Immune-Informatic Analysis and Design of Peptide Vaccine From Multi-epitopes Against *Corynebacterium pseudotuberculosis*

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ABSTRACT: Caseous lymphadenitis (CLA) is a disease caused by *Corynebacterium pseudotuberculosis* bacteria that affects sheep and goats. The absence of a serologic diagnose is a factor that contributes for the disease dissemination, and due to the formation of granuloma, the treatment is very expensive. Therefore, prophylaxis is the approach with best cost-benefit relation; however, it still lacks an effective vaccine. In this sense, this work seeks to apply bioinformatic tools to design an effective vaccine against CLA, using CP40 protein as standard for the design of immunodominant epitopes, from which a total of 6 sequences were obtained, varying from 10 to 16 amino acid residues. The evaluation of different properties of the vaccines showed that the vaccine is a potent and nonallergenic antigen remaining stable in a wide range of temperatures. The initial tertiary structure of the vaccine was then predicted and a model selected. Later, the process of CP40 protein and TLR2 receptor binding was performed, presenting interaction with this receptor, which plays an important role in the activation of the immune response.

KEYWORDS: Caseous lymphadenitis, endoglycosidase, modeling three-dimensional, TLR2

Introduction

Corynebacterium pseudotuberculosis is a causal agent of many diseases that can affect species such as camels, buffalos, cows, and horses causing ulcerative lymphangitis. It causes human lymphadenitis, presenting variations from subacute to chronic[1,2] or even as the chronic caseous lymphadenitis (CLA) in sheep and goat.[3,4] *Corynebacterium* genus is part of the Actinomycetales order known as Actinobacteria, mainly gram positives. This order is composed of 50 species, some of which are pathogens for animals and plants. The CMNR group (*Corynebacterium, Mycobacterium, Nocardia,* and *Rhodococcus*)[5–7] is very heterogeneous, with different features, such as peculiar cell wall composed of mycolic acid and arabinogalactose and a high content of guanine and cytosine (47%-74%) in its genome.[8] Due to the wide variety of hosts, there are CLA cases reported in several countries including Australia, New Zealand, South Africa,[59–61] United States, Canada, and Brazil, especially in ruminants.[9–11] According to the World Health Organization, from the 201 countries that reported sanitary conditions, 64 reported animals with CLA.[12] The *C pseudotuberculosis* is classified as facultative intracellular and gram-positive bacteria. It has the ability to survive for long periods in the environment, and due to the formation of abscess in the host, the antibiotics used do not surpass this boundary, which leads to granuloma maturing and hatching. This is a fundamental feature for the contribution of the high transmission rates of the disease inside the cattle.[13]

Nowadays, there is no efficient prophylaxis for CLA. Vaccines commercialized are based on the attenuated microorganism from the 1002 lineages. Therefore, there is the need to develop new prophylactic products for this disease. In the attempt to ensure an efficient vaccine, there is the need to design a vaccine according to the virulence factors, which are expressed in several stages of the disease. Recently, there are 3 virulence factors thoroughly reported in literature, which are phospholipase D,[14] a component of the iron uptake and secreted toxins (FagB)[15] and a endoglycosidase.[16,17] However, efforts for the development of an efficient prophylaxis are still needed. To design an effective vaccine, it is important to seek different methodologies from those commonly used against CLA.[16,18–21] Genomics aided in new vaccine strategies. Sequencing of pathogen genomes made it possible to know in silico the most likely protective antigens prior to conducting experiments to prove them. This is called reverse vaccination. It was used for the first time to identify antigens as probable candidate vaccines against serogroup B meningococcus.[22] Besides these obvious advantages, such as speed and low cost, the success of this approach depends on the accuracy of antigen prediction and many bioinformatics tools are available to facilitate this process.[23]

This increase is due to the bioinformatic tools which have helped many researchers in several areas[24,25–29] especially in the identification of adequate vaccine candidates, immunologic

information, and presenting important and satisfying analysis.[30] The peptide vaccine design based on immunogenic information enables another important aspect of the reverse vaccinology, being considered an evolving technology, as they reduce the vaccine development time, which lowers their financial cost and eases its projection.[31]

The initial step on the design of synthetic peptides for vaccines is the identification of potential epitopes.[32] The identification of peptides with antigenic and immunogenic potential is performed by a bioinformatics prediction of epitopes from B and T cells.[27] Therefore, the purpose of this study was to design in silico a new vaccine with several combined epitopes able to induce B cells, leading to the humoral and cellular immune response against *C pseudotuberculosis*.

Methods

B-cell epitopes prediction

Initially, the endoglycosidase *Corynebacterium* (CP40)[17] (accession number GenBank NC_017730.1), as a component of the virulence factors of *C pseudotuberculosis* and presenting an immune profile previously reported,[16] was used to map epitopes of immunodominant B cells using BepiPred software[33] which is based on propensity scale methods (http://www.cbs.dtu.dk/services/BepiPred/). Furthermore, ABCPred, based on learning methods of the machine, applies recurrent neural network (http://www.imtech.res.in/raghava/abcpred), and BCPreds, which are also based on the learning ability of the machine, applies to support vector machines (http://ailab.cs.iastate.edu/bcpreds/) and the Immune Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org/). The epitopes were predicted in different major histocompatibility complex (MHC) class II alleles (Iab, Iad, Ias, Ied, and Ies).

Vaccine properties

Allergenic potential. To analyze allergenic of the selected targets, software AlgPred (http://www.imtech.res.in/raghava/algpred/index.html) was used. The server executes prediction based on 6 different approaches. Combinations of the 6 approaches can be used to predict the allergenic proteins with high precision.[34]

Antigenic potential. To verify antigenicity of the proteins and the peptides predicted by the tools, 2 software were applied. The VaxiJen[25] v2.0 software (available at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) works based on independent alignment prediction of protective antigens. This software was developed to allow antigen classification based solely on the physical-chemical properties of the proteins without considering the sequence alignment. The server precision varies from 70% to 89% based on the target organism.[35] The other tool used was ANTIGENpro (available at http://scratch.proteomics.ics.uci.edu/). The server is an independent free

alignment predictor based on the sequence of 5 pathogens, which predict antigenicity based on the results obtained from the protein data by microarray.

Physical-chemical analysis. Different physical-chemical properties for the protein vaccines, including molecular weight (MW), isoelectric point (IP), stability index, half-life in vitro and in vivo, aliphatic index, and grand average of hydropathicity (GRAVY) were estimated by the ProtParam server (available at http://web.expasy.org/protparam).[36]

Location of the protein-peptide signal

To verify the peptide signal and consequently locate the *C pseudotuberculosis*, the SignalP 4.1 software was used (available at http://www.expasy.org/proteomics). This tool contains 2 neural networks, where it is possible to select sequence options with or without transmembrane sets. All analyses were conducted with gram-positive bacteria using 2 neural networks.[37] A second subcellular location software, PSORTb 3.0.2 (available at http://www.expasy.org/proteomics) was used consisting in multiple analytical modules, each one analyzing a biologic resource known for influencing or being typical of subcellular location.[24]

Three dimensional design

For the determination of tridimensional protein structure, 2 software were used: the 3Dpro (http://scratch.proteomics.ics.uci.edu) and the Galaxy TBM (http://galaxy.seoklab.org/index.html). 3Dpro uses predicted structural features, and the APB knowledge is based on statistic data considering energy. The conformational research uses a set of movements consisting on fragment substitution (using a fragment library built from the Protein Data Bank [PDB]), as well as random disturbance for the model. The Galaxy TBM develops modeling based on 2 stages. On the first stage, the more reliable structures are created selecting models that are aligned with the PROMALS3D and MODELLERCSA models. In the second stage, the loop areas or less reliable areas (called ULR, nonreliable local areas) are detected and remodeled using a refining method based on optimizations.[38]

Tertiary structure refinement. The refinement process was performed using a selected model by the 3Dpro and introduced in the server GalaxyRefine (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) to refine the entire protein, using the mild and aggressive relaxation methods.[39]

3-dimensional structure validation. To identify the possible error in the 3-dimensional (3D) structures predicted, the ProSA-web server and the Ramachandran validation were used. The ProSA-web calculates the general quality score for a 3D structure. If the calculated scores are outside the natural

protein range, an error in the predicted structure is indicated.[40] The Ramachandran validation is based on estimation of torsion angles for each residue in the structure, and these residues are classified as favorable, allowed, and isolated.[41]

Protein and TLR2 receptor binding

Exploring the interaction between protein and TLR2 receptor. To verify the anchoring between the protein and the toll-like receptor type 2 (TLR2) the SwarmDock software (http://bmm.crick.ac.uk/~SwarmDock/index.html) was used. This software uses a flexible protein-protein complex model. The server is divided into the following stages:

1. Entrance structure minimization and preprocessing.
2. Docking using a hybrid particle swam optimization/local research.
3. Minimization, reclassification, and anchored pose grouping.

The preprocessing stage involves the disordered bond mending, modeling absent atoms, posttransduction modifications, and using the CHARMM mechanics after minimizing the entrance structures.[42]

Exploring the interaction between peptide and TLR2 receptor. Seeking to verify the interaction between the predicted peptides and the design of an efficient vaccine, it was necessary to identify the peptides that present interactions with the TLR2 receptor. Therefore, the GalaxyPepDock was used, which predicts the protein-peptide complex structures and uses the 3D structure of the receptor and the peptide sequence combining information of the similar interactions found in the structure and optimization data based on energy (Figure 1).[43]

Results

Epitope determination

The sequences in the mapping indicate which epitopes from CP40 have a higher probability to interact with the antibodies, and the results showed 184 epitopes found for CP40. From these results, analyses were conducted to verify overlapping. Thus, only sequences present in the 3 different software analyses were used. In all software, 10 to 16 amino acid residues were chosen to compose the peptide because the MHC class II gap holds 10 to 30 amino acid residues.[44] Table 1 shows the immunodominant epitopes from CP40.

Vaccine properties

Allergenic potential. Vaccine was submitted to the AlgPred to verify the presence of binding sites for immunoglobulin E (IgE) and the possibility of allergic reactions. According to the AlgPred, the vaccine did not present IgE-binding sites being classified as nonallergenic.

Antigenic potential. Protein antigenicity was 0.5703% with VaxiJen 0.4% using a bacteria model, as for the AntigenPRO, a 0.951541% antigenicity was found. However, all sequences obtained in silico were submitted to VaxiJen at 0.4% and to AntigenPRO seeking to verify their antigenicity (Table 2). All results demonstrated antigenic potential according to the software used.

Physical-chemical analysis. The ProtParam server was used to verify some parameters. These parameters were MW and theoretic IP, which were 42 kDa and 6.48, respectively. The IP is the point where total liquid charge of the amino acid or protein molecule is 0, based on constant equilibrium points. Thus, the value 6.48 demonstrates that the protein is stable in pH 6.48. The total negative amino acid residues were of 55 (Asp + Glu) and the total positive amino acid residues were 53 (Arg + Lys). The total number of atoms was 5.997 with the chemical formula $C_{190}8H_{2967}N_{525}O_{588}S_9$. The vaccine half-life was predicted for 30 hours in mammal reticulocytes, in vitro; 20 hours in yeast, in vivo; and 10 hours in *Escherichia coli*, in vivo. The stability index was estimated as 21.20, which indicates that the vaccine is stable. The aliphatic index and GRAVY value from the vaccine construction were 68.94 and 0.692, respectively. The high aliphatic index indicates the protein stability in several temperatures, whereas the negative GRAVY values show that the protein is hydrophilic and presents strong interactions with water molecules.

Location of the protein-peptide signal

To determine the location of the protein, the SignalP-4.1 was used, indicating its position as transmembrane. This result is important as the success of a protein vaccine depends on the nature of the antigen and it needs to belong to the important proteins from the infection process. Also, a transmembrane protein is considered as a virulence factor due to the contact existing with the immunologic system. According to Shadnezhad et al,[17] the protein studied is classified as a endoglycosidase, which are enzymes that catalyze the hydrolysis of glycosidic binding inside the glycan chains.

Three dimensional design

The tridimensional structure of the vaccine was predicted with the 3DPro and Galaxy TBM software (Figure 2). A total of 6 models were obtained and they all were compared using the ProSA-web and Ramachandran analysis. The model with highest *z* score –5.26 (Figure 3) was predicted by the Galaxy TBM. According to Ramachandran analysis, this model presented 95.2% of amino acid residue in a favorable region. For this work, Galaxy TBM predicted the best 3D model, which was submitted to refining.
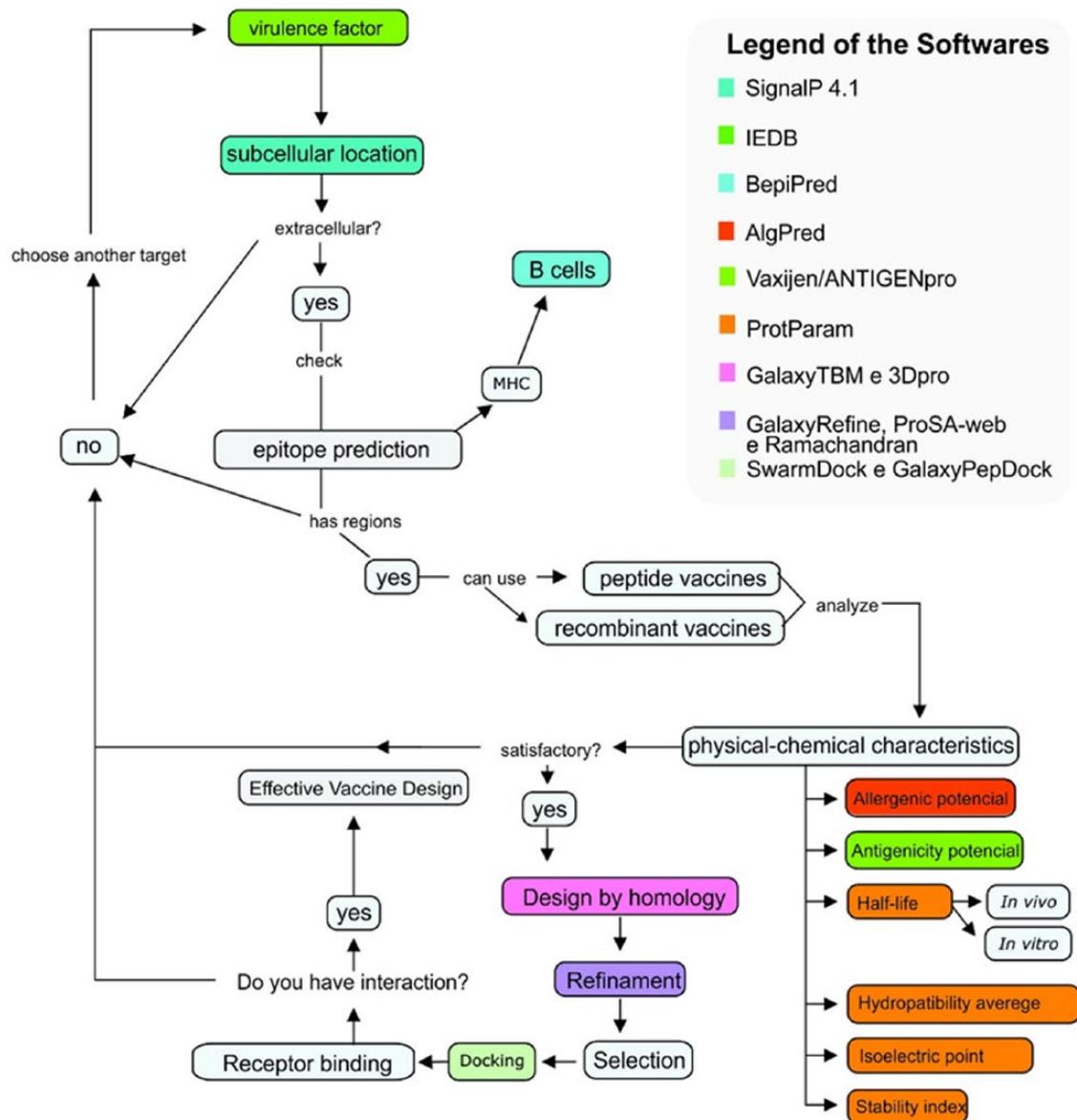
Figure 1. Schematic flow diagram for the screening of caseous lymphadenitis (CLA) vaccine candidate from the *Corynebacterium pseudotuberculosis* protein.

Table 1. Amino acid sequence (aa) of the immunodominant epitopes selected in silico.

	EPITOPES
CP40 protein	DRDGRTYDGDDFTT
	YKKDTKESVTQVWN
	AESATLSKEPLKASPG
	YKKDTKESVT
	ETFHREYQPELKKRGT
	AIELTTGESSTDLGKP

Refining and validation of the tertiary structure. The quality and potential errors of the design were calculated using ProSA-web.

The z score from the initial entrance model was –5.26, which is typically seen in native proteins found with similar sizes. The results obtained from the ProSA-web demonstrated that the initial model requires refining. Therefore, the initial model was submitted to refining with GalaxyRefine software. After all refinement procedures, the z score initial model reached –5.28 (Figure 4A). Furthermore, the Ramachandran analyses were performed before and after refining. In the initial model, 95.2% of the residues were in favorable regions. After refining, the residues in favorable regions increased to 96.6% (Figure 4B).

Protein and receptor TLR2 binding

Exploring the interaction between protein and TLR2 receptor. After obtaining the tridimensional structure of the CP40 protein, it was possible to use the SwarmDock software and verify

the interactions between the proteins and the TLR2 receptor. In Figure 5, it is possible to observe that there is interaction with the CP40 protein and the TLR2 receptor.

Exploring the interaction between peptide and TLR2 receptor. To verify whether or not the peptides predicted in this research have interacted with the TLR2 receptor, the GalaxyPepDock software was used. Then it showed that all peptides presented interaction with the TLR2. Figure 6 shows the binding of peptide AESATLSKEPLKASPG with the receptor.

Discussion

Corynebacterium pseudotuberculosis causes many diseases and therefore leads to several economical losses in Brazil and worldwide, especially in the sheep and goat growth caused by CLA.[45,46] The absence of serologic diagnosis of the disease contributes for its dissemination, and due to formation of granuloma, the treatment is very costly.[18] Thus, prophylaxis is the best cost-benefit strategy; however, an efficient vaccine able to protect the animals from this disease is still required. In this sense, several vaccine strategies have been tested applying purified toxins,[47] recombining proteins,[16,20,45,48] genetically modified

Table 2. Antigenicity determination by VaxiJen and AntigenPRO from the peptide sequence obtained in the in silico prediction.

EPITOPES	VAXIJEN AT 0.4%	ANTIGENPRO
DRDGRTYDGDDFTT	1.5316	0.4549
YKKDTKESVTQVWN	0.6637	0.3029
AESATLSKEPLKASPG	0.9483	0.5576
YKKDTKESVT	1.2187	0.0920
ETFHREYQPELKKRGT	0.5928	0.1591
AIELTTGESSTDLGKP	0.6488	0.5440

microorganisms,[19,20,49] and DNA vaccines,[18,46,50] presenting unsatisfactory results. Therefore, it is necessary to use distinct vaccine composition with the use of synthetic peptides based on the emergence of immunodominant epitopes.[33] The use of peptides is wide and growing due to the advent of bioinformatics, which enables the determination of epitopes from software presenting safe and promising results.[51,52] The immunodominant epitopes are specific regions with protein antigens that are bonded with immunologic receptors.[62,64] Thus, their use as vaccine and therapeutic composition has been widely used.[51,53–55]

The database of epitopes consists of a collection of epitopes from pathogens such as virus, bacteria, protozoa, and fungus.[29] These databases have become a commonly used information source in the search of antigen for the vaccines, diagnosis, and as immunotherapies.[52] However, the search needs to be totally directed to the type of immunologic response focused. The efficient response against *C pseudotuberculosis* is a T_H1[63] response, and therefore the epitopes from MHC class II for B cells are considered relevant. Therefore, this work had a purpose to design peptide vaccines against *C pseudotuberculosis* that were able to activate the immune system and in that sense immunoinformatics was used as support. Searching the antigen to formulate the vaccine, it is necessary to understand the pathogen and their virulence factors responsible for the host infection. The CP40 protein, classified as endoglycosidase, is part of the pathogen group of the *C pseudotuberculosis* and its immunogenic character was evaluated.[16,20,56] Therefore, the protein was the standard for the immunodominant design of the epitopes, which were obtained as 6 sequences between 10 and 16 amino acid residues, ideal size for bonding in the MHC II molecule gap. The choice of the MHC II epitope prediction is due to the direction of the immune response, which will lead to TCD4+ lymphocytes activation, important for the activation of T and B lymphocytes.[55]
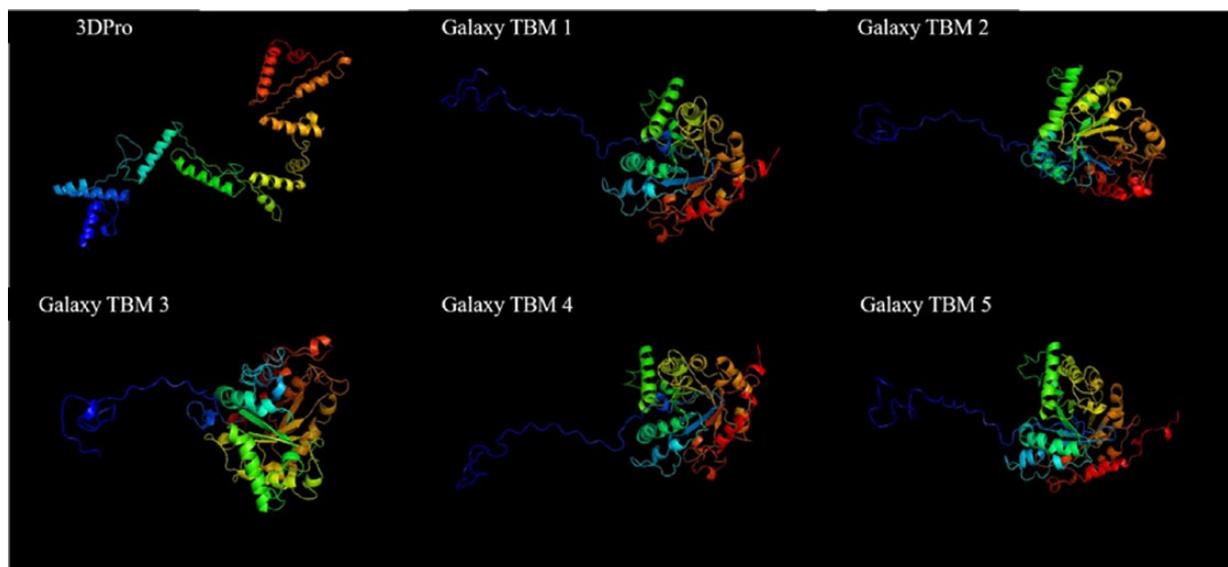

Figure 2. Modeling three-dimensional using 2 software: 3DPRo and Galaxy TBM.

The evaluations of different vaccine properties show that the vaccine is a potent and nonallergenic antigen with high aliphatic index, which shows its stability in a wide range of temperatures. VaxiJen was the software used to verify the vaccines' antigenicity and the z descriptors obtained indicate the main physical-chemical properties important for the recognition of antigens. The z value presents a threshold of 0.4.[25] Before this threshold and the obtained data, the antigenicity of all the vaccines explored in this work is verified.

It was possible to determine the cell location, where the transmembrane region is important due to the region being in contact with the external medium and defense cell. The initial
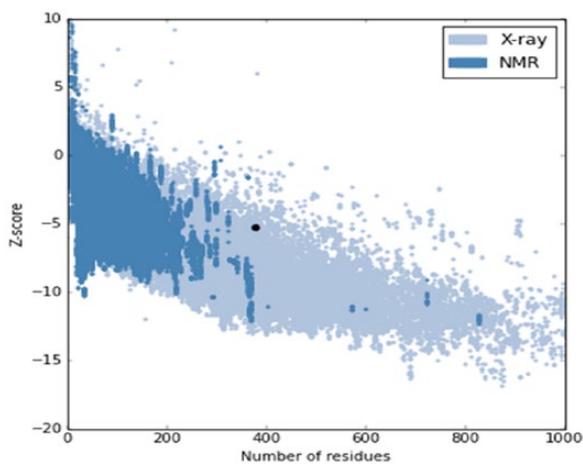
tertiary structure of the vaccine was predicted and the best model selected. A total of 6 models were predicted, one by 3DPro, whereas the remaining by Galaxy TBM. This makes the prediction in 2 steps. The first step is done using the models obtained by the software PROMALS3D and MODELLERCSA. These in turn have a pattern of 0.6, which indicates 60% of similarity between the sequences analyzed for modeling. In the second step, the less reliable regions called URL are detected and remodeled using an optimization-based refinement method. After the analyses, a table is generated giving the information of the Global Distance Test (GDT), which is most commonly used to compare the results of the prediction of protein structure with the experimentally determined structure, measured by determined models determined through x-ray crystallography or by nuclear magnetic resonance (NMR). All models predicted by Galaxy TBM presented GDT-HA between 0.8 and 0.9, which shows a great similarity between the sequence and its predicted model. Then, all models were evaluated using ProSA-web and Ramachandran analysis, which indicated that the model needs additional refining. Even with high identity prediction modeling obtained, the side chain structures may be less accurate than the backbone structure. Although predictions of the ab initio protein structure from primary sequences are difficult, refinement from an initial template structure tends to be easier. A successful refinement can provide more precise structures for functional study, molecular design, or experimental structure determination. Thus, GalaxyRefine was used, which primarily rebuilds all the side chain conformations and repeatedly relaxes the structure by simulations; these relaxations are gentle and/or aggressive. This method can improve the quality and accuracy of the global and local structure, as well as the physical correction in 59%, 67%, and 79% of the targets submitted to the refinement, using the
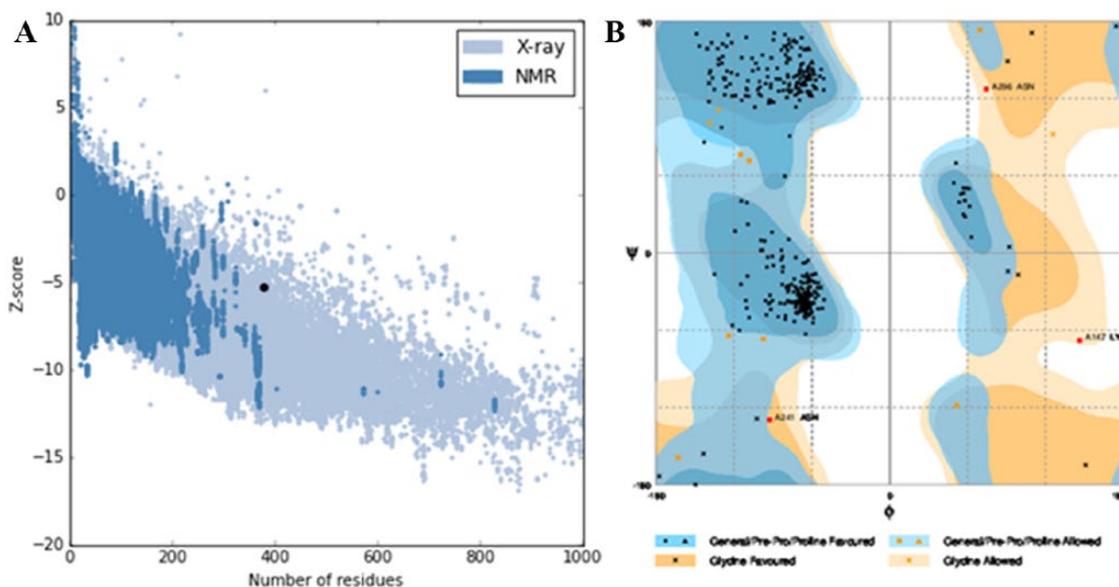

Figure 3. ProSA-web z score plot for the CP40 3D structure. The z score of the model is −5.26, which is considered in the conformational parameters of native proteins. The z score for the protein vaccine is shown with a black dot. The ProSA-web analysis consists of data from all the protein experimental chains in the PDB determined by nuclear magnetic resonance (NMR) (dark blue) and x-ray crystallography (light blue).

Figure 4. Tertiary structure validation after refining, using ProSA-web and the Ramachandran data. (A) The ProSA-web result that reveals a z score of −5.28; (B) Ramachandran data which reveals the amount of amino acid residue in favorable region increased to 96.6%.
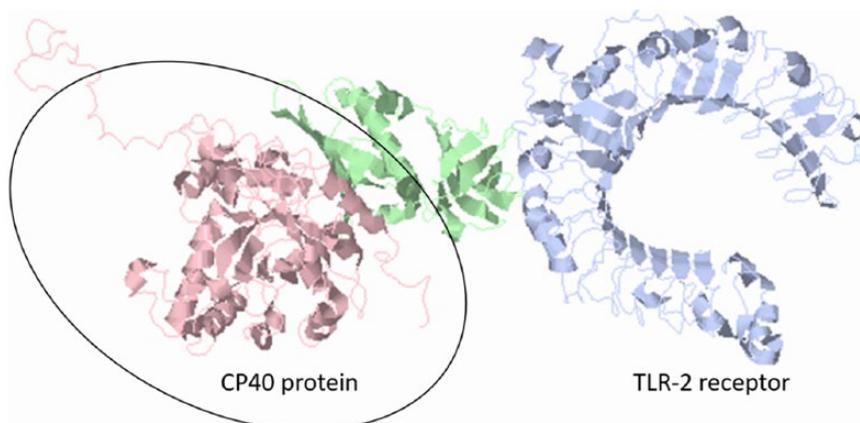
Figure 5. Interaction between CP40 and TLR2 receptor using SwarmDock software.
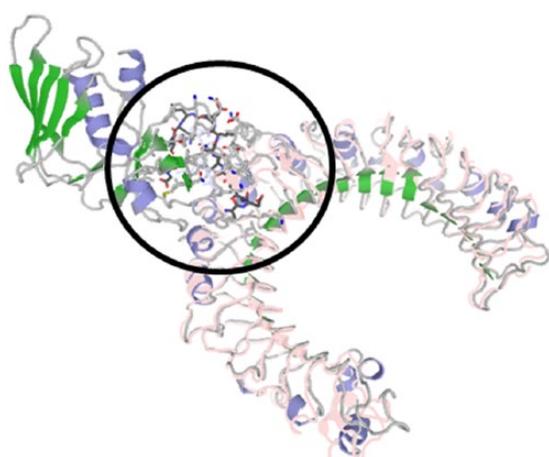

Figure 6. Interaction of AESATLSKEPLKASPG peptide with the TLR2 receptor using GalaxyPepDock software.

parameters determined by GDT-HA, GDC-SC, and MolProbity score.[39] After the refinement process, 5 models were provided, each presenting the parameters previously described. The first is made from smooth relaxation as the 4 additional models generated result in larger changes to compare with the structure of the initial model, which was made more aggressive relaxation. Both methods are done by molecular dynamics, with sequential repetitions of these relaxations. After obtaining the 5 models predicted by GalaxyRefine, all were submitted to ProSA-web and Ramachandran diagram.

The scores determined by ProSA-web compare the tridimensional models obtained with existing models predicted by x-ray or NMR and verify the probability of mistakes that may exist in these predictions. The quality index calculated by ProSA-web for a specific input structure is exhibited on a graphic that shows the scores of all experimentally determined protein chains and is currently available at PDB. This feature correlates the punctuation of a specific model with *scores* calculated from the all experimental structures deposited in PDB.[40] The *z scores* obtained in this study indicate that the predicted structures are located in the range of native

conformations, assisting in the choice of the best model, either the tridimensional structure or after refinement. The first selected model presented a *z* score of –5.26 and the *z* score changed to –5.28 after refinement, showing that after refinement the structure remains within the native conformations. This negative *z* score is a reflection of the amino acid residues, only presenting the N-terminal region with a positive character.

Then, the fitting process between the CP40 protein and the TLR2 receptor was performed. This showed that although the protein is a hydrophilic molecule, the same presents hydrophobic areas that are responsible for the interaction with the TLR2 receptor.[57] The same was predicted for all epitopes designed. The toll-like receptors (TLRs) are widely studied and found in the antigen-presenting cells (APCs).[58] With the interaction of vaccines and this receptor, it is possible to be certain that the vaccinal antigen will be recognized by the APC-specific receptors and these, after activation, will promote immune, innate responses and activate the adaptive immune response. Therefore, in this study, the hydrophobic areas of the CP40 protein and their respective immunodominant epitopes interact with TLR2, which plays an important role in the activation of the immune response.

Conclusions

In the past years, the peptide vaccine has gained attention due to its several advantages. In this work, several bioinformatic tools available were used to design efficient immunodominant epitopes for the formation of a peptide vaccine, seeking to activate the immune, innate, cellular, and humoral responses. After the determination of these epitopes, all were considered nonallergenic and with antigenic potential. The physical-chemical analyses presented relevant data on the stability of the 3 biologic systems proposed. When verifying the signal peptide for CP40 protein used as peptide standard, it was possible to confirm what is stated in the literature, that this protein is expressed and exported to the environment, being part of the virulence factors of *C pseudotuberculosis*.

By projecting the tridimensional structure of CP40 in silico, it was possible to verify an interaction with an important group of receptors, the TLRs, which are responsible for the activation of the immune response. In this context, it was possible to highlight the importance of bioinformatic software in the design of vaccines, which help to directly obtain immunodominant peptides, easing the design of modern vaccines with immune protective potential. Therefore, it is necessary to synthetize these peptides indicated by these tools as a manner to reinforce the importance of this area for vaccine studies, both for veterinary and human diseases.

Author Contributions

FFP conceived the idea for the project. DD-A performed most of the experiments, analyzed the results, and wrote the bulk of the paper. EF and FFP analyzed the results and wrote the article. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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