Isolation and Characterizations of A Novel Recombinant scFv Antibody Against Exotoxin-A of Pseudomonas Aeruginosa

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Abstract

Background: Pseudomonas aeruginosa is known as the leading cause of nosocomial infections especially in people with a compromised immune system. Targeting virulence factors by neutralizing antibodies is a novel paradigm for the treatment of antibiotic-resistant pseudomonas infections.

Methods: In this respect, exotoxin A is one of the most potent virulence factors in P. aeruginosa. The present study was conducted to identify a novel human scFv antibody against domain I of P. aeruginosa exotoxin A from a human scFv phage library. For this, the recombinant domain I of exotoxin A was expressed in E. coli and purified by Ni-NTA column. A novel screening procedure was conducted to prevent the elimination of rare specific clones. Based on polyclonal phage ELISA results, the fifth round of biopanning was selected to identify specific phage clones.

Results: Two positive clones were found by monoclonal phage. The phage clone with high reactivity was evaluated by ELISA and western blot. The purified scFv also showed high reactivity with full length exotoxin.

Conclusions: In conclusion, the purified anti-exotoxin A scFv displayed high specificity against exotoxin A. The human scFv identified can be the groundwork for development of a novel therapeutic agent for control of P. aeruginosa infections.

1. Background

Pseudomonas (p.) aeruginosa is the most common cause of nosocomial infections leading to a high mortality rate especially in people with cystic fibrosis, neoplastic disease and severe burns (1). Currently, the outbreak of antibiotic resistant strains has become one of the serious challenges for global health. Toxins play an important role in bacteria pathogenesis. P. aeruginosa produces several types of toxins including, exotoxin A, phosphorylase C, hemolysine, exoenzyme S which among them, exotoxin A plays a major role in the progress and prognosis of P. aeruginosa infections. Exotoxin A is a single-chain polypeptide with a molecular weight of 66.583, consisting of 613 amino acids comprised of three domains (2) (3). Domain I is responsible for the attachment of toxin to the cell receptor and composed of two subdomain 1a (amino acids 1 to 252) and 1b (amino acids 365 to 404). The function of Domain 1b is not well characterized but it may be necessary for the secretion of the toxin. The second domain is composed of 253 to 364 amino acids, consists of 6 alpha helix sequences, and is essential for the transfer of the toxin over the membrane. The third domain (405 to 613 amino acids) is the enzymatic and catalytic domain with ADP ribosyl transfer activity, which inhibits protein synthesis ultimately results in cell death (4). In addition to the above mentioned domains, there are two important motifs inside exotoxin A. The first motif (280 – 274 RHRQPRG amino acids) is located in domain II, appears on the external surface of the toxin, and is broken down by the eukaryotic proteases. The second motif (the REDLK-591 913 – 609) is located at the carboxylic end of the toxin and is responsible for retention of the toxin in the reticulum endoplasmic compartment. Both motifs are essential for toxicity (4). It has shown that
antibody-produced against Exotoxin A significantly can increase the survival rate of the infected subjects (5). Hence development of anti-exotoxin A antibody is of great interest for treatment of pseudomonas infections.

At present, high variety of human scFv phage libraries has provided a quick and reliable procedure to develop human antibodies against almost any antigen. This study was aimed to identify a human anti-exotoxin A scFv and evaluation of its specificity to \textit{P. aeruginosa} exotoxin A.

2. Methods

2.1. scFv-phage library, bacterial strains, and components

The human scFv phage libraries I & J (Tomlinson I & J), \textit{E. coli} strains (BL21, HB2151 and TG1), and KM13 helper phage (The Medical Research Council (MRC), Cambridge, were used for isolation and expression of specific scFv clones (6, 7).

2.2. Expression and purification of Exotoxin A Domain I

The expression construct encoding exotoxin A domain I (subdomains 1a and 1b) was transformed into \textit{E. coli} BL21. The transformed bacteria were cultured in LB media and induced with IPTG (0.5 mM) for 21 hours and the exotoxin A domain I expression was assessed by SDS-PAGE. For purification, the bacteria were cultured in 200 ml volume at 37 °C, lysed by sonication, and the inclusion body was washed by washing buffer and solubilized in 8M urea and purified by Ni-NTA column. After that, it was refolded by stepwise removal of urea by dialysis (8).

2.3. scFv phage library screening

After the amplification of Tomlinson I library, it was screened for 6 steps against exotoxin A Domain I (9). The biopanning process was started with 100 μg/ml on a Maxisorp 96-well plate in PBS buffer. (10). During biopanning rounds, to prevent the elimination of specific rare clones the concentration of domain I protein was kept constant. (10–12).

During biopanning to increase the screening stringency, the time of incubation of the phage pool with antigen was decreased and washing numbers between screening rounds were increased (Table 1). In beginning, the domain I - immobilized plate was blocked with 3% Bovine Serum Albumin (BSA) for 2 hrs then 10^{12–13} pfu phages were coated into the plates. After the incubation for 60 min at room temperature (RT), the plate was washed with PBS containing 0.1% Tween 20 (PBST) and trypsin-PBS (100 μl of 10 mg/ml trypsin stock solution in 10 ml PBS) was used for elution of the bound phages(Table 1). The biopanning rounds were continued to reach maximum OD in polyclonal phage ELISA. Totally, six rounds of biopanning were carried out to select domain I - specific phage clones.
Table 1
Data related to antigen concentrations, blocking buffers, Tween 20 percentage and washing numbers during seven biopanning rounds

| Rounds | I  | II | III | IV  | V   | VI  | VII |
|--------|----|----|-----|-----|-----|-----|-----|
| Protein(µg/ml) | 375 | 250 | 150 | 120 | 150 | 150 | 150 |
| Blocking buffers | %2 skim milk | %3BSA | %2 skim milk | %3BSA | %2 skim milk | %3BSA | %2 skim milk |
| % Tween20 | % 0.1 | % 0.1 | % 0.1 | % 0.1 | % 0.1 | % 0.1 | %0.1 |
| Washing numbers | 3 | 20 | 10 | 20 | 20 | 20 | 25 |
| The phage incubation periods(hr) | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

2.4. Investigation of the specificity of the phages clones by polyclonal phage ELISA

To investigate the specificity of selected phages from each round, a polyclonal phage ELISA against exotoxin A domain I was performed. For this purpose, 60 µg/ml exotoxin A domain I was coated into the ELISA plates and incubated at 4 °C overnight. After blocking with 3% BSA for 1 h, the eluted phages from each round (1:10 dilutions in 1% BSA–PBS) were added to the plates and incubated for 1hr. After that, the plates were incubated with anti-M13- HRP (1:2000 dilutions in 1% BSA–PBS) for 1hr. The reactivity was determined using TMB substrate. The optical density was recorded via ELISA Reader at 450 nm.

2.5. Selection of scFvs clones to exotoxin A domain I

The single colonies were randomly selected from the fifth round of screening and used to identify specific scFv clones by monoclonal phage ELISA. For this purpose, the individual colonies were inoculated into 2xTY medium (1% [w/v] yeast extract, 1.6% [w/v] tryptone, and 0.5% [w/v] sodium chloride) containing 4% glucose and 100 µg/ml ampicillin in a 96 well plate. The plate incubated at 37 °C for 2 hrs [18, 19]. After adding 10⁹ helper phages to the wells and incubation for 1hr at 37 °C, the plate was centrifuged at 3000 xg for 10 min. The supernatants were aspirated off and the bacterial pellet was resuspended in 2xTY medium containing 50 µg/ml kanamycin and 100ug/ml ampicillin. The cultures were continued overnight at 30 °C in a shaking incubator (250 rpm) and culture supernatants (1:2 dilution in1% BSA–PBS) were utilized for phage ELISA as described above.

2.6. Screening of clones via soluble fragment ELISA

All positive clones were initially confirmed by PCR with LMB3 and PHEN specific primers (13). A scFv ELISA was performed to evaluate the specificity of positive phage clones against exotoxin A Domain I. For this, a positive phage clone with high OD in phage ELISA was cultured in 2xTY medium containing ampicillin and 0.1% glucose media at 37 °C. The expression of antibody was induced by 0.5 mM IPTG at OD600 = 0.9 and the culture was continued at 200 rpm overnight at 37 °C. The soluble scFvs were
collected from the periplasmic fraction and used for ELISA. For this purpose, the ELISA plate was coated with exotoxin A Domain I (60 µg/ml overnight at 4 °C), and blocked with 3% BSA for 2 hrs and washed with PBST. Then soluble scFvs were added to the plate in different dilutions, incubated for 1 h, and washed with PBS-T. The plate was incubated with HRP conjugated Protein L (1:2000 dilutions in PBS-% BSA) for 1 h. The reaction was finally developed by TMB substrate and the OD values were measured with ELISA reader at 450 nm.

2.7. Expression of soluble anti-ExoA scFv in *E. coli*

To increase the expression of selected scFv, the sequence coding of scFv fragment with the highest reactivity was amplified by PCR and subcloned into the pET28a expression vector. The construct was transformed into BL21 (DE3) pLysS strain and cultured in LB media containing kanamycin (5mcg/ml). The antibody expression was induced by 0.5 mM IPTG at OD600 = 0.7, culture was continued overnight at 22 °C in a shaking incubator and expression was assessed with SDS-PAGE. The supernatant containing secreted antibody fragments was purified by Ni-NTA column and confirmed by 12% SDS gel, then the reactivity of the scFv was checked by ELISA technique.

2.8. Assessment of the reactivity of recombinant scFv with exotoxin A

The specificity of the purified scFv to Exotoxin Domain I was evaluated by western blot. For this, 25(µl/Lane) samples were electrophoresed through a 12% SDS-polyacrylamide gel and transferred onto PVDF membrane (Invitrogen, USA). The membrane was blocked with 5% Skim Milk PBS overnight 4 °C and incubated with the purified scFv followed by HRP conjugate Protein L (1-1000 dilutions) was added as the secondary antibody and developed by using the ECL Substrate.

3. Results

3.1. Enrichment and Specificity of anti-ETA phages during biopanning rounds

Enrichment rate of specific phages against Exotoxin Domain I was calculated during five rounds of biopanning. As expected, in the first round of screening, the yield of specific phages to exotoxin domain I was very low while, from the 3rd round, the elimination of nonspecific phages led to the enrichment of specific phages to $0.72 \times 10^{-4}$ that continued during rounds 4 and 5 (Table 2). Totally, the eluted phages were increased from $1.40 \times 10^{6}$ in the second round to $7.27 \times 10^{8}$ in the fifth round. However, the enrichment rate was again reduced in 6th round. These findings were indicating an effective screening to obtain specific phages against exotoxin domain I. To examine the specificity of the eluted phages against exotoxin domain I, a polyclonal phage-ELISA was performed after each biopanning round.
Table 2
Development of specific phages in five rounds of biopanning

| Round | Input (pfu)   | Output (pfu) | Enrichment(output/input) |
|-------|---------------|--------------|--------------------------|
| 1     | 3.52*10^{12} | 1.40*10^{6}  | 0.39*10^{-6}             |
| 2     | 1.40*10^{12} | 1.94*10^{9}  | 1.38*10^{-3}             |
| 3     | 2.52*10^{12} | 1.83*10^{8}  | 0.72*10^{-4}             |
| 4     | 1.62*10^{12} | 1.80*10^{8}  | 1.11*10^{-4}             |
| 5     | 2.908*10^{13}| 7.27*10^{8}  | 2.5*10^{-5}              |

The BSA was used as the negative control and displayed a weak signal in ELISA (Figure 1). Based on the results, the lowest OD was related to the wild type phages before panning. After six rounds of biopanning, the 5th round indicated the highest binding reactivity to exotoxin Domain I so it was selected for further studies.

3.3. Identification of scFv phage clones binding to Domain I by monoclonal phage ELISA

We examined more than 100 phage clones randomly from the fifth round and almost 18 specific phage clones were identified by monoclonal phage ELISA with optical densities range of 0.12 to 1.533 showing significant differences in the binding activity of selected phage clones. Among positive clones, one of the positive phage clones (C9) with higher reactivity to Domain I was used for further analysis (Fig. 2).

3.4. Sequence analysis of the positive clones

To confirm the presence of both VH and VL fragments in the positive clones, DNA was extracted from the 18 positive clones. PCR screening using PHEN and LMB3 specific primers results in the amplification of a specific PCR fragment that appeared as a single 950 bp band (Fig. 4).

In the next step, the integrity of the C9 scFv clone with the highest affinity was confirmed by sequencing. Blast analysis indicated that the nucleotide sequence is related to human VH and VL fragments. There was no amber stop codon and mutation in the selected clone. Analysis of CDR regions was performed by http://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi and displayed in Table 3.
3.5. Assessment the reactivity of selected scFv by western blotting

After purification of the selected scFv (c9) by Ni-NTA column, its purity was confirmed by SDS-PAGE gel that appeared as a single band of 25 kDa. The reactivity of scFv to Exo Do I was assessed by western blot. Results indicated a single band with a molecular Wight (MW) of 25 kDa, which was consistent with the calculated MW for the Exo Do I protein (14).

4. Discussion

Exotoxin A is one of the most potent virulence factors in *P. aeruginosa*. With increasing prevalence of multi drug resistant strains, it is necessary to develop alternative therapeutic approaches as a robust pipeline to combat resistant pseudomonas infections. Neutralizing virulence factors such Exo A can be an promising strategy to control bacterial infections. Moreover, this strategy might be affect drug resistance through maintaining the host endogenous microbiome and creating less selective pressure to the bacteria (15). Until now, various antibodies have been developed against different antigens of *pseudomonas*, but none of them have been approved for clinical use (16).

Theoretically, antibody libraries contain various synthetic and semi synthetic antibodies, facilitating the isolation of antibodies against any given antigens. At present, scFv phage libraries have impactful payload in the development of monoclonal antibodies without the need to experimental animals (17).

Pseudomonas exotoxin A domain I has been shown to be responsible for binding of toxin to animal cell receptors and plays a key role in the toxicity caused by pseudomonas exotoxin. The aim of the study was to develop a full human antibody against domain I of exotoxin A for potential use in neutralizing the toxic effects of Pseudomonas exotoxin to overcome the pseudomonas infection.

In this study, a novel screening strategy was used for elimination of nonspecific clones coupled with enrichment of specific clones during biopanning rounds. In this respect, results of output phage titers monitoring during screening rounds indicated a significant enrichment toward increasing specificity (64-fold) to exotoxin A domain I. During six biopanning rounds, two specific clones with VH-VL correct integrity were identified from fifth round. One of the scFv clones (C9) with high affinity was selected for further studies. In order to generate antibody with high efficiency, the expression and purification of C9 scFv was again performed in *E. coli*. In a 2019 study by Sirijan Santajit et al, they produced human
antibodies against subdomain Ia exotoxin A of *Pseudomonas* using phage display technology. However, in the present study, domain 1 subdomains (domains Ia and Ib) were targeted for antibody production, which had a greater neutralizing power (18). An older study also produced a mouse antibody against exotoxin A, but due to adverse immune responses we decided to produce a completely human antibody (19). S. NATHAN (20) used phage Display technology for isolation of the antibody against Burkholderia pseudomallei by several biopanning rounds. They observed high similarity (93%) among different clones while the main difference in the sequence was related to CDR3 area. In our study, the highest similarity was 93.9% and the different area in the sequence was related to CDRs (21).

The clone 9 anti-exotoxin A scFv was purified with high solubility from periplasmic fraction. Based on ELISA results, the resultant anti-exotoxin A scFv specifically recognized both recombinant exotoxin A domain I and supernatant of pseudomonas with high affinity. Also, western blotting results confirmed that human anti-exotoxin A scFv binds specifically to exotoxin A domain I protein.

5. Conclusion

In this study, we generated and characterized a human anti-exotoxinA scFv with a significant binding potency to exotoxin A of *Pseudomonas saeruginosa*. This scFv can potentially be considered for developing an alternative therapeutic agent against *pseudomonas* infections.

Declarations

Conflict of interest

There is no conflict of interest

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