

Cloning and expression of the immunogenic moiety of *Pseudomonas aeruginosa* exotoxin A

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Abstract

Introduction: *Pseudomonas aeruginosa*, as an opportunistic microorganism, is a major cause of nosocomial infections worldwide. Exotoxin A (ETA) is an extracellular enzyme that is produced by most clinical strains of *P. aeruginosa*. Although the pathogenesis of the diseases due to *Pseudomonas* are complex, clinical and experimental data linking ETA with the morbid and lethal consequences of *Pseudomonas* infection are accumulating.

Materials and methods: An immunogenic 490–bp DNA segment including translocation domain plus 1b domain of the ETA from *P. aeruginosa* strain PAO₁ were reproduced by PCR. The PCR product was cloned in *E.coli* DH5 α and expressed in *E.coli* BL21 using recombinant pET28a vector. The cloned polypeptide was found to have an electrophoretic mobility in sodium dodecyl sulfate- polyacrylamide gels (SDS -PAGE) of 18kDa.

Results: PCR and colony PCR results approved the cloning of immunogenic moiety of exotoxin A. Analysis of the location of cloned polypeptide by SDS- PAGE electrophoresis revealed that it was exported by *E.coli* into the bacterial periplasmic space.

Discussion and conclusion: Since the whole toxin is not necessary for enhancing the immune responses and this recombinant polypeptide has antigenic qualities, so it may serve as a useful vaccine to prevent *Pseudomonas* infections.

Key words: *Pseudomonas aeruginosa* PAO₁, Cloning, Exotoxin A, Immunogenic moiety

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen and is widespread in nature, inhabiting soil, water, plants and animals (including humans). This pathogen is an important cause of infection, especially in patients with compromised host defense mechanisms (1). Exotoxin A (ETA) is a potent cytotoxic protein secreted by *P. aeruginosa* and belongs to the well-characterized family of bacterial ADP-ribosylating toxins. The ability of ETA to covalently modify eukaryotic elongation factor II via ADP-ribosylation of a modified histidine residue has been extensively studied (2). ETA enters cells using receptor mediated endocytosis. After a series of intracellular processing steps, it is translocated to the cell cytosol and causes the inhibition of protein synthesis (3).

This 66-kDa toxin is composed of 3 major domains (I, II, III) and 1 minor domain (1b). Domain I is the cell binding domain, II is the translocation domain that enables toxin to reach the cytosol and III is the ADP-ribosylating domain that inactivates elongation factor II and causes cell death (4). A small epitope in domain 1b elicits enzyme-neutralizing antibodies (5). So both translocation domain and 1b domain have antigenic properties.

ETA production by *P. aeruginosa* is regulated by different environmental factors, including growth temperature and the level of iron in the growth medium. Iron represses ETA production. The production of ETA by *P. aeruginosa* also involves several positive and negative regulators. The most extensively analysed are the positive regulators namely *RegA* and *PvdS*. Negative regulation of ETA production occurs through the ferric-uptake regulator Fur. Iron-activated Fur represses *pvdS* transcription, thereby reducing *regA* and *toxA* transcription (6).

P. aeruginosa excretes ETA right after completion of its synthesis, and no

detectable pools are ever found in any of the subcellular compartments, including periplasmic space (7). Recent reports listed *P. aeruginosa* among the most serious antibiotic resistant bacteria and one for which effective vaccines are needed. ETA has been well studied as a virulence factor associated with the morbidity due to *Pseudomonas* infections and in human infections, high serum anti exotoxin A titers have been correlated with a greater chance of survival (2).

To complement biochemical and biophysical approaches in order to study the structure – function relationships in ETA and for genetic immunization against bacteria, recombinant DNA methods are usually used (2, 8).

In 1994, *P. aeruginosa* was detected from water samples by amplifying a small region of ETA structural gene by Khan and Cerniglia (9).

The ETA structural gene and gene fragments have been cloned and expressed in *E.coli* by many researchers ever since.

We report here the cloning of a segment of *P. aeruginosa* chromosomal DNA encoding a part of exotoxin A that contains the immunologically active site.

Materials and methods

Bacterial strains and plasmids

The source of chromosomal DNA for cloning was *P.aeruginosa* strain PAO₁, which was kindly provided by Dr Nima Hosseini Jazani, Urmia University of Medical Sciences, Urmia, Iran.

E.coli DH5 α was used as host for construction of recombinant plasmids and the plasmid used in the present study was pET28a which was resistant to kanamycin. Both *E.coli* strains (DH5 α and BL21) and plasmid were provided by Dr Bahman Hosseini, Faculty of Agriculture, Urmia University, Urmia, Iran.

Bacterial DNA preparation

P. aeruginosa was cultivated in LB broth (Sigma Alderich, L3022) at 37°C for 12 hours and then it was identified by standard biochemical tests (10).

The chromosomal DNA was extracted using DNA extraction kit according to the manufacture's manual (Fermentas, Germany).

Primer selection

The specific primers to the immunogenic moiety of ETA were chosen from published sequence (11). These primers were shortened and since the immunogenic moiety of ETA should be ligated into the *EcoRI* and *HindIII* sites of pET28a vector, primers were added restriction sites for *EcoRI* and *HindIII* enzymes using vector NTI software. So a 490 – bp region of the exotoxin A structural gene cocontaining translocation domain plus 1b domain, could be amplified. The final primer patterns were as below:

F(5'GAATTCATGCGCCTGCACTTTCCCGAGGGC-3')

R(5'-AAGCTTGTCTGCGTGCCGCGGGTGCTGAA-3')

Amplification of immunogenic moiety

The amplification reaction was performed using a DNA thermal cycler (Corbet, Australia). The reaction mixture (25 µL total volume) consisted of 12.5 µL of PCR master mix kit consisting of Taq DNA polymerase, PCR buffer, MgCl₂ and dNTPs, 5µL of template DNA, 1 µL of each primer (0.4 µmol) and 5.5 µL of sterile water (Cinna Gene, Iran). After being overlaid with mineral oil, samples were subjected to 35 cycles of amplification.

PCR cycles were run under the following conditions:

Denaturation at 94°C for 1min, primer annealing at 66°C for 1min and DNA extension at 72°C for 1 min in each cycle. After the last cycle, the PCR tubes were incubated for 7 min at 72°C (9).

After staining with etidium bromide, the PCR product was run in 1% agarose gel

electrophoresis and the expected size 490–bp DNA band was determined by exposing UV light onto the gel and the agarose gel band containing the desired DNA (490-bp) was cut and purified using Vivantis GF-1 gel DNA recovery kit (Vivantis, Malaysia).

Plasmid preparation

Bacteria, carrying plasmid (pET28a) was grown overnight in LB broth (Sigma Alderich, L3022) at 37°C. The plasmid was extracted using alkaline lysis method and was electrophoresed on agarose gel 1% at 80V for 45min (12).

Digestion process in order to obtain sticky ends in PCR product and pET28a

The purified PCR product and pET28a were double digested by *EcoRI* and *HindIII* restriction enzymes (According to the product datasheet of enzymes).

The first step of digestion process was done with 2.5 µL of buffer 10x, 10µL of both purified PCR product and pET28a, 12 µL of sterile water and 1 unit of *HindIII* enzyme, in a reaction volume of 25 µL for 2 hours at 37°C to create the sticky end.

The second step of digestion process was done with 3 µL of 10x buffer and 1 µL of *EcoRI* enzyme for 2 hours at 37°C to create the other sticky end.

Ligation of purified PCR product and pET28a

The combination of 3:1 cut PCR product (9 µL) and plasmid (3 µL) with sticky ends, 2 µL of 10x buffer, 1µL of ligase enzyme T4 and 5µL of sterile water were mixed and placed at 16°C for 8 hours (According

to the product datasheet of Fermentas T4 DNA Ligase enzyme).

Transformation

The recombinant plasmids were introduced into competent *E.coli* DH5α by transformation. Competent cells which were at -80°C, melted on ice slowly. Then, cells were mixed with 5µL of recombinant plasmid and placed on ice for 30min, at

42°C for 90 sec and then on ice for 2 min. After adding 0.5mL of LB broth in each vial they were cultivated for 1h at 37°C at 200rpm. The grown bacteria were centrifuged for 5min, at 3000rpm and were cultivated in LB agar medium with kanamycin (50µg /ml) at 37°C for 24h (13).

Cloning confirmation tests

The clones which grew in LB agar medium with kanamycin, were candidates to possess recombinant plasmids. To confirm recombinant plasmids, PCR was carried out on colonies which grew on the LB medium in the presence of Kanamycin. The reaction mixture (25 µL total volume) consisted of 18 µL of PCR master mix kit, 1 µL of the cloned gene primers (0.4 micromol), 5µL of sterile water. A small amount of each colony was added to each PCR tubes containing the PCR reaction and PCR was carried out as described previously. At the end of PCR process, the product was run on 1% agarose gel electrophoresis, and 490-bp DNA band was determined. As another screening test, the clones were grown overnight on LB broth medium containing kanamycin (50 µg/ml) at 37°C. Bacterial plasmids, were extracted using alkaline lysis method and again PCR was carried out with extracted plasmids as described previously and the 490-bp DNA band was observed on 1% agarose gel.

Expression of recombinant polypeptide

In order to express recombinant protein, a 100µL of transformed *E.coli* BL21 was grown in LB broth media overnight at 37°C in water bath shaker (12h), by adding 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside), (Fermentas, Germany).

The cells were collected by centrifugation (1000g,10min,4°C), washed twice with chilled PBS buffer and resuspended in 50mL of chilled PBS buffer, then they were subjected to sonication (14). Then, the samples were

centrifuged at 8000 rpm for 15min to pellet the debris. The supernatant containing solubilized proteins was collected for analysis by SDS PAGE electrophoresis. The samples were run at a constant voltage of 150V for 2.5 h until the loading buffer just runed off from the end, then proteins were visualized using Coomassie Brilliant Blue staining (15).

Results

In the present study, cloning and expression of the immunogenic moiety of *P. aeruginosa* exotoxin A was successfully done.

P. aeruginosa PAO₁ was identified using standard laboratory methods (10). PCR results of amplifying the immunogenic part of ETA by specific primers has been shown in Fig. 1.

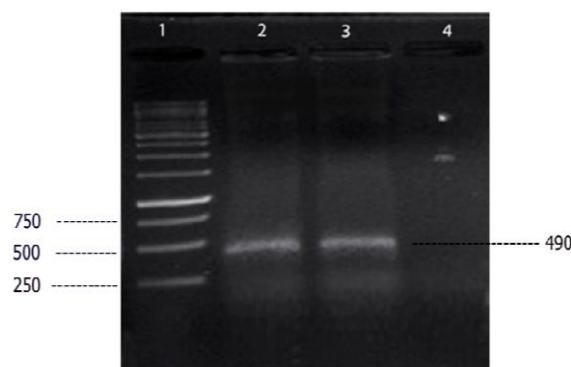


Fig 1- The electrophoresis of the PCR product of the immunogenic moiety of exotoxin A
Lane 1- Molecular weight marker DNA, 1kb (Fermentas, Germany); Lanes 2, 3 - positive sample (PCR product for immunogenic moiety of *P. aeruginosa* PAO₁ exotoxin A); Lane 4- negative control

The desired band (490-bp) was purified from agarose gel using GF-1Gel DNA recovery kit and electrophoresed in (1%) agarose gel (Fig. 2).

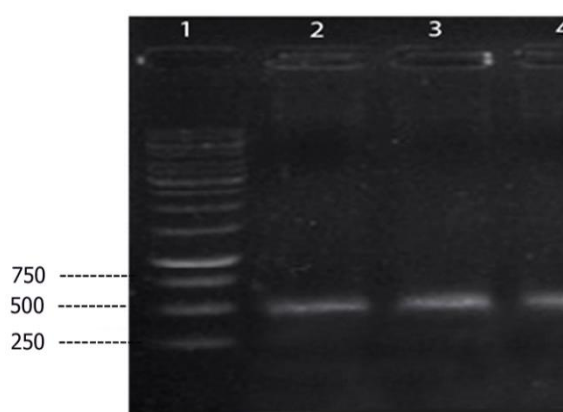


Fig. 2- The electrophoresis of the PCR product of the immunogenic moiety of exotoxin A using GF-1 Gel DNA recovery kit.

Lane 1- molecular weight marker DNA, 1kb (Fermentas, Germany); Lane 2- positive control (PCR product for immunogenic moiety of *P. aeruginosa* PAO₁ exotoxin A, 490bp); Lane 3, 4- positive sample (The electrophoresis of PCR product using GF-1 Gel DNA recovery kit, 490bp).

After transformation of competent *E. coli* DH5 α with ligation product of digested immunogenic fragment and pET28a, recombinant plasmids were extracted from single colonies appeared in LB agar plate. Presence of target gene in recombinant vector was confirmed by colony PCR and PCR detection of amplified 490-bp DNA fragment from recombinant colonies and extracted plasmids. (Fig. 3 and Fig. 4).

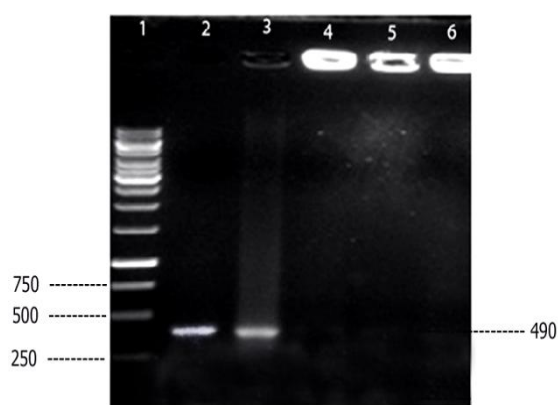


Fig. 3- The electrophoresis of recombinant colony PCR product plasmid

Lane 1-molecular weight marker DNA, 1kb (Fermentas, Germany); Lane 2- positive control (PCR product for immunogenic moiety of *P. aeruginosa* PAO₁ exotoxin); Lane 3, 4, 5 - Colonies of recombinant samples.

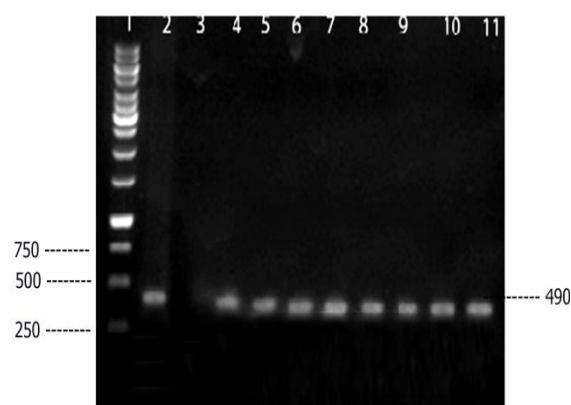


Fig. 4- The electrophoresis of recombinant PCR product plasmid

Lane 1- molecular weight marker DNA, 1kb (Fermentas, Germany); Lane 2- positive control (PCR product for immunogenic moiety of *P. aeruginosa* PAO₁ exotoxin A); Lanes 4 to 11 - Recombinant samples

Also in expression step, the BL21 competent cells were transformed with confirmed recombinant vectors and induced with IPTG to express target recombinant protein. Total protein was electrophoresed on 15% SDS PAGE gel and protein of interest with approximate molecular weight of 18 kDa was detected (Fig. 5).

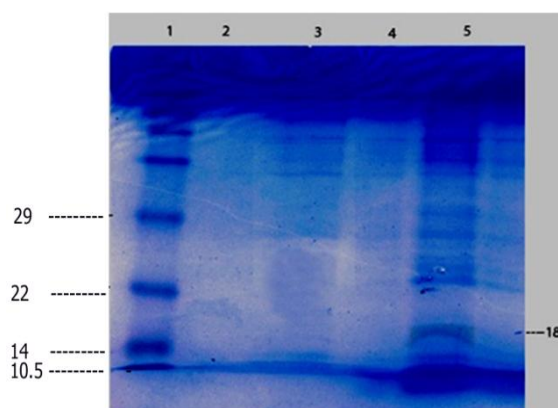


Fig. 5- SDS-PAGE of recombinant protein

Lane 1- molecular weight marker of protein (Vivantis, Malaysia); Lanes 2, 3, 4 - negative control of protein expression in *E. coli* BL21 without recombinant protein; Lane 5 - recombinant protein expression with IPTG stimulation

Discussion and conclusion

Exotoxin A of *P. aeruginosa*, is a secretion of virulence factor. Both clinical studies and animal models have demonstrated the importance of ETA in *P. aeruginosa* infections. For example, *P. aeruginosa* mutants defective in ETA production were less virulent in different animal models than their parents (6).

There were many problems in extracting ETA from *P. aeruginosa* and also secretion of proteases to culture media decreased the product efficiency. So using recombinant techniques for producing higher amounts of toxin, were developed (16).

In 1987, Chen et al cloned the exotoxin A genes from *P. aeruginosa* in a pUC9-derived plasmid. In a non-toxicogenic mutant of PAO₁ as host, the cloned genes directed the synthesis of intact exotoxin A (17).

Douglas et al in 1987 subcloned the structural gene for ETA of *P. aeruginosa* in front of the tac promoter in an *E. coli* expression vector. They found that the protein was predominantly in the periplasmic fraction (8). Since the hole toxin caused the inhibition of protein synthesis and cell death, we cloned a 490-bp segment of the structural gene which did not have toxic qualities.

In 1984, a 2760-bp DNA segment of *P. aeruginosa* exotoxin A structural gene was cloned in *E. coli* by Gray et al. Expression of the ETA coding sequence in *E. coli* under control of *E. coli* trp promoter, resulted in the production of enzymatically active protein (18). Trp promoter and its derivatives have been successfully used for heterologous protein expression, however, sometimes requires consideration of parameters other than transcription such as translation initiation, translation elongation and proteolysis (19).

Protein expression vectors that utilize the bacteriophage T7 polymerase/promoter system are capable of very high levels of protein production (20). Therefore, we used pET28a containing T7 promoter for increasing recombinant protein production.

Mozola et al (1984) cloned a segment of the gene from *P. aeruginosa* PA103

encoding the enzymatically active part of the exotoxin A protein. They used *E. coli* BamHI in the expression step (21).

Reintroduction of the plasmid into a T7 RNA polymerase-producing strain such as BL21 (λ DE3) reproducibly restored high level protein production (20). Therefore, we used this strain of *E. coli* for higher expression of recombinant protein.

Exotoxin A gene from *P. aeruginosa* was expressed in *E. coli* by Lory et al In 1988. Analysis of the location of exotoxin A in various bacterial compartments by immunoblotting revealed that exotoxin A was exported by *E. coli* into its periplasmic space, while less than 10% was found in the cytoplasmic and membrane fractions. No detectable toxin was excreted into the medium (7). The expression in *E. coli* of the gene for phospholipase C, another extracellular enzyme of *P. aeruginosa* was studied by Lory and Tai (1983). They found that this enzyme was outer-membrane associated but did not report whether it was processed from its precursor (22). Our results also showed that *E. coli* could not excrete the recombinant polypeptide into the medium and the recombinant polypeptide was found in the intracellular fraction of the bacteria.

In the present study, cloning and expression of a gene segment encoding translocation domain plus 1b of *P. aeruginosa* PAO₁ ETA, was done for the first time in order to produce much more potent immunogen. The two restriction enzymes (*EcoRI* and *HindIII*) were used for double digestion of pET28a vector and PCR product. In 2010, Bayat et al also used this vector and the same restriction enzymes (16).

We are presently screening new clones which we anticipate will contain the translocation domain plus 1b of exotoxin A structural gene. Such recombinant polypeptides or the DNA encoding them, can be used for the immunization purposes

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همسانه‌سازی و بیان قسمت ایمنی‌زای آگزوتوکسین A از سودوموناس آئروژینوزا

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چکیده

مقدمه: سودوموناس آئروژینوزا میکروارگانیسم فرصت‌طلبی بوده که به یکی از عوامل اصلی عفونت‌های بیمارستانی در سراسر جهان تبدیل شده است. آگزوتوکسین A یک آنزیم خارج سلولی است که توسط اغلب سویه‌های بالینی سودوموناس آئروژینوزا ترشح می‌شود. اگرچه پاتوژنز بیماری‌های ناشی از سودوموناس پیچیده است، ولی شواهد کلینیکی و تجربی فراوان نشان‌دهنده ارتباط آگزوتوکسین A با موارد ابتلا و مرگ و میر ناشی از عفونت‌های سودوموناسی است.

مواد و روش‌ها: در مطالعه حاضر، یک قطعه ایمنی‌زای ۴۹۰ جفت بازی شامل دومین انتقال‌دهنده و دومین I_b از آگزوتوکسین A سودوموناس آئروژینوزا سویه PAOI توسط PCR تکثیر داده شد. محصول PCR در اشرشیا کلی DH5 α کلون‌گشت و سپس در اشرشیا کلی BL21 با استفاده از وکتور پلاسمیدی (pET28a) بیان شد. وزن مولکولی پلی‌پپتید موردنظر در ژل SDS-PAGE، ۱۸ کیلو دالتون تعیین شد.

نتایج: نتایج PCR و colony PCR تایید‌کننده صحت همسانه‌سازی دومین ایمنی‌زای آگزوتوکسین A بود. بررسی موقعیت پلی‌پپتید کلون‌شده در الکتروفورز عمودی با استفاده از ژل SDS-PAGE نشان داد که پلی‌پپتید موردنظر توسط اشرشیا کلی به فضای پری پلاسمی تراوش می‌شود.

بحث و نتیجه‌گیری: از آنجایی که حضور توکسین کامل برای ایجاد پاسخ‌های ایمنی ضروری نیست و این پلی‌پپتید نو ترکیب از خاصیت آنتی‌ژنیک برخوردار است، بنابراین ممکن است به عنوان واکسن مؤثری در پیشگیری از عفونت‌های ناشی از سودوموناس عمل کند.

واژه‌های کلیدی: سودوموناس آئروژینوزا سویه PAOI، کلونینگ، آگزوتوکسین A، دومین ایمنی‌زا

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