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In Silico Prediction of B-Cell and T-Cell Epitopes of Protective Antigen of *Bacillus anthracis* in Development of Vaccines Against Anthrax

Research Article

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ABSTRACT

Protective antigen (PA), a subunit of anthrax toxin from *Bacillus anthracis*, is known as a dominant component in subunit vaccines in protection against anthrax. In order to avoid the side effects of live attenuated and killed organisms, the use of linear neutralizing epitopes of PA is recommended in order to design recombinant vaccines. The present study is aimed at determining the dominant epitopes based on multi-parameter and multi-method analysis. The epitopes were identified by the well-known online bioinformatics server and then they were selected and compared based on the highest score and the highest repetition rate. Further analysis on predicted epitopes has been carried out by online VaxiJen 2.0 and Protein Digest server. Among the selected epitopes, those with the highest antigenicity score (>0.9 threshold) and less susceptibility to gastrointestinal tract proteases, were selected as final epitopes. Final B-cell predicted epitopes were amino acid residues 292-308, 507-521 and 706-719; residues 17-31, 315-329 and 385-400 which were determined as the best major histocompatibility complex I (MHCI) class of T-cells epitopes; in addition, residues 455-464 and 661-669 were also considered the best MCHII class of T-cells epitopes. Since random coil structure had a high probability of protein forming of antigenic epitope, the results of secondary structure analysis of the final PA epitopes have shown that all these epitopes form a 100% random coil structure.

KEY WORDS *anthracis*, epitope prediction, protective antigen (PA).

INTRODUCTION

Anthrax is known as an epizootic and zoonotic disease in domestics which could spread through spore transmission via ingestion, inhalation or an open skin wound; it could also affect the humans who are in contact with the infected animals and their contaminated products (Leppla *et al.* 2002; Inglesby *et al.* 2002). *Bacillus anthracis* is a gram-positive, facultatively anaerobic and rod-shape pathogen with two different plasmids named pXO1 and pXO2 (Brey, 2005). PXO1 plasmid encodes toxin factors including protective antigen (PA) and two other catalytically active components; lethal factor (LF) and edema factor (EF). Exotoxins production was mediated by binary combinations of these three regions (Stanley and Smith, 1961). Using proteolytic cleavage of PA into a 20-kDa amino-terminal fragment and a 63-kDa polypeptide via furin (Gordon *et al.* 1995) along with the formation of heptameric oligomers, *B. anthracis* can bind with the cellular receptors via PA which later translocate LF and EF into the cytosol with enzymatic activity. With the use of zinc ion, LF inactivates mitogen-activated protein kinases (MAPKKs) which cause toxic shock and death (Vitale *et al.* 1998). EF factor, through high converting intracellular ATP into cAMP as an adenylate cyclase, stimulate rate of the intracellular cAMP levels and finally leading to edema (Leppla, 1982). PXO2's

encoded capsules enhance virulence in vivo by inhibiting phagocytosis of the organism (Little and Ivins, 1999). It has been proved that a truncated recombinant of PA could stimulate a protective immune response to anthrax (Abboud and Casadevall, 2008; Flick-Smith *et al*. 2002). In animal studies it has been demonstrated that protective immunity against anthrax is associated with the induction of neutralizing anti-PA antibodies (Farchaus *et al*. 1998; Little *et al*. 1997; Pitt *et al*. 2001). In recent researches, the purified recombinant PA (rPA) has been reported as an advanced anthrax vaccine. Therefore, PA with its four distinct domains could be considered the best choice for epitope prediction. Epitopes are specific sites of antigens as antigenic determinant which are classified into two major gropes; B-cell (continuous and discontinuous) and T-cell (major histocompatibility complex I (MHCI) and major histocompatibility complex II MHCII)) (Zhang *et al*. 2012). B- and T-cell epitopes of antigens can be identified and predicted using computational tools in order to design recombinant vaccines which are important in stimulation of antibodies. These predictor tools are as in-silico environment which are advantageous since they are inexpensive and noninfectious in vaccine designing, whereas viruses or bacteria could be harmful during experimental process. In contrast with the experimental methods which are costly and time-consuming, these tools are cheap and available (Ponomarenko and Van, 2009). The present study is aimed to identify B-cell and T-cell epitopes of PA antigen in vaccine designing in order to counter against anthrax using molecular biology software which could reveal the dominant epitopes of the protective antigen of *B. anthracis*.

MATERIALS AND METHODS

Amino acid sequence of the protective antigen protein of *B. anthracis*

Amino acid sequence of protective antigen protein of *B. anthracis* (Accession number: CAL49462) was obtained from GeneBank (http://www.ncbi.nih.gov/genbank/). PA protein is composed of 735 amino acid residues.

Prediction of the secondary and tertiary structure of the PA antigen

Different conformational states (helices, sheets, turns and coils) of PA antigen of *B. anthracis* protein were analyzed to predict the secondary structures using the improved self-optimized prediction method (SOPMA) software (http://npsa pbil.ibcp.fr/cgi bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon and Deléage, 1995).

The next step of augury of tertiary structure of PA antigen was accomplished using 3DLigandSite ligand banding sit prediction Server (http://www.sbg.bio.ic.ac.uk) (Wass *et al*. 2010).

Servers and software used for epitope prediction

Using in-silico softwares which have been listed in Table 1, the process of epitope prediction of PA antigen was carried out using antigen primary sequence. These softwares are designed for B-cell or T-cell epitopes and they use liner sequences of amino acids to determine antigenicity of hot spot regions, known as epitope region, and also to report them with different antigencity scores.

In T-cell prediction softwares, parameters of each server were adjusted as follows; 'MHC alleles (A-0101, A0201 and B-2705 alleles for MHCI class and DRB1-0101, DRB1-0401 and DRB1-0401 alleles for MHCII class)' and 'desired length' of related epitopes. The remaining parameters did not change.

Characterization of epitopes

The final predicted epitopes of B and T-cell were evaluated using VaxiJen 2.0 server, an alignment-independent prediction of protective antigens. VaxiJen server classifies antigens according to physicochemical properties of proteins without having recourse to sequence alignment (http://www.ddgApharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

The study on enzymatic digestion of final predicted epitopes PA protein has been done using (http://db.systemsbiology.net:8080/proteomicsToolkit/proteinDigest.html) server. Mass (Da) and point of isoelectric (pI) of each predicted epitopes were determined using this Protein Digest server.

Prediction of the secondary structure of the PA protein

The secondary structure of PA protein encompasses four conformational states; helices, sheets, turns and coils, which were analyzed by the improved self-optimized prediction method (SOPMA) software (https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon and Deléage, 1995).

Required parameters for prediction of the secondary structure such as threshold and window width were set to 8 and 17, respectively.

Tertiary structure was conducted using iterative threading ASSEmbly refinement (I-TASSER) site (http://zhanglab.ccmb.med.umich.edu/I-TASSER) which is a hierarchical approach to protein structure and function prediction.

And finally using PyMOLV1 Viewer software, the primary structure of the studied template from protein database (PDB) format was analyzed and viewed.

RESULTS AND DISCUSSION

Prediction of the secondary structure of PA protein

SOPMA software was used in order to identify some details of antigenic property of PA protein in its secondary structure. As it is shown in Figure 1, the number of extended strand, random coil and alpha helix were the most dominant region in PA protein (26.67, 35.65 and 26.26 respectively).

The existence of the extended strands and random coils in protein is the leading cause of the probability of protein formation as an antigenic epitope.

Prediction of B-cell and T-cell epitopes for PA antigen

B-cell and T-cell epitopes' prediction of PA antigen has been done using online software listed in Table 1. The predicted epitopes were selected based on the highest score and the highest repetition rate from all the softwares' outputs (data shown in supplementary file).

Pre-final B-cell epitopes which had the most conserved sequences among all proposed epitopes are being listed in Table 3. It is worth mentioning that the software utilizes different scoring systems. Using different online software the predicted epitopes of MHCI (A-0101, A0201 and B-2705 alleles) and MHCII (DRB1-0101, DRB1-0401 and DRB1-0401 alleles), as two separate classes of T-cell, were listed in Table 3. The high-scored regions which had a high potential, were selected as epitope region compared to all the other utilized softwares. The pre-final selection among T-cell epitopes was based on some sequences of epitopes which were presented in all MHCI as well as MHCII class alleles.

Antigenicity and characterization of Protein Digest selected epitopes

Further analysis to assign the best epitopes for PA antigen continued using the final results of the previously mentioned online software.

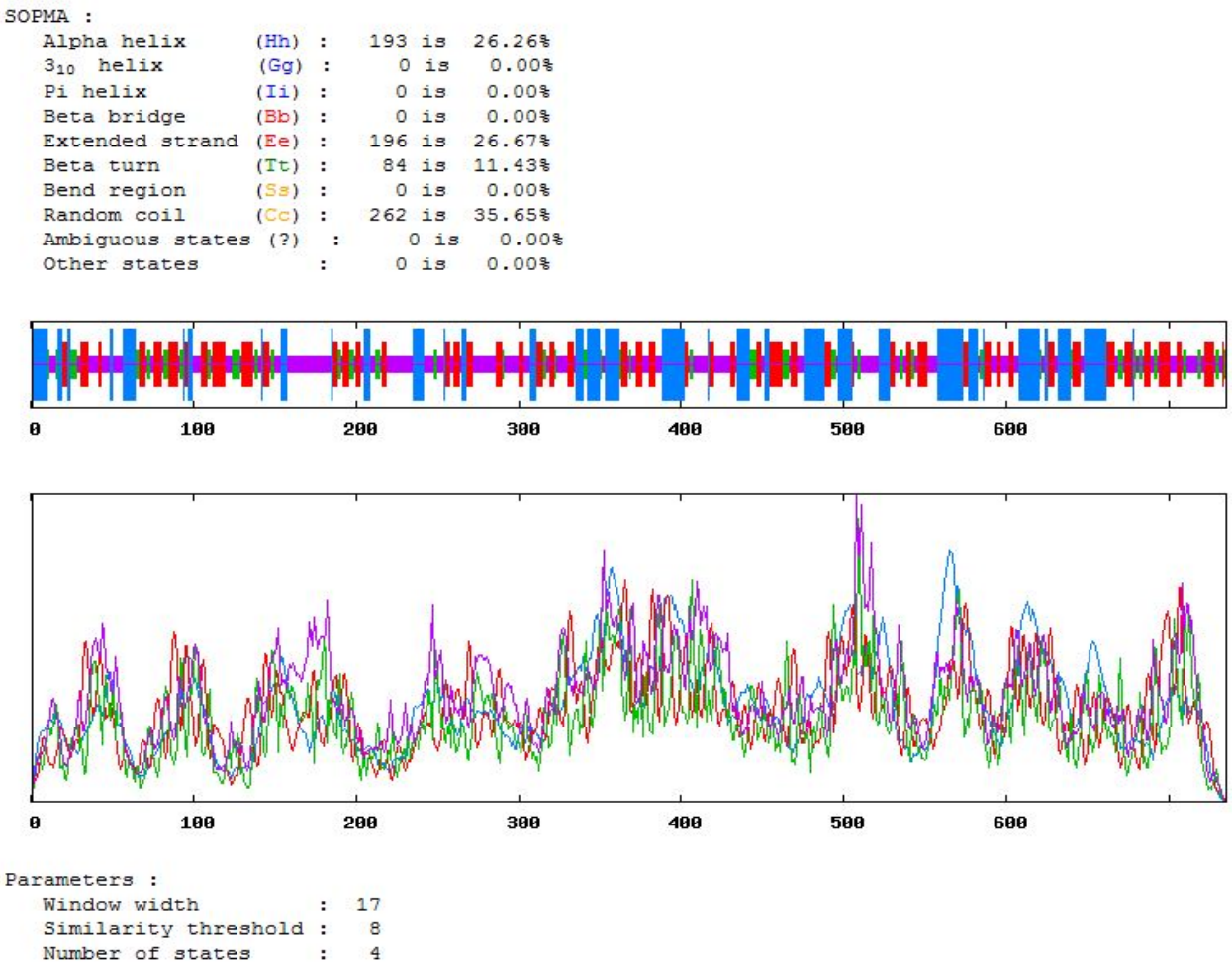

Figure 1 Secondary structure prediction results for the PA protein. An increased number of extended strands and random coils in the protein corresponded with an increased likelihood of the protein forming an antigenic epitope

Lines in different colors represent different secondary structures: blue, α helix; green, β turn; red, extended strand; and purple, random coil

Lines in different colors represent different secondary structures: blue, α helix; green, ß turn; red, extended strand; and purple, random coil

Table 1 Bioinformatics software that used in present study

Servers	Description	Link
T-cell epitopes prediction		
IEDB	SVM and ANN-based method for prediction	http://tools.immuneepitope.org/mhci
SYFPEITH	A database of MHC ligands and peptide motifs; predictive server for MHC binding peptide	http://www.syfpeithi.de
NetMHC	ANN-based method for prediction of HLA	http://www.cbs.dtu.dk/services/NetMHC
NetCTL	Prediction of T-cell epitope	http://www.cbs.dtu.dk/services/NetCTL
PropredI	Predict MHC class I binding peptides	http://www.imtech.res.in/raghava/propred1
Propred	Predict MHC class II binding peptides	http://www.imtech.res.in/raghava/propred
B-cell epitopes prediction		
Bcepred	Physio-chemical properties of amino acids based predictive server for linear B-cell epitope	http://www.imtech.res.in/raghava/bcepred
ABCpred	ANN based predictive server	http://www.imtech.res.in/raghava/abcpred
BepiPred	Predictor of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method	http://www.cbs.dtu.dk/services/BepiPred
BCPred	Prediction of linear B-cell epitopes using amino acid pair antigenicity scale and string kernels	http://ailab.cs.iastate.edu/bcpreds
SVMTrip	Predictor of linear B-cell epitopes using Support Vector Machine (SVM)	http://sysbio.unl.edu/SVMTriP
LEPS	Prediction of linear B-cell epitopes using Support Vector Machine classification and Amino Acid Propensity	http://leps.cs.ntou.edu.tw
IEDB	Physio-chemical properties of amino acids based predictive server for linear B-cell epitope	http://tools.immuneepitope.org/tools/bcell/iedb_input

Table 2 Training bioinformatics software that used in present study*

Antigen	Predicted epitopes	Experimental epitopes	Reference
GroEL[1] of *Yarsinia*	28-42, 78-92, 178-185, 275-290, **315-336**, 430-440, 526-545	316-326	Yamaguchi *et al.* (1996)
Dnak[2] of *Brucella*	40-67, 78-92, 210-227, 357-370, 523-537, **609-640**	617-637	Vizcaino *et al.* (1997)
Omp31[3] of *Brucella*	25-28, **46-73**, 122-127, 175-182	48-74	Wang *et al.* (2014) Cassaratro *et al.* (2005)
SOD[4] of *Brucella*	44-50, **70-86, 134-153, 147-165**	75-86, 136-150, 149-162	Tabatabai *et al.* (1994)

[1] Heat shock protein 60.
[2] Heat shock protein 70.
[3] Outer membrane 31.
[4] Sodium oxide dismutase.
* Similar epitopes between predicted epitopes using bioinformatics tools and experimental studies were specified by bold and were underline.

Antigenicity of the selected epitopes was determined along with the enzyme digestion position of each epitope using VaxiJen 2.0 and Protein Digest servers.

Epitopes mentioned in Table 3 were first filtered by the VaxiJen software with 0.5 threshold, and then scored more than 0.5 threshold and also checked for determination of their mass (Da), pI and enzymatic digestion (Table 3). Final B-cell predicted epitopes, based on the high score of antigenicity and maximum number of undigested enzymes, have been highlighted and ranged in 292-308, 507-521 and 706-719 amino acids (Table 3).

More investigations were carried out through determining the characterization of final MHCI and MHCII T-cell predicted epitopes. As it has been shown in Table 3, amino acids sequences in 17-31, 315-329 and 385-400 regions are the most suitable epitopes for MCHI class, and amino acids sequences in 455-464 and 661-669 range are the best ones for MCHII class of T-cells, according to their high VaxiJen score and the maximum number of undigested enzymes.

Prediction of the tertiary structure of PA protein

The tertiary structure of PA protein is predicted by I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and viewed by PyMOL Viewer software. This result demonstrated that our final predicted epitopes are exposed and located on the surface of the protein as it is shown in Figure 3; the red spheres represent the potential B-cell epitopes, the green spheres indicate stronger potential of MHCI class epitopes of T-cells, the blue spheres is related to MHCII class epitopes of T-cells and finally the white spheres represent the remainder of the protein.

Vaccine production based on live attenuated or killed organisms has long been criticized for its side effects; however, with the use of newly developed technique in fourth generation of recombinant DNA technology, vaccine production has been conferred by using some immunogenic component pathogens.

Table 3 Antigenicity ability of predicted epitopes using Vaxijen server and Protein Digestion analysis of pre-final B-and T-cell epitopes*

Final B-cell predicted epitopes				
Sequence	V.J score	Mass	pI	Undigested enzyme
3KQENRLLNESESSSQG19	1.0122	1902.99	4.79	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, AspN
35VTSSTTGDLSIPSSELENIP55	0.7880	2047.20	3.57	Trypsin, Chymotrypsin, Clostripain, IodosoBenzoate, Trypsin_K, Trypsin_R,
70KVKKSDEYTFATSADNHVTM90	0.9250	2272.51	6.75	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R
99KASNSNKIRLEKGRLYQIKI118	1.1331	2359.80	10.56	Cyanogen_Bromid, IodosoBenzoate, Proline_Endopept, AspN
115QIKIQYQREDPTEKGL131	0.9824	1946.19	6.18	Cyanogen_Bromid, IodosoBenzoate
155ELKQKSSNSKKRRS168	2.0535	1675.91	11.17	Chymotrypsin, Cyanogen_Bromide,IodosoBenzoate, Proline_Endopept, AspN
167RSTSAGPTVPDRDNDG187	0.8526	1644.68	4.43	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Staph_Protease, Trypsin_K, Chymotrypsin(modified)
241GRIDKNVSPEARHPLVAAYP261	0.6977	2190.49	8.60	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R
274NEDQSTQNTDSQTRTISKNTSTSRTHTSEVHGNAE308	1.6135	3862.91	5.48	Chymotrypsin, Cyanogen_Bromide, M IodosoBenzoate, Proline_Endopept, Chymotrypsin (modified)
292NTSTSRTHTSEVHGNA308	1.7064	1698.73	6.92	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_K, AspN, Chymotrypsin (modified),
422NAQDDFSSTPITMNYN438	0.6708	1817.90	3.56	Trypsin, Clostripain, IodosoBenzoate, Staph_Protease, Trypsin_K, Trypsin_R
465ENGRVRVDTGSNWSEVLPQI485	0.5150	2256.46	4.68	Cyanogen_Bromide, Trypsin_K,
507AVNPSDPLETTKPDM521	0.5455	1614.79	4.03	Trypsin, Chymotrypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_K, Trypsin_R
511SDPLETTKPDMTLKEALKIA531	0.8427	2201.56	4.78	Chymotrypsin, Clostripain, IodosoBenzoate, Trypsin_R,
530AAVNPSDPLETTKPDMTLKE550	0.8128	2157.42	4.54	Chymotrypsin, Clostripain, IodosoBenzoate, Trypsin_K, Trypsin_R
542YQGKDITEFDFNFDQQTSQN561	1.4173	2425.51	3.84	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R,
688YISNPNYKVNVYAVTKENTI707	0.7403	2330.62	8.38	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_R, AspN
706TIINPSENGDTSTN719	1.2134	1462.49	3.67	Trypsin, Chymotrypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_K, Trypsin_R, Chymotrypsin (modified)
705NTIINPSENGDTSTNGIKRI725	0.8542	2144.33	6.07	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Chymotrypsin (modified)
Final T-cell predicted epitopes (MHCI class)				
Sequence	V.J score	Mass	pI	Undigested enzyme
1EVKQENRLLNESESSS16	0.7739	1848.94	4.49	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept
17GLLGYYFSDLNFQA31	0.8413	1607.78	3.80	Trypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R
111GRLYQIKIQY121	1.8157	1281.52	9.70	Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, AspN
123EDPTEKGLDFKLYWTDS129	1.1810	2044.20	4.11	Clostripain, Cyanogen_Bromide, Trypsin_R,
153LPELKQKSSNSKKRRS168	2.0389	1886.18	11.17	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, AspN,
163SKKRRSTSAGPTVPDRD179	0.6143	1858.04	10.90	Chymotrypsin, Cyanogen_Bromide, IodosoBenzoate, Staph_Protease, Chymotrypsin (modified)
268NIILSKNEDQSTQNTD283	1.0858	1819.90	4.27	Chymotrypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R,
315DIGGSVSAGFSNSNS329	0.8265	1398.41	3.80	Trypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R, AspN
385LGKNQTLATIKAKENQ400	0.7593	1757.02	9.70	Chymotrypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, IodosoBenzoate, Proline_Endopept, Trypsin_R, AspN
563NIATYNFENGRVRV576	0.8575	1652.83	8.75	Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_K, AspN,
637KILSGYIVEI647	0.7649	1134.38	6.00	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R, AspN
644VEIEDTEGLKEVINDRYD684	0.8462	2137.28	4.08	Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept
687YISNPNYKVNVYAV700	1.0997	1643.86	8.43	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Staph_Protease, Trypsin_R, AspN
691NPNYKVNVYAVTKENT706	0.9906	1854.05	8.43	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_R, AspN
700AVTKENTIINPSENGDTSTNGI721	0.9138	2275.41	4.14	Chymotrypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_R, Chymotrypsin (modified)
Final T-cell predicted epitopes (MHCII class)				
Sequence	V.J score	Mass	pI	Undigested enzyme
118QYQREDPTEKGLDFKLYWTDSQNKKEVISSDNLQ152	1.0003	4104.46	4.66	Cyanogen_Bromide
168TSAGPTVPDRDNDGIPDSLEVEGYT193	0.7173	2605.71	3.66	Cyanogen_Bromide, IodosoBenzoate, Trypsin_K,
413APIALNAQDDFSSTPITM433	0.5277	1892.11	3.56	Trypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R,
455YGNIATYNF464	1.1019	1062.15	5.52	Trypsin, Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R, AspN,
533NEPNGNLQYQGKDITEFDFNFDQQ558	0.9275	2861.97	3.77	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Trypsin_R,
640GYIVEIEDTEGLKEV654	0.8338	1693.87	3.91	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Proline_Endopept, Trypsin_R,
661MLNISSLQQ669	0.54001	1033.21	5.28	Trypsin, Chymotrypsin, Clostripain, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R, AspN,
686LYISNPNYKY695	0.9188	1274.44	8.43	Clostripain, Cyanogen_Bromide, IodosoBenzoate, Staph_Protease, Trypsin_R, AspN,
701TKENTIINPSENGDTSTNGIKRIL725	0.6127	2615.88	5.85	Cyanogen_Bromide, IodosoBenzoate, Chymotrypsin (modified)

* Bold and highlighted sequences related to final selected epitopes based on higher score of antigencity given by Vaxyjen and more undigested enzyme.

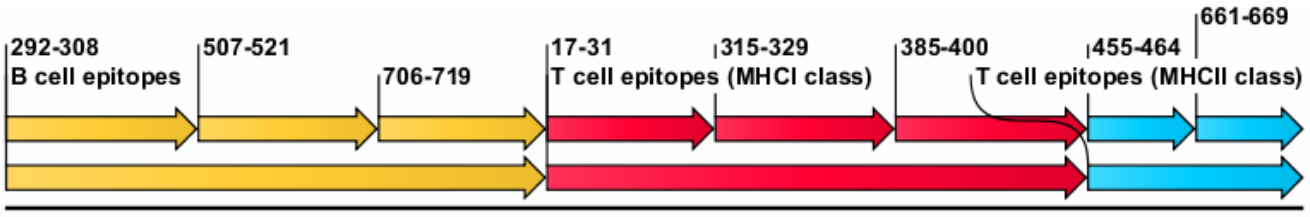

Figure 2 Final predicted epitopes
Yellow arrows represent B-cell epitopes: 292-308, 507-521 and 706-719 amino acids residual, red arrows represent T-cell (MHCI class) epitopes: 17-31, 315-329 and 385-400 amino acids residual, blue arrows represent T-cell (MHCII class) epitopes: 455-464 and 661-669 amino acids residual

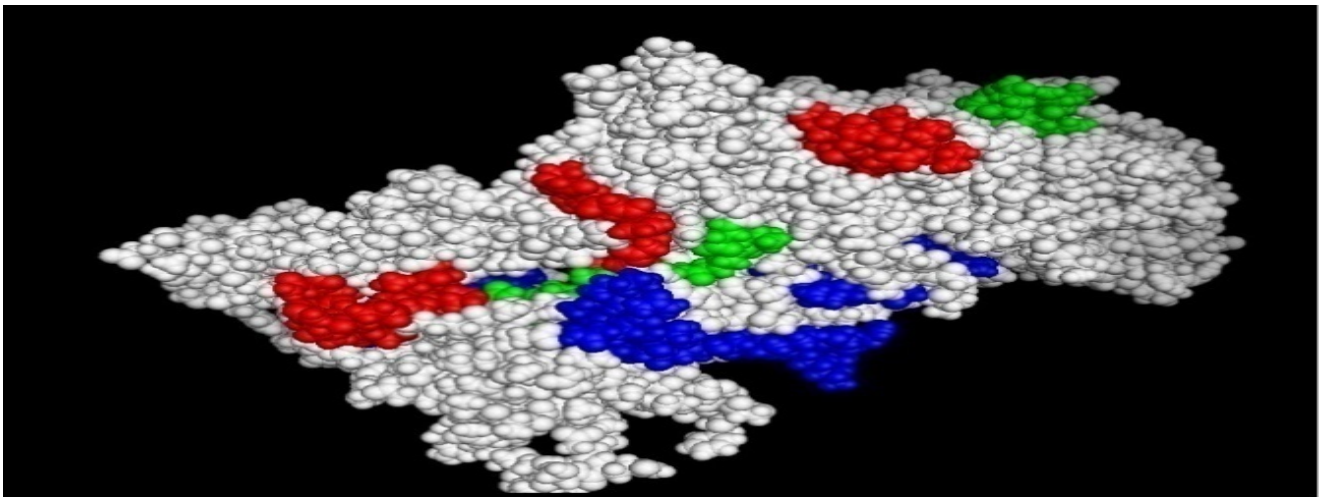

Figure 3 Tertiary structure of local situation of predicted epitopes in the PA protein
Red spheres are the B-cell epitopes, the green ones are the MHCI class of T-cell epitopes and the blue ones related to MHCII class of T-cell epitopes White spheres are the remainder part of PA protein

Selecting a proper antigens, rational adjuvant design and a good delivery system are the most important factors in a successful vaccine designing approach (Yang *et al*. 2013). Using developed bioinformatics tools in epitopes prediction which are based on multi-parameter and -method analysis methods, designing a dominant epitope through epitope prediction process seems to be more accurate and significant (Li *et al*. 2013).

In order to examine the functionality of the software used in this study, a comparison of the experimental epitopes of four antigens submitted in *ideb* server (http://www.iedb.org) with the results of epitope prediction of the selected antigens, has been done using bioinformatical tools (Yousefi *et al*. 2015). As it is demonstrated in Table 2, the reported experimental results overlap those outputs predicted by Bioinformatical tools.

The empirical epitopes in subunit vaccines are too costly and need molecular biology and immunological technologies. In this respect, in a study (Forouharmehr and Nassiry, 2015) B and T-cells epitopes, secondary and tertiary structures, and antigenicity prediction of P40 protein of mycoplasma agalactiae bacteria were analyzed using alternative online softwares.

And possible antigenic epitopes and their immunogenicity of predicted peptides were determined. Another research on epitope prediction belongs to Yousefi *et al*. (2015) which concentrate on the most desirable epitopes of OMP25 antigen of *Brucella melitensis* bacteria. In that study they used a wide range of on-line epitope prediction software and reported the most probable epitopes with high antigenicity and less restriction site for enzyme digestibility. In the current study, B and T-cell epitope prediction of PA antigen of *B. anthracis* has been conducted using well-known online epitope prediction servers.

As it is shown in Figure 2, final B and T-cell epitope prediction suggested three epitopic region for B cells, three epitopic region for MHCI T-cells and two for MHCII T-cells. Kaur *et al*. (2009) have identified three main regions; ID-I: 604-622, ID-II: 626-676 and ID-III: 707-723 residues as B-cell epitopes through BCPred, BcePRED servers; these regions are not the same as the predicted B cell epitopes in this study. Random coil regions, which are located on the surface, are essential in binding ligands, since they are both exposed and hydrophilic.

The high rate of random coil structures implies to most protein-forming antigenic epitopes (Li *et al*. 2013).

Through secondary analysis of final predicted epitopes, our findings revealed that all of our final predicted B cell epitopes contain 100% random coil structure, the same as MHCI and MHCII T-cell predicted epitopes. Consequently, these recommended epitopes (due to the suitable random coil structure) could be exposed to protein surface, making them appropriate candidates to be used in recombinant subunit of vaccines epitope with stronger antigenicity. In order to prevent degradation and decomposition of epitopes during antigen processing, epitopes with less restriction site of proteosomal should be selected (Toes *et al.* 2001).

Subsequently, the predicted B and T-cell epitopes have been analyzed based on the presence of enzymatic restriction sites. The results have demonstrated that some enzymes such as Trypsin, Clostripain, CyanogenBromide, IodosoBenzoate, Proline_Endopept, Staph_Protease, Trypsin_K, Trypsin_R and AspN, which are the central enzymes responsible for protein degradation, have no restriction sites in the final selected epitopes. Hence it could be concluded that these epitopes can be used not only in injective vaccines but also in oral ones. Bioinformatic analysis revealed that these epitopes have more antigenic effect on the body via their highest persistence in gastrointestinal tract and by avoiding enzyme digestion.

CONCLUSION

Finally, it can be concluded that, since the using recombinant vaccines has many advantages over the use of killed or live attenuated bacteria, the identification of epitopic zones of pathogenic bacteria and their use in the sub-unit vaccines consist of multiplex epitopic, can be an important step in creating a safe immunity in animals and humans. In the case of anthrax bacteria, based on the results obtained in this study, the use of final predicted areas with the highest immunogenic scores through bioinformatics processes can be a good alternative to initiating experimental experiments against this bacterium.

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