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# Evaluation of immune response to recombinant *Bacillus anthracis* LFD1-PA4 chimeric protein

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## Summary

**Background:** Anthrax is a particularly dangerous infectious disease that affects humans and livestock. Efficacious vaccines that can rapidly induce a long-term immune response are required to prevent anthrax infection in humans. Domains 4 and 1 of the protective antigen (PA) and lethal factor (LF), respectively, have very high antigenic properties. **Aims:** In this experimental study, the pET28a-*lfD1-pa4* expression vector was designed, constructed and transferred into *E. coli* BL21 (DE3) plysS. **Methods:** For this purpose, *pa4* gene was amplified by polymerase chain reaction (PCR) and cloned in a pGEM T-easy vector. The pGEM-pa4 and pGEM-lfD1 were digested by *XbaI* and *HindIII* enzymes. The ligation reaction was performed by ligase T4 enzyme and the gene cassette, lfD1-pa4, was subcloned in pET28a and transferred to *E. coli* BL21 (DE3) PlysS. Expression and purification of chimeric proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques. The chimera LFD1-PA4 and mixed LFD1+PA4 proteins were injected four times into mice and antibody production was relativity evaluated by enzyme-linked immunosorbent assay (ELISA) test. **Results:** The results showed that both chimeric and mixed proteins are immunogenic, but LFD1-PA4 has a higher potential to stimulate mice immune system. **Conclusion:** LFD1-PA4 chimeric protein induced a higher immune response than LFD1+PA4 mixed protein and elicited antibody responses to LF and edema factor (EF), therefore, it holds promise to be a more effective trivalent vaccine candidate to use in anthrax prevention.

**Key words:** Anthrax, Antibody titer, LFD1, LFD1-PA4, PA4

## Introduction

Anthrax is a particularly dangerous infectious disease that affects humans and livestock. It is characterized by intoxication, serosanguinous skin lesions, development of lymph nodes and internal organs, and may manifest itself in either a cutaneous or septic form. The pathogenic agent is *Bacillus anthracis*, a gram-positive, endospore-forming, rod-shaped aerobic bacterium (Schmidt *et al*., 2011; Ahmadi *et al*., 2015). Due to the production of heat resistant spores, very high mortality, easy production and distribution and also creating respiratory, gastrointestinal, cutaneous diseases and its potential use in biological warfare, *B. anthracis* is one of the most important fatal biologic agents (Knight, 2001; Ahmadi *et al*., 2015).

The size of *B. anthracis* genome is 5,227,419 bp and contains two plasmids, pOXI (181677 bp) and pOXII (94830 bp).

The principal virulence factors of *B. anthracis* consist of an anti-phagocytic capsule composed of poly-D-glutamic acid (PGA) and a secreted bacterial toxin. The former is encoded by genes located on pXO1 plasmid, and the latter is encoded by pXO2 plasmid (Bragg and Robertson, 1989; Bergman, 2010). The anthrax toxin, which is predominantly responsible for the etiology of anthrax, belongs to the family of bacterial binary AB-type toxins, which consist of a receptor-binding B subunit known as the protective antigen (PA) and two catalytic A subunits, i.e., the lethal factor (LF) and edema factor (EF). Protective antigen is combined with either LF or EF to form the lethal toxin (LeTx) and edema toxin (EdTx), respectively (Okinaka *et al*., 1999; Koehler, 2009; Schmidt *et al*., 2011; Honari *et al*., 2014).

Diagnosis in the early stages and administration of antibiotics treats the disease, but the symptoms of the disease do not always appear on time, so vaccination is the best approach for protection.

Currently, the standard approach for anthrax therapy is to kill the germinating bacilli by administering aggressive antibiotics. However, antibiotic therapy is ineffective once systematic anthrax symptoms appear because by that time, fatal concentrations of the anthrax toxin have accumulated in the patient's body (Schneemann and Manchester, 2009) and the emergence of antibiotic-resistant strains as a result of natural evolution or intentional modification by genetic engineering also poses a new challenge to traditional antibiotic treatment (Gilligan, 2002; Gilligan, 2004). Therefore, the development of an antitoxin for combined use with antibiotic therapy is a top priority.

At present, the process by which anthrax toxins enter cells and act is relatively well understood. Initially, the B subunit, i.e., the 83-kDa PA (PA83), binds to specific cell surface receptors through its C-terminal binding domain, and this is then proteolytically cleaved by furin

or furin-like protease into a 20-kDa N-terminal fragment (PA20) and an active 63-kDa C-terminal fragment (PA63). After dissociation of PA20, cell-bound PA63 self-assembles into a ring-shaped homo-oligomer (heptamer or octamer) termed a prepore (Young and Collier, 2007; Kintzer *et al*., 2009). Simultaneously, the prepore competitively binds up to three molecules of LF and/or EF to form toxin complexes (Klimpel *et al*., 1992; Gordon *et al*., 1997; Bradley *et al*., 2001; Liu *et al*., 2009a). The elucidation of the molecular mechanism of anthrax toxin action has provided us with new strategies for developing antitoxins for anthrax treatment and prevention.

Current vaccines for human administration are prepared in England and the United States based on cell extract deposition (Pannifer *et al*., 2001). The production of the new generation of anthrax vaccines has focused on various recombinant expression systems (Gorse *et al*., 2006; Campbell *et al*., 2007; Brown *et al*., 2010; Bellanti *et al*., 2012; Reed *et al*., 2015). Studies have shown that antibodies generated against four regions of PA (PAD4) are capable of neutralizing anthrax toxin (McComb and Martchenko, 2016). On the other hand, PA mixed with LF has been shown to increase the specific antibody response to PA (Pezard *et al*., 1995; Price *et al*., 2001). The purpose of this study was to express the *lfD1-pa4* gene of *B. anthracis* in *Escherichia coli* expression strain, purify the recombinant protein and produce polyclonal antibody in mice and compare the lethal factor domain 1-protective antigen 4 (LFD1-PA4) fusion protein and mixed LFD1 and PA4 recombinant proteins.

## Materials and Methods

### Plasmids, gene and primers

The complete sequence of *B. anthracis pag* gene sequence was elicited from GeneBank (NCBI; accession number NC_003980, 1). The recombinant PA4 proteins encompass C terminal domain of PA (510 bp of c-terminal). The forward and the reverse primers of *pa4* were designed by Primer3, Oligo and DNASIS software with *HindIII* and *XbaI* restriction sites, respectively and were synthesized by Cinnagen Co. (Iran). The primer's restriction sites were determined by BIOLABS_NEB-cutter software (the specific primer sequence is shown in Table 1).

**Table 1:** Primers used in the present study

| Primer | Sequence |
|---|---|
| *pa4*-For | 5´ATCTAGAGCGGAATTAAACGCAACTAAC3´ |
| *pa4*-Rev | 5´GTTCGAATTATCCTATCTCATAGCCTTTTT3´ |

### Cloning of *pa4* gene in pGEM T-easy cloning vector

Polymerase chain reaction (PCR) products were analyzed by the nucleic acid ladder (Fermentas, Ukraine) in agarose gel electrophoresis and were extracted from low melting agarose gel by Silica Bead DNA Gel Extraction Kit (Fermentas, Ukraine). Then, *pa4* PCR products were cloned in pGEM T-easy vector (Fermentas, Ukraine). For this purpose, in accordance with the instructions of Kit's manufacturer (Promega, US) ligation was performed with a linear vector by T4 DNA ligase enzyme and specific buffer followed by incubation for 13 h at 4°C.

The ligation product was transformed into *E. coli* DH5α competent cells which were prepared by standard cold $CaCl_2$ protocol (Joseph and David, 2001).

The transformed bacteria was grown in Luria Bertani (LB) broth medium antibiotic-free for 1 h and then was cultured in the LB agar (LIOFILCHEM, Italia) containing 100 µg/ml ampicillin.

### Ligation of the *pa4* to the *lfD1* gene in the expression vector

In order to ligate *pa4* (in a pGEM T-easy cloning vector) to pET28a-*lfD1*, the recombinant construct containing the *pa4* fragment (from the previous step) and pET28a(+) containing *lfD1* was double digested using *XbaI*, *HindIII* enzymes and digested products were isolated from 1% agarose gel with low melting temperature and extracted with gel extraction kit (Fermentas, Ukraine). Ligation of purified *pa4* gene fragment and the linearized pET28a(+) vector containing *lfD1* was performed according to the standard instructions using T4 DNA ligase enzyme and specific buffers followed by 13 h incubation at 4°C. The product of the reaction was transformed to *E. coli* BL21 and cultured in LB medium supplemented by 40 µg/ml final concentration of kanamycin. The recombinant pET28a(+) construct containing the *lfd1* and *pa4* genes (pET28a-*lfd1-pa4*) was confirmed by PCR, enzymatic digestion and sequencing.

### Expression of recombinant proteins

The recombinant LFD1 and PA4 proteins were expressed and purified as described elsewhere by this laboratory (Rezaee *et al*., 2014).

The recombinant strains (LFD1-PA4) were cultured in LB medium supplemented with 50 µg/ml kanamycin at 37°C until OD600=0.6. To induce protein expression, isopropyl1-thio-b-D-galactopyranoside (IPTG) (Roche, Germany) was added to a final concentration of 1 mM and incubated for 5 h. The bacteria cells were centrifuged at 5,000 rpm for 10 min and suspended in lysis buffer (100 mM $NaH_2PO_4$, 10 mM Tris-HCl, 8 M Urea, pH=8) at 4°C for 1 h and completely lysed by sonication (6 times for 10 s). The samples were analyzed before and after induction with IPTG in denaturing conditions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) with standard protein marker (Vivantis, Malaysia). The expressed proteins were eluted from nickel-nitrilotriacetic acid (Ni-NTA) column with buffer E (100 mM $NaH_2PO_4$, 10 mM Tri-HCl, 8 M Urea, and pH=4.5). The proteins purity was confirmed by SDS-PAGE and estimated by densitometry (Madanchi *et al*., 2012). The concentration of purified proteins were determined by Bradford method using BSA serum albumin (Cinagen) as standard

(Ranjbar *et al*., 2009).

## Western blot analysis

The identification of the expressed proteins was carried out by Western blot analysis. The purified proteins were transferred from polyacrylamide gel into nitrocellulose membrane (Roche, Germany) using Bio-Rad Protean II system and transfer buffer (192 mM glycine, 25 mM Trisbase, 0.1% SDS, 20% methanol, pH=8.3). To block the membrane, it was incubated in 3% bovine serum albumin (BSA)/phosphate buffered saline (PBS) blocking buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$ $7H_2O$, pH=7.3) and was gently shaken for 16 h at 4°C. After discarding the blocking buffer, the membrane was incubated in 1:10,000 dilution of a conjugated mouse antiHis-tag antibody (Abcam, UK) in the PBS containing 0.05% Tween 20 (PBST) with gentle shaking for 1 h at room temperature. The membrane was washed three times with PBST and stained with HRP staining solution, Diaminobenzidine (Sigma, USA). Chromogenic reaction was halted by rinsing the membrane twice with water (Bollag *et al*., 1996).

## Mouse injection and measurement of serum antibody titer

Ten male mice (20 g, 6-8 weeks old,) in two groups (5 mice each group) were separately immunized subcutaneously with 4 doses of recombinants lethal factor domain 1-protective antigen domain 4 (LFD1-PAD4), and lethal factor domain 1+protective antigen domain 4 (LFD1+PAD4) proteins at 2-week intervals and 5 male mice were considered as controls. In the first dose, 100 µL of the recombinant protein (containing 20 µg protein) was mixed with 30 µL PBS and then homogenized with 50 µL of the VAX-ORIENT IPA-70 Adjuvant (Pars Company). In the next boosters (doses 2, 3, and 4), 15, 10 and 10 µg recombinant protein was homogenized with ORIENT IPA-70 Adjuvant (Pars Company) and was injected (14 days gap between each stage). In association with each stage of injection, PBS was homogenized with ORIENT IPA-70 Adjuvant and injected into control mice (Bollag *et al*., 1996). Finally, the blood samples were taken from the mice and antibody titration was evaluated by enzyme-linked immunosorbent assay (ELISA). To perform ELISA, recombinant protein antigens of LFD1, PA4 and LFD1-PA4 were coated on a surface. Then, LF, PA and LF-PA antibodies were applied over the surface to bind to the antigens. These antibodies were linked to an HRP conjugated anti-mouse IgG. In the final step, a substance containing the O-phenilenediamine (OPD) was added. The subsequent reaction changes the color to yellow.

This method was performed according to the previous works by Ranjbar *et al*. (2004 and 2009) and Saadati *et al*. (2010) with modifications.

# Results

## Amplification of genes

The *pa4* gene was amplified with designed primer by PCR. The PCR products were analyzed in agarose gel and the related bands of *pa4* (524 bp) were observed (Fig. 1).

## Confirmation of gene cloning

To confirm the presence of the genes in cloned *E. coli*, sequencing (data not shown), PCR and restriction analysis were used. Figure 2 shows the related bands for *pa4*, *lfD1*, and *lfD1-pa4* in agarose gel.

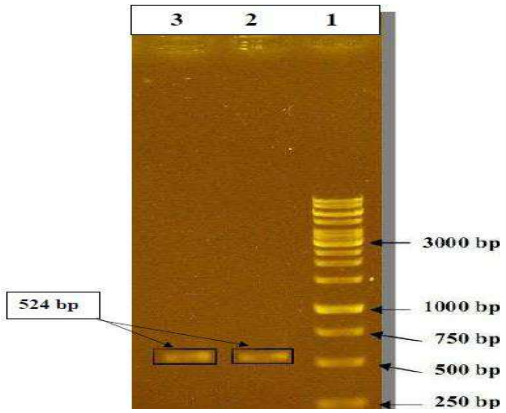


**Fig. 1:** The *pa4* gene was amplified in pX01 plasmid by PCR. Analysis of the PCR products was carried out by loading 3 µL of each samples onto a 1% agarose gel containing ethidium bromide, followed by electrophoresis and visualization via ultraviolet trans-illuminator. Lane 1: DNA ladder, and Lanes 2 and 3: PCR products of *pa4* (524 bp)

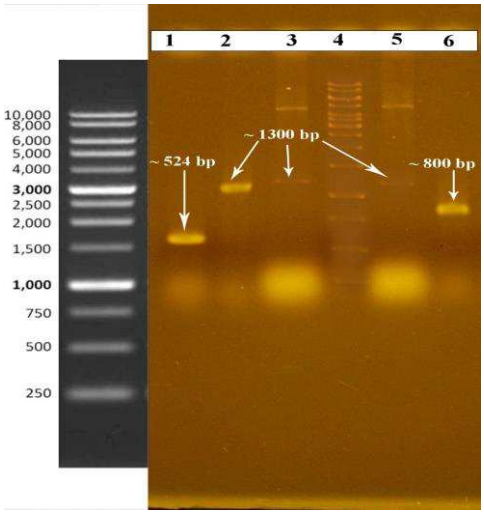


**Fig. 2:** The presence of the genes in isolated pET28a(+) vector were confirmed by PCR and restriction analysis. Electrophoresis pattern of the enzymatic digestion of pET28a(+) containing the *lfD1-pa4* gene by the *PstI* and *BamHI* enzymes and the PCR reaction on the 2% gel electrophoresis. Lane 1: The gene fragment of about 524 base pairs (*pa4*) of PCR reaction with forward and reverse primers designed for *pa4*, Lane 2: The product of the PCR reaction (*lfD1-pa4*) with reverse primer *pa4* and universal forward primer of pET (of about 1300 base pairs), Lanes 3 and 5: Enzyme digestion with the *PstI* and *BamHI* (1300 base pairs), Lane 4: DNA ladder (Fermentas, Ukraine), and Lane 6: The PCR product of the reaction with universal forward primer of pET and reverse primer for the *lfD1* gene (about 800 base pairs)

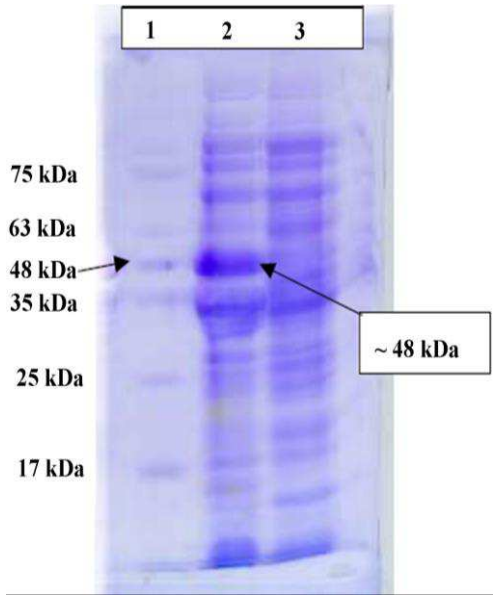


**Fig. 3:** SDS-PAGE electrophoresis of expressed recombinant protein LFD1-PA4. The expression host *E. coli* BL21 (DE3) plysS cells carrying pET28a-*lfd1-pa4* were induced with 1 mM IPTG at 37°C for 5 h, and the cells were collected and disrupted. Lane 1: Protein marker, Lane 2: Cell suspension of *E. coli*, transformed by pET28a-*lfd1-pa4*, induced with IPTG, and Lane 3: Cell suspension of *E. coli*, transformed by pET28a-*lfd1-pa4*; without IPTG

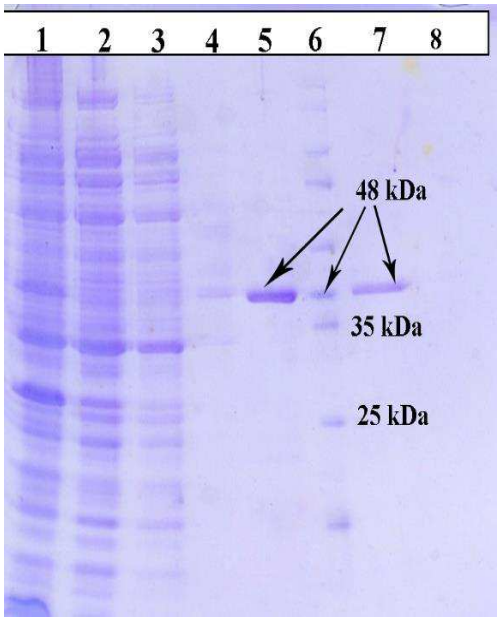


**Fig. 4:** SDS-PAGE gel electrophoresis of purified recombinant protein (LFD1-PA4) by Ni-NTA chromatography. Lane 1: Cell suspension of *E. coli*, transformed by pET28a-*lfd1-pa4*, Lane 2: The flow collected from the column, Lane 3: C buffer (pH=6.3), Lane 4: D buffer (pH=5.4), Lanes 5 and 7: E buffer (pH=4.5) containing recombinant protein LFD1-PA4, Lane 6: Protein marker, and Lane 8: MES buffer

## Expression and purification of the LFD1-PA4 protein

The recombinant strains were cultured in LB medium and protein expression induced by IPTG. After centrifugation and cell lysis, the proteins were analyzed by SDS-PAGE. As it can be shown in Fig. 3, the 48 kDa bands correspond to LFD1-PA4 recombinant proteins.

In small scale purification, the denatured protein was allowed to selectively bind to Ni-NTA agarose through His-tag. The protein was eluted (part 2-5), analyzed by SDS-PAGE and the relative bands corresponding to recombinant proteins were obtained (Fig. 4).

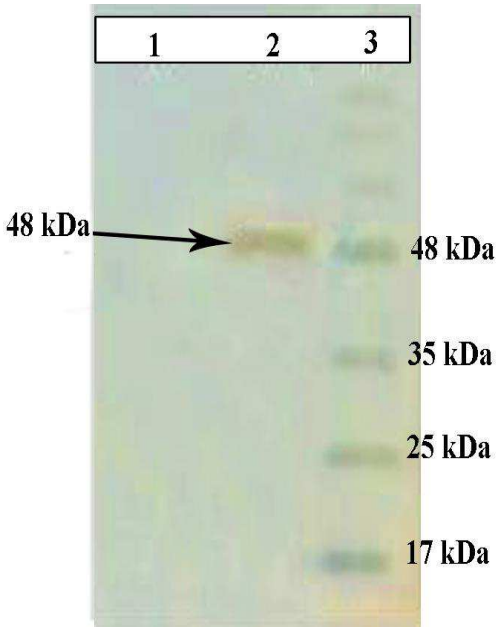


**Fig. 5:** Western blotting of purified recombinant protein PAD4. Lane 1: Collected suspension of flow, Lane 2: Purified recombinant protein LFD1-PA4 (& 48 kDa), and Lane 3: Protein marker

## Confirmation of expressed protein with western blotting

The eluted fractions containing expressed proteins were dialyzed overnight at 4°C against PBS and the protein concentrations were estimated by Bradford assay. The Western blotting analyses confirmed the recombinant proteins identity (Fig. 5).

## Serum antibody responses to the recombinant proteins

The antibody titers of collected serum from mice were measured with indirect ELISA. Blood samples were randomly taken from test and control mice, one week after the second, third, and fourth injection with LFD1-PA4 (fusion) and LFD1+PA4 (mixed) proteins. After serum isolation, indirect ELISA was performed. The antibody titration is shown in Figs. 6A-C and 7.

It should be noted that the obtained serum from LFD1-PA4 (fusion protein) was prepared through three times blood sampling and has been evaluated with LFD1, PA4 and LFD1-PA4 antigens and in case of LFD1+PA4 (mixed protein) serum, only the third one has been evaluated with LF, PA and LF-PA antigens.

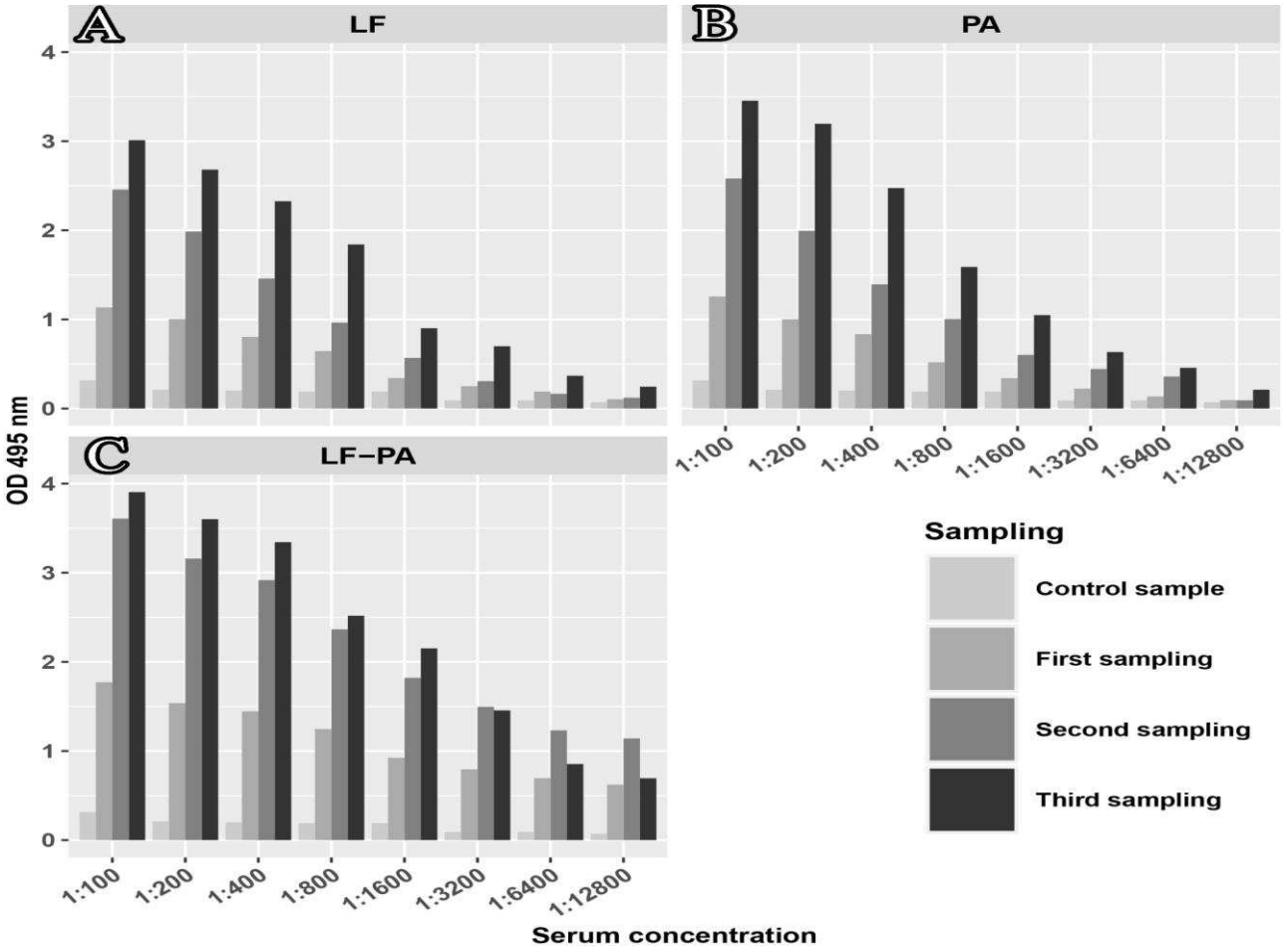


**Fig. 6:** Serum antibody responses in mice. Mice (5 mice per group) were immunized subcutaneously with 4 doses of recombinant LFD1-PA4 at 2-week intervals. Sera were collected 1 week after 2nd, 3rd and 4th injection and tested by ELISA for LF-specific (**A**), PA-specific (**B**), and LF-PA-specific (**C**) IgG Abs. The control group injected with PBS alone with alum adjuvant was used as the control. There is very significant difference between injection 3 and control group (P=0.009)

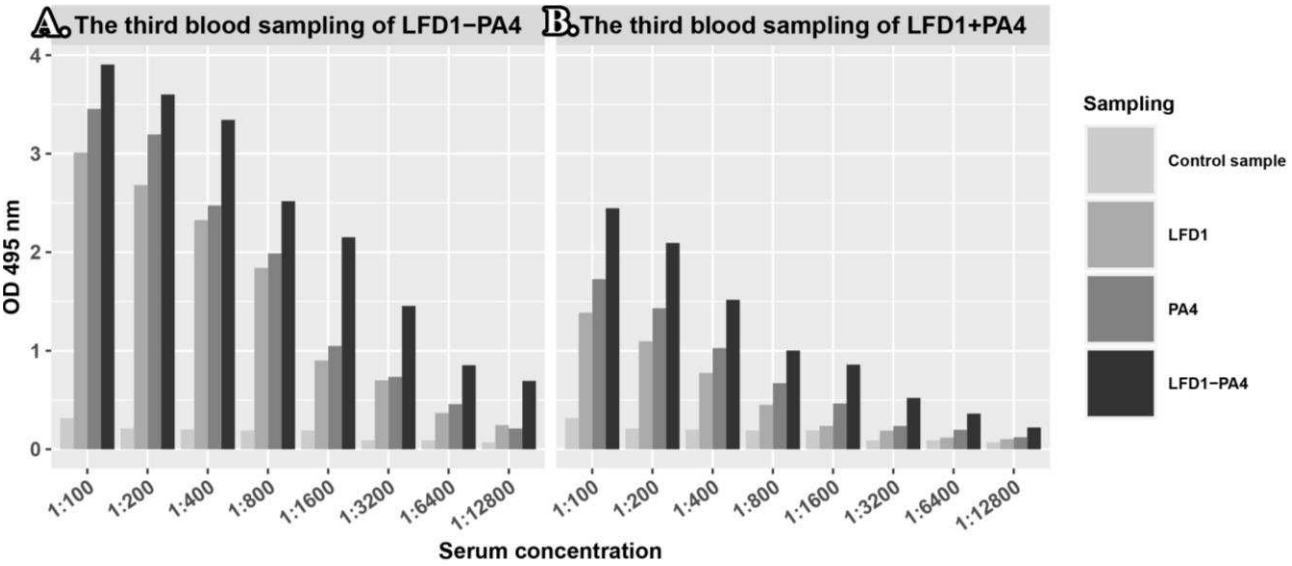


**Fig. 7:** Serum antibody responses in mice immunized subcutaneously with four doses of recombinant LFD1-PA4 and LFD1+PA4. **A**: LF-specific, PA-specific, LF-PA-specific, and control antibody responses in mice immunized with the fusion protein LFD1-PA4. **B**: LF-specific, PA-specific, LF-PA-specific, and control antibody responses in mice immunized with the fusion protein LFD1+PA4. This result denotes higher responses in mice immunized with LFD1-PA4 vs. other group (P<0.001)

## Discussion

The present vaccines against anthrax are based on PA such as anthrax vaccine adsorbed (AVA) in USA and anthrax vaccine precipitated (AVP) in Britain. The usage of the traditional AVA or the second-generation of recombinant protective antigen vaccine ((rPA)-based vaccine) is not ideal because multiple injections are required over a long period. Besides, neither AVA nor an rPA-based vaccine is suitable for the post-exposure vaccination in persons who have been freshly infected with *B. anthracis* (Aulinger *et al*., 2005; Bouzianas, 2010).

Recent studies on anthrax showed that LF is important and necessary for development of effective vaccines (Hepburn *et al*., 2007). Moreover, production of antibodies (toxin neutralization antibody-TNA) that cause immunity in animals are based on $CD4^+$ T cells plays a key role in production of neutralizing antibodies, class switching and maturity of lymphocytes.

In an experiment conducted on four volunteers vaccinated by AVP (75% PA and 25% LF proteins were used in this vaccine) results showed that T cells respond highly to LF protein than PA (18 folds). Therefore, LF has more antigenic potential (Baillie *et al*., 2004).

According to various studies carried out in this field, it has been determined that in order to obtain an effective and appropriate vaccine against anthrax, the use of LF and PA whether individually or in combination with other adjuvants is essential (Crowe *et al*., 2011; Madanchi *et al*., 2012). Hence, in this study PA4 and LFD1 was used among the other domains of anthrax toxin (Albrecht *et al*., 2007; Nguyen *et al*., 2009; Baillie *et al*., 2010).

In this research, *lfd1*, *pa4* genes and *lfd1-pa4* synthetic gene cassettes were expressed in *E. coli*-BL21 (DE3) PlysS cells and related proteins were purified by affinity chromatography. Nowadays, biotechnological methods are used for production of recombinant proteins and *E. coli* cells are utilized as a host for recombinant protein expression, both in research and industry. Also, in this research *E. coli* BL21 strain was used as host because it has no cytoplasmic proteases such as DegP, OmpT, Lon and HtpR, So PA4, LFD1 and LFD1-PA4 recombinant proteins can be effectively expressed (Hwang *et al*., 2007).

To evaluate the immunogenicity of recombinant proteins and production of polyclonal antibodies, mixed (LFD1+PA4) and chimeric (LFD1-PA4) proteins were injected into mice. Results obtained from this study showed that the antibody titer of chimeric proteins was more than mixed proteins, which indicates higher antigenicity potential of LFD1-PA4.

We expected the chimera LFD1-PA4, which lost the function of mediating anthrax intoxication, to be a better alternative to AVA or an rPA-based vaccine. On the other hand, an earlier study has shown that native LF (LFn) or catalytically inactive LF could augment the immunological response of PA-based vaccines against PA and enhance their immunoprotective efficiency, irrespective of whether immunization was carried out with DNA or protein (Price *et al*., 2001; Galloway *et al*., 2004; Liu *et al*., 2009b).

It is very likely that the individual moiety of LFD1-PA4 still possessed its immunogenicity and conferred an immune enhancement effect to PA, like in the isolated form. In other words, PA4 or LFD1, or both, might contribute to the stronger anti-PA antibody response elicited by LFD1-PA4 in comparison to that evoked by PA alone.

Additionally, we also noted that the antibody elicited by LFD1-PA4 cross-reacted with EF. This was probably due to the high similarity (55%) of the N-terminal amino acid sequences shared by EF and LF (Lacy *et al*., 2002). An earlier study has shown that the antibody elicited by LFn can neutralize the anthrax LeTx and protect the rabbit from the *B. anthracis* spore challenge (Galloway *et al*., 2004).

Moreover, it has been shown that certain anti-LF Abs that can abrogate LF-PA binding can also cross-react with EF by binding to the PA-binding domain of EF (EFn) (Little *et al*., 1990; Nguyen *et al*., 2009). All these findings indicate that Abs elicited by LFD1-PA4 might target both LF and EF and thus enhance the efficacy of LFD1-PA4 as a vaccine by blocking the binding of LF or EF to PA63.

Two antigens (LFD1-PA4) cooperate to raise antibody titers and hence raise the immunogenicity responses. Therefore, using such proteins as vaccines can decrease the dose of injection per vaccination. According to the results of this study and comparing them with the other studies in this field, these antigens can be considered as a new anthrax vaccine candidate formulation.

In conclusion, LFD1-PA4 induced a higher immune response than LFD1+PA4 mixed protein and rPA or AVA and elicited antibody responses to LF and EF, so it holds promise to be a more effective trivalent vaccine candidate than rPA or AVA for use in anthrax prevention.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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