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Design of a chimeric DNA vaccine against *Brucella spp.*

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Brucellosis, a common illness between domestic animals and humans, is mostly caused by *Brucella* (*B*) *abortus, B. melitensis* and *B.suis*. It has been shown vaccination of mice by Omp19 and Omp31 can induce protection against different *Brucella spp*. Furthermore, urease enzyme is considered a virulence factor in most members of *Brucella*. Here, we designed a chimeric DNA vaccine containing *omp19*, *omp31* and *urease* genes to obtain high protection against *Brucella spp*. The construct was analyzed using bioinformatics tools. Linear and discontinuous B-cell epitopes, MHC class I and II binding peptides of the chimeric vaccine were predicted. Results suggest the construct can be an appropriate vaccine candidate against brucellosis.

Key words: Brucellosis - Vaccines, DNA - Zoonoses.

Brucellosis is a zoonotic illness that is transmitted from domestic animals to humans. This illness is mostly caused by *Brucella*, a gram-negative, facultative intracellular bacterium.[1] Eight *Brucella* (B) species are: *B. suis*, *B. abortus*, *B. melitensis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. cetaceae* and *B. pinnipediae*. The first four species can cause brucellosis disease in humans.[2] The prevention of human infection needs dairy products pasteurization, suitable sanitation and control of the illness in the domestic animals by vaccination. Many efforts have been made for development of DNA and protein vaccines against brucellosis.[3-10] Unlike attenuated vaccines, subunit vaccines have some advantages such as safety, not infectious and not change to virulence form. In addition, use of DNA vaccine has already been promising in veterinary vaccination.[11-16] Advantages of DNA vaccines include safety, easy preparation in large scale, high stability and therapeutic applications.[17-20] Efficient immune response against *Brucella* is cell-mediated immunity. The interferon-gamma (IFN- γ) production by T helper (Th) 1 cells and Cytotoxic T Lymphocytes (CTLs) responses are crucial components for protection against *Brucella*, whereas Th2 responses are minor mediators.[21-23] Furthermore, it has been shown that IL-17 production plays a key role in immunity against this illness.[24] The success of a subunit vaccine is strongly related to its composition and route of administration. Recent study showed that vaccination of mice by *B. abortus* 19

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kDa outer membrane protein (Omp19) as a subunit vaccine can induce protection against *B. abortus*, *B. melitensis* and *B. suis*. Furthermore, this antigen had adjuvant property.[24]

In another study, vaccination with recombinant *B. melitensis* Omp31 could protect mice against *B. melitensis* and *B. ovis*.[5] Administration of this antigen as a protein vaccine induced antibody and cellular immune responses in sheep.[25] In general, this antigen was very immunogenic and induced neutralizing antibodies in both mice and sheep. Furthermore, application of Omp31 as DNA vaccine induced cytotoxic responses that have the key role to protection against *Brucella* infection.[26] Therefore, Omp31 could be a suitable vaccine component against *B. melitensis* and *B. ovis*.

Most *Brucella* members show strong urease activity.[27] Urease is a virulence factor for different human pathogens, and it plays a key role in gastrointestinal tract infections.[28] The sequence of Urease alpha subunit is highly conserved among *B. abortus*, *B. melitensis* and *B. suis* strains with 99% identity.[29, 30]

Determination of the binding affinity of major histocompatibility complex (MHC) molecules and antigenic peptides has been the main objective during epitope prediction process. However, this process is proved to be time consuming and difficult. Therefore, different *in silico* techniques have developed and used to identify antigenic epitopes. Use of bioinformatics tools along with experimental verification results in acceleration of the epitope discovery process by nearly 10-20-fold. Basically, *in Silico* prediction studies are very promising for development of new or improvement of existing vaccines.[31]

In this study, we designed a novel DNA vaccine containing *omp19*, *omp31* and alpha subunit of *urease* enzyme, fused together by hydrophobic linkers. For achieving the highest translation efficacy, codon optimization of this chimeric gene was performed. Finally, bioinformatics tools were utilized to analyze the structure of the chimeric construct.

Materials and methods

Sequence availability and similarity search

Related amino acid sequences for urease, *B. abortus* Omp19 and *B. melitensis* Omp31 were obtained from primarily from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were carried out using ClustalW software (EBI, UK) (http://www.ebi.ac.uk/Tools/clustalw2/) for identifying a conserved region in all the sequences.[32]

Construct design

An antigenic sequence was constructed by fusing Omp19 (Gln^{22} to Arg^{177}), Omp31 (Val^{22} to Phe^{240}) and middle fragment of Urease (Ala^{201} to Leu^{350}) by hydrophobic amino acid linkers. Gene bank codon data base and Swissprot reverse translation online tool (http://www.bioinformatics.org/sms2/rev_trans.html) was used for reverse translation.[33] SignalP server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences.[34]

Secondary and tertiary structure prediction

Chou and Fasman Secondary Structure Prediction (CFFSSP) server (http://www.biogem.org/tool/chou-fasman/) and GOR software (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_gor4.pl) were employed to predict the secondary structures of the chimeric protein.[35, 36] Raptor X (http://raptorx.uchicago.edu/predict/) was used for detection of disorder regions in the chimeric protein structure. The iterative threading assembly refinement (I-TASSER) server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and an integrated platform for automated protein structure and function prediction based on the sequence-to-structure-to -function paradigm were employed for tertiary structure prediction.[37]

Evaluation of structural stability and validation

3D structural stability of the synthetic protein was evaluated by Swiss-PdbViewer software for energy minimization and RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). C-score were used to estimate the accuracy of 3D model, quantitatively and qualitatively.

Primary sequence analysis

Various physicochemical parameters of the chimeric protein were estimated using ProtParam software (http://expasy.org/tools/protparam.html).[38] Prokaryotic and eukaryotic subcellular localization

of the protein was predicted by PSLpred (http://www.imtech.res.in/raghava/pslpred/) and WegoLoc servers (http://www.btool.org/WegoLoc), respectively.[39, 40] VaxiJen software (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and ANTIGENpro software (http://scratch.proteomics.ics.uci.edu/) were used to predict the antigenicity probability.[41, 42] PEST motifs were explored by epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind).[43] Allergenicity of the chimeric protein was predicted by APPEL (http://jing.cz3.nus.edu.sg/cgi-bin/APPEL).

Post-translational modification predictions

NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetGlycate (http://www.cbs.dtu.dk/services/NetGlycate/) servers were used for prediction of N-glycosylation sites in the protein.[44, 45] O-glycosylation sites were estimated using NetOGlycate server (http://www.cbs.dtu.dk/services/NetOGlycate/).[46] Prediction of N-terminal myristoylation sites was performed by Myristoylator server (http://expasy.org/tools/myristoylator/).[47] NetPhos server (http://www.cbs.dtu.dk/services/NetPhos/) was employed to predict phosphorylation sites.[48, 49]

Prediction of B-cell epitopes

Linear B -cell epitopes of construct were estimated using BepiPred 1.0 (http://www.cbs.dtu.dk/services/BepiPred/), and ABCpred servers (http://www.imtech.res.in/raghava/abcpred/ABC_submission.html).[50, 51] Discotope 1.2 (http://tools.immuneepitope.org/stools/discotope/discotope) and SEPPA (http://lifecenter.sgst.cn/seppa/) servers were employed to predict discontinuous B-cell epitopes. Additionally Ellipro server (http://tools.immuneepitope.org/tools/ElliPro/iedb_input) was used to predict both linear and discontinuous B-cell epitopes. This method predicts epitopes based upon solvent accessibility and flexibility.[52-54]

Prediction of T-cell epitopes

Proteasomal cleavage sites of the chimeric protein were predicted by Netchop 3.1 (http://www.cbs.dtu.dk/services/NetChop/), MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP/binding.html) and PCPS (http://imed.med.ucm.es/Tools/pcps/index.html). Peptides Binding affinity to TAP protein was computed by TAPPred (http://www.imtech.res.in/raghava/tappred).[55-57] ProPred-I (http://www.imtech.res.in/raghava/propred/), nHLAPred (http://www.imtech.res.in/raghava/nhlapred/) and CTLpred (http://www.imtech.res.in/raghava/ctlpred/) servers were used for prediction of peptides from the antigenic sequence binding with MHC class I.[58-60] HLApred (http://www.imtech.res.in/raghava/hlapred/), MHC2Pred (http://www.imtech.res.in/raghava/mhc2pred/help.html) and Propred (http://www.imtech.res.in/raghava/propred/) servers were employed to predict peptides from the chimeric protein binding with MHC class II.[58]

Codon optimization and CpG motif finding

The chimeric construct codons of mouse and human were optimized by GenScript service (https://www.genscript.com/ssl-bin/quote) and DyNAVacS (http://miracle.igib.res.in/dynavac/) server.[61, 62] Additionally, CpG motif evaluation was performed by EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/).

mRNA secondary structure prediction

The messenger RNA secondary structure of the chimeric gene was evaluated by the mfold software (http://www.bioinfo.rpi.edu/applications/mfold). Comparison of folding and thermodynamic features of native and optimized mRNA was performed.[63]

Results

Sequence availability and similarity search

Related amino acid sequences for Urease, *B. abortus* Omp19 and *B. melitensis* Omp31 were obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). After amino acid sequence alignment, one amino acid sequence of each protein was selected as a conserved sequence. The middle part of alpha subunit of Urease (Ala^{201} to Leu^{350}) showed 100% conservation among *B. abortus*, *B.melitensis* and *B.suis* strains. Therefore, this region was selected as a part of the chimeric structure.

The 156 amino acids from the carboxy terminus of *B. abortus* Omp19 (Gln^{22} to Arg^{177}) were selected. It has shown that the lipid moiety on Omp19

does not improve the protective immune response in the mice. Since the lipidation site of Omp19 is Cys21, its first 21 amino acids were removed. The 219 amino acids from the carboxy terminus of *B. melitensis* Omp31 (Val^{22} to Phe^{240}) were selected as a part of the chimeric structure. SignalP 4.0 software predicted first 21 amino acids of Omp31 as a signal sequence. Therefore, these first 21 amino acids were also removed.

Construct design

To separate different domains, linkers containing EAAAK repeats that are expected to form a monomeric hydrophobic α-helix were designed.[64] Four repeated EAAAK sequences were introduced between various domains for more flexibility and efficient separation. For accurate and high level of expression of mRNA in eukaryotic hosts, the Kozak sequence was introduced before the start codon. Additionally, histidine tag was added before the stop codon (Figure 1A).

Secondary and tertiary structure prediction

Two prediction methods were compared for assessing the structure of the chimeric protein. Results showed that helix structures are located in the regions of amino acids 151 to 170 and amino acids 327 to 346, which are related to the hydrophobic linkers inserted between different domains. The disorder regions in the protein structure located in Met^{1} to Glu^{10}, Ala^{155} to Ala^{170} and Ser^{326} to Ala^{341}. Forty three-dimensional models were created for this protein by I-TASSER software. The models were uploaded to the server to draw the tertiary structural illustrations with Accelrys Discovery Studio Visualizer 2.0 software for detecting the final structure of the protein. The results of tertiary structure prediction showed the three domains of this protein have been separated (Figure 1B).

Evaluation of structural stability and validation

The profile of energy minimization was computed by spdbv (Swiss-PdbViewer) (-4218.513 Kcal/mol) showing that the protein had acceptable stability compared to that of original structure of each domain. Furthermore, the data created by a Ramachandran plot verified the structural stability of the protein (Figure 2). The confidence score (C-score) for estimating the quality of predicted models by I-TASSER was -0.92. C-score is a confidence score for evaluating the quality of predicted models by

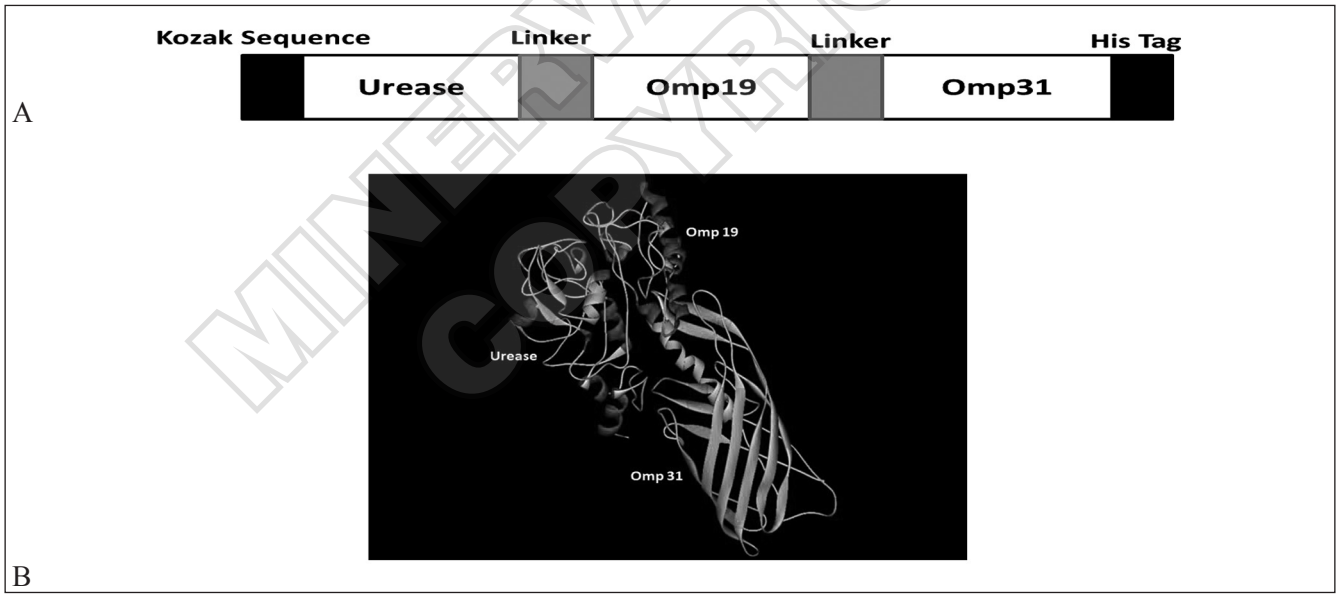

Figure 1. Schematic model and three dimensional model of chimeric protein. a) Schematic model that displays the construction of Urease, Omp 19 and Omp 31, bound together by the linkers for expression in eukaryotic cells. b) Ab initio and comparative modeling was employed to predict the 3D structure of the chimeric protein, Urease-Omp19-Omp31. The result was viewed by Accelrys Discovery Studio Visualizer 2.0 software. The linkers have been displayed in yellow color.

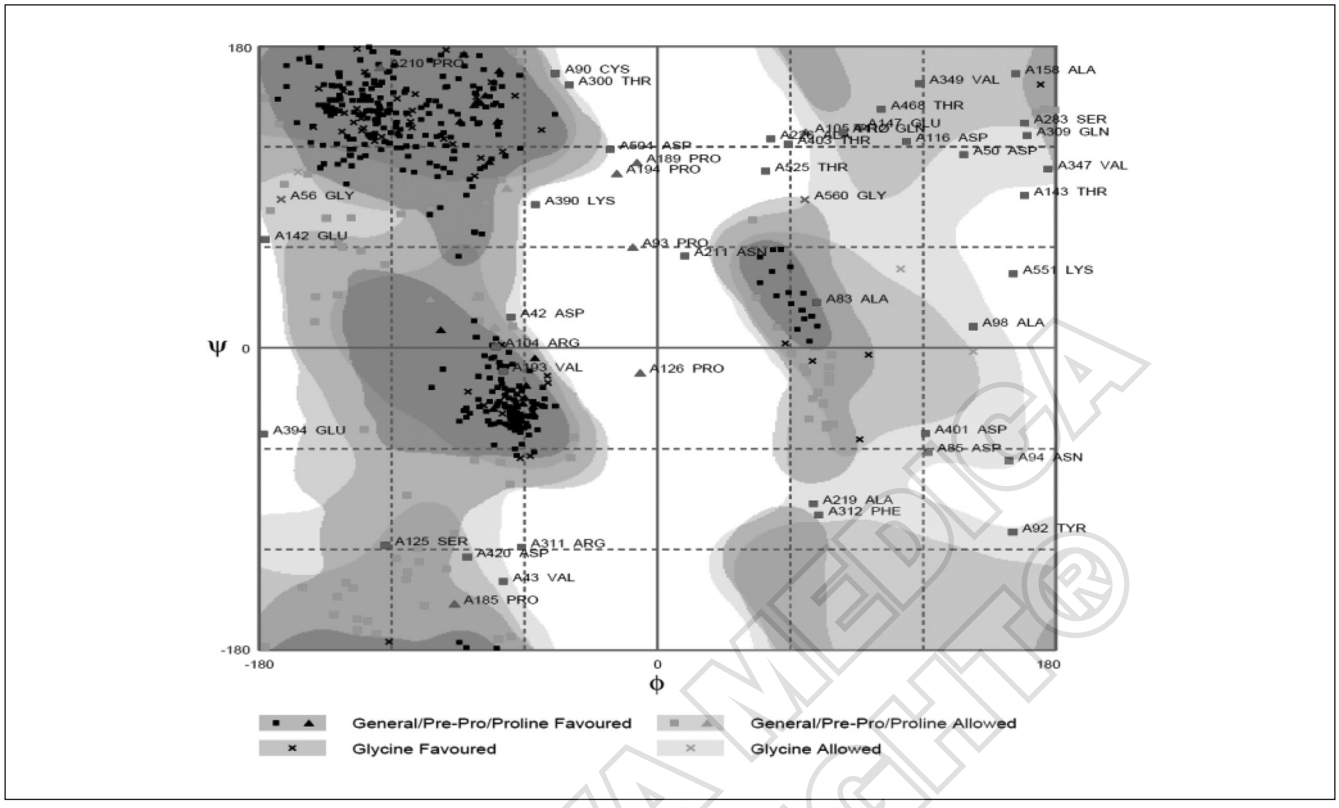

Figure 2.—Sample of Ramachandran plot for the chimeric protein, Urease-Omp19-Omp31.

I-TASSER. C-score is typically in the range of -5 to 2, where a C-score of higher value signifies a model with a high confidence and vice-versa.

Primary sequence analysis

Molecular weight, theoretical isoelectric point (pI), total number of negatively (Asp + Glu) and positively (Arg + Lys) charged residues, instability and aliphatic indices of the chimeric constructs are listed in Table I. The estimated half-life of this sequence was 4.4 h (*mammalian reticulocytes*, in vitro), more than 20 h (*yeast*, in vivo), more than 10 h (*E.coli*, in vivo). Both prokaryotic and eukaryotic sub-cellular localization of the protein was predicted to be the cytosolic. Results of antigenicity probability by Vaxijen and ANTIGENpro software have been listed in Table I. Seven poor PEST motifs were identified in the chimeric sequence. Based on prediction of APPEL, the construct was non-allergen.

TABLE I.—*Properties of the chimeric constructs given by "protparam"and antigen index of chimeric Urease-Omp 19-omp 31 protein given by Vaxijen and ANTIGEN pro.*

Molecular Weight	Theoretical pI	Total number of negatively (Asp+Glu) charged residues	Total number of positively (Arg + Lys) charged residues	Instability index	Aliphatic index
58.8 kDa	5.32	59	44	27.48	74.39
Antigen index					
Server	Urease	Omp19	Omp31	Chimeric Protein	
Vaxijen	0.48	0.71	0.71	0.68	
ANTIGENpro	0.78	0.90	0.78	0.90	

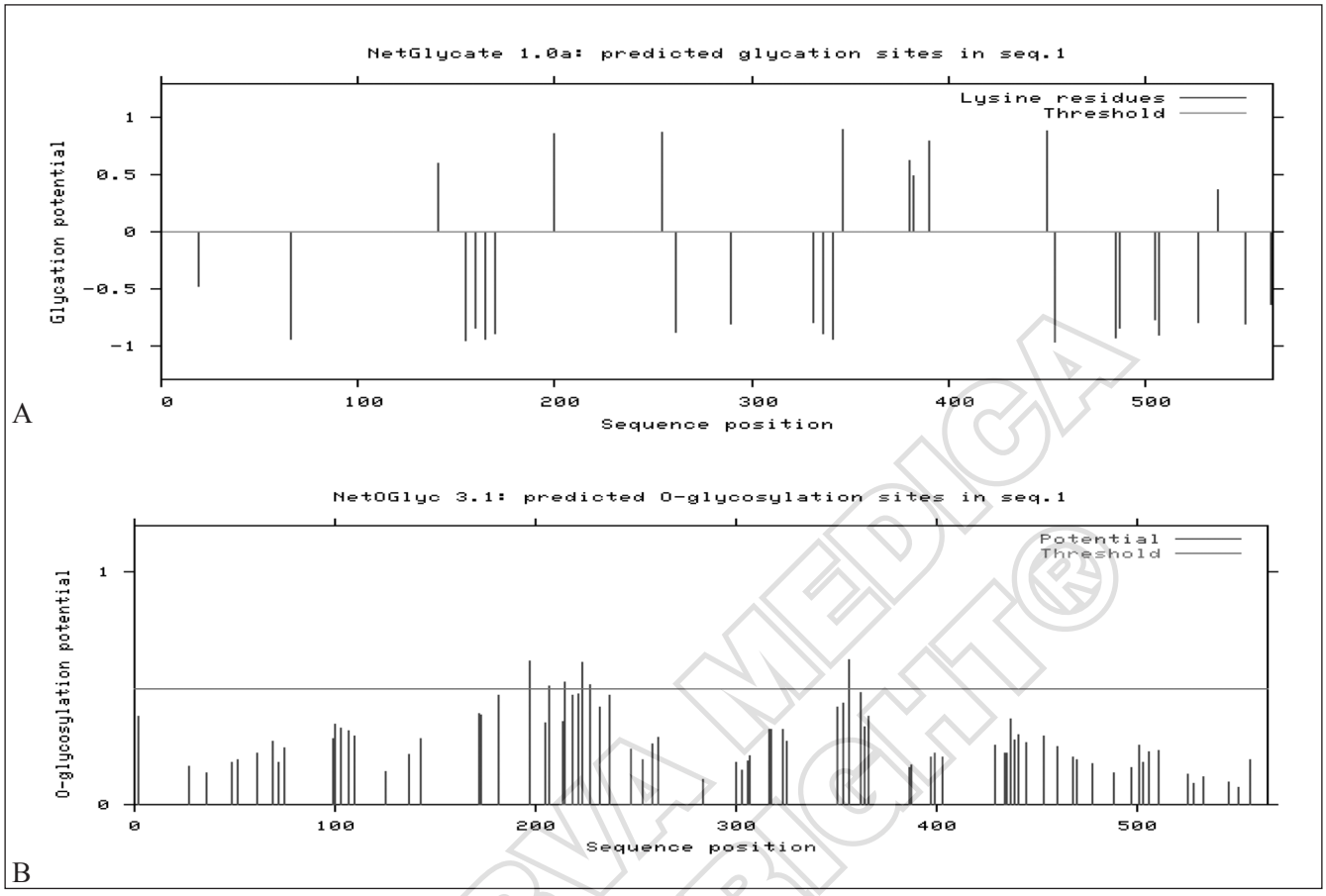

Figure 3.—Residues glycated in the chimeric constructs based on prediction of NetGlycate (A) and NetOGlycate (B). "Myristoylator" detected no site for N-terminal myristoylation. Based on prediction of "NetPhos' 26 sites undergo phosphorylation.

Post-translational modification predictions

NetGlycate predicted nine glycationsites on the chimeric protein (Figure 3A). NetNGlyc also predicted two asparagine amino acids at position 100 and 254 to be N-glycosylated. NetOGlycate predicted 6O-GalNAc (mucin type) glycosylation sites in mammalian proteins (Figure 3B). Myristoylator detected no site for N-terminal myristoylation. Based on prediction of NetPhos, 26 sites undergo phosphorylation.

Prediction of B-cell epitopes

Bcepred software was used to detect the linear B cell epitopes based on different features (Table II). Examination the 3D structure by Ellipro software showed four sets of discontinuous B-cell epitopes (Table II). According to SEPPA results, approximately 20% amino acids of the chimeric protein involved in discontinuous epitope formation.

Prediction of T-cell epitopes

Proteasome Cleavage Prediction Server (PCPS) predicted 58 proteasomal and 64 immune-proteasomal cleavage sites in the chimeric protein with a threshold of 0.6. The TAPPred results showed 23 and 142 peptides have high and intermediate binding affinity to TAP protein, respectively. The conserved peptide sequences with highest binding score to MHC class I and II predicted by different servers have been listed in Table III.

TABLE II.—*Continuous and discontinuous B-cell epitopes predicted in chimeric protein by various parameters based on Bcepred and Ellipro software.*

Continuous B-cell epitopes prediction	
Prediction parameters	Epitope positions
Hydrophilicity	49-56, 74-85, 98-105, 155-174, 196-205, 210-224, 260-266, 294-301, 305-311, 313-321, 326-342, 350-356, 386-399, 428-439, 448-454, 491-497, 503-509
Flexibility	96-102, 133-140, 166-175, 215-221, 247-253, 303-309, 311-317, 383-397, 430-437, 442-452, 500-507
Accessibility	47-54, 63-69, 97-110, 135-146, 155-177, 179-189, 198-216, 255-273, 305-317, 325-342, 350-356, 380-397, 415-421, 448-456, 466-473, 500-511, 517-540
Turns	518-526, 542-548
Exposed Surface	136-143, 388-396, 448-456
Polarity	4-14, 16-25, 65-77, 134-146, 155-171, 325-342, 378-396, 448-458, 549-555
Antigenic Propensity	16-22, 31-37, 39-45, 84-97, 115-126, 181-187, 286-295, 346-353, 396-402, 419-425, 526-532, 554-563

Discontinuous B-cell epitopes prediction			
N.	Residues	Number of Residues	Score
1	A:V349, A:S350, A:E351, A:P352, A:S353, A:A354, A:P355, A:T356, A:A357, A:A358, A:P359, A:V360, A:D361, A:T362, A:F363, A:S364, A:W365, A:T366, A:G367, A:G368, A:Y369, A:I370, A:G371, A:I372, A:F388, A:D389, A:K390, A:E391, A:D392, A:N393, A:E394, A:Q395, A:V396, A:F407, A:V408, A:G409, A:G410, A:V411, A:Q412, A:A413, A:G414, A:Y415, A:N416, A:W417, A:Q418, A:L419, A:D420, A:N421, A:G422, A:V423, A:V424, A:G426, A:V461, A:A463, A:R464, A:L465, A:G466, A:Y467, A:T468, A:A469, A:T470, A:E471, A:R472, A:L473, A:M474, A:V475, A:Y476, A:G477, A:T478, A:G479, A:G480, A:L481, A:L512, A:G513, A:A514, A:G515, A:A516, A:E517, A:Y518, A:A519, A:I520, A:N521, A:N522, A:N523, A:T525, A:L526, A:K527, A:S528, A:E529, A:L531, A:Y532, A:T556, A:V557, A:R558, A:V559, A:G560, A:L561, A:N562, A:Y563, A:K564, A:F565	101	0.770
2	A:A1, A:S2, A:L3, A:P4, A:G5, A:A6, A:E8, A:E9, A:R12, A:F41, A:G56, A:F57, A:V58, A:E59, A:D60, A:T61, A:L62, A:N63, A:A64, A:K66, A:T69, A:I70, A:H71, A:S72, A:F73, A:H74, A:T75, A:E76, A:G77, A:A78, A:G79, A:G80, A:G81, A:H82, A:I86, A:I87, A:R88, A:C90, A:Q91, A:Y92, A:P93, A:N94, A:V95, A:L96, A:P97, A:A98, A:S99, A:T100, A:N101, A:P102, A:T103, A:R104, A:M119, A:V120, A:C121, A:H122, A:H123, A:L124, A:S125, A:I128, A:P129, A:E130, A:D131, A:I132, A:A133, A:F134, A:A135, A:E136, A:S137, A:I139, A:R140, A:K141, A:E147, A:L150, A:E151, A:A153, A:A154, A:A157, A:A158, A:A159, A:K160, A:E161, A:A162, A:A163, A:A164, A:K165, A:E166, A:A167, A:A168, A:A169, A:K170, A:Q171, A:S172, A:S173, A:R174, A:L175, A:G176, A:L178, A:D179, A:N180, A:V181, A:S182, A:P183, A:P184, A:P185, A:P186, A:V198, A:K200, A:G201, A:N202, A:L203, A:D204, A:S205, A:P206, A:T207, A:Q208, A:F209, A:P210, A:N211, A:A212, A:P213, A:S214, A:A219, A:Q220, A:T223, A:Q224, A:V225, A:A226, A:S227, A:L228, A:P229, A:P230, A:A231, A:S232, A:A233, A:P234, A:D235, A:L236, A:T237, A:P238, A:G239, A:A240, A:V241, A:A242, A:G243, A:N246, A:A247, A:L249, A:G250, A:G251, A:Q252, A:S253, A:C274, A:P275, A:G276, A:E277, A:L278, A:A279, A:N280, A:L281, A:A282, A:S283, A:W284, A:A285, A:V286, A:N287, A:G288, A:K289, A:Q290, A:L291, A:V292, A:L293, A:Y294, A:D295, A:A296, A:N297, A:G298, A:G299, A:T300, A:V301, A:A302, A:S303, A:L304, A:S307, A:G308, A:G310, A:R311, A:F312, A:D313, A:G314	190	0.673
3	A:Q265, A:G266, A:A269, A:G270, A:P271, A:L272	6	0.582
4	A:D430, A:F431, A:Q432, A:G433, A:G449, A:K450, A:A451, A:E452, A:T453, A:K454, A:V455, A:E456, A:W457, A:F458	14	0.520

Codon optimization

GenScript optimized a series of factors that are essential to the efficiency of gene expression. We changed the codon usage bias in mouse and human as DNA vaccine by upgrading the codon adaptation index (CAI) from 0.70 to 0.84 and 0.72 to 0.86 respectively. The overall GC content of the mouse and human construct were reduced from 62.71 to 57.60% and 57.96% respectively, which may increase the overall mRNA stability from the synthetic gene. One CpG island was detected in both mouse and human constructs by EMBOSS CpGPlot.

mRNA secondary structure prediction

The minimum free energy for secondary structures formed by RNA molecules was also computed. All 13 structural elements obtained in this analysis revealed folding of the RNA construct. The results

TABLE III.—*Consensus MHC class I & II binding peptides predicted by different online servers.*

MHC Class I epitope prediction		
No	Epitope sequence	Position
1	ADHFDVQVA	38-46
2	FVEDTLNAF	57-65
3	TRPYTVNTI	103-111
4	AEHLDMLMV	112-120
5	SRLGNLDNV	173-181
6	NVSPPPPPA	180-188
7	TFSWTGGYI	362-370
8	KFKHPFSSF	380-388
9	KVEWFGTVR	454-462
10	TVRVGLNYK	556-564
MHC Class II epitope prediction		
No	Epitope sequence	Position
1	MVRAGACGL	10-18
2	IHTDTLNEG	47-55
3	LNAFKGRTIHSFHTEGA	62-78
4	IRVCQYPNVLPASTNPT	87-103
5	YTVNTIAE	106-113
6	MLMVCHHLS	117-125
7	IRKETIAAE	139-147
8	LVLYDAN	291-297
9	VVVSEPSAP	347-355
10	YIGINAGYA	369-377
11	QAGYNWQLD	412-420
12	QGSSVTGS	432-439
13	VEWFGTVRARLGYTATERL	455-473
14	VKSAFNLG	486-493
15	YAINNNWTLKS	518-528
16	YLYTDLGKR	530-538
17	VNFHTVRVGLN	552-562

showed the mRNA was stable enough for effective translation in the new hosts.

Discussion

Many efforts have been made for development of DNA and protein vaccines against brucellosis.[3-10] The components and administration route of the vaccine may affect its success. *B. abortus* Omp19 is a critical component of *Brucella* vaccine because the abrogation of its gene in the vaccine strain *B. abortus* S19 resulted in the loss of its protective potency in heifers.[65] Use of Omp19 has resulted in protection against three pathogen strains of *Brucella* in mice. Previous studies have shown recombinant *B. melitensis* Omp31 could be an appropriate vaccine candidate for the design of DNA vaccine against *B. melitensis* and *B. ovis*. In pathogenic bacteria such as *Brucella spp*, Urease is considered as a main virulence factor because its activity leads to bacterial survival in the acidic condition of the stomach.[27, 29, 66-68] In this study, we analyzed a novel construct of chimeric DNA vaccine containing *urease*, *omp19* and *omp31*genes. In the amino acid sequence of this construct two α -helix linkers (EAAAK repeats) were introduced between different domains. Our analysis showed these linkers have separated the three domains. Furthermore, the chimeric protein had acceptable stability. The results also indicated each of the three proteins and chimeric construct have good antigenicity.

Various distinct methods were used for B-cell epitope prediction.[69, 70] The results showed the chimeric protein have many linear and spatial B-cell epitopes. Based on prediction there are 10 consensus MHC class I binding regions. Additionally, the prediction shows that there are 17 consensus MHC class II binding regions in the chimeric protein sequence. Analysis of B cell and T cell epitope sites within the chimeric protein indicates that these epitopes are distributed in three different domains.

Expression of heterologous proteins is reduced due to existence of rare codons in gene structure. These rare codons can result in some errors in translation.[71] For avoiding this problem, codon optimization of the gene is essential. For high expression of the DNA vaccine in mouse and human, codon optimization was carried out on the chimeric gene. The mRNA stability at the 5' end has an effect on the translation efficiency of target gene. Existence of hairpin structures with low free energy in the 5' end results in high translation initiation efficiency.[72, 73] The analyses showed the optimized mRNAs have not any hairpin structures with high free energy in their 5' end.

Overall, different strategies have been employed to improve the efficiency of DNA vaccines. One of them is optimization of the translation initiation site for high protein production. In eukaryotic mRNA, the kozak sequence (5'GCCACCATGGC) surrounding the translation initiation site enhances the accuracy and efficiency of translation up to 10 fold.[74] Therefore, this consensus sequence was introduced at surrounding the initiation site.

Another strategy is codon optimization. This process results in more stimulation of the immune response.[75] CAI of mouse and human construct DNA vaccine after optimization was increased. Addition-

ally, there were not any stable stem loops in mRNA structure of these optimized genes.

Ensuring accurate termination of translation is very important. The ribosome passing from the stop codon in optimized gene may occur and result in disturbance in mRNA stability, size and folding of protein.[76] To avoid this problem, two stop codons were introduced at in the end of coding region. Un-methylated cytosine-phosphate-guanine (CpG) motifs in DNA vaccine construct or vector result in dimerization and activation of the Toll-like receptor 9. These sequences play an important role in induction of innate and acquired immunity.[77, 78] Our results show that both mouse and human DNA vaccine constructs have one CpG motif.

Another strategy for enhancing the DNA vaccine potency is fusion to heat shock proteins (HSPs). These proteins are strong stimulators of innate and antigen-specific immunity. HSP-antigen complexes are internalized by specific receptors on APCs.[79] Certain HSPs have key role on chaperoning antigen. Furthermore, these proteins have adjuvant property. These features intrigue researchers to use HSPs for vaccine development.[80] Previous study showed that using the polyhistidine in HSP-antigen complexes strongly induce the antigen-specific CTL responses compared with its protein vaccines.[80] So, addition of polyhistidine and HSP to the chimeric DNA vaccine is suggested.

Glycosylation may decrease antigenicity of the DNA vaccine product.[81, 82] According to predictions, the mouse and human constructs had few amino acids as targets for diverse glycosylation. Existence of myristoylation signal in N terminal enhances DNA vaccine efficiency.[83, 84] Since the mouse and human DNA vaccine constructs lack this signal, it may be introduced at the N terminal. Phosphorylation can lead to degradation of some proteins. Existence of various phosphorylation sites on DNA vaccine products enhances their degradation rate and subsequent presentation by MHC class I molecules.[85] The prediction showed that both the mouse and human constructs have many phosphorylation sites. Previous studies have indicated that the vaccination site and immunization method of DNA vaccine can affect the type of the immune response, for example the muscle and gene gun DNA immunization can boost Th1 and Th2 responses, respectively.[86] Since the main response against *Brucella* is Th1 responses, the muscle DNA immunization is suggested. Additionally, another promising DNA vaccine delivery vehicle is *Saccharomyces cerevisiae*. Recent study showed this organism can deliver DNA vaccine to murine macrophages and human dendritic cells with high efficiency.

This delivery method induced strong specific CTL responses.[87] Since CTL responses are crucial components for protection against *Brucella*, using the yeast *Saccharomyces cerevisiae* for delivery of the chimeric DNA vaccine may be a good strategy.

Conclusions

Our results shows that epitopes of the chimeric protein could induce both B-cell and T-cell mediated immune responses. The protein has proper stability, antigenicity and no allergenicity. The selection of administration route is a key factor for high efficacy of the DNA vaccine. In general, our data indicate this DNA vaccine can be an appropriate vaccine candidate against *Brucella spp*.

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