for decades to induce morphological changes in bacteria if not killed depending on binding patterns to the PBPs [**4**,176–178]. Sub-inhibitory concentrations of CAZ in growth medium cause elongation and filamentation leading to possible increased endotoxin release as it binds to PBP-3 at low levels [**4**,176,179]. MRP is used for treatment of ESBL-producing *K. pneumoniae*, and preferentially binds to PBP-2 inducing different morphological patterns in the bacteria with both spherical cells and slightly elongated cells with bulging middle zones [86,180].

Similar morphological changes of ESBL-producing *K. pneumoniae* isolates were observed when isolates were incubated with CAZ/MRP in NHS and HIHS, with CAZ inducing elongation (Fig. 8A-8C) and MRP inducing bulging oval centered bacteria (Fig. 8D) [**4**]. Synergy between complement and antibiotics have been suggested with some studies showing increased sensitivity of resistant bacteria to e.g. vancomycin and CAZ [**4**,149].

Increased capsular production of *K. pneumoniae* has been shown when using inhibitory and sub-inhibitory concentrations of CAZ, and as described in previous sections, complement mediates capsular changes in *K. pneumoniae* [**9**,181].

When we examined two CTX-M-15 producing, but MRP susceptible, *K. pneumoniae* isolates for minimum inhibitory concentrations (MIC) of CAZ and MRP, the MICs for the serum resistant isolate did not differ in NHS, HIHS and RPMI [**4**]. Thus, the effect of MRP did not change in serum despite possible binding to serum proteins [182–184]. The serum resistant isolate remained resistant to CAZ in NHS (Fig. 8A, 8C), supporting that MAC is essential for potentiating the antibiotic effect [**4**,149,185].

The proposed synergy of complement and beta-lactam seems dependent on generation of MAC after C4 activation, leading to increased access of the beta-lactam antibiotics to the periplasmic space [**4**]. This is in line with the observations of Bettoni et al. who showed restored antibiotic susceptibility of *Neisseria gonorrhea* upon C4-activation and subsequent synergy between complement mediated killing and antibiotics [185].

The complement enhanced effects of beta-lactam antibiotics seems dependent on the inherent serum susceptibility of the isolates, and stresses the significance of serum resistance in the selective pressure of antibiotic use, and the importance of adequate antibiotic treatment in order to avoid increased endotoxin release [**4**,149,176,179].

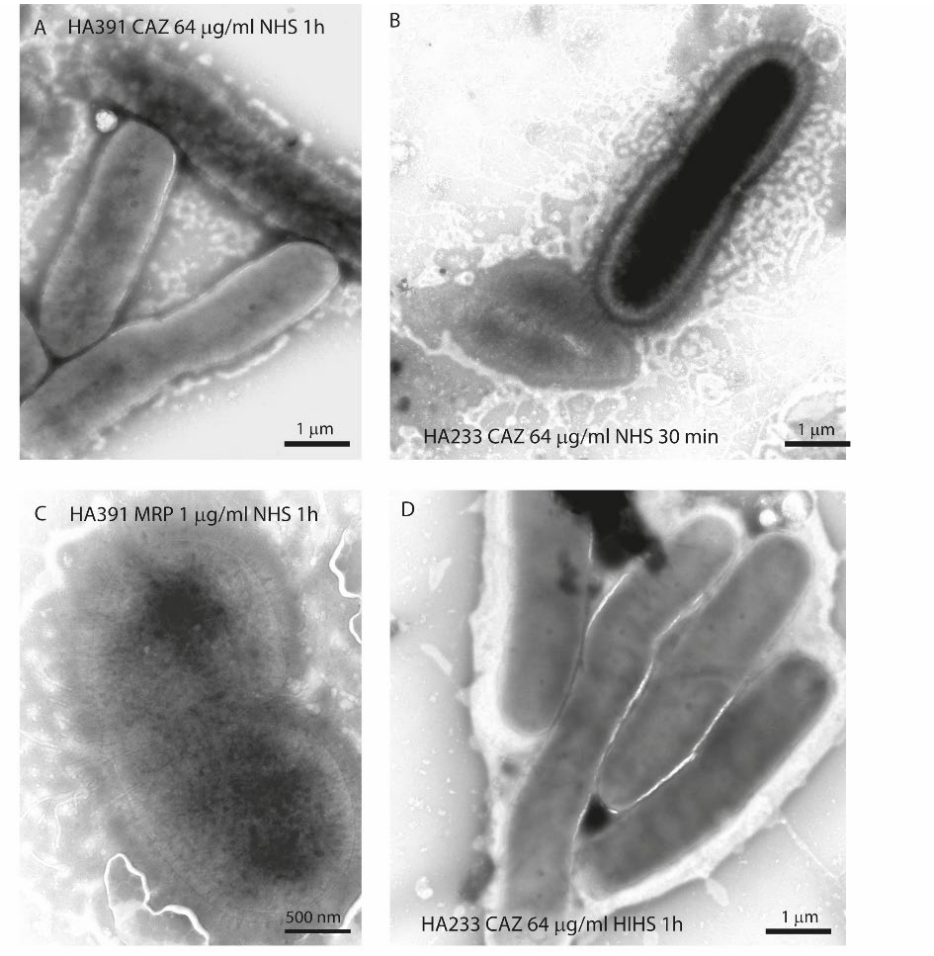

Figure 8. Ceftazidime and meropenem induce morphological changes in ESBL-producing K. pneumoniae. *HA233 and HA391 were incubated in 50% NHS or HIHS with the addition of either 64 μg/ml ceftazidime (CAZ) or 1 μg/ml meropenem (MRP) and processed for TEM or for serum susceptibility assay. A: Serum resistant HA391 NHS CAZ 1h. B: Serum sensitive HA233 NHS CAZ 30 min. C: Serum resistant HA391 NHS MRP 1h. D: Serum sensitive isolate HA233 HIHS MRP 1h.*

6 Concluding remarks

K. pneumoniae is a key player in disseminating antibiotic resistance, and with the increasing threat of multi-drug resistant isolates, knowledge of interactions with the innate immune system is needed in the search for new targets or treatment. Despite some clinical blood isolates being very serum sensitive in vitro, they have established both hospital-acquired and community-acquired infections in vivo. A possible explanation for the pathogenic success of serum sensitive isolates could be due to complement deficiencies, as complement C4 is pivotal for complement killing of *K. pneumoniae.*

The vast majority of clinical *Klebsiella* spp. isolates presented in this work grew unhindered when incubated in NHS from a healthy, non-immune donor. Nevertheless, most of these serum resistant isolates deposited iC3b, thereby possibly facilitating phagocytosis in vivo, if a proper functioning complement system is present in the patient. The differences in serum susceptibility of *K. pneumoniae*, even within closely related isolates, point to the direction of a complex stealth pathogen, which utilizes different mechanisms to avoid complement killing. The fact that *K. pneumoniae* sheds its capsule in NHS might facilitate access to other surface targets.

Some studies use low serum concentrations when investigating interactions between serum and pathogens. However, the possible impaired function of the AP in concentrations below 10% NHS, requires further insight as this might impact results.

The findings presented in this dissertation underlines the complexity of *K. pneumoniae* pathogenesis by demonstrating the heterogeneity in complement activation of clinical *K. pneumoniae* blood isolates. First, the capsule may present a physical barrier for complement killing as complement components are deposited away from the outer membrane. Second, C4-deposition is pivotal for killing of serum sensitive isolates. Third, beta-lactam antibiotics induce morphological changes in serum similar to what has previously been observed in growth medium, but synergy of complement and antibiotics is dependent on serum susceptibility.

It is noteworthy that serum resistance in clinical *K. pneumoniae* may present a selective advantage for antibiotic resistance, and it might be of interest for further studies to address the interactions of serum and antibiotics with clinical *K. pneumoniae* blood isolates, as the threat of antibiotic resistance increases in the future.

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