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Review

Development of immunization trials against *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae is the most common cause of nosocomial respiratory tract and premature intensive care infections, and the second most frequent cause of Gram-negative bacteraemia and urinary tract infections. Drug resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays, and are especially problematic in high impact medical areas such as intensive care units. Many investigations worldwide proved the increasing resistance of such pathogen, resulting in an average rate of 1.63 outbreak every year. A variety of preventive measures were applied to reduce such incidences. Immunotherapy and passive immunization researches as well found their way to the treatment of *Klebsiella*. During the last 40 years, many trials for constructing effective vaccines were followed. This up-to-date review classifies such trials and documents them in a progressive way. A following comment discusses each group benefits and defects.

Contents

1. Introduction

Klebsiella spp., as omnipresent bacteria, exist in the natural environment [1–5] and colonize the mucosa of mammals including humans. *Klebsiella pneumoniae* (also called *Klebsiella aerogenes*, according to Cowan British classification), especially drug resistant isolates remains an important hospital-acquired bacterial pathogen in the developed countries. These nosocomial insert hyperlink to glossary infections by *K. pneumoniae* adding significantly to hospital stays. They are especially problematic in high impact medical areas such as intensive care units, frequently populated by patients who have received intensive and expensive surgical management

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[6,7]. The control of *K. pneumoniae* is a complicated issue due to increasing resistance toward antibiotics, production of endotoxins that induce septic shock [8], bacterium capsular polysaccharide (CPS) that cause immuno-paralysis by inhibiting phagocytosis [9], and resistance to complement-mediated killing [10]. Therefore, immunizations and immunotherapy treatment appeared to provide a powerful tool to control *K. pneumoniae* infections.

2. Epidemiology of *K. pneumoniae*

K. pneumoniae is the most frequent cause of nosocomial respiratory tract infections and the second most common cause of Gram-negative bacteraemia and urinary tract infections [11,12]. Together with *Escherichia coli*, *K. pneumoniae* is the leading cause of serious infections in newborns, blood cancer patients, and other immunocompromised patients [8]. The patient's intestinal tract acts as a reservoir for *Klebsiella* within a hospital [13], which underlines the fact that *Klebsiella* was the etiologic agent of 13 out of 145 nosocomial epidemics reported between 1983 and 1991 [14]. The mortality rate of *Klebsiella* bacteraemia and pneumonia can exceed 50% [15–18]. It is an important hospital-acquired pathogen that causes severe morbidity and mortality particularly in intensive care units, premature intensive care units, as well as medical, pediatric and surgical wards [19–21].

2.1. Progressing antibiotics resistance

Bacteria may follow multiple mechanisms to resist antibiotics, either by modifying drug targets, or by producing antibiotics-hydrolyzing enzymes. Once this resistance is acquired, antibiotic pressure can enhance its transmission within a community, and transport may facilitate its dissemination to new communities [22]. *Klebsiella* spp. was reported to be an important source and receiver of transferable antibiotic resistance [7]. They produce several groups of β-lactamases such as SHV, TEM, and CTX.

K. pneumoniae is naturally resistant to amino-penicillins (Ampicillin and Amoxicillin) and carboxy-penicillins (Carbenicillin) due to the production of SHV-1, a potent penicillinase. However, these strains are usually susceptible to a combination of Amoxicillin and clavulanic acid. Co-amoxiclav resistance in *K. pneumoniae* results from overproduction of either SHV-1 or TEM-1/TEM-2 β-lactamase or biosynthesis of a clavulanic acid-resistant β-lactamase [23].

The initial description of Extended Spectrum Beta Lactamase (ESBL) emergence among *K. pneumoniae* was in 1983 [24–26]. The predominant ESBL types vary geographically [27,28]. The first propagated ones were CTX-1 [29], SHV-2 [30] and SHV-3 [31] which are able to hydrolyze oxyimino-β-lactam [32]. Afterwards, the number increased to include TEM-4, SHV-2, CTX-M-9 and CTX-M-10, and novel ESBLs emerged (TEM-110, SHV-11, SHV-12, CTX-M-14 and CTX-M-15) [33]. Dissemination of ESBL-producing *K. pneumoniae* (ESBL-KP) in a hospital is a complicated event that shows several single-strain and multiple-strains spread modes [7,24,34]. However, the successful management of these outbreaks remains controversial [35].

Starting from the 1990s, a new type of β-lactamases called CTX-M emerged in many countries of the world. The first organisms producing this type of β-lactamases were identified as clinical isolates in distant geographic regions (France, Germany and Argentina) [30,31,36]. More recently, a rapid increase in the proportion of multiple CTX-M variants to the TEM- and SHV-derived ESBLs has been reported in many hospitals in Spain [31–33,35], United Kingdom [37], Canada [38], China [39], and Korea [40]. Moreover, the frequency of ESBL-KP has increased steadily in Taiwan to reach 10–30% among clinical *K. pneumoniae* isolates [41–43]. CTX-M2 and CTX-M3 types also spread widely or even predominated in several countries, including Argentina [40], Japan [44], Poland [45] and Russia where they were possessed by 60.8% of all *K. pneumoniae* isolates [46].

A comparison analysis of ESBL co-resistance in Spain between strains isolated during 2001–2004 and resistant isolates previously identified during 1989–2000 revealed an increment in resistance to Trimethoprim (from 10.3% to 41.5%), Sulphonamide (from 29.3% to 54.7%) and the first generation quinolones (nalidixic acid – from 6.9% to 34.0%) [33]. For sometime, quinolones were thought to be potent therapeutic choice to control ESBL-KP infections. However, resistance among *K. pneumoniae* strains has been increasingly reported [41,47–51] till it reached 33% in Taiwan [52], and even approached 50% of isolates in some regions [41]. Concomitant second generation quinolones resistance with ESBL production in *K. pneumoniae* isolates that reached 18.5% in Taiwan would severely restrict treatment options. *K. pneumoniae* isolates also showed cross-resistance to the third and fourth generation quinolones, including Levofloxacin, Gatifloxacin, Gemifloxacin, and Garenoxacin, and otherwise than Sitafloxacin [53]. Simultaneously, since the 1970s, many articles discussed the emerging resistance of *K. pneumoniae* against aminoglycosides in neonates intensive care units in USA [43,54] and in Taiwan [55].

Subsequently narrowing the choice among antibiotic classes to carbapenems as effective antibiotics against ESBL-KP infections. Imipenem proved its potential as the most successful drug in several published reports [8,26,34,54]. However, over the past few years, a progressive increase in carbapenems resistance [9,56–58] mediated by class C cephalosporinase and loss of outer membrane porins were identified in isolates of *K. pneumoniae* [59–61]. These isolates are resistant to several antibiotic classes, exposing clinicians to a limited therapeutic choice [41]. Multidrug resistant isolates were reported in Nigeria [47], Scotland [62], and Tunisia [63].

2.2. Outbreaks due to K. pneumoniae

Due to the overuse of the cephalosporins and aminoglycosides, several outbreaks caused by *K. pneumoniae* have been recorded [22,24–26,28,62]. The appearance of ESBL-KP was reported at *Robert Debre* Pediatric Hospital in France [32], and at the Innsbruck University Hospital in Germany [64]. Another outbreak was detected in intensive care patients at the Hospital *de Bellvitge*, *Universidad de Barcelona* in Spain between May 1993 and June 1995. Likewise, an ESBL-KP outbreak especially among anti-cancer chemotherapy patients at the Seoul National University Children's Hospital was recovered from 1993 through 1998 and reached 52.9% of all identified *K. pneumoniae* isolates. The overall fatality rate of the ESBL group was 4.5 fold higher than that of the non-ESBL group [8]. Outbreaks due to multi-resistant *K. pneumoniae* (MR-KP) occurred in the Grampian Region of Scotland during 1992 [62], and in the neonatal ward at the '*Maternite Wassila Bourguiba*' in Tunisia during the first quarter of 1996 [63].

3. Antibiotics adverse reactions

Antibiotics beneficial usage may be limited for several reasons. In general, the common adverse drug reactions associated with antibiotics therapy include: diarrhea, nausea, rash, electrolyte disturbances, vomiting, headache, dizziness, mucous candidiasis, colitis, fever, and increased infection by resistant strains. Moreover, many antibiotics seem to be risky albeit powerful. Several cephalosporins may be associated with hypo-prothrombinemia [48,49], nephrotoxicity and ototoxicity [51]. Rare cases of peripheral neuropathy and tendon damage have been reported with quinolone therapy [50]. The potential of aminoglycosides for

ototoxicity and nephrotoxicity was well recorded. They are even correlated with irreversible vestibular damage, hearing loss and tinnitus [51].

4. Prevention and immunotherapy

The future advancement of *K. pneumoniae* infection control methods will be undoubtedly challenged by increasing antibiotic resistance as well as antibiotics adverse reactions. In addition, at least half of some bacterial cultures, such as those related to blood stream infection, show false-negative results [65]. Hence, only one possible measure remains by the immunotherapy of persons at risk, either by active or passive immunization trials to be discussed in more detail below.

Cranberry juice was used as a new approach to prevent or eradicate *K. pneumoniae* colonization in hospitalized patients' gut. Due to its fructose and macromolecules content, it inhibits type 1 pili and blocks mannose-resistant adhesion [56]. Obviously, it acts on the gastrointestinal organisms to eliminate the source rather than on the infection site [57].

Research on host defense mechanisms also found their way to reduce *K. pneumoniae* incidences. Lung surfactant protein type A (SP-A) showed to increase phagocytosis of *Klebsiella*, while SP-D has been reported to interact with bacterial lipo-polysaccharide (LPS), especially in lungs [9].

Most immunotherapy trials were performed to treat pneumonia that may develop to secondary septicemia, using many kinds of immuno-enhancers. An approach of recombinant adenoviral gene therapy that induces overproduction of IL-2 (Inteleukin-2) was established. It reduced pneumonia incidence by 12-fold [66]. Another approach was described using tumor necrosis factor (TNF), a pro-inflammatory cytokine that has beneficial effects on improving acquired host immunity, and reducing bacterial infection [59]. A much more recent trial in 2007 introduced the usage of cyclic di-GMP (c-di-GMP [c-diguanylate]), an immuno-stimulatory agent that boosts innate immunity. Its mucosal or dermal administration 24–48 h prior to the challenge of *K. pneumoniae* usually protects from infection, enhances neutrophils accumulation, and activates natural killer cells (NK) and T-lymphocytes [60]. Unfortunately, none of these preparations found their way to the market due to their high price and impractical approach.

5. Passive immunization

Many antibody preparations have been prepared to reduce bacterial counts in blood or lungs, and delay bacteraemia onset. The first attempts investigated the immunoprotective capacity of antibodies directed against *K. pneumoniae* capsular polysaccharide. These antibodies were elicited in response to immunization with inactivated whole cells [61], ribosomal preparations [67,68], or cell surface preparations (CSP) [69]. More precise preparations of purified capsular polysaccharide (CPS) were introduced by Cryz group at the Swiss Serum and Vaccine Institute between 1984 and 1985. Anti-CPS antibody administered passively [70] or raised by the immunization with purified antigen [71] was found to provide a high degree of protection against fatal *K. pneumoniae* KP1-O burn wound sepsis. Furthermore, this capsular antigen was found to be safe and immunogenic in human volunteers [72]. However, the above studies were limited to only a single capsular serotype. A further study proved the effectiveness of 18 different CPS serotypes [73].

More studies aiming at expanding the prepared antiserum effect were conducted in England by Roe and Jones group. *Klebsiella* vaccines were prepared from culture filtrates of six capsular serovars of *K. aerogenes* (K1, K2, K3, K20, K35 and K44). The passively administrated pooled antisera were said to protect the experimental animals against lethal infection of bacterial strains representing the 77 capsular types of *K. aerogenes* [74]. However, other studies showed limitations of using CPS-based passive immunization. In the Walter Reed Army Institute of Research, mAbs (murine monoclonal antibodies) specific for the K2 capsular polysaccharide (CPS) of *Klebsiella* were purified. These mAbs promoted *K. pneumoniae* K2 serovar killing and activated complement fixation. However, the degree of protection varied between the challenge strains [75]. The department of infectious diseases, Free University in Berlin of Germany, also reported the emergence of the *K. pneumoniae* as an urging nosocomial pathogen. A mixture of human CPS-specific mAbs against clinically urging *Klebsiella* serotypes was not found to prevent the invasion of virulent bacteria into the inter-alveolar space, but accelerated the resolution of infection [76].

Another trial was performed in intensive care units of sixteen Department of Veterans Affairs and Department of Defense hospitals. Intra venous administrated hyper-immune globulin (IVIG) was prepared in human volunteers previously immunized with a 24-valent *Klebsiella* CPS, plus an 8-valent *Pseudomonas aeruginosa* O-polysaccharide-exotoxin A conjugate vaccine. Although IVIG proved to decrease the severity of vaccine-specific *Klebsiella* infections, these reductions were not statistically significant. Unfortunately, those patients suffered more from adverse reactions (14.4% instead of 9.2%) [77]. For these reasons, the use of CPS directed immunoglobulin for the treatment of *K. pneumoniae* was not favored.

In 1994, Trautman et al. [78] studied a hybridoma producing a mAb against *Klebsiella* lipo-polysaccharide (LPS) derived from spleen cells of mice previously immunized with a *Klebsiella* strain lacking CPS. The mAb cross-reacted in vitro with LPS preparation of O1, O2ab, O2ac, O3, O4, O5 and O12. A multi-national in vitro study of the monoclonal antibody raised against the *K. pneumoniae* inner core was tested to bind to the bacterium LPS. It showed a reactivity with almost all known serotypes [79]. Later on, an O1-antigen (cross-reacts with O6 and O8) specific mAb was examined for its protective capacity against capsular and non-capsular *Klebsiella* causing sepsis [80] and proved its efficiency. This study confirmed that O-antigen-specific antibodies are more efficient than K-antigen-specific immunoglobulin preparations, due to the large number of *K. pneumoniae* K-antigens (77 antigens) compared to the O-antigens (8 antigens).

6. Active immunization

It is the introduction of a microbial epitope that the immune system recognizes and builds up antibodies against it. Upon searching for *Klebsiella* pathogenic mechanisms, five main classes of epitopes were identified: capsule, LPS, siderophores, adhesins (pili, fimbriae and aggregative adhesins), and exotoxins [9]. The possibility for immunization against *Klebsiella* spp. will be discussed in view of its pathogenesis and epitopes identification that can be applied in vaccine production [58]. However, it was anticipated that *Klebsiella* might not be simple to control by routine vaccines, due to its high degree of antigens variation including CPS epitopes in special.

6.1. Whole cell vaccines

Sometimes they are called the first generation vaccines using the whole inactivated or sub-burden dose of bacterial cells to induce an immune response.

6.1.1. Killed/attenuated vaccines

Many agents have been used to ensure pathogens attenuation. One of the first approaches was by using formalin [81]. Another attempt applied fine powder of acetone dried ground cells. The

dried vaccine was stable for over 2 years at room temperature [82]. These vaccines did not develop to reach the market, due to their limited safety and partial endotoxicity.

6.1.2. Digested bacterial lysates

Another safer technique applied the complete lysate of the bacterial cell to produce a cell-free vaccine containing all cell components in one mixture. This technique was mainly adopted by the Russian research centers to produce a vaccine against *K. pneumoniae*. One of the first trials was by using hydroxylamine disintegrated isolate (No. 204). This specific serovar was said to gather most *K. pneumoniae* serotypes and hence was capable of stimulating immunity to other heterologous isolates of K1, K9, K11, K16, K20, and K61 serotypes [83]. In 1988, immunization with hydroxylamine *Klebsiella* lysate ensured the removal of *K. pneumoniae* from blood, lungs, liver, kidneys and spleen of challenged mice [84]. A combined vaccine of *Staphylococcus aureus* aqueous extract and *K. pneumoniae* hydroxylamine vaccine was found to contain 53.5% of neutral monosaccharides (LPS), 0.7% of nucleic acids, and 11.63–14.0% of proteins of *Klebsiella*. This preparation was effective [85], faintly reactogenic and safe on morphology of peripheral blood, liver function, and IgE profile. It also increased *Klebsiella* antibody titers by 3- to 5-fold [86]. A more optimistic multi-component vaccine containing *K. pneumoniae*, prepared by the same previous method, showed antibody titers increase by 10–10^4 fold and over 5-year stability at 4 °C [87]. In 1997, the approach of making more pure vaccines was introduced. The fractional composition of the vaccine preparation obtained by hydroxylamine treatment of *K. pneumoniae* strain 204 was studied using gel chromatography [88]. This type of vaccines proved to be immunogenic and reduced reactogenic response.

Another attempt has been directed to use in vitro-produced extracellular toxic complex composed of CPS, LPS and a small amount of proteins. This media supernatant autolysate showed to induce protective antibody when injected in experimental animals with sub-lethal doses [89]. In a research study conducted at the Eijkman-Winkler Institute for Medical and Clinical Microbiology in the Netherlands, orally administrated hepta- and mono-valent *Klebsiella*-containing hydrolysates showed to be efficient in inducing a protecting antibody level through an intestinal pathway [90].

6.2. Ribosomal vaccines

These are composed of non-cell wall components, mainly the cytosol ones. The first attempt to apply such technique for *K. pneumoniae* vaccination was in 1978, a study of a ribosomal vaccine associated with *K. pneumoniae* glycoprotein cell walls administered by the aerosol route. The specific antibodies increased upon vaccination [91].

Then a series of researches were performed by the Riottot and Fournier group. The first one proved the challenge immunogenic-protective property of the subcutaneous injection of *K. pneumoniae* ribosomal preparations. It was noticed that its protective capacity was specific to the capsular serotype of the origin strain that endured purification steps by gradient centrifugation [67]. The second trial confirmed that ribosomal preparations extracted from a non-capsulated mutant *K. pneumoniae* were not immunogenic indicating that the ribosomal preparation was non-immunogenic, unless contaminated with cell wall components [92]. This remark was further confirmed on a dry-weight basis, where cell surface preparations provided better immuno-protective activity than ribosomal preparations did [69]. A final experiment resulted in turning a blind eye to ribosomal vaccines usage for *K. pneumoniae* prevention, when two peaks were obtained by cesium chloride density gradient ultracentrifugation of *K. pneumoniae* ribosomal preparations. The first peak contained the full cell wall components and was highly immunogenic, while the other peak contained nucleic acids, proteins and CPS, and was poorly immunogenic. These results provided evidence that ribosomal ribonucleic acid has no role in the immunogenic capacity to protect against *Klebsiella* infection [92].

6.3. Protein based vaccines

K. pneumoniae immunogenic proteins are mostly extracellular toxins or cell surface protein, such as outer membrane protein (OMP) and fimbriae proteins. The first experiments evaluated *K. pneumoniae* heat stable enterotoxin that showed immunological cross-reactivity with *E. coli* enterotoxin and protection against active challenge in rats immunized with the enterotoxin vaccine [93]. Later investigations on the protective efficacy of toxoids prepared from crude cytotoxin were found to be protective against *Klebsiella* challenges. The vaccine maternal capacity was proved when the offspring of immunized female rabbits acquired the specific antibodies, and were protected from *Klebsiella* infection up to 1 month of age [94].

Fimbriae proteins are well-recognized by the immune system. Therefore, two types of *K. pneumoniae* fimbriae were evaluated: mannose-specific fimbriae type 1 expressed in many enterobacterial species, and mannose-resistant type 3 fimbriae produced by the majority strains of *Klebsiella*. These surface antigens served as carrier proteins and as a common antigen for vaccines of broad specificity. Although both types do not stimulate TNF, type 3 and 1 fimbriae are moderate-to-weak IL-6 inductor; respectively [95]. More recent studies highlighted the use of type 3 fimbriae to protect against respiratory infections due to *K. pneumoniae* [96]. These finding on fimbrial proteins favored their use as carrier proteins.

The first trials to use outer membrane protein (OMP) in vaccine preparation were in 1983 at the Department of Immunology at Kyushu University in Japan. OMP immunized mice overcame lethal challenge with the wild *K. pneumoniae* [97]. More recently, a cloned recombinant protein derived from OMP (rP40) showed to induce significant antibody response without the need for an adjuvant [98]. A study conducted in 2001 ensured that P40 is a suitable carrier outer membrane protein for nasal immunization against *K. pneumoniae* [99] and might be used widely as a carrier protein in other vaccines. More recent researches, applied DNA vaccine techniques to produce OMP-based vaccines against *K. pneumoniae* [100].

6.4. Polysaccharide vaccines

The outermost layer of bacterial cells may be exopolysaccharide (EPS) or capsular polysaccharides (CPS). The cell wall of Gram-negative bacteria is composed of lipo-polysaccharides (LPS) that contribute to the microbial pathogenicity. *K. pneumoniae* has 77 different capsular serotypes (K-antigen), and 8 LPS serotypes (O-antigen, having 12 different chemical structures) [101]. Investigations revealed that every infection by *K. pneumoniae* is correlated to a specific antigen. The lipo-polysaccharide (LPS) (O antigen) is necessary for the colonization of the urinary tract, while the capsular polysaccharides are not of importance to colonize the gut [102–104]. Many sero-epidemiological studies investigated the frequency of polysaccharides-serotypes and their possible use in vaccine design [101,105–108].

6.4.1. Capsular polysaccharide vaccines

Klebsiellae usually develop capsules composed of complex acidic polysaccharides. The capsule consists of four to six sugars repeating subunits and frequently uronic acids. It can be classified into 77 serological serotypes. The capsular material forms a thick mass of fibrillose materials covering the bacterial surface.

This protects the bacterium from phagocytosis and bactericidal serum factors. CPS was the usual vaccine candidate for several reasons. They are highly immunogenic and nontoxic, represent the outermost contact with the milieu, and are produced by the majority of *Klebsiella* strains [73]. However, injection of large doses of *Klebsiella* CPS produce immunological paralysis manifested by decreased production of capsular antigen antibodies [9].

The Roe and Jones group in UK also contributed to *K. pneumoniae* CPS vaccine research. One dose of mono-valent vaccine prepared form the bacterial filtrate was able to protect sepsis-challenged mice from more than 40% of the 77 capsular types. The poly-valent preparation (2–12 mono-valent) was able to protect against 60–80% of the 77 capsular types of *K. aerogenes* [109]. Another *Klebsiella* vaccine was prepared from strains of *K. aerogenes* of CPS types K1, K36, K44 and K-Cross (a cross-reacting type with antibodies against many *Klebsiella* CPS types). All mice given one dose (1.0 μg/mouse) of K1 vaccine survived lethal intraperitoneal challenge for four days after vaccination, however this protection reduced to the half at the fourteenth day [110]. Later on, they proved that extracellular polysaccharide extracts from *K. aerogenes* K1 that contains autolysate contamination gives a longer protection [111]. This confirms that CPS alone does not induce memory cells immunity.

Scientists at *Institut Pasteur* of France compared the immunoprotective activity of *K. pneumoniae* K2 cell surface preparations and purified capsular polysaccharide in mice. CSP immunogenicity was referred to its association with other constituents like protein and LPS, which act as adjuvant, while pure CPS was not highly immunogenic. The two preparations lost their immunoprotective capacity after treatment with an alkali [112]. At the same institute, anti-*K. pneumoniae* K5 capsular polysaccharide vaccine was tested in squirrel monkeys. IgG antibody responses showed a marked increase with no side-effects [113]. Other trials in Latin America confirmed that *K. pneumoniae* polysaccharide capsular material mainly induces thymus-independent humoral immunity and confers protection against *K. pneumoniae* infections whether administrated passively or actively [114].

Many trials have been performed during the mid-1980s by Cryz SJ. Jr., with Fürer and Germanier group at the Swiss Serum and Vaccine Institute; again Cryz with Cross and Sadoff group at the University of Maryland, USA; and Cryz with others at the Department of Vaccine Production, National Bacteriological Laboratory in Stockholm, of Sweden. They confirmed that *K. pneumoniae* KP1-O capsular polysaccharide provided significant protection against fatal burn wound sepsis [70]. Pure CPS from 18 *Klebsiella* strains of different capsular types, containing traces of protein, nucleic acids, and lipo-polysaccharide, induced IgG production in human volunteers immunized with either native or NaOH-treated KP1-O capsular polysaccharide that were equally effective at preventing experimental fatal *K. pneumoniae* burn wound sepsis in mice [73]. All vaccinees showed a four-fold or more increase in IgG and IgM titers [72].

The main disadvantage of CPS vaccine is the great variability of K-antigens (77 different antigens) in *Klebsiella* spp. Therefore, the approach of using more than one K-antigen was introduced. Cryz evaluated a bivalent vaccine composed of purified serotype 2 CPS, as a second trial after that of Roe and Jones' [110]. Vaccination afforded protection against fatal pneumonia, promoted clearance of the bacteria from the lungs and prevented bacteraemia [115]. A fourfold increase of IgG offered a sufficient protection level against burn-wound sepsis in experimental animals immunized by a polyvalent *Klebsiella* vaccine composed of six serotypes of capsular polysaccharides (K2, K3, K10, K21, K30, and K55) either alkali-detoxified or crude [116]. However, in a study of the capsule types incidence among bacteraemic *Klebsiella* isolates, Cryz group observed that only 25 serotypes made up 70% of all bacteraemic strains [101]. Based on that sero-epidemiological findings, they formulated a 24-valent *Klebsiella* CPS that proved to be safe and immunogenic [116].

In the late 1980s, a 24-valent CPS vaccine was evaluated in seven adult volunteers in Stockholm. IgG increased more than 2 fold toward the eleven immunological related serotypes not included in the vaccine. Anti-CPS antibody was protective against bacteraemia [117]. Further studies proved that vigorous IgG and IgA responses were induced in humans, and hence the vaccine was not restricted to single antibody class or subclass [118]. This polyvalent vaccine composed of 24 different serotypes of *Klebsiella* spp. showed to be well tolerated and elicited functional antibody to 21–24 antigens. It was also active against additional 10 serotypes not included in the vaccine when applied passively. The produced immunoglobulin was protective and enhanced opsono-phagocytosis of bacteria [119].

It was mentioned in the mid-1990s that this vaccine seems to be the most promising approach for preventing sepsis caused by *Klebsiella* and has already passed Phase I in human trials [120]. The study of the 24-valent *Klebsiella* CPS vaccine demonstrated a protective antibody response after active immunization in traumatic patients [121]. Unfortunately, the maximum protection coverage offered by those vaccines never exceeded 70% of the *K. pneumoniae* strains. Due to its production complexity for the market, researches on this vaccine never continued after the Phase I trials on humans.

Further evaluation of the vaccine was performed at the University of Maryland after being mixed with a conjugate *Pseudomonas* vaccine 8-O-polysaccharide-exotoxin A. The antibody rise against the 33 antigens (24 CPS, 8-O antigens and exotoxin A) was the same after 2 months, and all declined after 18 months [120]. Vaccine potency in acute traumatic patients, who were vaccinated within 72 h of injury by the 33-antigens mixed vaccine, was further evaluated. Ninety percent of the patients responded to at least 18 of 24 *Klebsiella* antigens [121].

6.4.2. Lipo-polysaccharide vaccines

The primary lipid on Gram-negative bacteria surface and the main constituent of their cell wall is lipo-polysaccharide (LPS; endotoxin). LPS can be characterized by three structural regions: lipid A chain, oligosaccharide core, and O-polysaccharide repeated outer region. The lipid moiety of lipid A is embedded in the outer bacterial cell membrane, while the oligosaccharide core region lies between lipid A and the O-polysaccharide outer region. Lipid A has similar basic structure among Gram-negative bacteria and is the main endotoxic factor. Likewise, LPS oligosaccharide core regions show close similarity among Gram-negative bacteria and consist mainly of a limited number of heptoses. The O-specific antigen is highly variable and is composed of one or more oligosaccharide repeating units that is characteristic for each serotype [122–124].

In *K. pneumoniae*, an outer CPS massive layer was thought for a long time to mask the LPS from recognition by the immune system. However, more recent research studies proved that O-antigen extends from the majority of CPS [125–130]. Unlike K-antigen, the small number of *Klebsiella* O-types is a great advantage for vaccine production. Preparation of a multivalent LPS vaccine composed of the eight O-antigens or at least the inclusion of the most frequent O1-antigen should be a promising broad spectrum vaccine. Moreover, the passive administration of *Klebsiella* monoclonal antibodies showed to highly protect from lethal endotoxic shock [131]. The inclusion of O-specific antigens in a *Klebsiella* vaccine formulation may reduce the need for an adjuvant [132]. This finding was initiated by Robert et al. who compared the potency of CPS and whole cell surface preparations. The 50% protective dose (PD50), calculated on the basis of CPS concentration, was 2 ng for cell surface preparations and 50 ng for purified CPS. The two preparations lost their immunoprotective potency by alkaline treatment [112],

ensuring that the preparation immunogenicity mostly lies in the LPS that also acts as an adjuvant to the CPS.

Similarity of the lipo-polysaccharides core region, especially the common enterobacterial antigen, results in their shared cross-reactive antigens [122]. In 2000, a liposomal complete core lipo-polysaccharide vaccine isolated from *E. coli* K-12, *E. coli* R1, *P. aeruginosa* PAC608 and *Bacteroides fragilis* showed cross protection against *K. pneumoniae* serotypes O1, O2ab and O3 [123]. Another more recent study for the prevention of endotoxemia among Enterobacteriaceae by cross-reacting vaccine, was introduced [133]. This finding may be added to the use of LPS as a broad spectrum multivalent vaccine against all *K. pneumoniae* O-antigens with extended potency against other pathogens. However, cross-reactivity against normal microbial intestinal flora through secretory immunoglobulins should be considered. The obvious drawback of active immunization with LPS vaccines is the adverse endotoxic reactions.

A group working on pneumonia at the Punjab University in India initiated a liposome-incorporated LPS that was more effective (than free LPS) in protecting from pneumonia due to *K. pneumoniae*, and 10 times less toxic and non pyrogenic [124]. Further studies revealed that a remarked decrease in lung bacterial count and a fall in severity of lung lesions were observed [134]. A more recent approach to use 100 μg LPS preparation to prevent *Klebsiella* infection was performed by an Australian team [135].

6.5. Conjugate vaccines

A conjugate of fimbrial protein with a core oligosaccharide obtained from *E. coli* K-12 lipo-polysaccharide showed to contain an epitope common in several enterobacterial species. The produced antibody raised against both the oligosaccharide hapten and fimbrial carrier [95].

The first reported trials to construct a conjugate vaccine toward *K. pneumoniae* polysaccharide were by coupling 2 units of the repeating tetra-saccharide (octa-saccharide antigen) to bovine serum albumin (BSA) and keyhole limpet hemocyanin. Both conjugates produced thymus-dependent antigens [136].

Again at the Punjab University, a conjugate has been constructed from iron-regulated cell surface proteins and polysaccharide moiety of *K. pneumoniae* LPS. The conjugate immunogenicity was much higher than that of its individual components. It was safe, immunogenic and enhanced phagocytosis [137]. Later on at the same university, the polysaccharide (PS) extracted from *K. pneumoniae* NCTC 5055 lipo-polysaccharide was covalently linked to tetanus toxoid. The conjugate proved to be non-toxic and non-pyrogenic at 100 μg dose level, while being immunoprotective resulting in a decrease in bacterial colonization in lungs and activating alveolar macrophage [138]. A new easy and practical way for preparing conjugate vaccines developed in Egypt [130] on the base of an old method [139]. It depends on extracting the whole glyco-peptide molecule of the bacterial cell wall containing the LPS and OMP, then detoxifying the conjugate in order to remove the fatty acids. The prepared vaccine showed to be protective against *K. pneumoniae* urinary tract infection, pneumonia and even septicemia challenges. It proved to be broad spectrum, non-reactogenic, maternal, and showing an increase of antibody titer more than eight fold at a dose of 100 μg/kg. It manifested no cross-reactivity with the tested normal intestinal flora.

7. Comment

The majority of *Klebsiella* infections are acquired during inpatient hospital stays and accounts for 5–7.5% of all nosocomial infections. Moreover, *Klebsiella* spp. infections in pediatric wards have become an increasing concern. In newborn intensive care units, *Klebsiella* occupies more than 25% of the most common pathogens, and is the leading cause of serious infections in neutropenic and surgical patients [7,19,128]. Despite the use of the appropriate antibiotic therapy, the morbidity and mortality due to bacteraemia and pneumonia remain remarkably high. Fatality rates of 20–50% in bacteraemia and more than 50% in pneumonia due to *Klebsiella* have been reported [16–19,58,134]. This was referred to the tight pathogenesis of *K. pneumoniae* that is naturally resistant to complement-mediated killing and even some isolates (LPS masked isolates K1, K10 and K16) fail to activate complement [10]; bacterium CPS protection from phagocytosis [9]; and the release of LPS endotoxin that may cause renal scaring even after one episode of infection [56]. On the other hand, despite the usefulness of laboratory microbiological investigations, 20% of the patients with sepsis have negative-cultures [140].

K. pneumoniae is naturally resistant to penicillins even when coupled with β-lactamase inhibitors [23]. β-Lactames resistance mediated by different ESBL, including SHV, TEM and CTX, appeared in 1983 and always progressed. It reached 60.8% of isolates in Russia some years ago [46]. Co-resistance to sulfa drugs and quinolones has been reported with ESBL [33,51]. Resistance is continuously increasing and spreading through more antibiotic pressure and travel [22]. It was thought that ESBL-KP can be optimally controlled by carbapenems, fluoroquinolones and aminoglycosides [7,32,135,136]. However, enzymatic carbapenems resistance has been documented in *K. pneumoniae* [41]. During the 70s, there were frequent epidemics of gentamycin-resistance in hospitals [43], the fact that increased an alert for aminoglycosides usage. Antibiotics, as chemotherapeutic agents, have moderate to severe adverse reactions due to prolonged or high-dose therapy and in specific patient groups [50], especially aminoglycosides that cause renal toxicity [51].

Mal-diagnosis (leading to delay in proper patient management), resistance to antibiotics, inappropriate chemotherapy, or economic factors caused 13 epidemics between 1983 and1991 and 18 outbreaks between 1987 and1998 [14], with an average rate of 1.63 outbreak per year. These risk-factors together point to the need for immuno-prophylactic/immunotherapeutic agents for disease control. Prevention of infections with appropriate vaccine is increasingly urging [22].

Although immuno-enhancers showed to ameliorate the state of patients suffering from *K. pneumoniae* infection, none of them found their way to the market, due to their expensive prices and remarkable adverse reactions. The Immuno-globulin preparations, especially LPS specific ones, had a positive effect on such cases. However, reductions in bacterial infection were not statistically significant and patients receiving IVIG had more adverse reactions.

Whole cell vaccine trials were the first to be performed in the very early 70s, but their limited safety and adverse reaction (endotoxicity) has banned their practical use for protection. De-routed vaccine trials were absent since the bacterium colonizes almost all human mucosal surfaces, and is able to infect the blood stream. Low dose vaccines were also unsafe to be used and hence were never tried even on the veterinary level. Although digested bacterial vaccines showed to be efficient, only orally administrated experiments continued to reach the market [90]. Ribosomal trials were admitted between 1978 and 1981 to produce a vaccine toward the bacterium. However, evidences confirmed that the main epitopes of *K. pneumoniae* are in the cell wall and not the cytosol. Their slight effect on boosting the immune system was due to the traces of CPS impurities they contained [92]. Protein based trials were promising and applied a variety of surface protein epitopes, such as exotoxins, fimbriae and outer membrane proteins [95,96,99]. Albeit not cost-effective in large scale production for vaccine manufacture, such components can still be used as carrier proteins.

Polysaccharides (LPS and CPS) showed to be the most powerful epitopes of *K. pneumoniae*. However, findings indicated that a CPS-based vaccine should be multivalent at least against the major 24-CPS types, in order to cover 70% of all bacteraemic isolates [116]. The production of a 24-valent CPS vaccine was accomplished in 1986. It proved to increase IgG by 2 fold, protect from human bacteraemic cases [113–115], and have excellent antibody response in traumatic patients [121]. In addition, the produced IVIG may be used passively to ameliorate patients' status [119]. The main reticence of that vaccine is that it does not offer a full (100%) broad spectrum protection against the bacterium. On the other hand, it is composed of many CPS epitopes and, in practice, has not been released to market since its introduction in the late 80s. News on that vaccine, however, faded out since 1994 when its Phase I trials were announced [120]. Additionally, it only produces thymus-independent humoral immunity [114] that lasts for 18 months in immunocompetent individuals [120]. The role of the LPS was noticed in CPS preparations [73], and confirmed in Pasteur Institute where that CPS-vaccine lost its immunoprotective potency after alkali treatment [112], knowing that alkalis promote LPS dissociation. This confirms that LPS play an important role in immunogenicity, even when used as adjuvant in trace amounts.

Due to their endotoxic characteristics, LPS are well considered in septicemia pathology [126]. These facts attracted the attentions to LPS vaccines production that prevent LPS-caused endotoxemia [131]. Until early 1990s, *K. pneumoniae* LPS was thought to be masked by CPS, leaving it inappropriate as a vaccine candidate. However, recent studies demonstrated surface exposure of the LPS O-antigen [126]. The small number of different Klebsiellae O-antigens is a great advantage with respect to their applicability as broad spectrum vaccines (8 O-Ag against 77 K-Ag) [131,132]. Possible shared cross-reactivity with other Gram-negative bacilli is another advantage of using the LPS core region in vaccine preparation [122]. This can expand the action of LPS vaccine toward other blood stream infection pathogens, and reduce the need for adjuvants to induce a protective level of immunoglobulins [131,132]. The main drawback of active immunization with LPS is the adverse toxic reaction. However, liposomal preparations or alkali treatment of LPS reduces their toxicity significantly. This detoxified LPS induced a powerful immunogenicity against *K. pneumoniae*-mediated pneumonia [72,121,122].

The success in *K. pneumoniae* vaccine trials is the conjugate vaccines that elicit a thymus-dependent protection. The first conjugate vaccine toward *K. pneumoniae* LPS developed by coupling oligosaccharides to BSA and hence was of limited spectrum. Then, detoxified LPS were conjugated to tetanus toxoid to give an additional side-protection to tetanus toxoid. These were developed at the Punjab University to a more specialized broad spectrum vaccine, by coupling the detoxified LPS to a prepared iron-regulated cell surface protein, which requires many preparative and purification steps and was just restricted to respiratory infections. On the basis of an epitope mapping for *Klebsiella* in different patients' sera, studies revealed the involvement of all the major OMP in inducing early remarkable immune response [130]. A two-step easily prepared vaccine found its way to construction and evaluation. This new trend Egyptian vaccine against *K. pneumoniae* offered broad spectrum coverage of more than 85% to all tested clinical *K. pneumoniae* isolates, due to the O-antigen and the carrier proteins combination. It also showed to be safe, maternal, and reasonably cost effective.

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