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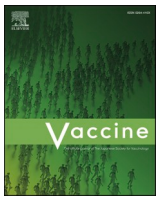

Pseudomonas aeruginosa vaccine identified by the AI-immunology™ platform improves outcomes in a murine biofilm lung infection model

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ABSTRACT

The Gram-negative opportunistic bacterial pathogen, *Pseudomonas aeruginosa* is considered by WHO as a "priority pathogen" for which new antibacterial strategies are urgently needed due to antimicrobial resistance development. In addition, *P. aeruginosa* is a cause of difficult to treat chronic infections due to its ability to form biofilms. Therefore, pseudomonal vaccines have been proposed as alternative strategies to combat these infections for the last 50 years, however, no vaccines are available on the market for human use. The aim of this study was to investigate the capacity of a vaccine composed of seven antigens, identified using EDEN™ (Efficacy Discriminative Educated Network) - a proteome-wide in silico antigen prediction model within AI-Immunology™ platform - in improving outcomes in a murine model of chronic *P. aeruginosa* lung infection. The primary endpoint was quantitative bacteriology (Colony forming units - CFU) in the lungs of immunized animals compared to control animals. The secondary endpoints were clinical signs (a clinical score), body temperature and weight loss. Mice immunized with the heptavalent combination vaccine had a significantly 1.2 log10 lower lung CFU compared to the control group. Furthermore, the vaccinated mice presented significantly fewer clinical signs of infection, had less reduction in body temperature and weight loss as, compared to control mice. There was a statistically significant correlation between the lung bacteriology and secondary endpoints. Antibodies against all seven antigens were measured by ELISA confirming their immunogenicity. The encouraging results obtained in this, and previous studies provide a proof-of-concept that EDEN™ is a useful tool in identifying vaccine antigens against *P. aeruginosa* and possibly other problematic pathogens.

1. Introduction

The burden of diseases caused by *Pseudomonas aeruginosa* biofilms is substantial, particularly for patients with chronic pulmonary disease, such as those with cystic fibrosis (CF), non-CF bronchiectasis, primary ciliary dyskinesia (PCD) and severe chronic obstructive pulmonary disease (COPD) [1]. In addition, critically ill patients, including those receiving mechanical ventilation at intensive care units as well as immunocompromised patients are also at significant risk of acquiring *P. aeruginosa* infections. In fact, in a worldwide epidemiology study of ventilator-associated pneumonia (VAP), *P. aeruginosa* was the most common pathogen, with a prevalence of 3 %–5 % in adults ventilated for longer than 48 h [2]. Treatment of *P. aeruginosa* infection is challenged by the organism's intrinsic resistance to several antibiotics, its capacity to readily acquire multi-drug resistance and its ability to form biofilms in the airway mucus and on medical device surfaces like airway and feeding tubes, further increasing their tolerance to antibiotics. Therefore, treatment regimens of *P. aeruginosa* infections often include a combination of antibiotics in increased dosage to be successful. The biofilm formation capacity leads to a range of persistent infections especially in chronic pulmonary disorders such as patients with CF who can acquire chronic *P. aeruginosa* infection which is associated with

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accelerated decline in pulmonary function and reduced quality of life. Similarly, the organism acts as a marker of severe disease and poor prognosis in the 10–30 % of patients with non-CF bronchiectasis [1]. According to the World Health Organization [3], chronic obstructive pulmonary disease (COPD) was the fourth leading cause of death worldwide in 2021, responsible for approximately 3.5 million deaths, which accounted for about 5 % of all global deaths. Consequently, both acute and chronic *P. aeruginosa* infections are associated with significant morbidity, increased mortality and considerable costs to the health system. Thus, specific patient groups are vulnerable to acute or chronic lung infection with this opportunistic pathogen, making a *P. aeruginosa* vaccine an attractive alternative to the challenging antibiotic treatment approach. An active immunization against *P. aeruginosa* infection could be administered to people at higher risk. This population would include those under hospitalization for patients with CF or COPD, PCD or non-CF bronchiectasis and patients who are admitted to ICUs. Despite a substantial effort over the past 50 years, a vaccine licensed for human use is still not available. Several *P. aeruginosa* antigens have been identified as potential vaccine candidates and pursued to different stages of development including lipopolysaccharides (LPS), O-antigen, polysaccharides, polysaccharide-protein conjugates, outer membrane protein F and I, the type III secretion system component PcrV, flagella, pili, DNA and whole killed cells in addition to a number of non-integral outer membrane protein candidate antigens [4,5].

EDEN™ (Efficacy Discriminative Educated Network) - a proteome-wide in silico antigen prediction model within the AI-Immunology™ platform - has been used to successfully identify vaccine candidates showing protection in preclinical challenge models, for seven different pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) [6] and *Neisseria gonorrhoeae* [7]. The aim of this study was to investigate the capacity of a vaccine composed of seven antigens, identified using EDEN™ in improving outcomes in a murine model of biofilm *P. aeruginosa* lung infection [8]. The primary endpoint was quantitative bacteriology in the lungs of immunized animals compared to control animals. The secondary endpoints were clinical signs of infection (a clinical score), body temperature and weight loss after infection.

EDEN™ is an AI model capable of rapid identification of novel, highly protective antigens for the use in pathogen-specific vaccines. EDEN™ has been designed to rapidly identify those B-cell antigens that will trigger a robust protective immune response. The model integrates a proprietary ensemble of machine learning algorithms trained on Evaxion's specialized dataset, which includes data from both clinical and preclinical studies. EDEN™ processes full-length protein amino acid sequences and transforms them into high-dimensional feature vectors capturing sequence-specific, structural, and immunologically relevant properties. These features enable the model to differentiate between proteins that are likely to induce protective immunity and those that are not. For this study, the complete proteome of *Pseudomonas aeruginosa* reference strain PAO1 was analyzed using EDEN™ version 4.1. Proteins ranked with the highest predicted protective potential and amenability to recombinant expression in *Escherichia coli* were selected for further evaluation as vaccine candidates.

We show here, in a mouse model of biofilm *P. aeruginosa* lung infection [8], the protective capacity of a heptavalent pseudomonal vaccine composed of outer-membrane antigens identified by the EDEN™ antigen discovery model.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with Danish law and approved by the Board of Animal Experiments under the Danish Animal Experiments Inspectorate of the Ministry of Environment and Food (2013-15-2934-00857). Experimental procedures were reviewed and approved by the animal welfare committee at University of Copenhagen and experiments performed by specially trained personnel. Animals were anaesthetized by an injection of ketamine and xylazine during the intranasal [9] challenge. Isoflurane was used when the temperature transponders were inserted. Every effort was made to minimize suffering. The clinical status of the mice was evaluated daily registering details of fur, posture, movement, eyes and breathing for each animal and a clinical score (suppl. Table 2) was calculated.

2.2. Antigen discovery

The EDEN™ model is an artificial intelligence-driven antigen discovery model based on an inverse vaccinology technique starting from the proteomic profile of the microorganism. EDEN™ has been developed to discover novel, protective antigens for vaccine development against pathogens. At its core, EDEN™ leverages a proprietary ensemble of artificial neural network algorithms that analyze immunologically relevant data to identify B-cell antigens with disease protective properties when used in vaccines.

The EDEN™ antigen discovery workflow involves four key steps: (i) selection of a relevant pathogen proteome, (ii) proteome-wide analysis using EDEN™, (iii) identification and design of high-potential antigen candidates, and (iv) evaluation of protective efficacy in preclinical in vivo models. *Pseudomonas aeruginosa* strain PAO1 [10] was chosen for analysis as a well-characterized laboratory reference strain widely used in studies of pathogenesis, biofilm formation, and antimicrobial resistance. Its complete proteome, comprising 5561 proteins, was processed using EDEN™ 4.1, resulting in a ranked list of candidate antigens. Proteins predicted to have the highest ability to elicit a strong protective antibody response and to express well in *Escherichia coli* were selected. The selected antigens were designed for recombinant production, e.g., by removing transmembrane regions and signal peptides, aiming to ensure solubility and yield.

2.3. Protein expression and purification

Eighteen peptides representing 14 different proteins identified by EDEN™ within the top 30 rankings were expressed in *E. coli* using an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible expression system, as described in the Qiagen protocol, the QIA*expressionist*™. In short, *E. coli* was transformed with plasmid DNA inserted into a pQE1expression vector (Genscript, USA). Successfully transformed *E. coli* were used to inoculate 1 L of Luria Broth (LB) Base (Invitrogen, CA, USA) with 25 μg/mL Kanamycin (Sigma-Aldrich, MO, USA) and 50 μg/mL Ampicillin (Sigma-Aldrich). The culture was incubated at 37 °C with vigorous shaking until reaching an $OD_{600\ nm}$ of 0.5–1.0, upon which IPTG (VWR, 43714 5×) was added to a final concentration of 1 mM. The cells were harvested by centrifugation (4500 x*g*, 20 min) after four hours of incubation and the pellet was dissolved in binding buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8.0). The lysate was centrifuged (10,000 x*g*, 20 min), and the supernatant incubated with Probond™ Nickel-Chelating Resin beads (Novex, 46–0019). The resin beads were washed with binding buffer and the proteins eluted using elution buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris, 300 mM NaCl, 300 mM imidazole, pH 8.0). The eluted proteins were dialyzed against decreasing concentrations of urea in saline, before finally being dialyzed against physiological saline. The protein solutions were filtered through a 0.45 μm pore-size filter into sterile tubes, and the quality of the purified proteins was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The approximate protein concentrations were determined by measurement of optical density at $OD_{280\ nm}$ and conversion by means of specific molar absorption coefficients calculated for the individual amino acid sequences.

The seven outer membrane antigen constructs, selected after pilot studies testing their immunogenic potential, are presented with their respective protein locus name, expressed amino acids and predicted

function in Table 1.

2.4. Protein conservation analysis

The protein construct conservation across the NCBI chromosomes library comprising 300 completely sequenced and translated strains was analyzed by sequence alignment (Smith-Waterman) algorithm [11] based on amino-acid sequence of the tested protein antigens. The pairwise alignment values (% similarity and % coverage) were calculated and the most similar protein to a given genome was selected and classified as homologous. Homology was defined as % similarity >75 % on minimum 3/4 of total protein length [12].

2.5. Immunization

Eight-weeks old female NMRI outbred mice (Janvier, France) were used for the studies. Prior to the lung infection with *P. aeruginosa*, the mice were divided into a control and a vaccinated group and were immunized subcutaneously (s.c.) three times at two-week intervals. To facilitate the experiments, the mice were separated in two staggered experiments following an identical protocol and performed by the same technical staff as presented in suppl. Table 1 with 21 mice in the first and 32 mice in the second experiment, in each of the two groups. All data obtained in these two experiments were pooled together and used for statistical analysis.

The mice in the vaccine group ($n = 53$ mice) received a heptavalent combination vaccine consisting of the recombinantly expressed *P. aeruginosa* antigens; PA1302, PA0931, PA2070 (long version), PA2070 (short version), PA2976, PA3901 and PA0041 (see Table 1). Antigen selection was based on a two-step strategy involving: (i) high-ranking predictions by the EDEN™ antigen discovery model, and (ii) evidence of strong antigen-specific antibody titers in preliminary screens. Proteins with high EDEN™ scores but poor immunogenicity were excluded. The multivalent formulation was tested in its entirety to maximize the likelihood of demonstrating protective efficacy in the biofilm lung infection model. Subsequent studies will evaluate the contribution of individual antigens to refine the vaccine composition.

Endotoxin (lipopolysaccharide, LPS) was not removed from the *E. coli* produced recombinant antigens prior to immunization, potentially contributing an additional adjuvant effect through activation of Toll-like receptor 4 (TLR-4). At all three immunizations, the mice in the vaccine group received 15 μg of each protein, in accordance with actual recommendations of mice immunizations [13]. Mice in the control group ($n = 53$ mice) received adjuvant alone and did not receive any *E. coli* derived LPS. For the first immunization the antigens were formulated with aluminum hydroxide ($Al(OH)_3$) (Brenntag, Denmark) at a ratio of 100 mL $Al(OH)_3$ per 125 mg protein and Freund's incomplete adjuvant (Sigma-Aldrich) at a 1:1 ratio to the total volume of protein and $Al(OH)_3$. For the following two immunizations, only $Al(OH)_3$ was used as adjuvant. Due to volume restrictions, the heptavalent combination vaccine was administered s.c. at both flanks. Three proteins in combination with adjuvant, were injected in the left flank of the mouse and the other four proteins were injected in the right flank. This routine was applied for all three immunizations.

Table 1
Antigens included in the heptavalent *P. aeruginosa* vaccine. The length of each antigen construct and the predicted function of the protein encoded by the respective genes are indicated.

Locus name	Expressed amino-acid sequence (length aa)	Predicted function
PA0041	34–550 (517)	TpsA2 (two-partner secretion system type Vb) [27] hemagglutinin extracellular
PA0931	29–742 (714)	ferric enterobactin receptor PirA (siderophore receptor)
PA1302	31–851(821)	haem receptor HxuA/C synonym (iron acquisition from haem, target for pyocins [14])
PA2070 (short)	173–880 (708)	TonB-dependent receptor OptM with function in metal uptake [15]
PA2070 (long)	29–880 (852)	TonB-dependent receptor OptM with function in metal uptake
PA2976	1–480 (480)	rneE ribonuclease E, a cytoplasmic protein identified in the proteome of outer membrane vesicles of *P. aeruginosa* PAO1 [14]
PA3901	33–784 (752)	fecA Fe(III) dicitrate transport protein FecA present in OM *P. aeruginosa* citrate-inducible FecA receptor [28]

2.6. Temperature transponders

Four days before the challenge, temperature transponders (Bio Medic Data Systems, DE, USA) were inserted s.c. on the lower back or on the side of each mouse. During the procedure, mice were briefly anaesthetized by inhalation of isoflurane. Using a compatible wireless scanner, Smart Probe (Bio Medic Data Systems) the body temperatures could be registered.

2.7. Preparation of PAO1 alginate beads for challenge

To mimic the biofilm growth mode of *P. aeruginosa* during biofilm lung infections, the bacteria were embedded in alginate beads prior to being administered to the lungs of the mice. A small aliquot of *P. aeruginosa* strain PAO1 was extracted from a frozen stock (stored at −80 °C) and streaked out on a LB agar plate. The plate was incubated at 37 °C overnight (O/N). The following day, one single colony was used to inoculate 100 mL sterile LB medium. The culture was incubated at 37 °C, with constant shaking, for 18 h. Thereafter, 50 mL of the bacterial culture was centrifuged at 5000 x*g* for 10 min at 20 °C and the resulting pellet was resuspended in 5 mL LB medium. The bacterial suspension was mixed with seaweed alginate (FMC BioPolymer, PA, USA) in a ratio of 1:24 in a controlled pressure chamber (encapsulation Unit Nisco Var J30 (Nisco Engineering AG, Zurich, Switzerland) as previously described [8]. The number of colony forming units (CFU) per mL alginate bead solution, was determined by dissolving the alginate beads in saline and subsequent plating on LB agar plates.

2.8. Challenge setup

The mice were housed at the Biocenter, University of Copenhagen, Denmark. The animals were kept in an environment characterized by a 12 h light-dark cycle and temperature and humidity control. The mice had access to chow and water ad libitum. Before challenge the mice were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Once sedated each mouse was inoculated intranasally with 1×10^7 CFU of *P. aeruginosa* PAO1 embedded in alginate beads. To avoid animals suffering from dehydration, the mice received 1 mL of physiological saline subcutaneously. The mice were assessed daily to register symptoms and development of disease. To ensure a consistent evaluation, each animal was scored individually following the score of clinical symptoms as presented in suppl. Table 2. The scoring was always done by the same researcher, and the experiments were blinded to ensure an unbiased evaluation. Animals were euthanized if the sum of clinical scores (suppl. Table 2) reached 9 or if the body temperature was below 32 °C. Blood samples for quantification of antibodies to the vaccine antigens were collected by cardiac puncture when the mice were sacrifice.

2.9. Bacteriology of the lungs

Following registration of weight, temperature and clinical signs on day four after challenge, the mice were euthanized by an intraperitoneal injection of pentobarbital and the lungs were removed aseptically. The

lungs were homogenized, the suspension serially diluted and 100 µL of each dilution was plated on *Pseudomonas* Isolation agar-plates (Sigma-Aldrich). The plates were incubated at 37 °C O/N, and the number of CFU was quantified the following day.

2.10. Measuring antibody titer

Antibody titers were measured using enzyme-linked immunosorbent assay (ELISA). MaxiSorp microtiter plates were coated with 1 µg/mL recombinant protein diluted in coating buffer (15 mM, Na_2CO_3, 35 mM $NaHCO_3$, MilliQ water, pH 9.6), and incubated O/N at 4 °C. The plates were washed three times in phosphate buffered saline with 0.05 % Tween-20, PBS-Tw (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, Milli Q water, pH 7.2, 0.05 % Tween 20; PBS-Tw) and the serum samples diluted 2-fold with PBS-Tw in the wells of the microtiter plates. The plates were incubated for two hours at room temperature (RT) with gentle agitation. After incubation the plates were washed three times with PBS-Tw, after which horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin diluted 1:10,000 in PBS-Tw was applied to the wells of the plates. Following one hour of incubation, at RT with gentle agitation, the plates were washed three times with PBS-Tw. The plates were then developed using a solution of developing buffer (38 mM $C_6H_8O_7$, 67 mM Na_2HPO_4, MilliQ water) with 0.4 mg/mL ortho-phenylenediamine (Kem-En-Tec Diagnostics, Denmark) and 0.4 µL/mL 35 % hydrogen peroxide (Sigma-Aldrich). The plates were incubated for 30 min in the dark and the reaction was stopped by the addition of 1 M H_2SO_4. The optical density was registered at 490–650 nm using the V_{max} Kinetic Microplate Reader (Molecular Devices, CA, USA).

2.11. Statistical analysis

The statistical analysis of the data obtained in this study was performed by GraphPad Prism 6 software, www.graphpad.com). The normal distribution of the values was tested by d'Agostino and Pearson omnibus normality test. For values that did not pass the test, a Mann-Whitney test was used to compare the measured parameters between the vaccinated and control animals. For the data with normal distribution, a *t*-test for unpaired samples was applied. Spearman rank correlation was used to investigate the degree of association between lung bacteriology (CFU) and clinical signs, temperature and weight loss *P*-values <0.05 were considered significant.

3. Results

3.1. Outcome of the infection in vaccinated and non-vaccinated mice

The number of mice at 96 h post inoculation was 44 in the control group and 42 in the vaccinated group. Eight mice from the control group and six from the vaccinated group have been sacrificed due to the high clinical score during the first 4 days after challenge and six mice died under anesthesia (five from vaccinated group and one mouse from

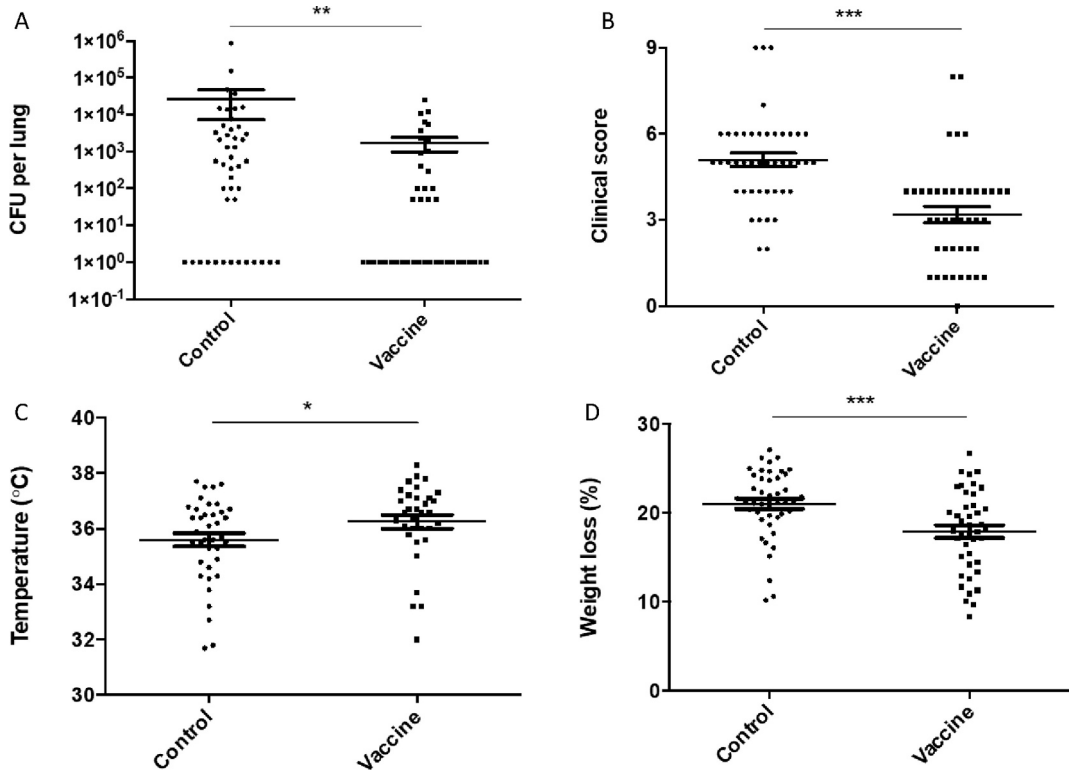

Fig. 1. Lung bacteriology, clinical score, temperature and weight loss four days after challenge. A) The mean lung CFU per lung of the vaccine treated animals was significantly lower compared to the mean CFU per lung in the control group having received adjuvant alone. The data is presented as mean ± SEM. The data was analyzed using a two-tailed Mann-Whitney test, *p*-value = 0.0041**. B) The physical appearance and behavior of the mice were evaluated daily by the same researcher and the mice were given a clinical score ranging from 0 to 9 accordingly. The treatment groups were blinded to the researcher. The data is presented as mean ± SEM. The data was analyzed using a two-tailed *t*-test, *p*-value; 0.0001***. C) There was no statistical difference between the temperatures measured in the animals of the two groups immediately before challenge. The mean temperature before challenge was 37.3 °C (SD = 0.56, SEM = 0.08). Four days after challenge, the group immunized with the combination vaccine had a significantly higher temperature compared to the control group. The results are presented as mean ± SEM. The data were analyzed using a two-tailed Mann Whitney test, *p*-value =0.0297*. D) The weight loss was calculated as the percent loss from the starting weight, measured immediately prior to challenge. There was no statistical difference in the weight registered for the two groups at 0 h. Four days after challenge, the group immunized with the combination vaccine had a significantly lower weight loss compared to the control group having received adjuvant alone. The data were analyzed using a two-tailed Mann Whitney test, *p*-value = 0.0008 ***)($p \leq 0.05$ *, $p \leq 0.005$ ** $p \leq 0.001$***).

control group).

3.2. Lung bacteriology

Animals were euthanized four days after challenge and their lungs were collected for bacteriological analysis. Mice immunized with the heptavalent combination vaccine had a significantly lower mean bacterial load per lung compared to the control group, with 1405 CFU ± 598 (SEM), vs. 24,493 ± 17,855 CFU, respectively (see Fig. 1A), representing an approx. 1.2 log10 reduction in CFU.

3.3. Clinical symptoms, temperature and weight loss

Clinical symptoms, body temperature and weight were registered daily as part of the overall assessment of animal welfare. Four days after challenge, the vaccine treated group presented significantly lower clinical score compared to the control group, suggesting that the mice in the vaccine group had fewer and less severe signs of infection compared to the control group (see Fig. 1B). Furthermore, the results of temperature registrations showed that animals immunized with the heptavalent vaccine had a significantly lower decrease in body temperature four days after challenge compared to adjuvant control group (see Fig. 1C). This is indicative of an improvement of disease symptoms, as the body temperature of infected mice is observed to decrease following infection, and gradually increases once the infection is being cleared. Weight loss was calculated for each mouse as a percentage of the starting weight. Mice immunized with the combination vaccine had a significantly lower percentage weight loss compared to the control group four days after challenge (see Fig. 1D). Weight loss can be observed in acutely infected animals, and hence a reduction of weight loss suggests amelioration of disease. Significant correlations between CFU and clinical score (*p*-value; 0.0014**), temperature (*p*-value; 0.0193*) and weight loss (*p*-value; 0.0003***) were found in the infected mice when using Spearman's rank correlation analysis.

3.4. Antibody response

The IgG levels to all seven antigens in the combination vaccine were determined in sera using ELISA (see Fig. 2). The mean ± SEM IgG levels in serum titers were as follows: PA0041: 8445 ± 1505; PA0931: 26642 ± 3835; PA1302: 45988 ± 5118; PA2070 (short): 35652 ± 5843; PA2070 (long): 71848 ± 8717; PA2976: 2428 ± 301; PA3901: 30314 ± 3907, confirming the heptavalent vaccine to be immunogenic. The lowest antibody response was observed for PA2976, which is a cytoplasmic protein identified in outer membrane vesicles of *P. aeruginosa* (see Table 1).

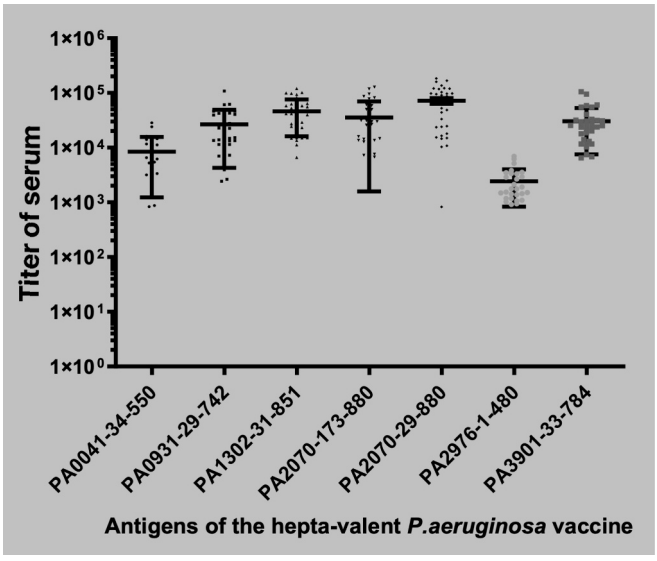

Fig. 2. Antibody response in immunized mice. Scatter plot representing the titers of serum IgG antibodies specific to each of the seven antigens of the vaccine. The mean IgG titers and SEM are indicated.

3.5. Antigens

Four of the identified antigens are TonB-dependent receptors (TBDR), which mediate substrate-specific transport across the outer membrane of *P. aeruginosa*, utilizing energy derived from the proton motive force. Three of the TBDR antigens are involved in iron acquisition (PA1302 heme receptor HxuA/C receptors [14] PA0931 ferric enterobactin receptor PirA; PA3901 citrate-inducible FecA receptor) and the fourth, OptM (PA2070), contributing with a short and a long peptide to the heptavalent vaccine, has a role in the active transport of metals [15]. In addition, the gene encoding for OptM (PA2070), has been found to have increased expression in biofilm cells and a putative role in antibiotic resistance and persistence of biofilms has been proposed. PA0041 encoding for Tps2, a two-partner secretion system (type Vb) has been shown to be part of contact-dependent growth inhibition system [16] involved in pathogenesis of acute and chronic infection formation and to be negatively regulated by quorum sensing [17].

3.6. Protein conservation study

Protein conservation is a key factor in developing a broadly protective vaccine, therefore we conducted a conservation analysis of the seven protective *P. aeruginosa* vaccine candidates across 300 completely sequenced strains in NCBI database. The 300 complete *P. aeruginosa* genomes were selected to represent the phylogenetic diversity of the species, ensuring broad coverage of its known genomic landscape. Genes coding for six of the proteins were present in 94–99 % of the 300 genomes analyzed, except for PA0041, which was found in 79 % of the genomes (see Table 2). Furthermore, all seven proteins had a mean of 98–100 % similarity to homologs.

4. Discussions

Our proof-of-concept study shows that a heptavalent *P. aeruginosa* vaccine identified by the AI-driven antigen discovery model EDEN™, induced protection against *P. aeruginosa* challenge in a mouse model of biofilm lung infection. The mice having received the vaccine had significantly lower bacterial load in the lung compared to controls, which was considered as the primary endpoint. Also, significant better recovery of the *P. aeruginosa* lung infection was observed in the immunized mice compared to the control groups. This was evaluated by secondary endpoint parameters represented by clinical score, body weight and temperature. The improvement in the secondary endpoint parameters correlated significantly with the reduction in the CFU/mL lung homogenate. The lack of endotoxin removal may have influenced the results by triggering immune responses that introduced confounding factors, potentially affecting the outcomes. Future studies are needed to clarify the potential impact of endotoxins and irrelevant antigens purified from *E. coli* on the immune response.

While in earlier studies known outer membrane proteins such as OprF, OprI or OprF/I fusion proteins [18,19] were selected as antigens based on the antibody-mediated opsonophagocytic killing- activity, the antigens selected by the EDEN™ model combine alternative surface proteins whose antigenic potential has not been previously tested.

Many of the six different proteins identified by EDEN™ (one protein PA2070 with a short and a long peptide version) are involved in iron or other metal ions acquisition and interestingly, these iron-uptake proteins were found to be highly expressed during acute pneumonia murine model [20]. In addition, it has been shown by high-resolution in situ transcriptomics of *P. aeruginosa* in cystic fibrosis lungs, that more than

Table 2
Analysis of protein construct conservation across 300 *P. aeruginosa* genomes. The library coverage indicates the percentage of available strain sequences that contain a homologue of the respective protein, calculated as the number of homologs divided by the total number of strains. Homology is defined as a percent similarity greater than 75 % to the protein sequence. The mean and standard deviation of similarity to homologs are also presented.

Locus ID (from paper)	Protein Construct	Length (aa)	% Library Coverage	[Number of Homologs/ Number of Strains]	Median of % Similarities to Homologs	Mean of % Similarities to Homologs	Standard Deviation of % Similarities to Homologs	Minimum % Similarity to Homologs	Maximum % Similarity to Homologs
PA0041	PA0041 (34–550 AA)	517	79 %	[236/300]	99 %	98 %	4 %	83 %	100 %
PA0931	PA0931 (29–742 AA)	714	96 %	[289/300]	100 %	100 %	1 %	96 %	100 %
PA1302	PA1302 (31–851 AA)	821	98 %	[295/300]	100 %	100 %	0 %	97 %	100 %
PA2070 (short)	PA2070 (173–880 AA)	708	94 %	[282/300]	100 %	100 %	1 %	90 %	100 %
PA2070 (long)	PA2070 (29–880 AA)	852	94 %	[282/300]	100 %	100 %	1 %	92 %	100 %
PA2976	PA2976 (1–480 AA)	480	96 %	[287/300]	100 %	100 %	0 %	100 %	100 %
PA3901	PA3901 (33–784 AA)	752	97 %	[291/300]	99 %	99 %	1 %	97 %	100 %

30 % of the genes differentially expressed were related to acquisition of iron [21]. Interestingly, among these, the gene PA1302 encoding the hem receptor HxuA/C, which is one of the antigens in our vaccine, was significantly up-regulated in several expression level studies [22]. Thus, the antibodies raised by the heptavalent vaccine will potentially impair iron-acquisition which is essential for bacterial growth during infection. The protective efficacy of the heptavalent vaccine was similar to other outer membrane-based vaccines (oprF vaccine) tested in a chronic *P. aeruginosa* lung infection model [23]. Although in our study the mice were sacrificed at an earlier stage of the biofilm lung infection compared to the study investigating the immunization with the outer membrane protein F (OprF) [23] (four days vs. eight days), several previous investigations [24] have shown that the inflammatory response in this mouse model using alginate embedded *P. aeruginosa* for challenge, recapitulates the in vivo situation of chronic biofilm infection.

The antibody response against receptors of iron acquisition systems and other transporter proteins in the outer membrane, might have an anti-virulence function by impairing peptides access to essential substances, such as iron. Through this general anti-virulence mechanism, the vaccine might also be effective in other models of *P. aeruginosa* infection.

The efficacy of several *P. aeruginosa* iron acquisition proteins as vaccine targets have been previously tested [22].

Vaccine targeting iron acquisition, such as anti-siderophore vaccines has been reported to protect against Gram-negative gastrointestinal pathogens [25] and recurrent urinary tract infections [26].

In this context, objectives for future research are to establish an optimal formulation by assessing the protective immunity induced by all seven antigens when administered individually and at highly pure quality, free from any endotoxins and possibly contaminating *E. coli* host cell proteins from the recombinant production. Furthermore, an alternative adjuvant to IFA/alum, suitable for clinical use need to be identified and the most optimal administration route (parenteral or mucosal) for the final vaccine formulation should be determined.

Despite more than four decades of study and clinical trials, no vaccine for *P. aeruginosa* has been licensed, suggesting that the fundamental approaches have been flawed. It is likely that multiple immune effectors will be needed to control this versatile pathogen. A major challenge lies in the fact that different immune effectors might be needed for different sites and types of infection (e.g., lung vs. burn wound vs. surgical wound vs urinary tract). Thus, it has been proposed that different vaccine antigens and adjuvants might need to be utilized in different patient populations [4].

Although our study focused on identifying antibody-mediated protective proteins using EDEN™ as a vaccine antigen discovery platform and alum-IFA as the adjuvant formulation, it is well established that humoral immunity alone is insufficient for protection against *P. aeruginosa.* Robust Th1 and Th17 responses are also required [5]. The emphasis on B-cell antigens represents a limitation of this study, as eliciting T-cell responses may enhance protective efficacy. We therefore recommend the inclusion of Th1/Th17-skewing adjuvants in future studies and propose incorporating T-cell-targeted antigens into multicomponent vaccine formulations against *P. aeruginosa*.

The encouraging results presented in this proof-of-concept study suggests EDEN™ to be a fast and useful tool when identifying vaccine candidates against *P. aeruginosa* and possibly other microorganisms. EDEN™ has been applied to identify vaccine candidates conferring protection in preclinical challenge models for seven distinct pathogens. In the *Neisseria gonorrhoeae* proof-of-concept study [18], a statistically significant correlation between EDEN™ prediction scores and reduction in bacterial burden underscores the model's predictive accuracy. These results support the utility of EDEN™ for rational antigen prioritization in vaccine development. Ongoing efforts are focused on deconvoluting the individual antigen contributions to protective efficacy to enable data-driven optimization of multicomponent vaccine formulations [6,7]. The aspiration is to apply the EDEN™ technology broadly to identify antigens from other multi-drug-resistant WHO priority pathogens and develop highly needed vaccines.

5. Conclusion

The encouraging results obtained in this study, and previous studies provide a proof-of-concept that EDEN™ is a useful tool in identifying vaccine antigens against *P. aeruginosa* and possibly other problematic pathogens.

CRediT authorship contribution statement

Stine Hansen: Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Kim Thomsen:** Writing – review & editing, Project administration, Methodology. **Andreas Holm Mattsson:** Writing – review & editing, Supervision, Software, Conceptualization. **Pär Comstedt:** Writing – review & editing, Supervision. **Claus Moser:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Oana Ciofu:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Reports a relationship with that includes: Has patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2025.127416.

Data availability

Data will be made available on request.

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