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ORIGINAL INVESTIGATION

Development of a novel multiepitope chimeric vaccine against anthrax

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Abstract

Bacillus anthracis (BA), the etiological agent of anthrax, secretes protective antigen (PA), lethal factor (LF), and edema factor (EF) as major virulence mediators. Amongst these, PA-based vaccines are most effective for providing immunity against *BA*, but their low shelf life limits their usage. Previous studies showed that B-cell epitopes, ID II and ID III present in PA domain IV possess higher toxin neutralization activity and elicit higher antibody titer than ID I. Moreover, N-terminal region of both LF and EF harbors PA-binding sites which share 100% identity with each other. Here, in this study, we have developed an epitope-based chimeric vaccine (ID–LFn) comprising ID II–ID III region of PA and N-terminal region of LF. We have also evaluated its protective efficacy as well as stability and found it to be more stable than PA-based vaccine. Binding reactivities of ID–LFn with anti-PA/LF/EF antibodies were determined by ELISA. The stability of chimeric vaccine was assessed using circular dichroism spectroscopy. ID–LFn response was characterized by toxin neutralization, lymphocyte proliferation isotyping and cytokine profiling. The protective efficacy was analyzed by challenging ID–LFn-immunized mice with *B. anthracis* ($pXO1^+$ and $pXO2^+$). ID–LFn was found to be significantly stable as compared to PA. Anti-ID–LFn antibodies recognized PA, LF as well as EF. The T-cell response and the protective efficacy of ID–LFn were found to be almost similar to PA. ID–LFn exhibits equal protective efficacy in mice and possesses more stability as compared to PA along with the capability of recognizing PA, LF and EF at the same time. Thus, it can be considered as an improved vaccine against anthrax with better shelf life.

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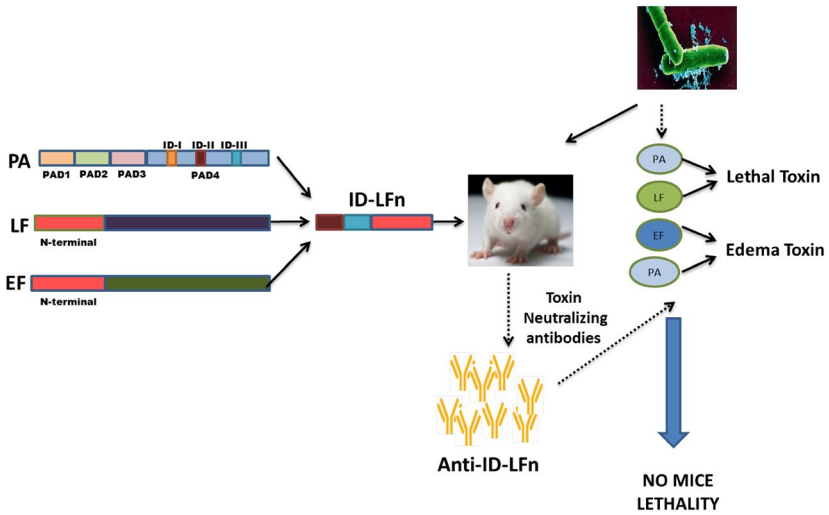
Graphical abstract



ID-LFn, a novel multiepitope chimeric anthrax vaccine: ID-LFn comprises of immunodominant epitopes of domain 4 of PA and N-terminal homologous stretch of LF and EF. The administration of this protein as a vaccine provides protection against anthrax.

Keywords *Bacillus anthracis* · Chimeric vaccine · Epitope · Protective antigen · Lethal factor

Abbreviations

BA	*Bacillus anthracis*
PA	Protective antigen
LF	Lethal factor
EF	Edema factor
ID	Immunodominant
LFn	N-terminal of LF

Introduction

Anthrax is a potential bioterror agent, classified as Category A organism by Centers for Disease Control and Prevention (CDC), USA [1]. Spores of *Bacillus anthracis*, a Gram-positive bacterium, are the main infectious agents for this disease. The spores can be easily transmitted or disseminated in a population leading to high mortality rates [2–4].

Current anthrax vaccines licensed in the United Kingdom and United States are Anthrax vaccine precipitated (AVP) and Anthrax vaccine adsorbed (AVA), respectively [5].These vaccines comprise protective antigen (PA), the most immunogenic protein of *B. anthracis* and a common component in both lethal toxin (LeTx) and edema toxin (ETx) [3]. Certain drawbacks such as undefined composition, batch to batch variation in PA, and lengthy course of immunization demand development of a next-generation vaccine conferring safer and rapid protection against this deadly pathogen [6].

Considering the immunogenic potential of PA, several PA-based subunit vaccines are also currently under development. But the main limitation associated with PA-based vaccine is its limited shelf life which makes stockpiling of this biodefense vaccine impossible reducing its suitability as biodefense vaccine [7, 8]. Failure in improving stability of recombinant PA needs an alternative with improved stability without compromising its protective efficacy.

Recently, epitope-based vaccines have been shown to provide several advantages over conventional full-length antigen vaccines including production ease, and affordability [6]. These vaccines are highly stable due to the presence of only protective epitopes reducing the chance of structural changes in the protein associated with storage of conventional vaccines that lead to altered immunogenicity and efficacy [9]. Considering these advantages, we have tried to engineer a better vaccine candidate against anthrax using protective epitopes.

According to the crystal structure of PA (PDB-1ACC), it has four major structural domains [10]; amongst these, PA domain 4 (PAD4; aa 596–735) plays a crucial role in mediating anthrax toxin binding to cellular receptor as well as pH-dependent pore formation in host cells [11]. Moreover, PAD4 has also been shown to protect mice against anthrax infection [12]. It is also known to possess immunodominant protective epitopes, namely ID I (aa 604–622), ID II (aa 626–676) and ID III (aa 707–723) which mediate

neutralization of anthrax lethal toxin and elicitation of cytokine as well as protective responses [9]. ID II has been shown to generate high antibody titer and also possesses high lethal toxin-neutralizing activity while ID III induces antibodies with high affinity as well as avidity [9].

Being a part of anthrax toxin, portions of EF and LF proteins also contribute towards generation of toxin-neutralizing antibodies, as shown in a study by Price et al., where DNA vaccination with a plasmid encoding the truncated form of LF has shown 100% protection against an anthrax lethal toxin challenge [13]. In addition, LF has also been found to enhance the magnitude of the PA-specific antibody response in mice [13]. The N-terminal domain of LF (LFD1) which facilitates binding of LF to PA prior to membrane translocation also possesses extensive sequence homology with N-terminal domain of EF [14, 15]. In another study, LFn has not been found to neutralize anthrax toxins [16]; however, it has been shown to recognize LF and EF [14].

Here, in this study, we have assessed the immunogenic as well as stability potential of a novel fusion chimeric protein comprising immunodominant epitopes (ID II and ID III) of PA and N-terminal domain of LF (LFn) towards development of better vaccine candidate against anthrax. Our studies suggest that the proposed chimeric vaccine, by virtue of its stability and protective efficacy, is a prospective next-generation vaccine.

Materials and methods

Bacterial strains, growth conditions and common chemicals

Escherichia coli DH5α and BL21 (λDE3) strains were procured from Novagen. LB media containing kanamycin (50 µg/ml) was used to grow transformed *E. coli* strains.

Cloning of *id–lfn* fusion construct

The fusion construct containing ID-II and ID III of PAD4 and LFn was amplified from genomic DNA of *B. anthracis* Sterne using overlapping PCR employing specific primers (Table 1). After 20 amplification cycles, the final fusion construct was amplified using primers P1 and P4 and amplicons from the above two reactions as a template. The resulting fusion construct was then cloned into pET-28a (+) vector and its sequence was confirmed by Sanger's dideoxy DNA sequencing.

Expression and purification of recombinant proteins

The recombinant pET-28a–*id–lfn* plasmid was expressed in *E. coli* BL21 (λDE3) strain by inducing it with 1 mM IPTG at OD_{600}~0.6 at 37 °C with shaking at 160 rpm. The recombinant protein was then purified from inclusion bodies as described previously [17]. Briefly, the induced culture was repeatedly sonicated in buffer A [20 Mm Tris (pH 8), 200 mM NaCl] followed by washing with buffer W1 [buffer A containing 2M urea, 0.1% Triton X-100 and 2 mM EDTA]. The suspension was kept at 4 °C for 5–10 min followed by centrifugation at high speed under refrigerated condition. The pellet was again washed with buffer W2 [buffer A containing 0.1% Triton X-100 and 2 mM EDTA] and held for 5–10 min followed by centrifugation. The pellet was resuspended in 20 mM Tris (pH 8.0), incubated for 5–10 min and again centrifuged. The resulting pellet was then resuspended in 8M urea in buffer A and held for 1 h at RT for solubilization. The purified protein was then obtained by performing Ni–NTA chromatography followed by dialysis in PBS with 10% glycerol. The purity was assessed by SDS-PAGE. The purified fraction was then stored at − 80 °C until further use. The purification of PA was done as described previously [9].

Immunization of mice

Each experimental group comprised 12 6–8-week-old female BALB/c mice. Each group was administered 25 µg of ID–LFn and PA intraperitoneally, resuspended in PBS and emulsified in complete Freund's adjuvant (CFA) for prime dose at day 0. Subsequent boosters emulsified in incomplete Freund's adjuvant (IFA) were administered at day 15 and day 30. The group immunized with PBS with CFA or IFA served as negative control. Blood samples from mice of each group were collected at days 0, 14, 28, 35 and 42 and stored at − 20 °C until further analysis. All the animal experiments were performed under standard laboratory condition

Table 1 Primers used in the study

S. no.	Primer name	Primer sequence (restriction sites are marked as bold underlined sequences)
1	P1	5′GGCC**GGATCC**GGATTATTGTTAAATATTGATAAGGATATAAGAAAAATATTATC3′
2	P2	5′TTTTCTTGATCCCGTTGGTACTAGTATCCCCATTCTCACTAGGATTAATAACTATAATGTTTTTCC ATCTTGCCGTA3′
3	P3	5′AGTGAGAATGGGGATACTAGTACCAACGGGATCAAGAAAAGCGGGCGGTCATGGTGATGTAGG3′
4	P4	5′GGC**GTCGAC**TTTAAGTTCTTCCAAGGATAGATTTATTTCTTGTTCG3′

at animal facility of Jawaharlal Nehru University and were approved by Institutional Animal Ethical Committee of the university.

Reactivity of ID–LFn fusion protein antisera with PA and LF and vice versa

Direct binding assay

The reactivity of ID–LFn antisera with PA, LF and EF was analyzed by direct binding assay as described previously [9]. Briefly, 500 ng/well of PA, LF and EF or ID–LFn were coated in the ELISA plates overnight at 4 °C followed by subsequent blocking with 2% BSA and washed with PBS. The plates were then incubated with respective antisera for 2h at RT, washed with PBS and incubated with HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology Inc.) for 1 h at RT. The plate was washed and OD_{630} was recorded. Binding of different proteins with their respective antisera were taken as reference binding.

Immunoblotting

10 µg of PA, LF and EF was resolved on 12% SDS-PAGE followed by electroblotting on nitrocellulose membrane. The membrane was blocked with 2% BSA. After washes, it was incubated with ID–LFn antisera. For determining the reactivity of ID–LFn with PA/LF/EF antisera, purified ID–LFn was electroblotted and probed with polyclonal anti-PA, anti-LF and anti-EF antisera generated in mice. The binding was analyzed using AP-conjugated goat anti-mouse IgG and NBT–BCIP substrate.

End-point antibody titer and IgG isotyping

As a measure of humoral immunity, total antigen-specific IgG antibodies and their isotypes (IgG1/IgG2a) were determined using ELISA (enzyme-linked immunosorbent assay). The absorbance of control mice immune sera at 1:1000 dilutions plus 2.5 times its standard deviation was taken as the cut-off [18]. Anti-mouse IgG1/IgG2a HRP-conjugated antibodies (Santa Cruz Biotechnology) were used at 1:10,000 dilution of the supplied initial concentration of 100µ g/ml.

In vitro splenocyte stimulation and cytokine estimation

Twenty-five days post-last immunization, two mice from each group were euthanized and their spleens were removed aseptically and splenocytes were isolated in RPMI complete medium. 5×10^5 cells were seeded per well in a 24-well plate and allowed to stimulate with or without 6 µg/ml of PA, ID–LF_n or 5 µg/ml of Concanavalin A at 37 °C, 5% CO_2 and 95% humidity. Media supernatant were then collected at 24 h, 48 h and 72 h. Quantitative analysis of IL-4, and IFN-γ from media supernatant was done using BD Opt EIA™ kits as per the manufacturer's protocol. Concentrations of different cytokines were determined by employing linear regression equations calculated from absorbance of standards as provided by the manufacturer.

Lymphocyte proliferation

For lymphocyte proliferation, two mice from each group were killed after 25 days of last immunization. Isolation of splenocytes and in vitro stimulation were done as described above. The cells with or without protein were incubated for 24 h at 37 °C and 5% CO_2. The proliferative effect was calculated by MTT incubating the stimulated as well as unstimulated cells with 0.5 mg/ml MTT. DMSO was used to dissolve the formazan crystal formed as a reaction of MTT with mitochondrial succinate dehydrogenase of live cells. The absorbance was recorded at 540 nm.

In vitro toxin neutralization assay

Lethal toxin-neutralizing potential of antibodies was assessed on murine macrophage RAW 264.7 cell line. 5×10^4 cells were seeded in each well of 96-well plate and kept at 37 °C and 5% CO_2 until they reached confluency. For LeTx activity, 1000 ng/ml of PA and 500 ng/ml of LF were used. Different dilutions of polyclonal sera from differently immunized groups were incubated with PA for an hour. This was followed by incubation with LF for another hour. The mixture was then added to the cells and kept for 4–6 h. The percent survival was analyzed by incubating cells with 100 µg MTT per well for 1 h at 37 °C. Resulting formazan crystals were then solubilized by DMSO. Absorbance was then taken at 540 nm to determine cell viability.

Stability analysis by circular dichroism spectroscopy

Thermal scan of PA and ID–LFn (0.2 mg/ml) was carried out in 10 mM phosphate buffer (pH 7.4) at a temperature range from 25 to 80 °C. The molar ellipticity was measured in a 1-mm cell at 220 nm using J-710 spectro-polarimeter (JascoCorp.), as described previously [19, 20].

pH-dependent stability analysis

Effect of pH on stability of proteins was determined by incubating proteins for 4 h at RT in varying buffer compositions such as sodium acetate buffer (10 mM; pH 3.0–5.0), sodium phosphate buffer (10 mM; pH 6.0–7.0) and Tris–HCl buffer (10 mM; pH 8.0–10.0). The result was analyzed by SDS-PAGE.

Protective efficacy

Challenge experiments were executed after 25 days post-last immunization. To assess the protective efficacy, BALB/c mice (6–8 weeks old; female) from each group were challenged 25 days post-last immunization with 10^4 spores of *B. anthracis* (pXO1$^+$ and pXO2$^+$) intraperitoneally. The mice were analyzed for 12 days for any morbidity or mortality.

Statistical analysis

All data are expressed as mean ± standard deviation (SD) of three different sets of experiments. Two-tailed Student's *t* test was done for different groups and p value < 0.05 was considered to be statistically significant.

Results

Cloning, expression and purification of recombinant ID–LFn protein

The fusion construct of *id–lfn* was amplified using overlapping PCR (Fig. 1a, b). The desired amplicon was cloned into pET-28a expression vector to get His-tagged fusion protein (Fig. 1c) and is expressed in *E. coli* BL21 (DE3) expression cells. The purification of ID–LFn was done using Ni–NTA chromatography from inclusion bodies as described in "Materials and methods". The purified protein migrated at expected size of 40 kDa on SDS-PAGE (Fig. 1d).

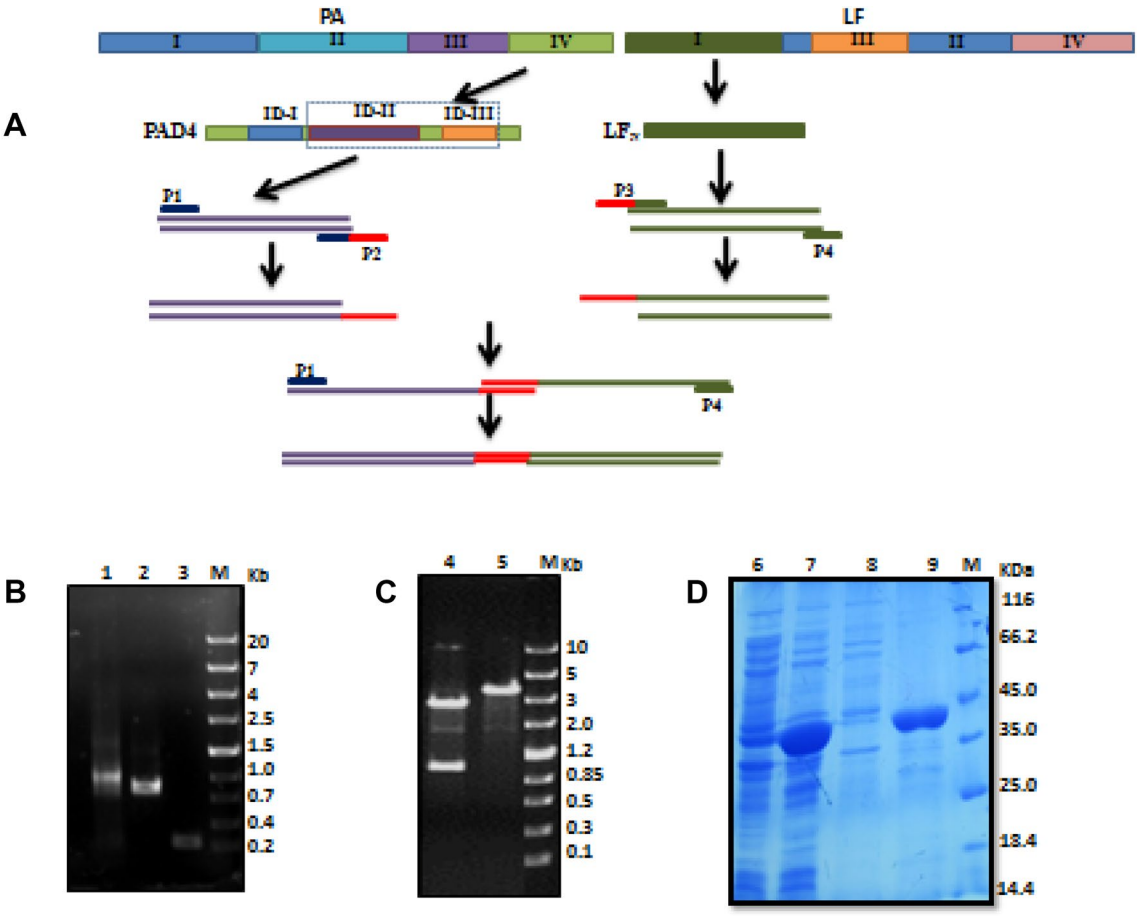


Fig. 1 Cloning, expression and purification of ID–LFn. **a** Schematic representation of the strategy used for cloning chimeric gene *id–lfn*; **b** PCR amplicons. Lane 1, *id–lfn* (983 bp); 2, *lfn* (780 bp); 3, *idII–idIII* (203 bp); M, DNA ladder; **c** clone confirmation by restriction digestion. Lane 4, fall out corresponding to *id–lfn;* 5, undigested pET-28a-*id–lfn;* M, DNA ladder; **d** expression analysis of rID–LFn by SDS-PAGE after induction with 1 mM IPTG. Lane 6, uninduced; 7, induced; 8, cytosolic fraction; 9, inclusion bodies; M, protein ladder.U

Reactivity of anti-ID–LFn with PA and LF and vice versa

Antibody pools generated after immunization of mice with ID–LFn, PA, LF and EF were analyzed for their cross reactivities with the individual proteins.

ID–LFn antiserum recognized PA, LF and EF

The reactivity of ID–LFn antiserum with PA, LF and EF was analyzed by direct binding assay. The data showed ~ 60%, 70% and 50% binding with PA, LF and EF, respectively (Fig. 2). This result indicates that polyclonal serum raised against ID–LFn has antibodies against PA, LF as well as EF.

To further confirm our analyses, the binding was also analyzed by immunoblotting and it was found that ID–LFn antiserum could recognize PA, LF as well as EF (Fig. 2b). FlotP, the raft marker protein of *B. anthracis* was taken as negative control [19].

PA, LF and EF antisera recognized ID–LFn

The recognition of ID–LFn by PA, LF and EF antisera was also demonstrated by direct binding assay employing ELISA. We found ~ 88% binding with anti-PA antibody (Fig. 3a–i), whereas ~ 63% and 58% binding was observed with anti-LF and anti-EF antibodies, respectively (Fig. 3a-ii, iii). This suggests that the antiserum generated against full-length PA and LF contain the significant proportion of antibodies against IDII, IDIII and LFn epitopes, respectively. Immunoblotting of ID–LFn with anti-PA/LF/EF antibody further confirmed our analysis (Fig. 3b).

ID–LFn generated mixed Th1–Th2 response

To compare the antigen-specific humoral response generated by ID–LFn and other proteins, determination of IgG response was done using indirect IgG ELISA. For this, mice were intraperitoneally immunized with ID–LFn and PA. A high antibody titre approaching ~ 3×10^5 was observed after immunization with ID–LFn whereas in consistence with the previous studies, PA showed titer of ~ 3.5×10^5, as observed 15 days after the second booster (Fig. 4).

As reported previously, the immune response against *BA* primarily depends on the differential IgG subclass response [21, 22]. Thus, to investigate the specific humoral response, IgG isotyping was done. Mixed IgG1 and IgG2a response was observed in ID–LFn suggesting mixed immune response. PA also showed relatively similar IgG1/IgG2a response indicating mixed Th1–Th2 response as reported previously (Fig. 4) [13].

ID–LFn elicited secretion of mixed Th1–Th2 cytokines

To further confirm whether the mixed IgG1 and IgG2a antibody subtype response as observed in ID–LFn is due to induced Th1 and Th2 heterogeneity, the splenocytes from immunized mice of different groups were isolated after 25 days post-immunization for cytokine profiling. ID–LFn was found to elicit mixed Th1–Th2 response as depicted by IFN-γ levels (240 ± 26.4 pg/ml) and IL-4 level (92 ± 18.9 pg/ml) and PA-immunized mice showed IFN-γ and IL-4 to be 181.7 ± 22.3 pg/ml and 98.7 ± 19.6 pg/ml, respectively, indicating mixed immune response (Figs. 5).

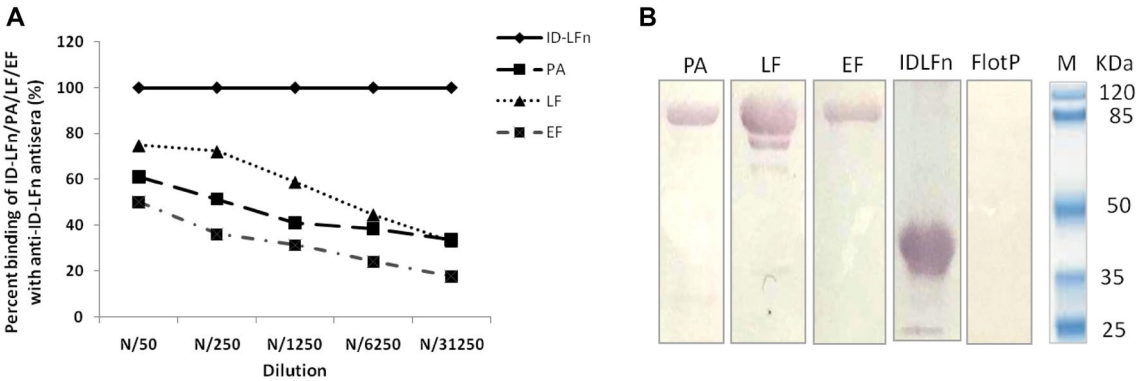


Fig. 2 Direct binding assay of anti-ID–LFn antisera with ID–LFn/PA/EF/LF. **a** ID–LFn/PA/LF/EF were coated on ELISA plate and incubated with increasing dilution of anti-ID–LFn. Goat anti-mouse IgG–HRP at 1:10,000 dilution was added after washing the plate with PBST. Data are plotted with respect to binding of anti-ID–LFn with ID–LFn and are considered as reference binding for the analysis; **b** Immunoblotting of PA/EF/LF/ID–LFn/FlotP with anti-ID–LFn antisera. Lanes: (1) PA; (2) EF; (3) LF; (4) ID–LFn; (5) FlotP, where FlotP, the *B. anthracis* raft marker protein (M.wt.: 59 kDa), was taken as negative control

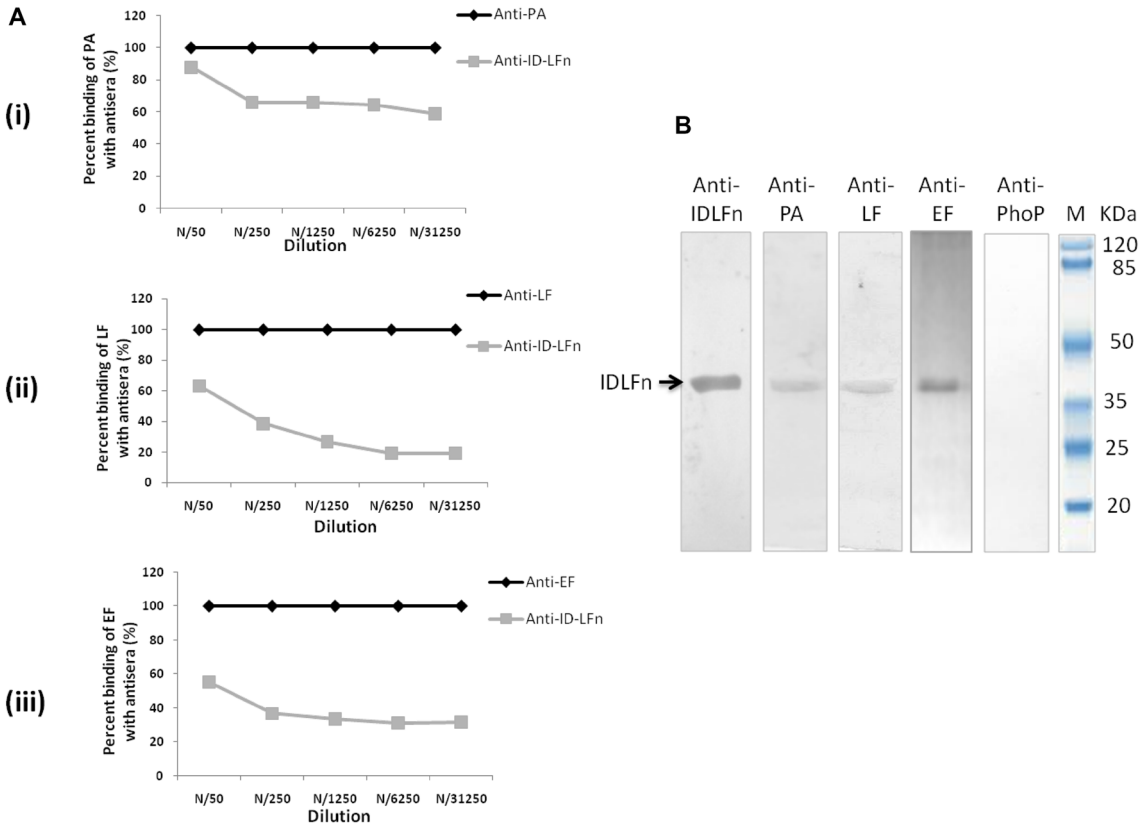


Fig. 3 Direct binding assay of ID–LFn with anti-PA/LF/EF antisera. **a** ID–LFn,PA, LF and EF were coated on the ELISA plate and incubated with increasing dilution of anti-PA/LF/EF in respective wells. Colorimetric analysis was done by adding goat anti-mouse IgG–HRP at 1:10,000 dilution after washing the plate with PBST. Data are plotted with respect to binding of anti-PA/LF/EF with their respective proteins PA/LF/EF and are considered as reference binding for the analysis: **i** with anti-PA, **ii** with anti-LF and **iii** with anti-EF; **b** immunoblotting of ID–LFn with anti-ID–LFn/PA/LF/EF antisera. Anti-PhoP was taken as negative control

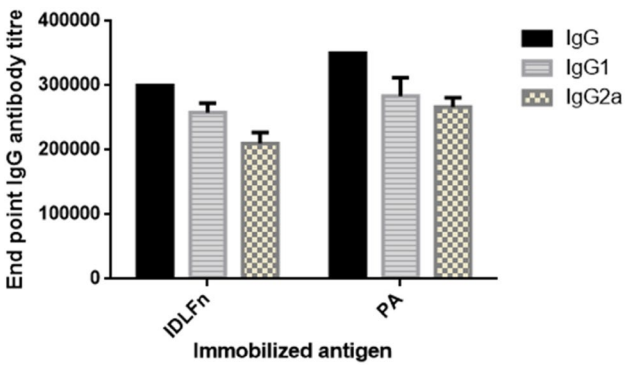


Fig. 4 Determination of IgG response in immunized mice. End point antibody titer was calculated through ELISA by serially diluting the sera obtained from mice after 15 days of last booster. Total antibody titre was calculated by taking goat anti-mouse IgG–HRP at the dilution of 1:10000 whereas for isotypic analysis, goat anti-mouse IgG1–HRP and goat anti-mouse IgG2a–HRP at 1:10,000 dilution were used. Data are expressed as mean ± SD of three independent experiments

Lymphocyte proliferation

Cellular response generated by the antigen can be determined by estimating lymphocyte proliferation which is an acute response of lymphocytes to antigenic stimulation by increasing inflammatory cell or pathogen-specific responder cells. The difference in the proliferation index of ID–LFn (3.4) was insignificant as that of PA (3.5) with $p > 0.05$ (Fig. 6).

Anti-ID–LFn sera neutralize lethal toxin

Antibodies against PA or LF have the capability to neutralize lethal toxin, in vitro. In our study, to determine the LeTx neutralizing potential of antibodies generated against ID–LFn, the standard in vitro cytotoxicity assay was performed using RAW 264.7 macrophage cell line. Anti-ID–LFn antibodies was found to exhibit ~ 80% protection while anti-PA antibodies showed ~ 96% protection from LeTx-mediated cell killing when diluted 100 times ($p = 0.03$). Serum from PBS-immunized mice showed no protection from LeTx-associated cell death (Fig. 7).

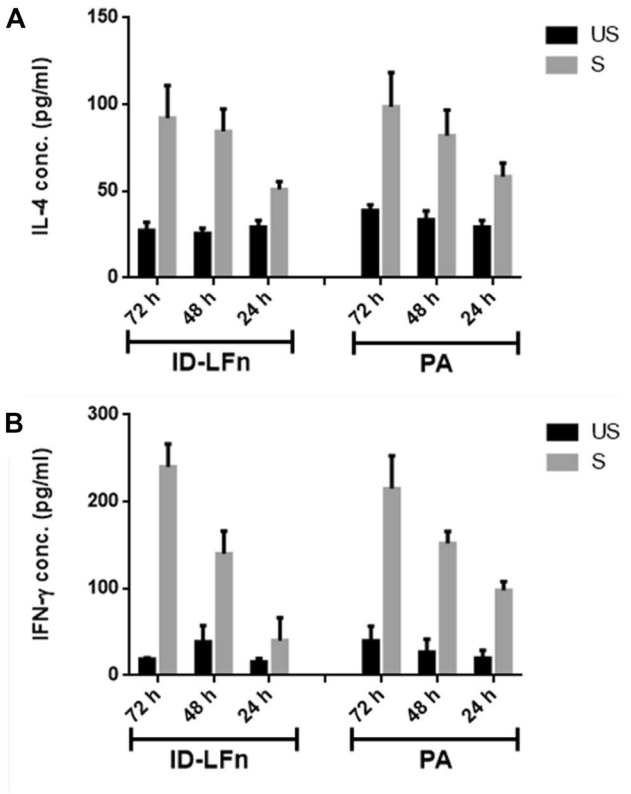


Fig. 5 Cytokine profile secreted from splenocytes. Results are expressed as mean ± SD of cytokine concentration (pg/ml) in triplicate. **a** IL-4; **b** IFN-γ. *US* unstimulated (without protein), *S* stimulated (with protein)

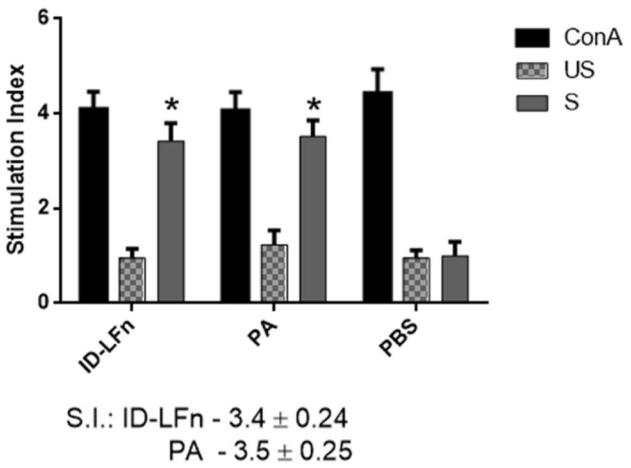


Fig. 6 Determination of lymphocyte proliferation from splenocytes of immunized mice: 25 days post-last immunization, splenocytes from immunized mice were isolated aseptically and cultured in the absence (unstimulated) or presence (stimulated with 6 µg protein). Stimulation by Concanavalin A was taken as positive control. The analysis was done using MTT dye by taking absorbance at 540 nm. *ConA* Concanavalin A, *US* unstimulated, *S* stimulated

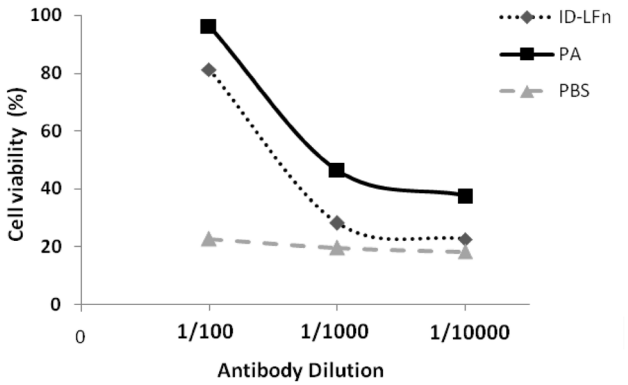


Fig. 7 Toxin neutralization assay. RAW264.7 mouse macrophage cell line was incubated with anthrax lethal toxin (PA + LF) together with pooled anti-ID–LFn or anti-PA or anti-PBS. The cytotoxicity was analyzed by MTT assay. Data are the representative of three independent experiments

ID–LFn is more stable than PA

Our study revealed that ID–LFn is significantly more stable than PA. Stability of the studied proteins was analyzed at different temperature and pH values. Thermal stability was determined by measuring ellipticity (θ_{220}) at different temperatures by circular dichromism (CD) spectroscopy. It was observed that θ_{220} of PA increases with the rise in temperature from 25 to 80 °C whereas ID–LFn did not show any variation in θ_{220nm} on subsequent temperature change (Fig. 8a).

According to WHO guidelines for stability evaluation of vaccines, pH is another important aspect to be considered [23]. To have more insight into such critical feature, stability of ID–LFn was also compared with PA at different pH values ranging from 3 to 12. PA showed stability only at 7–8 whereas it got degraded at lower pH (3–5) and higher (9–12) pH range (Fig. 8b). However, ID–LFn showed no degradation throughout the entire pH range. Thus, ID–LFn is a better vaccine candidate with better stability at different temperature and varied pH range.

ID–LFn showed protective efficacy comparable to PA

Immunized mice were challenged with 10^4 spores of virulent strain of *BA* 2 weeks post-immunization. Survival analysis revealed ID–LFn to be equally protective as PA with approximately 80% whereas mice administered with PBS died in 5–6 days (Table 2). All the survived mice were healthy without any apparent symptom of illness.

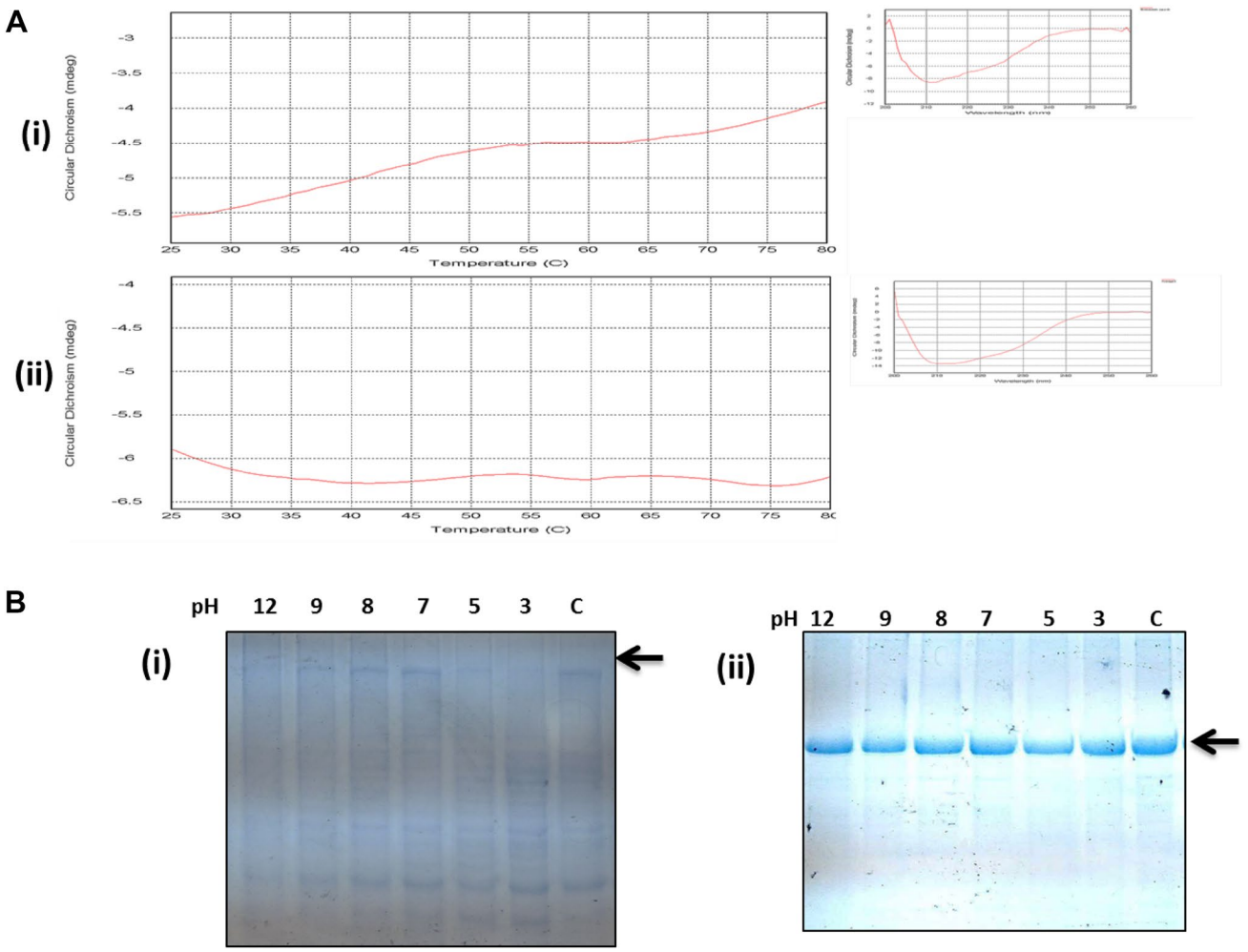


Fig. 8 Stability analysis of ID–LFn w.r.t. PA. **a** Change in circular dichroism (mdeg) was calculated by analyzing CD spectra of rID–LFn and PA at 220 nm under different temperatures, **i** PA; **ii** ID–LFn. CD spectra of PA and rID–LFn is shown in inset. **b** Effect of pH on ID–LFn and PA was analyzed by incubating proteins at different pH for 2 h and then analysis was done by SDS-PAGE. **i** PA; **ii** ID–LFn

Table 2 Protection conferred by ID-LFn and PA against *B. anthracis* spore challenge

Number of animals per group	Number of mice survived/total number of mice		
	ID-LFn	PA	PBS
10	8/10	8/10	0/10

The data is from one the representative experiment of three different independent experiments

Discussion

Although several approaches have been taken by different research groups to make a better anthrax vaccine [24, 25], lack of effective treatment strategy against anthrax and limited shelf life of existing PA-based vaccines demand efforts towards development of an effective vaccine against anthrax [6]. Over recent years, epitope-based subunit vaccines proved to be promising prophylactic agents in comparison to traditional vaccines as they are more specific as immunogen and have more flexibility to form chimera (due to their small size). Further, epitope-based subunit vaccines possessed improved stability, resilient immunity, cost-effectiveness along with fewer side effects [9].

Previous studies have demonstrated ID II and ID III as the most immunodominant epitopes of PA as they have the potential to generate LeTx neutralizing antibodies [9]. Also, the N-terminal region of LF and EF share PA-binding residues [15]. On this basis, we have developed a recombinant chimeric protein comprising immunodominant epitopes (ID II and ID III) of PA and LFn. This chimeric vaccine has been investigated for various attributes including stability upon pH and temperature variation and protective efficacy against virulent anthrax challenge in mice model. A special

emphasis on the stability factor has been given because the existing PA-based vaccine of anthrax cannot sustain temperature fluctuation which is encountered during transportation and storage for longer.

In accordance with our hypothesis, antisera generated against ID–LFn were able to recognize PA, LF and EF, as observed by both immunoblotting and ELISA. The result emphasizes that anti-ID–LFn polyclonal sera comprise a pool of antibodies against specific epitopes of PA as well as LFn. Similarly, anti-PA, anti-LF and anti-EF were found to recognize rID–LFn by ELISA as well as immunoblotting indicating the presence of antigenic determinants specific to PA, LF as well as EF.

Our analysis demonstrated that this chimeric vaccine is immunogenic, producing IgG titers indicating a significant humoral response. Previously, Th2 response have appeared to be important for providing protection against anthrax [26]. In our studies, end-point titer analysis for IgG1 and IgG2a levels indicated generation of TH2 type immune response. This is important because stimulation of an appropriate antibody isotype may be a prerequisite for obtaining protection against anthrax though the titer was found to be lower than PA which could be explained by the fact that full-length PA is more immunogenic than its domains.

Although to combat anthrax, humoral response is a prerequisite, but cytokines and costimulatory signals mediating cellular immunity may also be important for obtaining complete protection [27]. The cytokines produced by in vitro restimulation of splenocytes with the respective proteins were assessed. It was found that ID–LFn elicited mixed Th1–Th2 cytokines. The concentration of cytokines secreted by ID–LFn stimulated cells was even higher than PA itself. IFN-γ performs microbicidal function by inducing antibodies for opsonization followed by phagocytosis. Similarly, high levels of IL-4, principal cytokine of Th2 cells, were also secreted which enhances humoral immune response by suppressing T-cell-mediated immunity.

Toxemia along with bacteremia is the prime cause of lethality in anthrax infection [28]. Thus, toxin neutralization is one of the major aspects to be taken into consideration during the development of post-exposure prophylactics against anthrax [29]. We found that anti-ID–LFn sera exhibit significant toxin neutralization potential but lower than PA which might be accredited to complex polyclonal antibody pool generated in response to PA. While, quantity of toxin-neutralizing antibodies raised in response to ID–LFn is lower as compared to PA, but challenge studies demonstrated equal protective efficacy.

Considering the immunogenic potential of PA to combat *BA* infection, several research groups are trying to develop PA-based chimeric vaccines [30–32]. But the foremost challenge in their path is its unstability which prompted the search for novel antigen or molecules that could confer better stability to PA without losing its protection potential. In this study, stability analysis of ID–LFn revealed that the molecule is quite stable over a broad range of temperature as well as pH and provides comparable protective efficacy as PA in mice model.

In summary, our results show that immunization with chimeric protein (ID–LFn) provides protection against anthrax together with better shelf life compared to existing PA-based vaccination. Thus, we are proposing ID–LFn as a better prospective vaccine candidate in comparison to all other designed subunit vaccines against BA where ID–LFn was found to exhibit equal protective efficacy and more stability as compared to recombinant PA with the capability of neutralizing PA, LF and EF at the same time. Thus, this can be a reliable next-generation vaccine against anthrax with better shelf life.

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References

1. Lamps LW, Havens JM, Sjostedt A, Page DL, Scott MA (2004) Histologic and molecular diagnosis of tularemia: a potential bioterrorism agent endemic to North America. Mod Pathol 17(5):489
2. Riedel S (2005) Anthrax: a continuing concern in the era of bioterrorism. Proc Baylor Univ Med Center. 18(3):234
3. Grundmann O (2014) The current state of bioterrorist attack surveillance and preparedness in the US. Risk Manage Healthcare Pol 7:177
4. Cogliati S, Costa J, Ayala F, Donato V, Grau R (2016) Bacterial spores and its relatives as agents of mass destruction. J Bioterror Biodef 7:141
5. Cybulski RJ Jr, Sanz P, O'Brien AD (2009) Anthrax vaccination strategies. Mol Asp Med 30(6):490–502
6. Kaur M, Bhatnagar R (2011) Recent progress in the development of anthrax vaccines. Recent Patents Biotechnol 5(3):148–159
7. Lu S, Wang S (2009) Technical transformation of biodefense vaccines. Vaccine 27:D8–D15
8. Merkel TJ, Perera P-Y, Lee GM, Verma A, Hiroi T, Yokote H et al (2013) Protective-antigen (PA) based anthrax vaccines confer protection against inhalation anthrax by precluding the establishment of a systemic infection. Hum Vaccines Immunother 9(9):1841–1848
9. Kaur M, Chug H, Singh H, Chandra S, Mishra M, Sharma M et al (2009) Identification and characterization of immunodominant B-cell epitope of the C-terminus of protective antigen of *Bacillus anthracis*. Mol Immunol 46(10):2107–2115
10. Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC (1997) Crystal structure of the anthrax toxin protective antigen. Nature 385(6619):833
11. Williams AS, Lovell S, Anbanandam A, El-Chami R, Bann JG (2009) Domain 4 of the anthrax protective antigen maintains structure and binding to the host receptor CMG2 at low pH. Protein Sci 18(11):2277–2286
12. Flick-Smith HC, Walker NJ, Gibson P, Bullifent H, Hayward S, Miller J et al (2002) A recombinant carboxy-terminal domain of

the protective antigen of *Bacillus anthracis* protects mice against anthrax infection. Infect Immun 70(3):1653–1656
13. Price BM, Liner AL, Park S, Leppla SH, Mateczun A, Galloway DR (2001) Protection against anthrax lethal toxin challenge by genetic immunization with a plasmid encoding the lethal factor protein. Infect Immun 69(7):4509–4515
14. Kulshreshtha P, Bhatnagar R (2011) Inhibition of anthrax toxins with a bispecific monoclonal antibody that cross reacts with edema factor as well as lethal factor of *Bacillus anthracis*. Mol Immunol 48(15–16):1958–1965
15. Lacy DB, Mourez M, Fouassier A, Collier RJ (2002) Mapping the anthrax protective antigen binding site on the lethal and edema factors. J Biol Chem 277(4):3006–3010
16. Nguyen ML, Terzyan S, Ballard JD, James JA, Farris AD (2009) The major neutralizing antibody responses to recombinant anthrax lethal and edema factors are directed to non-cross-reactive epitopes. Infect Immun 77(11):4714–4723
17. Robinson A, Farrar GH, Wiblin CN (2003) Vaccine protocols. Springer, New York
18. Singh D, Somani VK, Aggarwal S, Bhatnagar R (2015) PLGA (85: 15) nanoparticle based delivery of rL7/L12 ribosomal protein in mice protects against Brucella abortus 544 infection: a promising alternate to traditional adjuvants. Mol Immunol 68(2):272–279
19. Somani VK, Aggarwal S, Singh D, Prasad T, Bhatnagar R (2016) Identification of novel raft marker protein, FlotP in *Bacillus anthracis*. Front Microbiol 7:169
20. Aggarwal S, Somani VK, Gupta V, Kaur J, Singh D, Grover A et al. Functional characterization of PhoPR two component system and its implication in regulating phosphate homeostasis in *Bacillus anthracis*. Biochimica et Biophysica Acta (BBA)-general subjects. 2017;1861(1):2956–2970
21. Quinn CP, Sabourin CL, Niemuth NA, Li H, Semenova VA, Rudge TL et al (2012) A three-dose intramuscular injection schedule of anthrax vaccine adsorbed generates sustained humoral and cellular immune responses to protective antigen and provides long-term protection against inhalation anthrax in rhesus macaques. Clin Vaccine Immunol 19(11):1730–1745
22. Manish M, Rahi A, Kaur M, Bhatnagar R, Singh S (2013) A single-dose PLGA encapsulated protective antigen domain 4 nanoformulation protects mice against *Bacillus anthracis* spore challenge. PLoS One 8(4):e61885
23. Organizaion WH (2009) Guidelines on stability evaluation of vaccines. Biologicals 37(6):424–434
24. Friedlander AM, Little SF (2009) Advances in the development of next-generation anthrax vaccines. Vaccine 27:D28–D32
25. Kaur M, Singh S, Bhatnagar R (2013) Anthrax vaccines: present status and future prospects. Expert Rev vaccines 12(8):955–970
26. Little SF, Leppla SH, Cora E (1988) Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. Infect Immun 56(7):1807–1813
27. Sinha K, Bhatnagar R (2010) GroEL provides protection against *Bacillus anthracis* infection in BALB/c mice. Mol Immunol 48(1–3):264–271
28. Aggarwal S, Somani VK, Bhatnagar R (2015) Phosphate starvation enhances the pathogenesis of *Bacillus anthracis*. Int J Med Microbiol 305(6):523–531
29. Pitt M, Little S, Ivins B, Fellows P, Boles J, Barth J et al (1999) In vitro correlate of immunity in an animal model of inhalational anthrax. J Appl Microbiol 87(2):304-
30. Baillie LW, Huwar TB, Moore S, Mellado-Sanchez G, Rodriguez L, Neeson BN et al (2010) An anthrax subunit vaccine candidate based on protective regions of *Bacillus anthracis* protective antigen and lethal factor. Vaccine 28(41):6740–6748
31. Suryanarayana N, Verma M, Thavachelvam K, Saxena N, Mankere B, Tuteja U et al (2016) Generation of a novel chimeric PALFn antigen of *Bacillus anthracis* and its immunological characterization in mouse model. Appl Microbiol Biotechnol 100(19):8439–8451
32. Varshney A, Puranik N, Kumar M, Goel A (2016) Immunogenecity of a chimeric protein of *Bacillus anthracis* protective antigen and lethal factor in murine model. Int J Infect Dis 45:426