Pseudomonas aeruginosa: The potential to immunize against infection

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Abstract

Pseudomonas aeruginosa remains a serious pathogen for specific cohorts of patients where chronic infection is a poor prognostic indicator such as those with cystic fibrosis, burn wounds or who are immune-compromised. Significant disease burden is associated with a diverse spectrum of both nosocomial and community acquired infections.

To date vaccines against *P. aeruginosa* have shown limited and often conflicting efficacy data especially against heterologous strains, which are increasingly identified as co-colonisers of biofilms. While few studies have gone beyond Phase II Clinical Trials, a particular concern is the ability of *P. aeruginosa* to evade the immune system while provoking an immune response that contributes to the destructive nature of infection. Vaccine development therefore needs to focus on preventing attachment and colonisation as well as preventing conversion to a mucoid phenotype that is characteristic of the chronic condition that promotes pathology.

Keywords

Pseudomonas aeruginosa, virulence factors, disease burden, infection, cystic fibrosis, burn, immunization, human vaccine trials

1.0 Introduction

The Family Pseudomonadaceae includes three genera involved in chronic and often fatal infections: *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas*. Whilst this review will focus on *Pseudomonas aeruginosa* there is increasing evidence that members of the other genera in the Pseudomonadaceae Family have significant pathogenic potential, and have occurred in mixed biofilms with *P. aeruginosa* [1,2].

P. aeruginosa is an aerobic Gram negative bacterium and type species for the *Pseudomonas* genus. Considered a common human saprophyte, *Pseudomonas* is opportunistic, becoming infectious in the compromised host with a dysfunctional or immature immune system, or where physical barriers are disrupted. The organism has been isolated in burn wounds, trauma, intravenous lines, urinary catheters, dialysis catheters, endotracheal tubes, neonates, cystic fibrosis (CF), acquired immune deficiency syndrome (AIDS), neutropenia, complement deficiency, hypogammaglobulinemia and iatrogenic immunosuppression. The large and complex genome varying according to strain from 6.3 to 7.1 Mbp, is presumed to attribute to *P. aeruginosa's* survival in a wide range of ecological niches [3,4]. Studies of the genomic organisation of clonal populations belies the phenotypic diversity found in this organism and serological identification of strains, based on the immunogenic nature of surface exposed antigens, is not always supported by molecular genetics (DNA-DNA hybridisation and ribotyping) [5-9]. Gene clusters were often found in different portions of the genome, and the changing clonal morphology of sequential isolates from infected patients appears to reflect the phenotypic plasticity of this organism [4,6,10]. This variability of gene expression and heterogenic phenotype is of importance when considering the role of proteins in pathogenesis and as vaccine targets. Added to this is the increasingly evident problem of gene transfer or DNA exchange between co-colonizing serogroups and even genera [11,12]. The multiple typing systems used to identify the different *P. aeruginosa* strains, of

which approximately 10% remain untypeable and represent the mucoid O-polysaccharide deficient strains, is a problem when comparing the outcomes between vaccine studies [13].

1.2 Disease Burden

Cystic fibrosis is one condition that is highly susceptible to *Pseudomonas* infections, and the subsequent lung damage remains the main cause of morbidity and mortality. The cause of this damage has been attributed not only to virulence factors from the microbe, but significantly to the host's own immune response. Neutrophilic elastase and altered levels of host cytokines, such as increased tumour necrosis factor alpha, interleukin 1, interleukin 8 (inflammatory) and reduced interleukin 10 (anti-inflammatory) have been documented in the CF patient, and may contribute significantly to host related tissue damage [14,15,16]. The means by which *Pseudomonas* establishes itself in the CF lung and its subsequent clearance has long been a contentious issue. *P. aeruginosa* expresses several different surface ligands that can recognise epithelial receptors, in particular type IV pilin that has an affinity for the glycolipid asialoganglioside 1 (asialo-GM1) and asialo-GM2, but not to the sialylated homologues [17,18,19]. However, some strains of *P. aeruginosa* have been reported to be able to bind to sialylated residues by non-pilus adhesions [17]. The CF lung is characterized by the expression of abnormal transmembrane conductance regulator protein (CFTR), which interferes with the glycosylation of epithelial cells resulting in increased asialo-GM1 expression on the apical surface. It has been suggested that this increased expression may directly contribute to the colonization of the CF lung by *P. aeruginosa* [20,21]. Different CFTR mutations expressed in CF account for varying CFTR function and hence levels of sialylation providing a range of phenotypes observed with respect to susceptibility and severity of *P. aeruginosa* infections [18,22]. There appears to be a strong association between adherence of *P. aeruginosa* to respiratory epithelial cells and homozygosity for the delta 508 mutation, while epithelial cells of heterozygote carriers and carriers of other mutations bind significantly less bacteria [23]. Antibodies directed at asialo-GM1 receptors compete with pilated bacteria for

epithelial binding sites, however, there is a lack of evidence to support the concept of preferential binding to asialo-GM1 receptor sites in CF subjects and CF mice [22,24]. Furthermore, the physiological nature of a number of the *in vitro* experimental parameters is of concern [25]. Pier and co-workers [26-29] have advanced the hypothesis that the intact CFTR acts as an epithelial cell receptor involved in the uptake of *P. aeruginosa* by respiratory epithelia, and that shedding of these cells is an important component of host defence against *P. aeruginosa* colonization. The evidence for this concept has been demonstrated in a mouse model in which CFTR-mediated internalization of *P. aeruginosa* was a key factor for host resistance to infection [24]. Finally, the highly viscous and dehydrated nature of mucous in the CF lung results in failure of the mucocilliary escalator and an inability to clear invading microbes. This viscosity of the pulmonary mucous is further contributed to by the ongoing recruitment and subsequent destruction of polymorphonuclear neutrophils (PMNs) during the inflammatory process, which releases DNA [30]. The hallmark of CF is the progressive bronchopulmonary damage associated with chronic *P. aeruginosa* infection, which, despite strong antibody responses in serum, saliva and pulmonary secretions, is unable to clear infection once established [31,32,34].

A study of CF patients found that a period of intermittent colonisation usually preceded the onset of chronic *P. aeruginosa* lung colonisation, and may occur as young as three years of age [34]. Recently it has been established that while most CF patients carry the same clone for many years, almost all patients infected with *Pseudomonas* carry multiple strains at some time [17,35]. The prevalence of *Burkholderia cepacia* infection in CF patients varies from 3-8% and has become a significant problem in CF patients as it is highly transmissible and more resistant strains are rapidly emerging [36]. Of concern is the recent discovery that *P. aeruginosa* and *B. cepacia* inhabit mixed biofilms in the lungs of CF patients and possibly influence each other through exchange of genetic information and quorum sensing mechanisms [37,38].

Burn wounds represent another major risk factor for *P. aeruginosa* infection, and the region below the eschar becomes a nidus for bacterial growth with resultant bacteremia, a lethal complication. The incidence of *P. aeruginosa* colonization is often proportional to the extent of the burn wound, the age of the patient and duration of stay in a burn unit where acquisition from other patients and the surrounding environment can occur [39]. In a longitudinal study of Indian burn cases *P. aeruginosa* was the predominant isolate in 59% of culture positive cases, with *Staphylococcus aureus* the next most common isolate at 17.9% [40]. The predominant serotypes also vary according to geographic location and local transmission. The most common serotype appears to be IATS serotype O11, however in Italy a multidrug resistant outbreak was attributed to serotype O12, which is reportedly rare in northern Europe and USA. [41,42]. Meanwhile local transmission was attributed to an epidemic in a Belgian burn unit with the epidemic strain suddenly disappearing when the occupancy rate of the burn unit dropped to 10%, suggesting a threshold effect for cross-transmission [39].

P. aeruginosa accounts for up to 70% of soft contact lens-associated cases of keratitis with the high incidence correlating to increasing popularity and unintended abuse of soft lenses [43]. Infection of the cornea can occur as a result of trauma or abrasion, where a mucin forms the main protective layer against potential pathogens. In addition, isolated clones of *P. aeruginosa* from keratitis cases have displayed increased activity levels of elastase, alkaline protease, cytotoxic exoenzyme U (ExoU) as well as numerous other mucin degrading enzymes [44,45]. The ability of *P. aeruginosa* to form biofilms on biotic as well as abiotic surfaces suggests that the lens material itself may be conducive to bacterial colonisation [46].

The ubiquitous nature of *Pseudomonas* has also led to community acquired infections which can often lead to serious complications or outbreaks. Whirlpool, spas, water-slides and lakes have contributed to outbreaks of *Pseudomonas* associated dermatitis and folliculitis as well as otitis,

with the largest single outbreak of folliculitis (265 cases) reported from a waterslide in Utah in 1983 [47,48]. Recent trends in body piercing, especially through poorly vascularised cartilage, has seen an increased risk of infection with multidrug resistant *P. aeruginosa* often resulting in serious deformation and even septic shock [49,50,51].

The spectrum of disease caused by *P. aeruginosa* is diverse, ranging from the compromised host, nosocomial infections as well as community acquired infections where the immune system is compromised, physical barriers are disrupted or abiotic surfaces, such as indwelling catheters are available to facilitate colonization.

2.0 Vaccination against *P. aeruginosa*

Given the high degree of antibiotic resistance and the difficulties inherent in long-term chemotherapy particularly against inevitable chronic and persistent infections, the development of a vaccine against *P. aeruginosa* is an appropriate and challenging strategy to pursue. A range of antigens based on surface components, proteins, enzymes, and toxins have been isolated and tested as vaccine candidates. Human trials using some of these antigens are summarised in tables 1-4.

2.1 Flagella: Pseudomonal motility is characterised by the presence of polar flagella, essential for overcoming the repulsive forces and flow associated with pulmonary airways, and in the establishment of acute infection, with flagella cap proteins having a role in mucin-specific adhesion in the absence of pili [52-55]. Hence flagella are of interest for their role in virulence and as potential vaccine targets. Distinct antigen types identified from *P. aeruginosa* flagella include the heterologous Type-a antigen with five subtype antigens, and the serologically uniform Type-b antigen [56,57]. Antigen specific murine monoclonal antibodies (MAb) inhibited flagella motility and were protective against lethal challenge in a mouse burn wound sepsis model [56]. Significantly this study demonstrated differences in flagella antigen expression among isolates.

MAbs specific to Type-b antigen recognised only 31% of isolates tested, whilst MAb against Type-a recognised 67% of isolates tested, hence a vaccine against flagella must incorporate both antigen types for broad coverage [56,58]. Human trials incorporating flagella antigen Type-b have been undertaken in Europe as a multicenter Phase III clinical trial in non-colonised CF patients [59]. Although the results of this trial have not been reported. The studies in animals and healthy volunteers show promise by inducing long lasting antigen-specific antibodies in serum and secretions [60].

2.2 Pili: *P. aeruginosa* adhesion to epithelial cells is predominantly through Type IV pili with specific receptor-binding sites located in a C-terminal disulphide-bonded loop of each pilin monomer [10,61]. *In vitro* studies have demonstrated that *P. aeruginosa* anti-pilin antibodies are cross-reactive despite poor sequence conservation in the pilin genes, but only partially blocking adhesion (56% of adhesion), indicating that other adhesins may be involved [62,63,64]. Whilst the C-terminal region of *P. aeruginosa* pilin displays a degree of sequence and structural homology, MAb directed against this region result in variable cross-reactivity and adhesion blocking activity [10,21,65,66,67]. It has been proposed that the C-terminal putative binding site may be a hidden structure becoming externalized only on contact with the target receptors, or be structurally fragile [61,64], thus a vaccine based on this region of the protein would be difficult to envisage. Various synthetic peptide consensus sequences have been examined and manipulated with limited success, in an attempt to develop a single peptide that will induce cross-reactive antibodies [67,69,70]. Structural studies (70,71,72) have demonstrated that the putative binding site may be conserved independently of sequence, provided the main-chain conformation is conserved. Whilst this would explain antibody cross reactivity across diverse type IV pilins it is an added complexity in developing a vaccine. Recently mice immunised with glycosylated pili produced antibodies that recognised pili strain specific LPS [73]. The identification of glycan on pili with similarities to the

O-antigen repeating unit of LPS suggests a vaccine candidate with the potential to afford pilus based and LPS based protection [73].

The role of the epithelial surface asialo-GM1 receptor site in pilin adherence has been demonstrated experimentally in a study whereby anti-asialo-GM1 MAb significantly reduced *P. aeruginosa* adhesion on scarified corneas of mice [74].

2.3 Lipopolysaccharide (LPS) and O-polysaccharide: LPS plays a key role in pathogenesis with the lipid A portion being a potent stimulator of macrophage activity, contributing much of the toxic effects seen in vaccine trials. While serotype-specific immune responses post infection are directed mainly at LPS, there is significant structural and antigenic heterogeneity in the LPS even among homologous strains, contributed to largely by the O-polysaccharide side chain in the outer core, with the inner core being much more conserved [75].

Heptavalent LPS vaccines incorporating the seven Fisher-Devlin-Gnabasik immunotypes, and polyvalent LPS vaccines incorporating IATS serotypes, have been tested in clinical trials involving leukemia patients, colonised and non-colonised CF patients as well as burn patients [76-82].

Longitudinal studies in leukemia patients, which represent a cohort at significant risk of *Pseudomonas* associated infections, found that despite an initial significant increase in hemagglutinating antibody titre, with 85% seroconversion reported in children, immunisation afforded only minimal protection although the results may be confounded by dose variation, possible pre-existing infections and immuno-suppressive therapy [81,82]. Indicative of possible pre-existing exposure is the variability in cross-reactive antibodies elicited, with the highest hemagglutinating response to immunotype 2 in leukemia patient sera and immunotype 6 in CF sera [80]. An increase in antibody titres specific to LPS was demonstrated in colonised and non-colonised CF patients following immunisation with a polyvalent LPS vaccine, with the latter

cohort remaining non-colonised 25-28 months post vaccination [76]. The baseline antibody levels in colonised CF patients, however, exceeded that induced by vaccination in non-colonised patients, and were of low affinity compared to vaccine induced antibodies [76].

A polyvalent (16 strain) vaccine comprising LPS and outer membrane proteins, was trialed on severe burn patients with >20% surface area burn and where other chemoprophylaxis was unavailable [77,78,79]. While protective efficacy results are inconclusive, increased antibody titres and reduced mortality was observed particularly if vaccinated within 72 hours of injury. A similar vaccine administered to a rat model of chronic pulmonary infection induced a significant increase in antibody titres (predominantly IgM) and reduced the severity of lesions, but afforded no effective clearance of bacteria [83].

All LPS vaccine trials report significant component related side-effects, and in some studies microbe replacement has been reported. An increased incidence of fungal and non-*Pseudomonas* infection has observed in leukemia patients [80] and *Klebsiella* in burn patients following vaccination [78]. In an attempt to reduce the side effects associated with parenteral immunisation, intranasal administration of a purified LPS vaccine was trialed in CF patients resulting in slight increases in serum antibody titres, but no appreciable changes in specific antibody levels in parotid saliva or sputum [84]. In contrast liposomal complete core LPS vaccines trialed on rabbits were non-pyrogenic and elicited cross-reactive antibodies to a large panel of rough and smooth *P. aeruginosa* so may offer a suitable alternative [85].

In general LPS vaccines have provided inconsistent results with limited protection, high toxicity with marked pyrogenicity, pain at the administration site, immunosuppressive effects, and have been abandoned in human studies for less toxic immunogens [80,82,86,87,89].

During acute infections, the smooth-type LPS with complete O-polysaccharide side-chains attached to core lipid A, predominates and protects the organism from complement-mediated killing [89]. In chronic infections the infecting strain undergoes an alteration to the rough-type mucoid LPS form, with shortened or completely lacking O-polysaccharide side chains, making it untypeable by O-antigen serotyping. MacIntyre et al. [90] correlated protective immunity to the O-polysaccharide chain length, with molecules over 18 O-antigen repeating units inducing a 50-100 fold increase in protective antibodies in immunised mice. Monovalent high molecular weight polysaccharide vaccines appear to be more immunogenic at low doses eliciting opsonophagocytic killing activity against the homologous strain while higher doses induce a heterologous response [91,92]. The complex nature of the O-polysaccharide side chain may limit its use as a cross-protective antigen, as different serotypes express subtype variants. In addition animal studies indicate different subtype-specific and cross-reactive antibody responses between mice and rabbits vaccinated with a heptavalent vaccine [93].

In a series of studies in which the control group was retrospectively identified it has been demonstrated that conjugation of the O-polysaccharide with exotoxin A or tetanus toxoid enhances immunogenicity, resulting in strong anti-LPS and anti-toxin responses [94-100]. Longitudinal studies of non-colonised CF patients immunised with polyvalent O-polysaccharide-exotoxin A conjugate vaccines, demonstrated strong opsonic and anamnestic antibody responses, with up to 75% of patients remaining free of infection after 6 years, with newly infecting strains belonging predominantly to the smooth typeable strains instead of the rough non-typeable strains [99,100,101]. This compared favourably against a non-conjugated O-polysaccharide vaccine trial of Langford & Hiller [101]. Currently, a Phase III, double-blind, randomised, placebo-controlled, multicenter efficacy trial using a polyvalent O-polysaccharide exotoxin A conjugate vaccine in non-colonised CF patients is in its fourth year [102].

Immunisation with O-polysaccharide vaccines are well tolerated. However, their use may be limited as a result of poor immunogenicity and the existence of over 20 serotype specific antigens.

2.4 Mucoïd Exopolysaccharide (MEP): *P. aeruginosa* transforms from planktonic forms to slow growing, non-motile mucoid colonies accompanied by the excess production of mucoid exopolysaccharide (MEP) also referred to as alginate. MEP production appears to inhibit both opsonic and non-opsonic phagocytosis and is indicative of chronic infection [89]. The presence of high levels of opsonic anti-MEP antibodies in older CF patients who are free of *P. aeruginosa* infection, as well as the observation that early colonizing strains produce small amounts of MEP, suggest that MEP may be a useful vaccine candidate [103-106].

In animal and human studies MEP vaccines preferentially elicit either opsonic or non-opsonic antibodies dependent on the molecular size and dose of the MEP antigen and the presence of pre-existing non-opsonic antibodies to MEP [103,105,107,108]. Low molecular weight MEP appears to be poorly immunogenic, whereas high molecular weight MEP elicited opsonic antibodies in 80-90% of healthy volunteers [107,109]. The linking of MEP to carrier proteins such as exotoxin A or keyhole limpet hemocyanin enhanced the immune response in rabbits and mice, specific to MEP and elicited opsonic antibodies that recognised heterologous MEP [103,104]. In a mouse model of acute pneumonia, monoclonal antibodies specific to alginate afforded protective efficacy and phagocytic killing against *P. aeruginosa* strains that had the genetic capability to synthesis alginate *in vivo* whether or not the strains were phenotypically mucosal or non-mucosal *in vitro* [110]. As most clinically isolated strains of *P. aeruginosa* are able to produce MEP, particularly in chronic infections, immunoprophylaxis or therapy is plausible.

Despite good antibody responses being observed following immunization with MEP, and the encouraging passive immunization results in an animal model, caution must be exercised that an

immune response is not induced to a secreted component that would result in widespread pathology at the site of infection. Further studies are needed to determine whether or not such fears are justified.

2.5 Exotoxin and Enzymes: *P. aeruginosa* produces several extracellular enzymes and toxins, which are implicated in localized and systemic pathologies [111]. These compounds are disruptive to eukaryotic signal transduction through the action of an ADP-ribosyltransferase and all are dependent on a Type III secretion system, except exotoxin A [112,113,114]. Exotoxin A is the most toxic extracellular protein produced by *P. aeruginosa*, however, its expression varies between strains [115,116]. Through manipulation of specific amino acid residues, exotoxin A is modified to a non-toxic form for use in conjugate vaccines and induces antibodies capable of blocking the exotoxin binding to its receptor [117-121]. Immunisation with a recombinant exotoxin A, or plasmid constructs of a mutant exotoxin A, induced neutralising antibodies and protected mice from the effects of the toxin following lethal challenge [114,122]. Passive immunisation with sera obtained from a detoxified exotoxin A vaccine was also able to neutralize the toxic effects of exotoxin A and effectively protect mice [123].

Elastase and alkaline protease contribute not only to pathogenesis, particularly lung injury, but also impair the immune response of the host by cleaving immunoglobulins and inactivating cytokine activity [124,125]. Immunisation of rats with an elastase peptide induced high antibody titres in the lung which neutralised proteolytic activity and reduced the severity of lung injury by both *B. cepacia* and *P. aeruginosa* [124]. Immunisation with mutant or detoxified elastase and alkaline protease are reported to be protective against corneal ulcerations in mice, haemorrhagic pneumonia in mink, and severe lung infection in rabbits, however, no human studies have yet been undertaken [126,127,128]. Combination vaccines incorporating toxoids of exotoxin A and alkaline protease have increased survival to 60% against *P. aeruginosa* sepsis in mice, though not all studies using

vaccines derived from enzymes and toxins have demonstrated protective outcomes in animal models [129,130]. Vaccines comprising elastase, exotoxin A and outer membrane protein (Opr)F in combination or alone, when compared for efficacy in reducing lung injury in rats following *P. aeruginosa* infection, demonstrated variable outcomes [131]. The OprF significantly reduced lung lesions, elastase afforded some protection, a combination of OprF and elastase afforded no protection, and exotoxin A with OprF increased the incidence of lesions. Surprisingly, all three components when combined gave the same level of protection as OprF alone.

Invasive strains infiltrate host cells by injecting type III effector molecules (ExoS, ExoT, ExoU) causing rapid cell apoptosis, fatal sepsis in burn injury, and epithelial injury in acute lung infections [31,132,133,134]. A vaccine targeting PcrV (a type III secretion system translocating protein) has been used in normal, burn and immune compromised animal models of *P. aeruginosa* infections resulting in significantly reduced bacterial load and inflammatory damage resulting in enhanced protection and survival [114,135,136,137].

While most vaccine candidates comprise surface exposed antigens or virulence factors, the cytosolic derived enzymes, catalase A, aminopeptidase and amidase have also demonstrated significant immunogenicity and enhanced lung clearance of *P. aeruginosa* in immunized animals [138]. Hence, vaccines based on bacterial enzymes and toxins, were generally successful in inducing antibodies capable of neutralising the *Pseudomonas* associated toxic effects and pathology, but not all demonstrated bacterial clearance capabilities.

2.6 Outer Membrane Proteins (Oprs) and Chimeric Vaccines: Outer membrane proteins (Opr) contribute to the structural integrity of the cell membrane and also form trans-membrane channels allowing selective permeability into the periplasm. A number of Oprs have been consistently identified in *P. aeruginosa*, (Oprs D, E, F, G, H & I), while others are expressed only

under growth limiting conditions (Oprs P, G, E, C, IROMP, FBP) [139,140]. Those considered antigenic determinants for vaccine use are the more highly conserved and abundant forms, OprF, OprI and OprH.

Phase I/II clinical trials using a polyvalent Opr vaccine (10-100kDa) in healthy volunteers and burn patients induced a rapid increase in antibody titre (100% seroconversion) with complement specific opsonophagocytic activity, and increased survival through reduced bacteremia [141-144].

An OprF vaccine comprising OprF from seven Fisher-Devlin-Gnabasik immunotypes, afforded significant protection in mouse burn wound and rat pulmonary infection models with high titres of cross-reactive, antigen specific antibodies, increased clearance from lungs and reduced severity of pulmonary lesions [145,146,147]. While LPS contamination of OprF vaccines was suspected of contributing to the resultant immunogenicity, a recombinant OprF produced similar vaccine efficacy in the absence of LPS, and was further explored through the use of OprF DNA vaccines [145,148,149,150]. The OprF DNA vaccine induced opsonophagocytic activity and reduced severe lung lesions in immunised mice, but passive transfer of immune sera failed. Specific amino acid segments of OprF (peptide 9 and peptide 10) have been identified as affording the highest probability of surface exposure, and have been incorporated in recombinant and hybrid vaccines [150,151]. Animal studies using monovalent recombinant Opr I induced cross-reactive antibodies, that recognised all serotypes in the IATS schema, as well as high titres of sIgA antibodies in the gut mucosa after oral immunisation [152,153,154]. Vaccination of healthy volunteers using recombinant OprI expressed in *E. coli* induced increased antibody titres with a significant dose dependant variation in response and complement mediated opsonization, with 90% of volunteers seroconverting [154,155]. This was compared to 75%-88% seroconversion in healthy volunteers and burn patients following vaccination with an OprF-I fusion protein [156,157]. The OprF-I

hybrid vaccine, however, afforded greater protection against systemic infection in immune suppressed mice than was afforded using a single Opr vaccine [154].

Hybrid OprF-I vaccines have been evaluated for safety and immunogenicity in animal and human studies and have been shown to induce cross-protective antibodies against all 17 IATS serotypes [156,158]. Phase I and Phase II studies in burn patients using this hybrid vaccine determined the need for two doses to reach the equivalent antibody titre of healthy individuals after a single dose [159]. Bacterial clearance was predominantly mediated by increase titres with an IgG1 isotype and complement dependent opsonisation, augmented by antigen-specific sIgA antibodies [156]. Intranasal administration of a OprF-I hybrid vaccine, formulated in emulgel and sodiumiododecylsulfate, to healthy volunteers demonstrated significant increases in IgA and IgG antibodies in serum [157]. These researchers have indicated that a Phase III trial is proposed for patients with CF using the same formulation.

Recently, hybrid proteins combining specific epitopes of interest have been used as chimeric vaccines. These have included epitopes of Type IV pilin combined with exotoxin A, which when tested on rabbits reduced adherence and neutralised exotoxin A in immunised animals [160]. Most chimeric vaccines incorporate Opr F and I, either as hybrid OprF/I or as OprF-viral chimeras with the viral components derived from Cowpea Mosaic Virus (CPMV), Tobacco Mosaic Virus (TMV) or Influenza Virus with promising results in animal studies of both chronic and acute pulmonary infection [161-165]. These vaccines have induced high titres of epitope specific IgG, demonstrated opsonophagocytosis, afforded protection against challenge and reduced lung lesions in vaccinated mice. A particular drawback appears to be the significant variation in immune response to chimeric vaccines, dependent on the vector used, and the length of the peptide and locality of its insertion [166,167,168]. It has been fairly well established from outer membrane protein vaccine studies that the most effective and protective epitopes on OprF are in the peptide 9 and peptide 10

domains. These have proven difficult to join onto carrier proteins limiting peptide 10 to Influenza virus, and peptide 9 to Tobacco Mosaic Virus [164]. A chimeric protein vaccine composed of receptor binding epitopes of exotoxin A with Opr I and F, had variable results in ability to neutralise cytotoxicity and increase opsonophagocytic uptake [123].

While chimeric vaccines hold exciting prospects extensive animal studies are still pending. Oprs remain the most promising vaccine candidates for immunisation against the diversity of infections caused by *P. aeruginosa*.

2.7 Whole cell sonicate, live and dead *Pseudomonas aeruginosa*: *P. aeruginosa* vaccines comprising, whole-cell sonicates, formalin or heat killed bacteria, temperature sensitive mutant strains and attenuated live bacteria have been trialed. The array of human subjects included in vaccine trials is also diverse encompassing numerous medical conditions, invariably where other chemoprophylactic therapies are unavailable or have failed, or where prophylactic benefit is expected as in pre-surgery cancer patients. A purified soluble antigen extract from seven Fisher-Devlin-Gnabasik immunotypes administered to burns patients induced increased antibody titres that correlated with resistance to infection, and was variably cross-reactive, with the strongest reaction to immunotype 2 [167]. A polyvalent heat killed *Pseudomonas* vaccine, which was administered to a cohort of 287 burn patients induced anti-*Pseudomonas* antibodies by day 4-5 with a maximum increase in titre by day 10, however, *Pseudomonas* positive cultures were evident by day 15 [168]. Trials in non-colonised CF patients followed over a 3-year period, reported an initial vaccine induced rise in antibody titre. This decreased over time and was associated with positive *Pseudomonas* culture in sputum but no overall change in the disease process [98,101].

Mucosal intestinal immunisation with a whole cell formaldehyde killed *P. aeruginosa* vaccine in animal studies afforded significant protection against acute lung challenge with live bacteria.

Specific antibody responses as well as macrophage and PMN recruitment to the lung were observed [169,170,171]. In addition it appears the inflammatory response in the lungs was also down regulated in immunised animals following challenge with live bacteria [170]. This research group also demonstrated that specific immunisation against *P. aeruginosa* could be transferred using CD4⁺ T-helper lymphocytes and by passive immunisation using donor cells and sera from animals immunised with this vaccine [172,173]. Most importantly it was demonstrated that enhanced bacterial clearance from the lung following immunisation of animals was not vaccine strain specific, suggesting that cross-protective immune responses were induced. This vaccine has been trailed in patients with *P. aeruginosa* associated bronchiectasis [174] and in healthy volunteers [175]. In bronchiectasis patients, oral vaccination resulted in the detection of circulating antigen-reactive peripheral blood leukocytes as well as a significant reduction in the levels of *P. aeruginosa* in sputum. In normal healthy volunteers the vaccine resulted in a strong anti-LPS specific antibody response and opsinophagocytic killing. In both bronchiectasis patients and healthy volunteers no side effects to oral immunisation occurred.

An alternative approach to mucosal immunization is to administer a live, attenuated *P. aeruginosa* strain intranasally. Although no human studies have been conducted at this stage, intranasal immunization of mice with an *aroA* deletion mutant demonstrated protection against acute pneumonia when challenged with homologous but not heterologous strains [176,177]. Of interest, as in whole killed cell intestinal immunization, intranasal immunization included both specific humoral and cellular immune responses suggesting that a combination of both is required for optimal vaccine induced protection against *P. aeruginosa* infection.

3.0 Conclusion

P. aeruginosa is the most common and persistent pulmonary pathogen in CF, and a significant contributor to nosocomial infections. Due to its diverse phenotypic and serological nature, *P.*

aeruginosa presents a significant challenge to vaccine development, which will require a multicomponent vaccine to attain cross-reactive and cross-protective immunity against all serotypes. The presence of pre-existing antibodies to *P. aeruginosa* in non-immunised healthy and infected individuals from prior exposure may also inhibit vaccine efficacy, particularly through boosting existing and perhaps non-efficacious immune responses.

The importance of the flagella and pili in adhesion has been proven yet their suitability as vaccine candidates remains uncertain in view of non-conserved epitopes and their inconsistent presence or loss in colonizing strains. Early promising vaccines which included heptavalent or polyvalent LPS, or components of whole killed cells were either inconsistent with their protective efficacy, toxic or poorly tolerated, and ultimately have been withdrawn from routine clinical use. The O-polysaccharide vaccine only provides homologous protection against the vaccine strain, and is among the least immunogenic of components. The use of MEP is likely to be limited to strains that have the genetic capability to produce MEP, and further studies are required to determine whether MEP specific responses induced by immunization, exacerbate pathology at the site of infection. Anti-toxoid vaccines with potential for their effectiveness in neutralising the damaging effects of bacterial enzymes and toxins, appear useful for reducing pathogenicity but are limited in disease prevention. Outer membrane proteins that are highly conserved among all serotypes represent a good choice for inducing cross-reactive and cross-protective immune responses. These can be produced in ample quantities through recombinant technology and specific protective epitopes hybridised to various carrier proteins as chimeric vaccines. These have been proven to be safe and well-tolerated, immunogenic, and induce antibodies that enhance phagocytosis, however, they have not been trialed in CF patients. Oral immunization with whole killed cells has been shown to be effective in an animal model of acute lung infection as well as in patients with bronchiectasis and healthy subjects. Strong anti-LPS antibodies were induced and no vaccine side

effects were observed. Intranasal immunization with live attenuated organisms require study in human subjects.

A consistent stumbling block for vaccine success is the inherent problem of lack of cross-reactivity to the many serotypes strains and genomovars. An ideal protective *Pseudomonas* vaccine would have to include antigens that induce cell mediated and humoral responses with opsonisation and effective phagocytosis of the pathogen without compounding immune-complex associated tissue damage.

Opinion

With respect to vaccine candidates for use in immunization against *P. aeruginosa*:

- The major outer membrane proteins hold the most promise,
- Non-integral proteins are worthy of further investigation and human trial,
- Whole killed cell vaccines, particularly administered orally, are well tolerated, show promise and require further study,
- Live attenuated organisms administered intranasally are worthy of further study. A vaccine consisting of multiple serogroups may be required,
- LPS is considered too toxic for further clinical trial at this time, while the O-polysaccharide is poorly immunogenic and induces immune responses only against homologous strains,
- Both LPS and O-polysaccharide require a polyvalent vaccine formulation,
- MEP (alginate) has potential, although further studies are required, to determine that immune-complex associated tissue damage is not enhanced by antibodies to this widely dispersed, secreted component.
- Toxin and virulence-associated enzyme vaccines may be suitable for reducing pathology but not preventing infection.

Thus the major challenge for any vaccine is that its efficacy is not compromised by the existence of hypermutable strains, mixed colony biofilms (including the co-existence of both *P. aeruginosa* and *B. cepacia*), the range of serotypes and genomovars, existing strain-specific immune responses and the diversity of the types of infection.

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Table 1: *P. aeruginosa* vaccine trials conducted in healthy volunteers

ACTIVE COMPONENT	OUTCOME	REFERENCE
Flagella (Type-b)	Tolerated; long lasting antibody titre	[59,60]
Hmw MEP (strain 2192)	Subcutaneous, IMI Larger polymer elicited long lived opsonic antibodies in 80-90% of vaccinates	[109]
Oprs (Immunotypes 1,2,3,6)	Subcutaneous, IMI Significant Opr specific antibody response; 100% seroconversion via IMI	[143,144]
Opr I (Recombinant)	Dose response study; Tolerated & safe ↑ in antibody titre with subsequent does; lasted 30 wks; Antibody mediated complement dependent opsonisation; 90% seroconversion Significant individual variation in response	[154]
Opr F-I	75% (6/8) seroconversion after IN administration; individual variation in response Dose response study; local side effects; higher antibody titre with higher dose Sera IgA IgG; complement binding; opsonisation	[156,157,158,178]
Hmw O-polysaccharide (Immunotype 1)	Dose dependent ↑ in binding and opsonic antibody (IgA & IgG); lasting 6 mos Cross-reactive Ab	[92,179,180]
O-polysaccharide-exotoxin A conjugate (Immunotype 5)	Mild reactions; Anti-LPS and anti-toxin antibody response; IgG1 + IgG2 specific to LPS with delayed IgG4 specific to toxin A. Anamnestic response on booster; mainly anti-toxin antibody response	[95,96,97]
O-polysaccharide-tetanus toxoid conjugate	Mild reaction to subcutaneous administration 19 fold ↑ mean antibody titre; ↑ IgG to LPS + Ttxd; opsonic activity; 82% seroconversion	[94]
Whole cell <i>P. aeruginosa</i> (Formaldehyde killed)	Safety and immunogenicity study Mucosal immunisation induced anti-LPS antibodies	[175]
<i>P. aeruginosa</i> Exotoxoid A	Safety and Immunogenicity trial	[187]

Abbreviations

IMI = intramuscular injection; IN = intranasal; Hmw = high molecular weight; Wks = weeks; ↑ = increase; Ttxd = tetanus toxoid.

Table 2: *P. aeruginosa* vaccine trials conducted in burn patients

ACTIVE COMPONENT	OUTCOMES	REFERENCES
Oprs (Immunotypes 1,2,3,6)	IMI in patients with >10% body surface burns. Significant antibody titre at highest dose; overall ↓ <i>P. aeruginosa</i> ; opsonophagocytic- killing activity; cross-reactive antibodies against heterologous strains	[141,142]
OprF-I	IMI in patients with 35-55% body surface burns 88% (7/8) seroconversion with 3 fold ↑ in antibody titre	[157,160]
LPS (Polyvalent) Anti-LPS Immunoglobulins	↓ mortality; ↓ <i>P. aeruginosa</i> positive culture In non-infected - 100% survival compared to 40% in controls. In infected - Reduced mortality from 40.6% to 6.6% in adults; 20.8% to 4.8% in children Lowest mortality in children passively immunised at 0% and 21% in controls Combined passive and active immunisation gave less protection (13.6% mortality) than vaccine alone; Infection less common in passive immunised patients ↑ <i>Klebsiella aerogenes</i> infections in immunised	[77,78,79]
LPS (polyvalent) Immunoglobulin	Patients with 5-70% body surface burns 4-fold reduction in mortality in adults and 2- fold in children Species replacement with <i>Klebsiella</i> <i>pneumoniae</i>	[181]
"Heptavalent purified soluble <i>Pseudomonas</i> antigen"	↑ antibody titres; Variable cross-protection to all immunotypes; Immunotype-2 highest pre & post vaccination. in response.	[167]
Whole cell <i>P. aeruginosa</i> (Polyvalent)	Single dose not sufficient for early protection	[182]
Whole cell <i>P. aeruginosa</i> (Heat inactivated)	↑ <i>Pseudomonas</i> specific antibody titre by day 4-5 with max. on day 10, however, <i>Pseudomonas</i> positive cultures by day 15	[168]

Abbreviations

IMI = intramuscular injection; ↓ - decrease; ↑ - increase.

Table 3: *P. aeruginosa* vaccine trials conducted in CF patients

STATUS	ACTIVE COMPONENT	OUTCOME	REFERENCES
Non-colonised CF	LPS (Polyvalent)	<p>↑ anti-LPS IgG; ↑ affinity antibodies</p> <p>Remained non-infected 25-28 months post vaccination</p> <p>Slight ↑ in serum antibodies, less in saliva and sputum after IN administration</p> <p>Toxicity</p>	[76,80,84]
	O-polysaccharide	<p>3 year study (n=34)</p> <p>No difference in overall disease progress or colonisation</p>	[101]
	O-polysaccharide-exotoxin A conjugate	<p>Anti-LPS, anti-exotoxin A response</p> <p>High affinity anti-LPS antibodies; cross-reactive to vaccine strains</p> <p>Opsonic and neutralising antibody response</p> <p>4yr follow-up: Anti-LPS antibodies comparable between infected and non-infected patients; 61.5% remained free of infection</p> <p>4 doses over 3 years (n=30). Decline in Ig titre in 3rd year associated with ↑ isolation of <i>P. aeruginosa</i></p> <p>6yr follow-up: smooth type in immunised vs rough type in non-immunised; anamnestic response at 12 + 36 months booster</p> <p>10yr follow-up: mucoid infection higher in controls (44% compared to 25% in vaccinates)</p> <p>Mild adverse reactions</p>	[98,99,100,102,183]
Colonised CF	LPS (Heptavalent)	Haemagglutinating antibody response	[80]
	LPS (Polyvalent)	<p>High baseline antibody titre exceeding that induced in non-colonised vaccinates</p> <p>↓ opsonophagocytic killing activity</p> <p>↑ anti-LPS IgG; ↓ affinity antibodies</p>	[76]
	LPS-free OMPs (34.5kDa, 37.5kDa, 58.5kDa)	Did not protect CF patients against further lung infections with <i>P. aeruginosa</i>	[184]

Abbreviations

IN = intranasal; n = number; yr = year; ↑ = increase; ↓ = decrease; kDa = kilo Daltons

Table 4: *P. aeruginosa* vaccine trials conducted on patients with conditions other than burns or CF

CONDITION	ACTIVE COMPONENT	OUTCOME	REFERENCES
Trauma	O-polysaccharide-exotoxin A	>4 fold ↑ antigen specific antibody titre; some cross-reactivity	[185]
Leukemia/lymphoma Leukemia children Acute leukemia	LPS (heptavalent)	Side effects; cross-reactive; no significant difference in bacteremic mortality; however, overall ↓ in <i>P. aeruginosa</i> bacteremia 85% seroconversion which declined rapidly; predominantly IgM ↑ fungal and non- <i>Pseudomonas</i> infections	[80,81,82]
Chronic infected mastoid (n=13)	Whole cell <i>P. aeruginosa</i>	↑ in antibody titre; good immune response over 6 months.; ↑ IgG, IgA, IgM; some improvement but recurrence did occur	[186]
Sepsis & bacteremia	Hyperimmune <i>P. aeruginosa</i> -IgG (Tetavalent) (IVI)	↑ Antibody titre associated with clinical improvement; 70% survival	[188]
Bronchiectasis patients	Whole cell <i>P. aeruginosa</i>	Specific lymphocyte responses after oral immunisation	[174]

Abbreviations

IVI = intravenous injection; ↑ = increase; ↓ = decrease.