



A recombinant chimeric protein of protective antigen and lethal factor from *Bacillus anthracis* in polymeric nanocapsules showed a strong immune response in mice: a potential high efficacy vaccine against anthrax

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ABSTRACT

Anthrax is a serious infectious disease caused by *Bacillus anthracis*, rod-shaped gram-positive bacteria. The disease infects both humans and animals and causes severe illness. Many vaccines have been developed for anthrax, but the vaccine with very high efficacy is yet to be developed. To overcome the problems of efficacy posed by the existing vaccines, a recombinant chimeric fusion protein containing domain 1 of lethal factor (LFD1) and domain 4 of *Bacillus anthracis* protective antigen (PA4) was used as antigen in copolymeric nanocapsules (NCs). Accordingly, the solvent evaporation double emulsion method was used to produce NCs containing recombinant chimeric fusion protein (LFD1-PA4). Zeta sizer and potential of nanoparticles, nanoparticle loading efficiency, release pattern of recombinant protein, and the possible effect of polylactic acid-polyethylene glycol (PLA-PEG) nanoparticle production method were investigated. Mice were used to test and evaluate the immune response. The mean titer of antibody produced against loaded LFD1-PA4 compared to free form showed a significant difference. The difference in antibody titer between the groups of once injected, twice injected, and free antigen was significant, and the highest antibody titer was found in the mice twice injected. In addition, a single-time loaded injection showed significantly higher antibodies than the free form injection indicating that loaded LFD1-PA4 into PLA-PEG nanoparticles elicits a stronger immune response. This study showed that LFD1-PA4 fusion protein from *Bacillus anthracis* served as an active antigen in mice. Also, the nanocarrier (PLA-PEG) containing the antigen can stimulate the immune system in the mice, owing to their controlled release property.

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Introduction

Anthrax is a serious infectious disease resulting in severe illness in humans and animals. The disease is caused by a rod-shaped gram-positive bacteria known as *Bacillus anthracis*. In the past several years, anthrax has been an economically devastating disease in domestic animals, it is currently controlled by developing appropriate health services such as the Texas Department of State Health Services (DSHS) and the Texas Animal Health Commission (TAHC). Moreover, anthrax poses a threat as it could be potentially used as a bioweapon (1). If diagnosed early anthrax can be treated with antibiotics, but the symptoms do not always appear in time for antibiotic treatment to be effective. So, vaccination is, therefore, a necessary measure to counter the disease (2).

To design an effective vaccine, several antigens from *Bacillus anthracis* have been studied for their ability to induce protective immunity against the disease (3). Some well-known antigens are the capsule, the S-layer, surface polysaccharides, and other proteins. However, only the proteins that together make the anthrax toxin produce detectable antibodies (2).

Anthrax toxin is a binary A-B toxin comprised of protective antigen (PA) and two enzymatic moieties, edema factor (EF) and lethal factor (LF). The antigen is adsorbed onto aluminum hydroxide in a vaccine licensed under the Anthrax Vaccine Adsorbed (AVA) (4). Current human vaccines are low in efficacy, expensive to produce, require repeated doses, and may cause transient side effects in some people (5). There is also evidence that complete protection against all strains of this bacterium may not be provided. The main

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problem with the existing vaccines is that a long-term vaccination schedule (six vaccinations in eighteen months) is required and lacks a broad spectrum of immunity (6).

The reason for the low immunogenicity of the vaccine is PA instability and low resistance to free PA in harsh and unfavorable conditions (7). Due to the mentioned reasons, different types of delivery solutions such as polymeric micro and nanospheres, biodegradable implants, injectable hydrogels, and PEG carriers have been widely presented and considered (6). One of the advantages of these drug delivery systems is that they maintain appropriate drug concentration in the blood for a long time, avoiding the requirement of additional doses of the drug (8).

The use of polymers as vaccine carriers will protect against acidic conditions and gastrointestinal proteases and result in an effective immune response with the slow and long-term release of the antigen (9). For this purpose, various biodegradable and biocompatible polymers have been used that are produced naturally or synthetically.

Despite the benefits of vaccines containing live organisms, there are some drawbacks; For example, many of the components of these organisms are not needed to stimulate the immune system response, but the whole organism is transmitted to the vaccinated person (10). These additional components can reduce the effectiveness of vaccination by decreasing the level of protection created against antigens. In some cases, can even lead to damage to the vaccinated person. In some rare cases, the weakened organism becomes pathogenic and causes disease in the person (2, 11).

To overcome the problems above, nowadays there is an attempt to develop vaccines containing one or more components of a specific organism (3). Those vaccines are called subunit vaccines and have significant advantages over traditional vaccines that include improved immunity (if the suitable antigen is selected), specificity, compatibility, accessible storage and maintenance (12). However, that leads to increased demand for determining the appropriate formulation to produce an immune response (13).

Recent studies have shown that lethal factors are essential for effective vaccination (14, 15). The titer of antibody produced (induced) against this antigen is high due to the presence of epitopes in the structure of the lethal factor (LF) (16). Also, it has been shown that

monoclonal antibodies produced against LF have the most interaction with lethal factor domain1 (LFD1), which indicates the importance of this region (15). LFD1 epitopes are also almost twice as involved in antibody production as any other region (17). To address the shortcomings of the existing vaccines including the improved effectiveness, a fusion construct of two essential domains LFD1 and PA4 was made. After purification was used as an antigen in combination with PLA-PEG copolymeric nanocapsules (NCs). A more robust immune response was observed in the laboratory animal models (mice) compared to the antigen's free f.

Materials and methods

Cloning of LFD1-PA4 fusion protein in pGEM T-easy vector

The complementary gene fragment contains the LFD1 (amino acid sequence 1-257 from the amino terminus of the lethal factor) and PA4 (510bp from the 1786-2296 base sequence carboxylic terminus of the protective antigen) were transferred to *E. coli*, and it was expressed in a bacterial host.

The PA4 primers were designed by Primer3 software with *HindIII* and *XbaI* restriction sites which were synthesized by Thermo Fisher Scientific Co. (USA). The sequences of PA4 forward primer was 5'ATCTAGAGCGGAATTAACGCAACTAAC3' and the sequences of PA4 reverse primer were 5'GTTCGAATTATCCTATCTCATAGCCTTTTT3'.

The PA4 PCR products were cloned in the pGEM T-easy vector (Fermentas, Ukraine). For this purpose, by 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* DH5 α competent cells were prepared by standard cold CaCl₂ protocol (18).

Purification of the recombinant LFD1-PA4 fusion protein

For protein purification, 250 ml of fresh LB medium was induced by 1mM IPTG and kept at 37°C for 3 hours. The cells were then centrifuged at 9000 rpm, and 10 ml of lubricating buffer was poured onto the precipitate and pipetted. The above buffer contains 50 mM sodium phosphate and 300 mM sodium chloride and is called LEW (lysis equilibration wash) for short.

The resulting suspension was frozen three times in a liquid nitrogen tank and melted in water at 37°C, and then sonicated nine times for 80 seconds, each time for 20 seconds. After every 20 seconds, the suspension was kept in ice water for 1 minute, then centrifuged at 1000 rpm for 20 minutes, and the supernatant was removed from the residue. Then 4 ml of LEW buffer containing 8 M urea was added to the residue, and sonication was performed as in the above steps. Finally, the supernatant from the final centrifuge step containing the recombinant LFD1-PA4 protein was collected for purification with a nickel column.

The recombinant LFD1-PA4 protein was purified using a Ni-TED 2000 packed protino® column (MACHERY-NAGEL). Thus, the prepared samples obtained from the extraction were added to the column. The column was then washed with a 12 ml wash buffer and 2 ml Eluting buffer (LEW + Im) containing 250 mM imidazole to remove the protein. Then the chimeric antigen was loaded into the PEG-PLA double-block copolymer nanospheres.

Production and characterization of LFD1-PA4 loaded PLA-PEG NCs

The dual emulsion method of solvent evaporation was used to produce NCs (19). The produced nanoparticles were measured by the Dynamic Light Scattering (DLS) technique to evaluate the size of Zeta Potential. The PLA-PEG nanoparticles containing 3mg of protein in 500µl of PBS solution were poured into a 2ml microtube and dispersed by stirring to investigate the release pattern of recombinant chimeric LFD1-PA4 protein from NCs.

Immunization in mice models and antibody titer measurement

The recombinant LFD1-PA4 protein was injected subcutaneously on days 1, 14, 28, and 42, respectively, on four experimental and control mice with five replications (female *Mus musculus* weighing 20-25 g and four weeks old). Also, the recombinant LFD1-PA4 protein encapsulated in PLA-PEG nanoparticles was administered as one injection (day 1) and two injections (days 1 and 28) subcutaneously (Table 1). Non-protein nanoparticles and PBS were used instead of antigens for control animals.

Table 1. Groups, time, and amount of injected protein in the immunization process

Injected Protein	1 st Injection (20µg)	2 nd Injection (15µg)	3 rd Injection (10µg)	4 th Injection (10µg)	Injection method
	1 st day	14 th day	28 th day	42 nd day	
Free LFD 1-PA4 dimer protein	Done	Done	Done	Done	Subcutaneously
Chimeric LFD1-PA4 protein loaded on nanoparticles	Done	Not Done	Done	Not Done	
Non-protein nanoparticles and PBS	Done	Done	Done	Done	

Blood samples were taken three times from the corner of each mouse's eye one week after the second injection (day 21), one week after the third injection (day 35), and one week after the fourth injection (day 49). The indirect ELISA method was used to determine the antibody titer in rat serum, as follows:

Antigen protein at a concentration of 5 µg / ml in 100 µl PBS buffer was added to ELISA plate cavities (Nunc Maxsorb, Denmark). The plate coated with antigen protein was kept overnight at 4°C. Then it was washed three times with washing buffer (PBS buffer containing 0.5% Tween-20). PBS buffer containing 1% bovine serum albumin (BSA) was used for blocking. A volume of 200µl of this buffer was added to each well and then kept at laboratory temperature for one hour. The plate was washed three times again with the washing buffer, and serum samples were diluted 1 to 200, 1 to 400, and 1 to 800 in 100µl of each well, and the plate was kept at 25°C for 2 hours.

After washing the plate, antibodies of the mouse conjugated with peroxidase 1: 1000 were added to all wells and stored for 2 hours. After washing the plate, OPD-specific substrate was added to the wells. 100µl of 0.5 M sulfuric acid was added as a reaction stop in each well. Optical Density (OD) at 495nm was recorded using the ELISA Reader Stat Fax-2100. Antibody concentration was measured from the standard curve (21).

Statistical analysis

ANOVA and T-tests were used to analyze data obtained from antibody titers produced by indirect ELISA in serum. This test was performed to compare the mean antibody titers of the samples (Duncan test to examine the differences between groups) at a probability level of 5% using SPSS software version 24.

Results and discussion

The LFD1-PA4 protein was stable inside the PLA-PEG NCs

The antigen-loaded PLA-PEG nano-capsules prepared as described in the Methods section were tested for the effect of the PLA-PEG NCs on protein stability. Subjected to NCs the stability of recombinant LFD1-PA4 protein was studied. Finally, the cumulative percentage curve of protein released from nanoparticles was plotted at specified times. The LFD1-PA4 protein release pattern from PLA-PEG NCs showed slowed antigen release over time.

Protein release from PEG-PLA nanoparticles was studied for 49 days (the time of the last stage of blood sampling). Based on the cumulative percentage of protein release, as shown in Figure 1, about 79% of the release of PEG-PLA nanoparticles was done after this time.

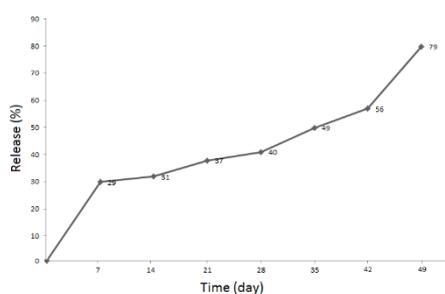


Figure 1. Cumulative release percentage of recombinant chimer protein from PEG-PLA nanoparticles during 49 days

After sample preparation, PEG-PLA nanoparticles produced in the presence of LFD1-PA4 chimeric protein and the absence of protein were examined by DLS. In terms of zeta potential and scattering index (PDI), the above nanoparticles were in the appropriate range (Table 2).

Table 2. Physicochemical properties of nanoparticles using dynamic light scattering

Sample	Zeta Potential (mV)	Size (nm)	PDI
Control	-32.2	14.01	0.341
Nanoparticles contain LFD1-PA4 chimeric protein	-24.3	94.1	0.279

In the present study, the chimeric LFD1-PA4 antigen nanoparticles loaded in PEG-PLA with an average size of 94.1 nm, a zeta potential of -24.3 mV, and PDI = 0.279 were prepared by changing the effective parameters in the nanoparticle fabrication process.

Figure 2 shows the size and zeta potential characteristics of PEG-PLA nanoparticles containing LFD1-PA4 chimeric protein and free of protein under optimal conditions by DLS.

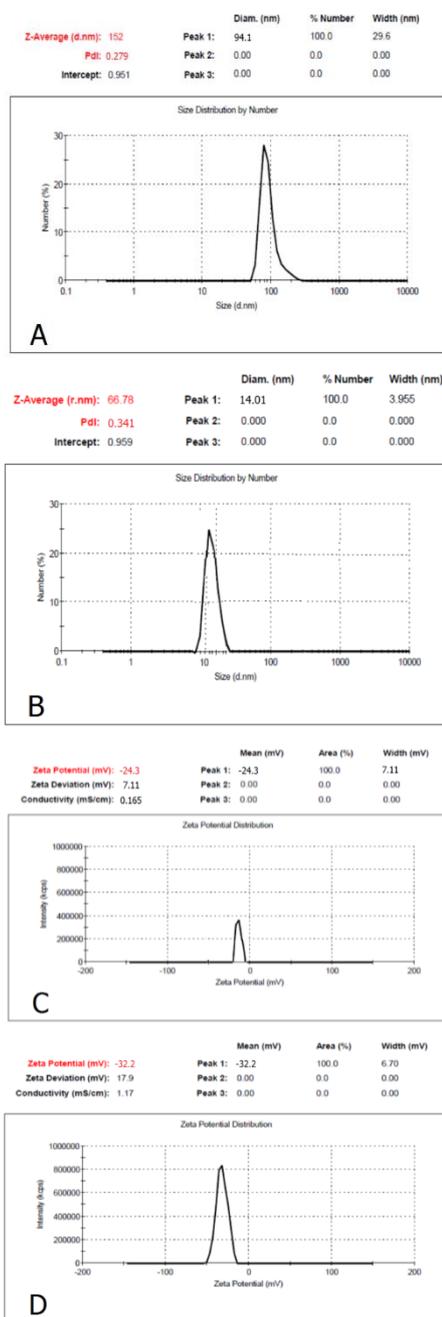


Figure 2. Graph of PEG-PLA nanoparticle size and zeta potential distribution range containing LFD1-PA4 chimeric protein (A and C) and no protein (B and D)

The mouse model showed a strong immune response to LFD1-PA4 loaded PLA-PEG NCs

The results showed that the highest antibody concentration in all administrations was related to the third blood sampling taken from mice one week after

the last injection. Also, in the final blood sample, the highest antibody concentration was measured against nanoparticles loaded with LFD1-PA4 chimeric antigen. To evaluate the antibody titer produced by injection of LFD1-PA4 protein in three forms, free (four injections), loaded once the injection, and loaded twice injection, and calculate its amount in each injection step, the method Indirect ELISA was used against PA4, LFD1-PA4, and LFD1 antigens. The antibody concentration was measured based on the optical absorption OD of the third injection (dilution 1/200) and the standard curve and repeated three times, measured. The results were compared in Figure 3.

The results of the Duncan test show the difference between the groups including free antigen, loaded once the injection, loaded twice injection, and control groups including PBS and PLA - PEG at the five percent error level. The comparison of means is shown in Figure 4.

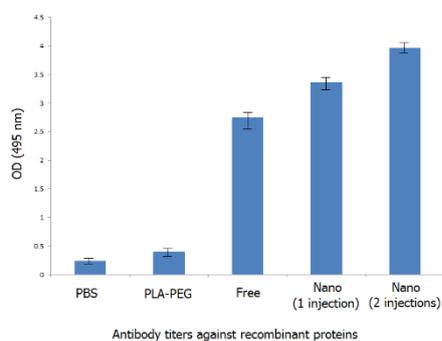


Figure 3. Antibody concentration chart produced after the third injection (1/200 dilution) of LFD1-PA4 chimeric protein in three forms: free, loaded once the injection and loaded twice injection against PA4, LFD1-PA4, and LFD1 proteins

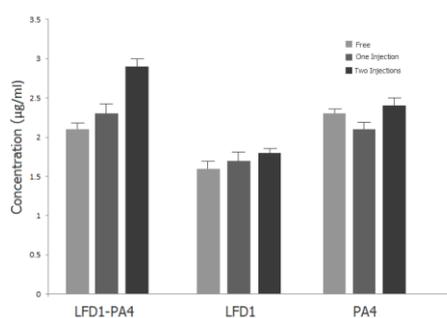


Figure 4. Mean comparison of antibody titers produced by injection of LFD1-PA4 chimeric protein into three forms: free, loaded once injection and loaded twice injection against LF antigen

Many vaccines have been developed for anthrax, but the ideal vaccine has not yet been developed (22). Studies have shown that to achieve an effective and

appropriate vaccine, it is necessary to use protective antigen (PA) and lethal factor (LF) in combination with each other and adjuvants (23). The nucleotide sequence of different strains for *Bacillus anthracis* shows that all strains are identical in LEF and PAG genes (genes responsible for producing lethal factors and protective antigen) and amino acid sequence (24). Researchers are looking to develop recombinant protective antigen (rPA) vaccines for anthrax. The protective antigen is one of the most important proteins of the three anthrax toxins, consisting of 735 amino acids with four domains (3). The protective antigen binds to its receptor at the host cell's surface via domain 4, and this domain contains most of the protective PA epitopes. Domain 4 includes 139 amino acids from the carboxyl terminus of the protective antigen (amino acid sequence from 596 to 735) (25).

In studies performed to make recombinant protective antigen or chimeric protein, different base sequences of the carboxyl terminus of the protective antigen have been selected. Baillie *et al.* (26) studied 552 bp and Makam *et al.* (27) studied 411bp at the carboxyl terminus of the protective antigen.

The triple nature of anthrax toxin raises the expectation that other parts of the toxin, such as lethal factor and PA, will also be involved in producing toxin-neutralizing antibodies (28). Various studies have shown that co-administration of lethal factors and PA increases the number of antibodies against PA in mice. This adjuvant effect of a lethal factor is related to its amino terminus, LFD1 (28, 29). Researchers have used this region to transmit various antigens and have been shown to elicit immune responses to CD4+ and CD8+ T cells (29).

After reviewing similar studies in this case and considering that the domain-1 lethal factor at the amino terminus of the protein and PA4 are naturally located at the carboxylic terminus of the protective antigen, finally, the chimeric protein LFD1-PA4 was selected.

Baillie *et al.* (26) designed the chimeric protein from the domain of a lethal factor and the domain-4 protective antigen with *Bacillus anthracis*. They placed the domain 4 protective antigen at the carboxylic end of the chimeric protein. In the design and fabrication of the chimeric protein from the protein fragments of the domain of a lethal factor and the 63 kDa protective antigen (PA63), the domain of a lethal factor was placed at the amino terminus (30). Makam *et al.* (27)

placed domain 4 at the amino terminus to make chimeric protein from four protective antigens and a protein fragment from the carboxyl terminus of the extractable antigen. Shcherbinin *et al.* (31) placed IgG2a 4 at the carboxylic terminus of the chimeric protein to produce chimeric protein from domain 4 of the protective antigen and the FC portion of the antibody. However, placement at the amino terminus of chimeric protein elicited a similar antibody response. In another similar study, Varshney *et al.* (32) placed domain one at the amino terminus to make the chimeric protein from 2-4 domains of protective antigen and domain 1 killer factor.

The results of IgG antibody evaluation by indirect ELISA showed that in all injection groups, the amount of antibody increased at each stage of blood sampling, and there was a statistically significant difference between the injected and control groups. Increasing the antibody titer in the groups that received nanoparticles containing chimeric antigen indicates that the nanoparticles could act as adjuvants and did not produce more antibodies with the proper and long-term release of the antigen and exposure to the immune system. The mean antibody concentration made against loaded chimeric proteins shows a significant difference compared to free antigens ($P=0.043$). Also, the difference in antibody titer between the groups of once injected and twice injected, and free antigen was significant at the probability level of 5%. The highest antibody titer was related to twice-injected. Also, a significant difference was observed between the loaded once injection and free injection. It indicates that more antibodies can be produced by loading the chimeric antigen into PLA-PEG nanoparticles and injecting once (instead of four injections). Since nanoparticles containing chimeric antigen have produced an acceptable amount of antibody by gradual release and supply to the immune system, ELISA results showed that in the group of nanoparticles containing chimeric antigen, a higher antibody level was produced than free antigen with adjuvant.

Conclusions

Traditional low-load antigen delivery systems destroy part of the antigen (33). Low stability results in the rapid release of antigen, and transferring the required amount of antigen to the animal's body requires repeated injections. Therefore, nanoparticles

are an excellent option to replace conventional systems due to less degradation and more protection of antigen activity in nanoparticles than traditional methods and less injection repetition, reduction of side effects, lower cost, and slow-release (34). On the other hand, antibodies to inactivate immunity can be a perfect way to deal temporarily and quickly with disease cases (1). Also, the development of nanoparticles containing recombinant chimeric protein for people who are more prone to anthrax can significantly reduce the fatal effects of the disease by providing effective immunity (6). This study showed that the second four protective antigens and lethal factors integrate and produce active antigens. This chimeric antigen is dynamic and can stimulate the immune system of the laboratory animal. Also, nanocarriers containing antigens produced by the controlled release can stimulate the immune system of the laboratory animal.

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Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

References

1. Bower WA, Schiffer J, Atmar RL et al. Use of anthrax vaccine in the United States: recommendations of the Advisory Committee on Immunization Practices, 2019. *MMWR Recomm Rep* 2019; 68(4): 1.
2. Tsai M-h, Chuang C-c, Chen C-c et al. Nanoparticles assembled from fucoidan and trimethylchitosan as anthrax vaccine adjuvant: In vitro and in vivo efficacy in comparison to CpG. *Carbohydr Polym* 2020; 236: 116041.
3. Chuang C-C, Tsai M-H, Yen H-j et al. A fucoidan-quaternary chitosan nanoparticle adjuvant for anthrax vaccine as an alternative to CpG oligodeoxynucleotides. *Carbohydr Polym* 2020; 229: 115403.
4. Skoura N, Wang-Jairaj J, Della Pasqua O et al. Effect of raxibacumab on immunogenicity of Anthrax Vaccine Adsorbed: a phase 4, open-label, parallel-group, randomised non-inferiority study. *Lancet Infect Dis* 2020; 20(8): 983-991.
5. Modi T, Gervais D, Smith S et al. Characterization of the UK anthrax vaccine and human immunogenicity. *Hum Vaccines Immunother* 2021; 17(3): 747-758.

6. Smiley MA, Sanford DC, Triplett CA et al. Comparative immunogenicity and efficacy of thermostable (lyophilized) and liquid formulation of anthrax vaccine candidate AV7909. *Vaccine* 2019; 37(43): 6356-6361.
7. Schneider JC, Chen HC, Bautista E, Retallack D. Safety and immunogenicity of Px563L, a recombinant anthrax vaccine candidate, in a two-dose regimen for post-exposure prophylaxis in healthy adults. *Vaccine* 2021; 39(42): 6333-6339.
8. Dogan E, Merhan O, Erdag D, Karamanci E, Bozukluhan K, Dogan Anc. The Effect of Vitamin C on Oxidant and Antioxidant Parameters in Anthrax Vaccine Administered Cattle. *Van Vet J* 2021; 32(3): 109-113.
9. Nevagi RJ, Skwarczynski M, Toth I. Polymers for subunit vaccine delivery. *Eur Polym J* 2019; 114: 397-410.
10. Han J, Zhao D, Li D, Wang X, Jin Z, Zhao K. Polymer-based nanomaterials and applications for vaccines and drugs. *Polymers* 2018; 10(1): 31.
11. Aziziarum Z. C3953T genetic variation in interleukin 1 β and idiopathic male infertility: a systematic review and meta-analysis. *Cent Asian J Med Pharm Sci Innov* 2021; 1(6): 242-249.
12. Malik A, Gupta M, Mani R, Gogoi H, Bhatnagar R. Trimethyl chitosan nanoparticles encapsulated protective antigen protects the mice against anthrax. *Front Immunol* 2018; 9: 562.
13. Legler PM, Little SF, Senft J et al. Treatment of experimental anthrax with pegylated circularly permuted capsule depolymerase. *Sci Transl Med* 2021; 13(623): eabh1682.
14. Hardenbrook NJ, Liu S, Zhou K, Ghosal K, Hong Zhou Z, Krantz BA. Atomic structures of anthrax toxin protective antigen channels bound to partially unfolded lethal and edema factors. *Nat Commun* 2020; 11(1): 1-10.
15. Zhou K, Liu S, Hardenbrook NJ, Cui Y, Krantz BA, Zhou ZH. Atomic structures of anthrax prechannel bound with full-length lethal and edema factors. *Structure* 2020; 28(8): 879-887. e873.
16. Gallegos-Candela M, Boyer AE, Woolfitt AR et al. Validated MALDI-TOF-MS method for anthrax lethal factor provides early diagnosis and evaluation of therapeutics. *Anal Biochem* 2018; 543: 97-107.
17. Machen AJ, Fisher MT, Freudenthal BD. Anthrax toxin translocation complex reveals insight into the lethal factor unfolding and refolding mechanism. *Sci Rep* 2021; 11(1): 1-10.
18. Liu J, Chang W, Pan L et al. An improved method of preparing high efficiency transformation *Escherichia coli* with both plasmids and larger DNA fragments. *Indian J Microbiol* 2018; 58(4): 448-456.
19. Jain AK, Goyal AK, Mishra N, Vaidya B, Mangal S, Vyas SP. PEG-PLA-PEG block copolymeric nanoparticles for oral immunization against hepatitis B. *Int J Pharm* 2010; 387(1-2): 253-262.
20. Singh NA, Mandal AKA, Khan ZA. Fabrication of PLA-PEG nanoparticles as delivery systems for improved stability and controlled release of catechin. *J Nanomater* 2017; 2017.
21. Isho B, Abe KT, Zuo M et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. *Sci Immunol* 2020; 5(52): eabe5511.
22. Tao P, Mahalingam M, Zhu J et al. A bacteriophage T4 nanoparticle-based dual vaccine against anthrax and plague. *M Bio* 2018; 9(5): e01926-01918.
23. Kondakova OA, Nikitin NA, Evtushenko EA, Ryabchevskaya EM, Atabekov JG, Karpova OV. Vaccines against anthrax based on recombinant protective antigen: problems and solutions. *Expert Rev Vaccines* 2019; 18(8): 813-828.
24. Bylaiah S, Shedole S, Suresh KP, Gowda L, Patil SS, Indrabalan UB. Analysis of Codon Usage Bias in Cya, Lef, and Pag Genes Exists in px01 Plasmid of *Bacillus Anthracis*. *ICT Analysis and Applications*: Springer; 2022: 1-9.
25. Béguin EP, van den Eshof BL, Hoogendijk AJ et al. Integrated proteomic analysis of tumor necrosis factor α and interleukin 1 β -induced endothelial inflammation. *J Proteomics* 2019; 192: 89-101.
26. Baillie LW, Huwar TB, Moore S et al. An anthrax subunit vaccine candidate based on protective regions of *Bacillus anthracis* protective antigen and lethal factor. *Vaccine* 2010; 28(41): 6740-6748.
27. Makam SS, Kingston JJ, Harischandra MS, Batra HV. Protective antigen and extractable antigen I based chimeric protein confers protection against *Bacillus anthracis* in mouse model. *Mol Immunol* 2014; 59(1): 91-99.
28. Castanon I, Hannich JT, Abrami L et al. Wnt-controlled sphingolipids modulate Anthrax Toxin Receptor palmitoylation to regulate oriented mitosis in zebrafish. *Nat Commun* 2020; 11(1): 1-14.
29. Bachran C, Leppla SH. Tumor targeting and drug delivery by anthrax toxin. *Toxins* 2016; 8(7): 197.
30. Wu G, Hong Y, Guo A et al. A chimeric protein that functions as both an anthrax dual-target antitoxin and a trivalent vaccine. *Antimicrob Agents Chemother* 2010; 54(11): 4750-4757.
31. Shcherbinin D, Esmagambetov I, Noskov A et al.

Protective immune response against *Bacillus anthracis* induced by intranasal introduction of a recombinant adenovirus expressing the protective antigen fused to the Fc-fragment of IgG2a. *Acta Natr* 2014; 6(1 (20)): 76-84.

32. Varshney A, Puranik N, Kumar M, Goel A. Immunogenicity of a chimeric protein of *Bacillus anthracis* protective antigen and lethal factor in murine model. *Int J Infect Dis* 2016; 45: 426.
33. Fu P, Zhang J, Li H, Mak M, Xu W, Tao Z. Extracellular vesicles as delivery systems at nano-/micro-scale. *Adv Drug Deliv Rev* 2021; 179: 113910.
34. Dykman L, Khlebtsov N. Gold nanoparticles in biomedical applications: recent advances and perspectives. *Chem Soc Rev* 2012; 41(6): 2256-2282.