Genome-Scale Screening of Vaccine Candidates Against Pseudomonas Aeruginosa

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Research

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Abstract

Background

Infections due to Pseudomonas aeruginosa (PA) are becoming a serious threat to patients in intensive care units. A PA vaccine is a practical and economical solution to solve the problems caused by PA infection successfully. In recent years, several antigen candidates have been tested in animal and human clinical trials, but none of them has been approved to date. An alternative strategy for antigen screening and protective antigens is in urgent demand.

Methods

In this study, we generated a genome-wide library of PA protein fragments tagged with maltose-binding protein (MBP). Using sera from patients who recovered after PA infection, we identified novel protective antigens and investigate the mechanism of these antigens induced protections.

Results

we identified a novel protective antigen, FlgE, which is the structural component of the flagella hook. Vaccination with recombinant FlgE (reFlgE) induced a Th2-predominant immune response and reduced bacterial load and inflammation in PA-infected mice. Anti-reFlgE antibodies recognized native FlgE on the bacterial membrane in vitro and conferred protection in mice, which may be due to the mediation of opsonophagocytic killing and inhibition of bacterial motility. In addition, the combination of reFlgE with rePcrV\textsubscript{NH}, an engineered antigen we reported previously, provided elevated protection against PA infection.

Conclusion

Our data demonstrate that FlgE is a promising vaccine candidate for PA and provide a new strategy for the efficient screening of antigens of other pathogens.

Background

Pseudomonas aeruginosa (PA) is one of the most common causative agents for nosocomial infections [1, 2]. A systematic review of 49 outbreaks of PA infections between 2000 and 2015 presented a mortality rate as high as 23% [3]. In particular, patients with mechanical ventilation [4] and compromised immunity [5, 6] are at high risk for PA infection in intensive care units. However, the conventional application of antibiotics is less effective when treating PA-infected patients due to increasing drug resistance [7, 8]. A vaccine is a cost-effective and practical way to combat PA infection.

Identifying protective antigens is the initial and crucial step when developing a vaccine. To date, a number of PA antigens have been tested in animals and even patients in recent years [9]. Traditionally, lipopolysaccharide (LPS) is a preferred vaccine candidate vaccine for bacteria. However, there are twenty
major serotypes and various subtypes, and the poor immunogenicity of lipopolysaccharide limits the protection of LPS vaccines, as observed in clinical trials [10]. The genetically engineered components of flagella, pilin, the type 3 secretion system (T3SS) and other virulence factors have also been tested [11-13]. A phase 2/3 clinical trial for a promising new vaccine, IC43, has been completed in patients in intensive care units [14]. However, no PA vaccine is currently on the market, despite recent progress.

In recent years, several proteomic tools have been used to discover the protective antigens of PA. To date, 52 potential antigens have been predicted by reverse vaccinology, and recombinant PA5340 combined with PA3526-MotY conferred the maximum protection in an acute respiratory infection model [15]. From a library of in vitro translated proteomes, PopB was found to elicit Th17-dependent immunity against PA infection [16]. The currently reported antigens meet the requirements of a successful PA vaccine, which requires the discovery of novel protective antigens [9].

In this study, we generated a genome-wide library of PA protein fragments tagged with maltose-binding protein (MBP). Using sera from the recovered patients after PA infection, we found a novel protective antigen, FlgE, the structural component of flagella in PA. The mechanism of FlgE-mediated protection was elucidated.

**Methods**

**Animals and strains**

Specific pathogen-free Balb/c mice aged six to eight weeks were purchased from Beijing HFK Bioscience Limited Company (Beijing, China). PA XN-1 (CCTCC M2015730) was isolated from pneumonia patients in Southwest Hospital of China. Sera were collected from ten PA-infected patients at Southwest Hospital in China.

**Construction of the genomic DNA library and screening of putative antigens**

The strategy to construct the screen for the PA genomic DNA library is shown in Figure 1A. In brief, the genomic DNA of PA XN-1 was extracted using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing). To produce random fragments, 0.4 units of Sau3A I (TaKaRa) restriction enzyme were used to digest 40 μl of genomic DNA at 37 °C for 30 minutes. After separation using 1% agarose gel electrophoresis, fragments were removed under UV illumination and collected using the Gel Extraction Kit (OMEGA). In addition, after digestion by BamH I, the pMal-c5x plasmid was incubated with shrimp alkaline phosphatase (TaKaRa) to avoid self-ligation. Furthermore, 4 μl of DNA fragments, 2 μl of vector and 4 μl of the DNA Ligation Kit (TaKaRa) were mixed and incubated at 16 °C overnight. Then, 10 μl of the ligation mixture was transformed into *E.coli* XL-Blue competent cells, which were then dispersed on the LB plate with 100 μg/ml ampicillin. LB plates were incubated overnight at 37 °C.

All positive clones were picked up, and the recombinants were induced by 0.5 mM IPTG in LB culture medium at 16 °C. Then, cells were collected and lysed with lysozyme (Diamond). The supernatant of the
lysate was added to 96-well plates and incubated at 37 °C for 1 hour. The 96-well plates were precoated with purified rabbit anti-MBP IgGs to capture the MBP fusing proteins. In addition, PcrV fused with an MBP tag (MBP-PcrV) and MBP were used as positive and negative controls, respectively. After washing the plates three times, the mixture of sera from 10 PA-infected patients was added at a dilution of 1:1000 and incubated at 37 °C for an additional 1 hour. After washing, HRP (horseradish peroxidase)-labeled goat anti-human Fc antibodies (1:5000, Abcam) were added and incubated at 37 °C for 1 hour. The optical density (OD) was measured after addition to BeyoECL (Beyotime). The following formula was used to calculate the relative strength of each fragment to MBP-PcrV: fragments protein% = (OD\textsubscript{X} - OD\textsubscript{MBP}) / (OD\textsubscript{MBP-PcrV} - OD\textsubscript{MBP})\times 100\%. The OD\textsubscript{X} \text{ and } OD\textsubscript{MBP} indicate the ODs of MBP and MBP-PcrV, respectively. The sequences of clones of interest were determined and aligned with the genome sequence using BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Production of recombinant vaccine candidates**

The top 8 fragments with the highest relative strength were selected for further investigation. The full-length recombinant antigens corresponding to these fragments were produced in *E. coli*. First, the genes encoding the full-length protein were amplified by PCR and cloned into the pMal-c5x vector using corresponding primers (Table S1). Then, the 8 recombinants were transformed into *E. coli* and induced by 0.5 mM IPTG at 16 °C overnight. Cells were collected by centrifugation and lysed by sonication. The supernatant of the lysate was passed through a column of maltose resin (NEB). Finally, the 8 full-length proteins were quantified by BCA assay and stored at -80 °C for future assays.

To produce recombinant tag-free FlgE (reFlgE), the gene encoding FlgE was amplified using primers 5'-CGCGGATCCATGAGTTTCAACATCG-3' and 5'-CCGG AATTTCAGCGCAGGTGATG-3' and then cloned into the pGEX-6p-2 vector using restriction sites BamH I and EcoR I, which were then transformed into *E. coli* XL-Blue. These cells were grown in LB culture medium at 37 °C. Protein was induced by 0.5 mM IPTG at 16 °C for 16 hours when the OD\textsubscript{600} reached 0.5~0.6. Cells were collected and lysed in lysis buffer (20 mM PB, pH 7.0, 150 mM NaCl) by sonication. The supernatant of the lysate was passed through a column of glutathione resin (GE Healthcare). After washing with washing buffer (20 mM PB, pH 7.0, 1 M NaCl), PreScission protease (GE Healthcare) was added and incubated at 4 °C overnight. The reFlgE was eluted from glutathione resin and further purified by a Hi\textsuperscript{Trap} Q column and G25 desalting column (GE Healthcare). The purified reFlgE proteins were finally stored in PBS buffer and stored at -80 °C for future use.

**ELISA**

To evaluate the immunogenicity of reFlgE, the titers of anti-reFlgE IgGs in the sera of immunized mice were determined by ELISA. Briefly, 96-well plates were coated with 0.2 μg of purified reFlgE at 4 °C overnight and blocked with 1% BSA (bovine serum albumin) diluted in PBST (PBS buffer containing 0.5% Tween-20) at 37 °C for 2 h. After washing three times, serially diluted serum was added to the plates and incubated at 37 °C for 2 h. Then, horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000,
Abcam) was added to the wells for 40 minutes at 37 °C. 3,3',5,5'-Tetramethylbenzidine (TMB) as the substrate and 2 M H$_2$SO$_4$ as stopped buffer were used to determine the OD at 450 nm. In addition, to determine the subtype of antibody, horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (1:5000, Abcam) was added into wells for 40 minutes at 37 °C. Similarly, OD$_{450}$ was also measured, and the titers of the subtypes were determined.

**Immunization and challenge of mice**

To evaluate the protection of the 8 full-length proteins, 110 mice were divided equally into eleven groups. Eighty mice were intramuscularly injected with 20 μg of each protein. The other 30 mice were injected with PBS, PcrV$_{NH}$ and MBP proteins as control groups. A lethal dose of PA XN-1 was intratracheally injected into the mice in each group. The mortality was recorded every 12 hours during an observation period of 7 days. To evaluate the protection of reFlgE protein, 30 mice were equally divided into 3 groups and immunized with 20 μg of Al(OH)$_3$ formulated reFlgE, PcrV$_{NH}$, PBS. The other ten mice were intramuscularly immunized with reFlgE + PcrV$_{NH}$ to evaluate the protection of the combination of reFlgE with PcrV$_{NH}$. Al(OH)$_3$ as an adjuvant was applied in these experiments. The immunization interval times were 0, 14, and 21 days. At 7 days after the last immunization, mice were challenged by intratracheal instillation of a lethal dose of PA XN-1 (1.0×10$^7$ CFU/mouse). Afterward, the deaths were recorded every 12 hours for 7 days. In addition, to further evaluate the difference in protection effect brought by reFlgE, PcrV$_{NH}$, reFlgE + PcrV$_{NH}$, another 40 mice were vaccinated and challenged in the same procedure as above. Three-fold lethal doses of PA XN-1 were intratracheally administered to mice (3.0×10$^7$ CFU/mouse).

**Observations of global disease**

To further investigate the protection mechanism, ten mice were randomly divided into two groups. Five mice immunized with reFlgE were challenged intratracheally with a sublethal dose of PA XN-1 (0.8×10$^7$ CFU/mouse). The body weights of the mice were monitored every 24 hours for 7 days. In addition, the disease score was determined every 12 hours according to movement, breathing, piloerection, nasal secretion and posture. The global disease score was recorded based on the following scoring criteria: unaffected (0-1), slightly affected (2-4), moderately affected (5-7), and severely affected (8-10).

**Quantification of proinflammatory cytokines**

The bronchoalveolar lavage fluids (BALF) were collected from mice immunized with reFlgE or PBS at 6 hours after challenge with a sublethal dose of PA. The levels of proinflammatory cytokines, such as IL-1β and TNF-α, were quantified using the Mouse ELISA Kit (Dakewei) according to the manufacturer's instructions.

**Histological characterization of infected lung**
Twenty-four hours after challenge with a sublethal dose of PA, the lungs of mice immunized with reFlgE and PBS were collected, saturated with 4% paraformaldehyde and cut into slices. Tissues were stained with hematoxylin and eosin (HE). Then, the sections were viewed at 100-fold and 400-fold magnification by light microscopy. The score (0-10) depending on the criteria, such as hemorrhage edema, hyperemia and neutrophil infiltration, represents the degree of infection.

**Quantification of bacterial burden**

Mice immunized with reFlgE and PBS were sacrificed at 24 h after challenge. Briefly, the lung and spleen tissue were separated, weighed and homogenized. The homogenization was serially diluted and dispersed on LB plates at 37 °C overnight. The amounts of bacteria colonized per mg of tissue were calculated.

**Passive immunization**

The rabbit was immunized with reFlgE (500 μg) and 625 μl of Freund's adjuvant (Sigma) at day 0, day 14, and day 21. ReFlgE-specific antibodies were purified by Protein A affinity chromatography from sera at 7 days after the last immunization. To evaluate the prophylactic effect of reFlgE-specific antibodies in vivo, 15 mice were equally divided into 3 groups. Each group was intraperitoneally injected with 6 mg of anti-FlgE antibodies, nonspecific antibodies and PBS. One hour later, all mice were challenged with a lethal dose of PA XN-1, and mortality was recorded every 12 h for 7 days.

**Motility assays**

To further evaluate the effect of anti-reFlgE antibodies on bacterial motility, antibodies and PA XN-1 were incubated on semisolid medium in vitro. In brief, sterile swimming medium including tryptone, NaCl, 0.2% agar and 0.5% glucose was made into swimming plates. PA O1 and PA XN-1 were inoculated on swimming plates containing 4.5 mg of anti-reFlgE antibodies, 4.5 mg of nonspecific antibodies, or 300 μl of PBS and incubated for 12 hours at 37 °C. Swimming areas were determined using ImageJ software.

**Immunofluorescence**

To test whether anti-FlgE antibodies are able to recognize native FlgE on the membrane, PA XN-1 cells were grown and fixed with 4% paraformaldehyde. The pellets were collected and then incubated with anti-FlgE antibodies for 30 min. After washing three times, pellets were resuspended in 100 μl of buffer containing Alexa Fluor 594 (Abcam)-labeled goat anti-rabbit antibodies and DAPI (Thermo Fisher). After incubation for 30 min and washing, cells were deposited on microslides and visualized using a laser scanning confocal microscope. Images were captured with a camera at 630x magnification.

**Opsonophagocytic killing assay**

HL-60 cells (ATCC, CCL-240) were differentiated into granulocyte-like cells in medium containing 100 mM N’N-dimethylformamide for 5 days. Anti-reFlgE serum and nonspecific serum were heat-inactivated (56
°C, 30 min) and diluted with opsonization buffer. In 96-well plates, a mixture of 40 µl of 4×10^5 HL-60 cells, 10^3 CFUs of PA XN-1 dissolved in opsonophagocytic buffer, 20 µl of serum, and 10 µl of 1% infant rabbit serum as a complement source were added into the well. Ten microliters of each mixture was evenly smeared on the plates after incubation at 37 °C for 2 hours. The decrease in CFUs was used to identify the killing effect when compared with the CFUs in nonspecific serum.

**Statistical analysis**

All data are shown as the mean ± SE. The significance of differences was analyzed by unpaired parametric tests (Student’s t-test for two groups or one-way ANOVA for more than three groups). The survival rate was analyzed using Kaplan-Meier survival curves. SPSS 21.0 and GraphPad Prism 7.0 were used to analyze the data. P<0.05 is represented for significant differences.

**Results**

**FlgE is a protective antigen against PA**

To broadly screen the protective antigens, the DNA genomic library of PA XN-1 was constructed as shown in Figure 1A. Ultimately, 7628 colonies were successfully constructed. The expression of the clones was induced in *E. coli*, and the reactions to the sera of PA-infected patients were determined by ELISA. After sequencing and BLAST analysis, the top 8 fragments were from RtcA, PscF, FlgE, PscD, HlyD, HmgA, OprP and RecG. The relative strengths of RtcA, PscF and FlgE were even higher than that of PcrV, whereas the relative strengths of PscD, HlyD, HmgA, OprP and RecG were lower than that of PcrV (Fig. 1B). The general information on the 8 fragments is shown in Table 1. The full-length proteins corresponding to the 8 fragments fused with the MBP tag were produced in *E. coli* to further evaluate protective effects (Fig. S1). Mice immunized with the 8 recombinant proteins were intratracheally challenged with a lethal dose of PA XN-1. No significantly improved survival was observed in mice vaccinated with HmgA, PscD, HlyD, RecG, OprP, PscF and RtcA compared with MBP- and PBS-immunized mice (*P* > 0.05) (Fig. 1C). However, the survival rate of mice vaccinated with MBP-FlgE was 40%, which was significantly higher than that of mice immunized with MBP and PBS (*P* < 0.05). No significant difference in survival was observed between mice vaccinated with MBP-tagged FlgE and PcrV (*P* > 0.05) (Fig. 1C). These results indicate that FlgE is a protective antigen against PA infection in mice. (*P* < 0.05).

| Table 1 General information of the top 8 fragments |
| No. | Gene | Protein description | Insertion sequence |
|-----|------|---------------------|--------------------|
| 1#  | hmgA | homogentisate 1,2-dioxygenase | Leu276-Phe312 |
| 2#  | pscD | type III export protein PscD | Ile79-Arg235 |
| 3#  | hlyD | HlyD family secretion protein | Ile131-Glu205 |
| 4#  | recG | ATP-dependent DNA helicase | Ile149-Glu279 |
| 5#  | oprP | phosphate-specific outer membrane porin OprP | Glu319-Ser369 |
| 6#  | pscF | type III export protein PscF | Ala34-Lys56 |
| 7#  | flgE | flagellar hook protein FlgE | Ile98-Phe174 |
| 8#  | rtcA | RNA 3’-terminal-phosphate cyclase | Ile119-Arg239 |

**Vaccination of reFlgE protects mice against acute infection**

To explore the mechanism of protection mediated by FlgE vaccination, recombinant tag-free FlgE (reFlgE) was produced. Then, mice immunized with reFlgE were challenged with a sublethal dose of PA XN-1. As expected, the global disease scores of reFlgE-immunized mice were significantly lower than those of PBS-immunized mice from 36 hours to 96 hours post infection ($P < 0.05$) (Fig. 2A). In addition, the loss of body weight of reFlgE-immunized mice was significantly less than that of PBS-immunized mice ($P < 0.05$) (Fig. 2B). Moreover, the body weight of mice recovered faster than that of PBS-immunized mice. Furthermore, the lungs and spleens of mice immunized with reFlgE were detached and homogenized to evaluate their bacterial burden at 24 hours postinfection. Bacterial loads per mg of lung and spleen in the reFlgE-immunized group were clearly less than those in the PBS-immunization group ($P < 0.05$) (Fig. 2C). Then, there was a significant decrease in the proinflammatory cytokines TNF-α and IL-1β in the BALF of mice immunized with reFlgE ($P < 0.05$) (Fig. 2D). The histological analysis suggested that bacteria caused less damage (infiltration of inflammatory cells, bleeding, alveolar rupture) to the lungs of mice in the reFlgE-immunization group (Fig. 2E), which was consistent with the inflammatory score. Notably, the inflammatory score of reFlgE-immunized mice was significantly lower than that of PBS-immunized mice ($P < 0.05$) (Fig. 2F). Therefore, these results indicate that protection induced by reFlgE may be mediated by decreasing bacterial burden, inflammation, and the production of proinflammatory cytokines in lung tissue.

**Anti-reFlgE IgGs conferred prophylactic protective effects**

The levels of specific antibodies in sera were determined to clarify the immune response after reFlgE immunization. Obviously, the levels of anti-reFlgE-specific antibodies were significantly higher than those preimmunization ($P < 0.05$) (Fig. 3A). Furthermore, the subtypes of induced IgGs were evaluated. The levels of both antigen-specific IgG1 and IgG2a were elevated ($P < 0.05$). The levels of IgG1 were significantly higher than those of IgG2a ($P < 0.05$), suggesting a Th2-predominant immune response (Fig. 3B). Building on these results, we then tested whether the anti-reFlgE IgGs alone conferred protection.
Mice were given immunized anti-reFlgE-specific antibodies before being challenged with a lethal dose of PA XN-1. As expected, the survival of mice vaccinated with anti-reFlgE IgGs was significantly higher than that of mice vaccinated with PBS or nonspecific antibodies ($P < 0.05$) (Fig. 3C). These results show that anti-reFlgE-specific IgGs play a crucial role in prophylactic infection of PA.

To clarify the mechanism of the protection mediated by anti-reFlgE IgGs, we first determined whether anti-reFlgE IgGs were able to recognize native flgE on the membrane of PA. PA XN-1 was clearly observed by immunofluorescence microscopy after staining with anti-reFlgE IgGs, indicating the binding of anti-reFlgE IgGs to native FlgE on the membrane (Fig. 4A). Because the flagella hook assembled by FlgE was critical for the motility of PA, we next tested whether the anti-reFlgE IgGs were able to block flagellin-mediated motility. As expected, the growth areas of PA 01 and PA XN-1 incubated with anti-reFlgE IgGs were smaller than those treated with nonspecific IgGs and PBS ($P < 0.05$) (Fig. 4B). The results from the quantitative analysis of areas confirmed the trend ($P < 0.05$) (Fig. 4B). In addition, we determined the in vitro opsonophagocytic activities of anti-reFlgE antibodies. Obviously, the anti-reFlgE antibodies showed a potent killing effect on PA in a dose-dependent manner ($P < 0.05$). However, no bactericidal activity was observed when adding the nonspecific antibodies (Fig. 4C). Taken together, these results suggest that the blocking of PA motility and opsonophagocytosis could be the mechanism for anti-reFlgE IgG-mediated protection.

**Combined vaccination with reFlgE and PcrV$_{NH}$ significantly improves the protective effect**

These results lead us to combine PcrV$_{NH}$, a vaccine candidate invented previously to improve protection against PA[13]. As shown in Figure 5A, the levels of anti-reFlgE and anti-PcrV$_{NH}$ were significantly elevated after immunization with the two recombinant proteins. Then, the immunized mice were challenged with a lethal dose of PA XN-1 to test the efficiency. Obviously, the survival of mice immunized with PcrV$_{NH}$ and reFlgE + PcrV$_{NH}$ was significantly better than that of mice immunized with reFlgE alone and PBS ($P < 0.05$). However, no significant difference in survival was observed between the PcrV$_{NH}^{-}$-vaccinated group and the reFlgE + PcrV$_{NH}^{-}$-vaccinated group ($P > 0.05$) (Fig. 5B). Next, a two-fold lethal dose of PA XN-1 was used to verify the protection induced by PcrV$_{NH}$ and reFlgE combined with PcrV$_{NH}$. Clearly, the survival of mice vaccinated with reFlgE plus PcrV$_{NH}$ was significantly higher than that of mice immunized with PcrV$_{NH}$ or reFlgE alone ($P < 0.05$) (Fig. 5C). Collectively, these results suggest that the vaccination of reFlgE plus PcrV$_{NH}$ improves the protective effect against PA infections.

**Discussion**

The genomic DNA library has been used as an effective tool to perform in-depth analyses of unknown proteins for the screening of antigens and the diagnosis or therapy of tumors [17-19]. Traditional methods for antigen screening are time consuming and limited by solubility based on the evaluation of a single protein. Previous data showed that MBP tags can promote fused protein folding properly into its biologically active conformation [20, 21]. MBP is a far more effective solubilizing agent than glutathione
S-transferase (GST) and thioredoxin (TRX) [22]. Consequently, we used the pMal-C5x vector to increase the solubility of the fusion partners [23]. Consistently, we found that the application of the MBP tag promoted the expression of genomic fragments, which accelerated the screening of antigens. All eight full-length candidates of interest can be produced in soluble form. These data provide an efficient strategy for screening the antigens of PA and other pathogens.

One interesting finding is the discovery of antigen FlgE despite a protection rate of 40%. The flagella is composed of three parts: basal body, hook, and filaments. FlgE polymerizes and forms the hook, which joins the basal body and filament to facilitate the structure and function of PA flagella [24]. One reason for FlgE-induced protection could be the impairment of the flagella because we note that the anti-FlgE antibodies bind to FlgE on the membrane and inhibit the swimming of PA. Another explanation could be that the anti-FlgE antibodies mediated opsonophagocytic killing, as we observed. Additionally, FlgE binds to caveolin-1 and promotes lung inflammation [25]. Whether the proinflammatory effects of FlgE can be blocked by anti-FlgE antibodies warrants further investigation.

Because flagella are critical for bacterial motility and pathogenesis, many PA vaccine candidates targeting flagella have shown protection in animal models and in clinical trials [26, 27]. The monomer FliC flagellin longitudinally assembles and forms the filament, which is the main target of the vaccine reported previously [28, 29]. However, the sequence of FliC, especially the a-type FliC from clinical isolates, is highly variable [30], which may be the reason for the limited effectiveness observed in the clinical trial [27]. In this study, for the first time, we found that FlgE, the elementary component of the flagella hook, is able to induce a protective immune response. Because FlgE is genetically conserved from the available database, the FlgE-based vaccine should provide a broad spectrum of protection.

The combined immunization of FlgE and PcrV_{NH} provided a better superposed protective effect than any of them alone. In light of the fact that both the FlgE and PcrV_{NH} vaccines are immunogenic and induce a Th2-predominant immune response, there are mechanisms for how anti-FlgE and anti-PcrV antibodies mediate protection against PA infections. First, the interaction between anti-flagella-specific antibodies and flagella inhibits the motility of PA, leading to the reduction in bacterial adhesion to host cells and inflammation. Second, anti-PcrV_{NH} antibodies blocked the type three secretion system (T3SS), a key and conservative virulence factor in PA. Third, high-titer antibodies lead to opsonophagocytic killing effects. Taken together, these results show that the combination of protective antigens is a practical direction for the development of PA vaccines.

Notably, the relative strength of the eight tested antigen candidates was not positively correlated with protection in the mouse pneumonia model. One reason could be the subcellular location of antigen candidates. For example, RtcA, which accounts for the highest relative strength, provides a protection rate as low as 30%. It is likely that the RtcA protein is a member of the RNA 3'-phosphate cyclase family and is located in the cytoplasm [31], so anti-RtcA immunity has little chance of recognizing bacterial RtcA. Another possible reason could be that the MBP tag (~ 40 kDa) affects the folding of the target antigen. To date, the conserved residues D76, P47, and Q54 are essential for the folding and polymerization of
PscF, which ensures the needle structure of the T3SS of PA [32]. However, the folding and polymerization of PscF are not correct after fusion with MBP, which results in little protection. In addition, proteins with strong reactivity may not be critical for the pathogenesis of PA.

Conclusion

In this study, we described an effective strategy to screen vaccine candidates against PA of antigen at a genomic scale. For the first time, we found that FlgE, the element component of the flagella hook, is able to induce protective immunity in mice. Anti-FlgE antibodies were able to recognize native FlgE, which mediates opsonophagocytic killing activities and the inhibition of bacterial motility. In addition, combined vaccination with FlgE and PcrV_{NH} significantly improved the protective effect. Our results demonstrate a promising subunit vaccine candidate for the control of PA infections

Declarations

Authors’ contributions:

C.W., C.G. performed the substantial experiments. Z.L. and Q.X. collected the sera. Y.F. and W.Z. analyzed the data and draw the figures. Q.Z., G.L and J.G. supervised the experiments. C.G. and Q.Z. draft and revised the manuscript. G.L. and J.G. designed the project and supervised the experiments. All authors read and approved the final manuscript

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal care and experiments abided by the ethical regulations and were approved by the Animal Ethical and Experimental Committee of the Army Military Medical University (No.: TMMU0190). Patients were enrolled after written informed consent was obtained from them or their legal representatives.

Consent for publication

Consent for publication has been obtained.

Competing interests

We declare that there is no competing interest.
References

1. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, Molin S: Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. Nature reviews Microbiology 2012, 10(12):841-851.

2. Murray CK, Wilkins K, Molter NC, Li F, Yu L, Spott MA, Eastridge B, Blackbourne LH, Hospenthal DR: Infections complicating the care of combat casualties during operations Iraqi Freedom and Enduring Freedom. The Journal of trauma 2011, 71(1 Suppl):S62-73.

3. Wieland K, Chhatwal P, Vonberg R-P: Nosocomial outbreaks caused by Acinetobacter baumannii and Pseudomonas aeruginosa: Results of a systematic review. American Journal of Infection Control 2018, 46(6):643-648.

4. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR, Sievert DM: Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. Infection control and hospital epidemiology 2016, 37(11):1288-1301.

5. Thirumala R, Ramaswamy M, Chawla S: Diagnosis and management of infectious complications in critically ill patients with cancer. Critical care clinics 2010, 26(1):59-91.

6. Carratala J, Roson B, Fernandez-Sevilla A, Alcaide F, Gudiol F: Bacteremic pneumonia in neutropenic patients with cancer: causes, empirical antibiotic therapy, and outcome. Archives of internal medicine 1998, 158(8):868-872.

7. Petersen K, Riddle MS, Danko JR, Blazes DL, Hayden R, Tasker SA, Dunne JR: Trauma-related infections in battlefield casualties from Iraq. Annals of surgery 2007, 245(5):803-811.

8. Aronson NE, Sanders JW, Moran KA: In harm's way: infections in deployed American military forces. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2006, 43(8):1045-1051.

9. Merakou C, Schaefers MM, Priebe GP: Progress Toward the Elusive Pseudomonas aeruginosa Vaccine. Surg Infect (Larchmt) 2018, 19(8):757-768.

10. Pier GB: Promises and pitfalls of Pseudomonas aeruginosa lipopolysaccharide as a vaccine antigen. Carbohydrate research 2003, 338(23):2549-2556.

11. Behrouz B, Hashemi FB, Fatemi MJ, Naghavi S, Irajian G, Halabian R, Imani Fooladi AA: Immunization with Bivalent Flagellin Protects Mice against Fatal Pseudomonas aeruginosa Pneumonia. J Immunol Res 2017, 2017:5689709.

12. Gholami M, Chirani AS, Razavi S, Falak R, Irajian G: Immunogenicity of a fusion protein containing PilQ and disulphide turn region of PilA from Pseudomonas aeruginosa in mice. Lett Appl Microbiol 2017, 65(5):439-445.

13. Wan C, Zhang J, Zhao L, Cheng X, Gao C, Wang Y, Xu W, Zou Q, Gu J: Rational Design of a Chimeric Derivative of PcrV as a Subunit Vaccine Against Pseudomonas aeruginosa. Frontiers in immunology 2019, 10:781.
14. Adlbrecht C, Wurm R, Depuydt P, Spapen H, Lorente JA, Staudinger T, Creteur J, Zauner C, Meier-Hellmann A, Eller P et al.: Efficacy, immunogenicity, and safety of IC43 recombinant Pseudomonas aeruginosa vaccine in mechanically ventilated intensive care patients—a randomized clinical trial. *Critical Care* 2020, 24(1):74.

15. Bianconi I, Alcalá-Franco B, Scarselli M, Dalsass M, Buccato S, Colaprico A, Marchi S, Masignani V, Bragonzi A: Genome-Based Approach Delivers Vaccine Candidates Against Pseudomonas aeruginosa. *Frontiers in Immunology* 2019, 9(3021).

16. Wu W, Huang J, Duan B, Traficante DC, Hong H, Risseh M, Lory S, Priebe GP: Th17-stimulating protein vaccines confer protection against Pseudomonas aeruginosa pneumonia. *American journal of respiratory and critical care medicine* 2012, 186(5):420-427.

17. Galvao CE, Fragoso SP, de Oliveira CE, Forner O, Pereira RRB, Soares CO, Rosinha GMS: Identification of new Corynebacterium pseudotuberculosis antigens by immuno screening of gene expression library. *BMC microbiology* 2017, 17(1):202.

18. Hou Q, Chen K, Shan Z: The construction of cDNA library and the screening of related antigen of ascitic tumor cells of ovarian cancer. *European journal of gynaecological oncology* 2015, 36(5):590-594.

19. Ogunremi O, Benjamin J, MacDonald L, Schimpf R: Construction of a complementary DNA library for Parelaphostrongylus tenuis and identification of a potentially sero-diagnostic recombinant antigen. *The Journal of parasitology* 2008, 94(6):1402-1409.

20. Raran-Kurussi S, Keefe K, Waugh DS: Positional effects of fusion partners on the yield and solubility of MBP fusion proteins. *Protein expression and purification* 2015, 110:159-164.

21. Raran-Kurussi S, Waugh DS: The ability to enhance the solubility of its fusion partners is an intrinsic property of maltose-binding protein but their folding is either spontaneous or chaperone-mediated. *PloS one* 2012, 7(11):e49589.

22. Kapust RB, Waugh DS: Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein science: a publication of the Protein Society* 1999, 8(8):1668-1674.

23. Waugh DS: Crystal structures of MBP fusion proteins. *Protein science: a publication of the Protein Society* 2016, 25(3):559-571.

24. Minamino T, Imada K: The bacterial flagellar motor and its structural diversity. *Trends in microbiology* 2015, 23(5):267-274.

25. Shen Y, Chen L, Wang M, Lin D, Liang Z, Song P, Yuan Q, Tang H, Li W, Duan K et al.: Flagellar Hooks and Hook Protein FlgE Participate in Host Microbe Interactions at Immunological Level. *Scientific reports* 2017, 7(1):1433.

26. Progress Toward the Elusive Pseudomonas aeruginosa Vaccine. *Surgical Infections* 2018, 19(8):757-768.

27. Doring G, Meisner C, Stern M, Flagella Vaccine Trial Study G: A double-blind randomized placebo-controlled phase III study of a Pseudomonas aeruginosa flagella vaccine in cystic fibrosis patients.
28. Döring G, Pfeiffer C, Weber U, Mohr-Pennert A, Dorner F: Parenteral application of a Pseudomonas aeruginosa flagella vaccine elicits specific anti-flagella antibodies in the airways of healthy individuals. *American journal of respiratory and critical care medicine* 1995, 151(4):983-985.

29. Campodónico VL, Llosa NJ, Grout M, Döring G, Maira-Litrán T, Pier GB: Evaluation of Flagella and Flagellin of <em>Pseudomonas aeruginosa</em> as Vaccines. *Infection and immunity* 2010, 78(2):746-755.

30. Spangenberg C, Heuer T, Bürger C, Tümmler B: Genetic diversity of flagellins of *Pseudomonas aeruginosa*. *FEBS Letters* 1996, 396(2):213-217.

31. Tanaka N, Shuman S: Structure-activity relationships in human RNA 3’-phosphate cyclase. *RNA (New York, NY)* 2009, 15(10):1865-1874.

32. Lombardi C, Tolchard J, Bouillot S, Signor L, Gebus C, Liebl D, Fenel D, Teulon JM, Brock J, Habenstein B et al: Structural and Functional Characterization of the Type Three Secretion System (T3SS) Needle of Pseudomonas aeruginosa. *Frontiers in microbiology* 2019, 10:573.

**Figures**
Figure 1

Screening of vaccine candidates for Pseudomonas aeruginosa. A. Schematic representation of the strategy to identify vaccine candidates at a genomic scale. The DNA fragments from the genome of PA after random digestion with Sau3 I were cloned into pMAL-c5x by the BamH I site. Then, the recombinants were transformed into E. coli and induced by the introduction of IPTG. The 96-well plates were precoated with anti-MBP antibodies to capture the MBP-tagged recombinants. The reaction of each
inserted fragment was determined by ELISA. PcrV, a diagnostic marker for PA infection, was used as a control after fusion with MBP. B. The bar represents the relative strength of the inserted fragment to MBP-PcrV. The top 8 recombinants, namely, RtcA, PscF, FlgE, PscD, HlyD, HmgA, OprP and RecG, are shown. C. The survival of mice immunized with full-length RtcA, PscF, FlgE, PscD, HlyD, HmgA, OprP and RecG after challenge with a lethal dose of PA XN-1. MBP-tagged PcrV was used as a control. FlgE provided the highest protection among the eight recombinants.

Figure 2
The mechanism for reFlgE (recombinant tag-free full-length FlgE)-induced protection. A. The bar represents the global disease score of reFlgE-immunized mice after challenge with a sublethal dose of PA XN-1 (0.8×10^7 CFU/mouse). B. The bar represents the global disease score of reFlgE-immunized mice after challenge with a sublethal dose of PA XN-1. C. The bacterial loads of the lung (left panel) and spleen (right panel) in reFlgE-immunized mice 24 hours after challenge with a sublethal dose of PA-XN-1. D. The levels of TNF-α (left panel) and IL-1β (right panel) in reFlgE-immunized mice 6 hours after challenge with a sublethal dose of PA-XN-1. E. Histological analysis of lungs from reFlgE-immunized mice 24 hours after challenge with a sublethal dose of PA XN-1. Images were obtained at 100-fold and 400-fold magnification by light microscopy. F. The inflammatory scores of the lung sections according to the degree of hemorrhage edema, hyperemia and neutrophil infiltration. The "*" indicates a significant difference when P<0.05.

Figure 3

The prophylactic protection of anti-reFlgE IgGs. A. The bar represents the titer of anti-reFlgE antibodies in the sera of mice immunized with reFlgE. B. The subtypes of anti-reFlgE IgGs in the sera of mice immunized with reFlgE. C. Mice were intraperitoneally injected with anti-FlgE IgGs or nonspecific IgGs before being challenged with a lethal dose of PA XN-1 (1.0 ×10^7 CFU/mouse). The survival of the mice was recorded every 12 hours.
Figure 4

The mechanism for anti-reFlgE IgG-mediated protection. A. The anti-reFlgE IgGs were able to recognize native FlgE on the membrane of PA XN-1, as observed by immunofluorescence. B. The anti-reFlgE IgGs inhibited the motility of PA 01 and PA XN-1. The growth of PA 01 and PA XN-1 in the semisolid medium was recorded (upper panel). The areas were quantified using ImageJ software (lower panel). C.
Opsonophagocytic killing assay of anti-reFlgE antibodies. The bar represents the percentage of killed PA XN-1 at different dilutions. The "*" indicates a significant difference when $P<0.05$.

**Figure 5**

A. ReFlgE + PcrVNH antiserum

B. Combined vaccination with reFlgE and PcrVNH significantly improved the protective effect. A. The titers of anti-reFlgE and anti-PcrVNH IgGs in sera from mice immunized with reFlgE plus PcrVNH. B. The survival of immunized mice with reFlgE plus PcrVNH after challenge with a lethal dose of PA XN-1 (1.0...
×107 CFU/mouse). C. The survival of immunized mice with reFlgE plus PcrVNH after challenge with a three-fold lethal dose of PA XN-1 (3.0 ×107 CFU/mouse).

Supplementary Files

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