Research article

Genome-level therapeutic targets identification and chimeric Vaccine designing against the *Blastomyces dermatitidis*

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A R T I C L E   I N F O

A B S T R A C T

Blastomyces dermatitidis is a thermally dimorphic fungus that can cause serious and sometimes fatal infections, including blastomycosis. After spore inhalation, a pulmonary infection develops, which can be asymptomatic and have lethal effects, such as acute respiratory distress syndrome. Its most common extra-pulmonary sites are the central nervous system, bones, skin, and genito-urinary systems. Currently, no vaccine has been approved by the FDA to prevent this infection. In the study, a peptide-based vaccine was developed against blastomycosis by using subtractive proteomics and reverse vaccinology approaches. It focuses on mining the whole genome of B. dermatitidis, identifying potential therapeutic targets, and pinpointing potential epitopes for both B- and T-cells that are immunogenic, non-allergenic, non-toxic, and highly antigenic. Multi-epitope constructs were generated by incorporating appropriate linker sequences. A linker (EAAAK) was also added to incorporate an adjuvant sequence to increase immunological potential. The addition of adjuvants and linkers ultimately resulted in the formation of a vaccine construct in which the number of amino acids was 243 and the molecular weight was 26.18 kDa. The designed antigenic and non-allergenic vaccine constructs showed suitable physicochemical properties. The vaccine's structures were predicted, and further analysis verified their interactions with the human TLR-4 receptor through protein-protein docking. Additionally, MD simulation showed a potent interaction between prioritized vaccine-receptor complexes. Immune

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simulation predicted that the final vaccine injections resulted in significant immune responses for the T- and B-cell immune responses. Moreover, in silico cloning ensured a high expression possibility of the lead vaccine in the E. coli (K12) vector. This study offers an initiative for the development of effective vaccines against B. dermatitidis; however, it is necessary to validate the designed vaccine's immunogenicity experimentally.

1. Introduction

Blastomyces dermatitidis (*B. dermatitidis)*, the pivotal factor of blastomycosis, is a dimorphic fungus thermally that arises as filamentous molds in the environment at 25 °C but as yeasts in the tissues of the human body at body temperature (37 °C) [1,2]. It belongs to the genus Blastomyces and the family Ajellomycetaceae [1]. It grows in moist, acidic soil, especially in densely cultivated regions near water sources such as rivers, etc [3]. PCR technology has successfully identified *B. dermatitidis* in expected ecological niches, such as vegetation, dense woodland with moist soil, or animal tissues that are exposed to these environments [2]. When moist soil or decaying matter that contains micro-foci of *B. dermatitidis* mycelia is disrupted, pathogenic spores are released and breathed in by sensitive individuals [4]. Upon spore inhalation into the lungs*, B. dermatitidis* converts into pathogenic yeast, allowing the spores to avoid host immune defenses, which can result in acute respiratory distress syndrome (ARDS), ultimately caused by a pulmonary infection [5].

Its most common extra-pulmonary sites are the central nervous system, bones, skin, and genitourinary systems. *B. dermatitidis* is commonly found in the Eastern, South Eastern, Central, and US Great Lakes regions. Since *B. dermatitidis* is caused by spores, blastomycosis cases are found worldwide, from the Mississippi and Ohio River valleys [6] to Africa, India, South America, China, and the Middle East. Currently, blastomycosis is reported in five US states: Wisconsin, Minnesota, Michigan, Louisiana, and Arkansas [7]. *B. dermatitidis* also infects immunocompetent individuals, but they typically experience less illness and can be fully recovered [8]. However, adult men, mainly males, are more likely to develop this systemic infection due to occupational exposure. Patients with weak immune systems, such as those with AIDS, solid organ transplants, and hematologic malignancies, have a higher risk of developing severe disease, pneumonia, and death from blastomycosis [1,7].

Other mammals, particularly dogs, have a 10-fold higher incidence rate of *B dermatitidis* than humans, making them potential hosts [9]. *B. dermatitidis* was separated from the soil of a single riverbank during an investigation of two blastomycosis outbreaks in Central Wisconsin countries in the summer of 1985. It was found that riverbanks are natural habitat for *B. dermatitidis, and* the environment around waterways is the major source of its transmission [10,11].

The primary signs of symptomatic pulmonary blastomycosis are a slow, chronic process: dyspnea, chest pain, low-grade fever, weight loss, cough, and fatigue. At this stage, patients are less likely to develop an acute pulmonary infection. However, pulmonary blastomycosis can cause sub-clinical pneumonia and ARDS in their severe forms. Rapid ARDS has a fatality rate of 50 %, which can be even higher if the diagnosis is delayed [12].

An estimated 25–40 % of individuals who develop symptomatic infection develop extra-pulmonary disease, which is usually caused by pulmonary disease. Extra-pulmonary sites commonly affected by disseminated disease include the genitourinary system (less than 10 %), bones (5–25 %), and skin (40–80 %). Extra-pulmonary symptoms may include subcutaneous nodules on the skin, joint pain and arthritis in the bones, prostatitis in the genitourinary tract, and meningitis in the CNS [7]. Infection of the CNS is uncommon, accounting for 5–10 % of disseminated disease cases, with populations containing people with weak immune systems being more vulnerable [3,13].

Currently, there are no effective treatments available to treat the infections caused by this fungus. Amphotericin B and itraconazole are somewhat effective, but these drugs can cause serious side effects [4,14]. Amphotericin B proved to be the first-line treatment for the patients with severe, fatal CNS involvement or immunocompromised blastomycosis, but it showed adverse reactions such as side effects related to infusion, reduced rate of glomerular filtration, hypomagnesaemia, anemia, and hypokalemia [12,15]. Moreover, itraconazole and other azoles [16] are widely used for effective treatment against blastomycosis, but these drugs are effective only for immunocompetent patients without CNS disease [4]. Azole antifungal drugs can cause hepatic toxicity, typically resulting in asymptomatic elevated aminotransferases and gastrointestinal upset, including nausea, vomiting, and diarrhea [12,17]. Furthermore, vaccination has the potential to help minimize the growing frequency of fungal diseases in immunocompromised patients by inducing CD8$^+$ T cells (Tc 17 cells) to persist and provide resistance to *B. dermatitidis*, even without CD4$^+$ T-cell assistance [18]. But there is no effective and licensed vaccine to prevent blastomycosis [4]. Evaluating thousands of macromolecules and conducting *in vivo* tests in a wet lab is time-consuming and costly for vaccine development. Computational biology and bioinformatics analysis have made significant progress, reducing time and costs by utilizing various approaches to discover vaccine candidates, new drug targets, understand interactions among the host and the pathogen, design drugs that are structure-based, and conduct genome-based comparisons while reducing the need for traditional laboratory experiments [19,20].

In this study, we applied immunoinformatics strategies to construct peptide vaccines against *B. dermatitidis* by using B- and T-cell epitopes, linkers, and adjuvants. The developed constructs with negligible toxicity and allergenicity may provoke a potent immune response. The developed vaccine was efficiently reverse translated and expressed in *E. coli* (K12 strain) through *in-silico* cloning. This research aims to develop an effective, novel, and dynamic immunizations against *B. dermatitidis* by utilizing an exclusive blend of the fungus's antigenic peptides through specific immunoinformatics tools.

2. Material and methods

Subtractive proteomics and reverse vaccinology strategies were used to determine potential therapeutic targets for *B. dermatitidis* and construct an immunogenic vaccine. The methodology followed for this study is given in Fig. 1.

2.1. Retrieval of core proteome & host non-homologous proteins identification

The entire proteome of *B. dermatitidisER-3* was retrieved from the NCBI database, a valuable resource for biological information, as well as the human proteome from the UNIPROT database, a collection of protein sequences and their annotations. The proteins with a significant similarity with the proteins of humans were excluded from the *Blastomyces* proteome using the BLASTp [21] by implementing the thresholds of query coverage <35 %, E-value > 1e-20, sequence identity <35 %, and bit-score <100 [22,23]. The resulting non-homologous proteins were subjected to downstream analysis.

2.2. Determination of essential and host-pathogen interacting proteins

Proteins required for fundamental cellular processes such as transcription, translation, metabolism, and DNA replication are encoded by essential genes. In the context of drug development or vaccine target identification, essential genes are especially intriguing because inhibiting or changing their function may result in the disruption of crucial cellular processes, which may ultimately lead to the elimination of infections or the treatment of diseases [24]. Genes necessary for the pathogen's survival are listed in the DEG (Database of Essential Genes). Experimentally verified essential genes from bacteria, archaea, and eukaryotes are included in the DEG library. DEG consists of proteins responsible for an organism's survival [24]. Non-essential proteins of *B. dermatitidis* were excluded, while those with sequence similarity to DEG essential proteins were studied further. The HP-PPI database was used for screening due to the importance of the host-pathogen interaction proteins for the infectious pathogenic organism to survive within the host system [25]. To determine the essential proteins of *B. dermatitidis* and those involved in the interaction of the host and pathogen, the proteins from the previous step were analyzed using BLASTp by using query coverage ≥50 %, sequence identity ≥50 %, bit-score ≥100, and E-value < 1e-4 against the Database of Essential Genes (DEG) and Host-Pathogen Protein-Protein Interaction (HPPPI) databases, respectively. The prioritized protein targets were determined through a combination of findings from these analyses.

2.3. Analysis of metabolic pathways

The metabolic pathways of the proteins from the previous analysis were determined using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [26]. The KO (KEGG Orthology) numbers were assigned to these proteins by the KAAS server (KEGG Automatic Annotation Server), which annotates the functions of the genes via metabolic pathways [27]. The host and pathogen pathways were manually compared, which led to the identification of the unique pathogen pathways and the host-pathogen common pathways. Moreover, the proteins associated with pathogen-unique pathways were identified, and their FASTA sequences were downloaded from the NCBI database for further analysis [28].
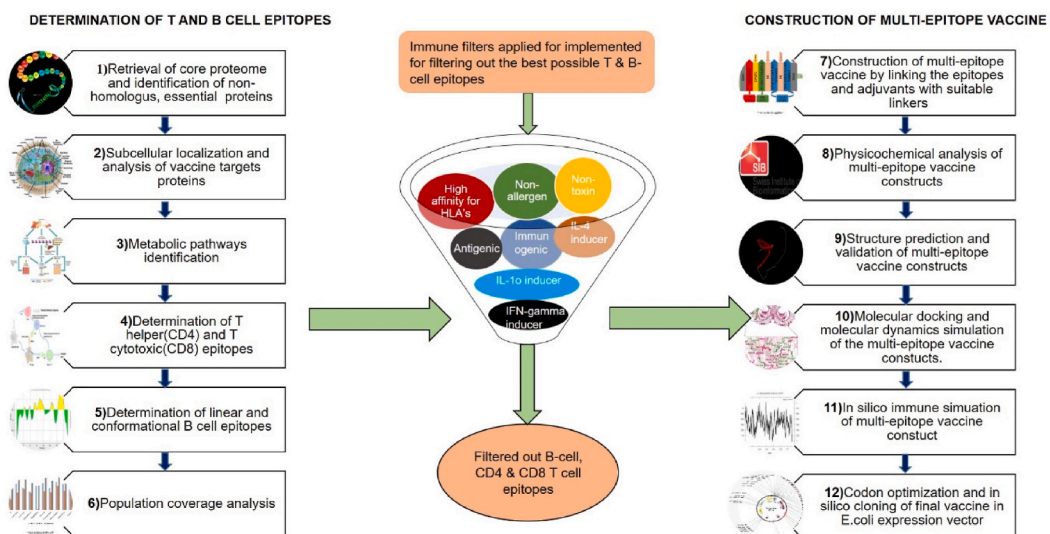

Fig. 1. Workflow followed for the development of a potential peptide vaccine against *B. dermatitidis*.

2.4. Subcellular localization prediction

A specific location is necessary for all proteins to perform their optimum function [29]. Recognizing the roles that proteins play in cells requires an understanding of their subcellular locations. The subcellular localization of the Kegg-dependent and independent proteins was determined by using three tools: BUSCA [30], Cello v.2.5 [31], and WOLF PSORT [32]. Identification of the subcellular localization is important to identify potential drug and vaccine targets [33].

2.5. Vaccine candidate selection

The predicted vaccine targets were analyzed for their ability to be used as vaccine candidates. For this purpose, the VaxiJen v2.0 server [34] was used to identify their antigenicity, while the Allertop server [35] was used to determine their allergenic profile. Furthermore, the physicochemical properties, topology values, and water solubility of these proteins were determined using the ProtParamExpasy, TMHMM [36], and SolPro [37] servers, respectively. The non-allergenic and higher antigenic proteins were chosen as the vaccine candidates.

2.6. Determination and analysis of T cell epitopes

T-cells are made up of MHC (Major Histocompatibility Complex) I and II immune cells. Antigens have the smallest immunogenic peptides called epitopes, which are able to provoke either HTL (Helper T lymphocytes) or CTL (Cytotoxic T lymphocytes) cells [38]. CTL epitope prediction is important for developing a potential vaccine [39]. CTLs have the potential to stimulate cell-mediated immunity and memory cells [40], which detect and reduce the antigen with the assistance of helper T lymphocytes [41]. The IEDB (Immune Epitope Database) MHC-I binding epitope is an estimation tool used to determine epitopes of MHC-I. Multiple epitopes ranging from 9 to 10 residues were picked [42]. An epitope selected for vaccine construction must be non-allergenic, antigenic, immunogenic, and non-toxin. VaxiJen v2.0 [34] was used to assess the antigenic properties of the predicted epitopes. Immunogenicity was determined by using the IEDB MHC-I immunogenicity tool. Toxicity and the allergenicity of the epitopes were determined through the Toxinpred server [43] and the AllerTOP v.2 server [35], respectively. Helper T-cells are important in all adaptive immune responses because they produce antibodies and help macrophages engulf and absorb pathogens [44]. HTLepitopes were identified using the IEDB server [45] and the epitopes of the lowest rank were selected and analyzed for their antigenic, non-allergenic, and non-toxin properties. Furthermore, HTL epitopes are important because they produce cytokines like interferon-gamma (IFN-), interleukin-10 (IL-10), and interleukin-4 (IL-4), which stimulate T_c and other immune cells [46]. Therefore, the IFN epitope server [47], IL-4 pred [48], and IL-10 pred [49] servers were used to find the IFN-gamma, IL-4, and IL-10 inducing characteristics of the preferred epitopes, respectively [46].

2.7. B-cell epitopes prediction

During the development of multi-epitope vaccines, B-cell epitopes play critical roles in immunological reactions induced by antibodies [50]. BepiPred Linear Epitope Prediction 2.0, available in the IEDB-AR [51], is the most efficient and precise tool for determining linear B-cell epitopes. B-cell epitopes with 10–40 amino acid residues are favored [33]. For analyzing the B-cell epitopes, toxicity, antigenicity, and allergenicity were determined.

2.8. Population coverage analysis

A population coverage tool is used to analyze epitope frequencies across different ethnicities [52]. The IEDB server's population coverage tool [53] estimated the abundance of specific epitopes of T-cells with their relative MHC HLA-binding alleles. So, the deviations in HLA allele distribution and expression in the global population help to develop an efficient multi-epitope vaccine. *In-silico* vaccine design takes population coverage into account to ensure broad protection against a pathogen and effectiveness across diverse populations [54].

2.9. Construction of multi-epitope vaccine

For vaccine construction, adjuvants, linkers, and epitopes are required [55]. Adjuvants are necessary to lower the dose number of antigenic material, which leads to strong immune responses [56]. Epitopes are non-allergenic, non-toxin components with an antigenic nature that trigger the immune response [57]. Linkers connect epitopes and ensure vaccines function properly [58]. Commonly used linkers to join the epitopes are KK (bi-lysine), GPGPG (Gly-Pro-Gly-Pro-Gly), and AAY (Ala-Ala-Tyr) for B-cell, MHC-II, and MHC-I. In this study, an AAY linker was not used because of the selection of one MHC-I. Four different adjuvants, namely beta-defensin, L7/L12 ribosomal protein, granulocyte-macrophage colony, and HBHA adjuvant, were incorporated to formulate four vaccine constructs (V1, V2, V3, and V4), respectively.

2.10. Physicochemical analysis of vaccine constructs

The developed constructs were examined for their allergenicity, antigenicity, solubility, and toxicity. For this purpose, VaxiJen

v2.0, Allertop, SolPro, and Toxinpred servers were used. The instability and aliphatic index, amino acid count, molecular weight, theoretical Pi, and GRAVY value were determined by using the ProtParamExpasy server [59,60]. The molecular weight influences antigenicity, the instability index provides information about the stability of vaccine constructs, the theoretical Pi indicates the hydrophilic and hydrophobic nature of a vaccine, the aliphatic index assures thermostability, and the hydropathy index (GRAVY value) indicates the polarity of vaccine constructs.

2.11. Prediction of vaccine structures

Secondary structure was generated by using the PDBsum [61] and SOPMA [62] servers. hese servers determine the random coil, beta-turn, alpha-helix, and extended strand structures in the constructs. The tertiary structures of the constructs were generated by the SWISS-MODEL structure modeling server [63]. Protein three-dimensional structures offer insights into their molecular function and are useful in various life science research applications [64]. The PyMOL software was used for the visualization of the predicted structures.

2.12. Refinement and validation of the3D structure

GalaxyRefine [65] is a server for the refinement of protein models and improves the conformations and quality of the local structure. It improves the structural backbone quality slightly and refines the models given as input, with a possibility of greater than 50 %. For the protein models that are computed or predicted experimentally, the validation can be done by the Ramachandran plot. For an efficient protein structure, the residue count in the favorable regions should be greater than 90 % [66]. The PROCHECK tool was used to validate the refined 3D using the Ramachandran plot. The ERRAT plot is also a confirmation algorithm for the structures of proteins that show non-bonded interactions, which is beneficial for observing the advancement of crystallographic modeling and refinement. The higher the ERRAT score, the higher the protein quality, which was determined by the SAVES server [67].

2.13. Determination of conformational B-cell epitopes

Conformational B-cell epitopes are usually composed of one to five linear segments of amino acid residues. These epitopes are irregular, distantly spaced apart in order, but adjacent in space, which forms interaction sites with antibodies [68]. MEVs are designed to fold differently, resulting in discontinuous B-cell epitopes that provoke adaptive immune responses [69]. That's why the determination of discontinuous epitopes is important to refine the structure of MEVs. The ElliPro server [70], a well-known discontinuous B-cell epitope prediction server, was utilized to determine discontinuous epitopes at the top level for any protein.

2.14. Molecular docking of the vaccine protein & NMA evaluation

Molecular docking is a method for determining the ligand-receptor binding potential. The ClusPro2.0 server was utilized to conduct docking analysis to verify the interaction between the vaccine and the human immune receptor TLR4 (PDB ID: 48GA). The MOE software was used for the preparation of both the vaccine and receptor structures. Their structures were then uploaded in PDB format by a protein-protein docking tool, ClusPro2.0 server, that works based on the Fourier correlation algorithm and clears out models with a combination of electrostatic and desolvation energies [71]. The binding capacity of all the resulting docked complexes was identified, and the Pymol program was used to display and investigate their interactions. The complex with the best binding affinity and a greater number of interactions was subjected to molecular dynamic simulations and normal mode analysis (NMA) to check its stability [17,40]. The IMODs server performed the normal mode analysis of the best docked vaccine receptor complex to check its stability [72]. This server predicts NMA in internal (dihedral) coordinates to determine the collective motions of macromolecules, such as proteins. In this server, eigenvalues, B-factor, covariance map, deformability, variance, and elastic network for the docked complex were analyzed, and results were obtained as graphs.

2.15. MD simulation

The molecular dynamics (MD) simulation [73] was conducted to examine the stability of the prioritized vaccine-receptor complex. 100-ns-long molecular dynamics simulations were run with Schrödinger LLC's Desmond software package [74]. The docked complex was preprocessed using the Protein Preparation Wizard or Maestro software, including optimization and minimization, before the start of the simulations. The System Builder tool performed the system construction. An orthorhombic simulation box with the TIP3P solvent model was used to imitate realistic environmental conditions. The OPLS_2005 force field [75] was utilized to run the simulations, and as needed, counter-ions were incorporated into the models to neutralize them. In order to simulate physiological circumstances, a 0.15 M solution of sodium chloride was added. The NPT ensemble was also used to ensure mole conservation (N), temperature (T), and pressure (P) at 300 K and 1 atm, respectively, over the duration of the simulation. Pre-simulation relaxation techniques were used for the docked complex. The root mean square deviation (RMSD) and root mean square fluctuations (RMSF) were calculated in order to assess the stability of the simulation.

2.16. Immune simulation

The prioritized vaccine-receptor complex's immune profile was checked through the C-ImmSim server [76]. It determines the immune response of the vaccine using a position-specific score matrix and machine learning techniques. This server was developed by simulating three distinct anatomical locations: the thymus, bone marrow, and lymph node, all of which are necessary for the function of the healthy immune system. For vaccination, the minimal possible interval between the first and second injections is 4 weeks. During immunological simulation, three injections that contained 1000 units of the vaccine were applied with a gap of four weeks, and the injections were set at intervals of 1, 84, and 168 h to predict the immune response. The other variables were kept at their default values [39,54].

2.17. Codon optimization and cloning of final vaccine

The JCAT (Java Codon Adaptation Tool) server was utilized for cloning and optimization of the sequence of amino acids in the vaccine [77]. The tool was used to reverse-transcribe the vaccine sequence, optimize its codons, and express it in an *E. coli* K12 strain expression vector. The JCAT tool estimated the codon adaptation index score (CAI) and GC content for DNA sequences by selecting three different options: prokaryotic ribosome binding sites, rho-independent transcription, and restriction enzyme's cleavage sites [78]. The optimal CAI value is 1.0, while the optimal GC content is 30–70 %. The SnapGene tool [79] was used for the incorporation of the optimized vaccine sequence into the expression vector pET-28a (+) due to its wide use in expression studies [80].

3. Results

3.1. Identification of human non-homologous proteins

The complete genome of *B. dermatitidis* consists of 11538 genes. After excluding the identical copies of proteins, a total of 1420 non-paralogous core proteins were identified. The data was screened to remove human homologs, and 371 human host non-homologous proteins were selected for further analysis.

3.2. Determination of pathogen essential and host-pathogen interacting proteins

The DEG provides comprehensive information on protein essentiality based on experimental analysis. Essential proteins were selected due to their significant similarities with the proteins present in DEG. The 371 human non-homologous proteins from the previous analysis were screened against the DEG by BLASTp with a sequence identity of 50 %, which resulted in 50 proteins that were found to be essential. Furthermore, infectious diseases are caused by protein-protein interactions between the pathogen and the host, which involve molecular communication between hosts and pathogenic organisms. Identifying these proteins can lead to the determination of more effective therapeutic targets in order to treat infectious diseases. BLASTp analysis resulted in 86 interactive homologs with the HPPPI database. These essential and interactive proteins were combined, and duplicates were removed. As a result, 131 proteins of *B. dermatitidis* were found to be important and proceeded further.

3.3. Analysis of metabolic pathways

Metabolic pathways for *B. dermatitidis* (129 pathways) and the human host (356 pathways) were obtained from the KEGG server.

Table 1
Unique metabolic pathways of *Blastomyces dermatitidis*.

Sr. No.	Unique Pathways	Pathway ID
1	Monobactam biosynthesis	bgh00261
2	Lysine biosynthesis	bgh00300
3	Penicillin and cephalosporin biosynthesis	bgh00311
4	Carbapenem biosynthesis	bgh00332
5	Cyanoamino acid metabolism	bgh00460
6	C5-Branched dibasic acid metabolism	bgh00660
7	Methane metabolism	bgh00680
8	Indole alkaloid biosynthesis	bgh00901
9	Carotenoid biosynthesis	bgh00906
10	Sesquiterpenoid and triterpenoid biosynthesis	bgh00909
11	Biosynthesis of various plant secondary metabolites	bgh00999
12	Biosynthesis of secondary metabolites	bgh01110
13	beta-Lactam resistance	bgh01501
14	MAPK signaling pathway - yeast	bgh04011
15	Cell cycle - yeast	bgh04111
16	Meiosis - yeast	bgh04113
17	Autophagy - yeast	bgh04138
18	Mitophagy - yeast	bgh04139

The metabolic pathways of humans and B. dermatitidis were compared to identify their common and unique pathways. This resulted in 111 common pathways in humans and B. dermatitidis, while 18 pathways in B. dermatitidis were found to be unique (Table 1). The 131 pathogen-important proteins identified by comparative subtractive genomics were functionally annotated using the KAAS server (KO assignment) through metabolic pathway enrichment. Three proteins were found to be KEGG-independent, while 37 proteins were identified as being involved in the pathogen's unique pathways.

3.4. Subcellular localization

Vaccines and drugs are designed against specific protein targets based on their localization with the pathogen. Cytoplasmic and organellar proteins may serve as drug targets, whereas membrane and extracellular proteins may serve as possible vaccine targets. Among the 40 proteins (KEGG-dependent and independent), 37 were found as drug targets and 3 as vaccine targets, among which one variant protein was discarded (Table 2).

Table 2
Subcellular localization of vaccine and drug target proteins.

Sr. No.	Accession No.	Protein Name	Localization	Targets
1	XP_045280810.1	superoxide dismutase [Blastomyces dermatitidis ER-3]	Extracellular	Vaccine
2	XP_045276112.1	superoxide dismutase, variant [Blastomyces dermatitidis ER-3]	Extracellular	Vaccine
3	XP_045276441.1	aldehyde dehydrogenase [Blastomyces dermatitidis ER-3]	Extracellular	Vaccine
4	XP_045273533.1	malate dehydrogenase, NAD-dependent, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
5	XP_045274731.1	malate dehydrogenase, mitochondrial [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
6	XP_045282827.1	malate dehydrogenase, NAD-dependent [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
7	XP_045275794.1	isocitrate dehydrogenase [NAD] subunit 1, mitochondrial [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
8	XP_045275850.1	isocitrate dehydrogenase [NAD] subunit 2, mitochondrial [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
9	XP_045273396.1	aldehyde dehydrogenase (NAD+) [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
10	XP_045278133.1	amidophosphoribosyltransferase [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
11	XP_045281821.1	amidophosphoribosyltransferase, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
12	XP_045275548.1	biotin synthase, variant 1 [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
13	XP_045280487.1	biotin synthase [Blastomyces dermatitidis ER-3]	Mitochondrial	Drug
14	XP_045280488.1	biotin synthase, variant 2 [Blastomyces dermatitidis ER-3]	Mitochondrial	Drug
15	XP_045278510.1	3-deoxy-7-phosphoheptulonate synthase [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
16	XP_045278899.1	phospho-2-dehydro-3-deoxyheptonate aldolase, phenylalanine-inhibited [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
17	XP_045275730.1	enolase [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
18	XP_045280588.1	enolase, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
19	XP_045274527.1	succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
20	XP_045279955.1	succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial, variant [Blastomyces dermatitidis ER-3]	Mitochondrion	Drug
21	XP_045282045.1	glucosamine-6-phosphate isomerase [Blastomyces dermatitidis ER-3]	Cytoplasmic or Mitochondrial	Drug
22	XP_045282046.1	glucosamine-6-phosphate isomerase, variant 1 [Blastomyces dermatitidis ER-3]	Cytoplasmic or Mitochondrial	Drug
23	XP_045282047.1	glucosamine-6-phosphate isomerase, variant 2 [Blastomyces dermatitidis ER-3]	Cytoplasmic or Mitochondrial	Drug
24	XP_045282529.1	CMGC/MAPK protein kinase [Blastomyces dermatitidis ER-3]	Nucleus	Drug
25	XP_045282530.1	CMGC/MAPK protein kinase, variant [Blastomyces dermatitidis ER-3]	Nucleus	Drug
26	XP_045274151.1	14-3-3 family protein [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
27	XP_045275734.1	14-3-3 family protein [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
28	XP_045274461.1	GTP-binding protein 1 [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
29	XP_045279211.1	ribosome-interacting GTPase 1 [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
30	XP_045279212.1	ribosome-interacting GTPase 1, variant 2 [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
31	XP_045279213.1	ribosome-interacting GTPase 1, variant 3 [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
32	XP_045282586.1	peptidyl-prolyl cis-trans isomerase H [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
33	XP_045282587.1	peptidyl-prolyl cis-trans isomerase H, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
34	XP_045280124.1	peanut-like protein 1 (cell division control like protein 1) [Blastomyces dermatitidis ER-3]	Nucleus	Drug
35	XP_045280125.1	peanut-like protein 1 (cell division control like protein 1), variant [Blastomyces dermatitidis ER-3]	Nucleus	Drug
36	XP_045274662.1	calcium-binding protein NCS-1 [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
37	XP_045280056.1	calcium-binding protein NCS-1, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
38	XP_045280382.1	methylthioribulose-1-phosphate dehydratase, variant [Blastomyces dermatitidis ER-3]	Cytoplasmic	Drug
39	XP_045276874.1	serine/threonine protein phosphatase [Blastomyces dermatitidis ER-3]	Nucleus	Drug
40	XP_045277618.1	GTP-binding protein rhoC [Blastomyces dermatitidis ER-3]	Nucleus, Mitocndrion, cytoplasm	Drug

3.5. Vaccine target proteins analysis

Two extracellular proteins (superoxide dismutase and aldehyde dehydrogenase) were identified as vaccine targets and evaluated for their vaccine-candidate properties. The antigenicity, allergenicity, aliphatic index, instability index, topology value, gravy value, and water solubility were determined by different servers. The proteins with a topology value of 0 and a negative GRAVY value were selected. The vaccine target should also be stable, non-allergenic, water-soluble, and antigenic. After monitoring all the parameters, superoxide dismutase was determined as a potentepitope prediction candidate and vaccine construction (Table 3).

3.6. Determination and analysis of T and B-cell epitopes

T-cells consist of two kinds of immune cells, i.e., MHC-I and MHC-II; therefore, their epitopes were determined and analyzed. In the case of MHC-I-binding epitopes, a total of 7857 epitopes were determined by the IEDB server. Out of these epitopes, the top 10 epitopes with the lowest rank were selected for further examination. A single MHC-I antigenic, non-allergenic, and immunogenic epitope was chosen for vaccine construction (Table S1), whereas for MHC-II binding epitopes, a total of 3618 MHC II epitopes were determined by the server. Between these, the top 7 had been chosen for further assessment, and the one non-allergenic, most antigenic, and non-toxin epitope was chosen for vaccine formulation (Table S2). The BepiPred Linear Epitope Prediction Server was used to determine the B-cell epitopes. The results showed three epitopes for superoxide.

dismutase, with lengths ranging from 10 to 40. After analysis, 2 B-cell epitopes that showed non-allergenicity, non-toxicity, and optimal antigenicity were prioritized for the development of a vaccine (Table S3).

3.7. Population coverage analysis

The IEDB population coverage tool was utilized to determine the T-cell binding epitopes' population coverage, which was chosen for the vaccine construction. Each epitope's population coverage was estimated, assessing its frequency across various ethnic groups. After analysis, the epitopes showed allele coverage of 88.3 % worldwide, which suggests that the vaccine is efficient globally. Since then, blastomycosis cases have been found worldwide, from the Mississippi and Ohio River valleys to China, India, Africa, and South American states like Wisconsin, Minnesota, Michigan, Louisiana, and Arkansas. So, the coverage of the MHC-I and MHC-II combined epitopes in these regions individually was also determined and was found to be 91.9 %, 81.94 %, 74.0 %, and 72.65 %, respectively. Moreover, the population coverage graph indicates the coverage of epitopes globally in 16 different regions (Fig. 2).

3.8. Construction and analysis of multi-epitope vaccines

Four vaccine constructs are developed by combining the non-allergenic, most antigenic, and non-toxin epitopes. Different linkers are used for this purpose, providing elasticity to the vaccine constructs. In this study, the selected B-cells, MHC-II, and MHC-I epitopes were linked through appropriate linkers. To provide strength at the ends, an EAAAK linker was integrated at the C- and N-terminals of the construct (Fig. 3). Moreover, to improve the construct's immunogenicity, four different adjuvants, namely beta-defensin, L7/L12 ribosomal protein, granulocyte-macrophage colony, and HBHA protein, were incorporated to form the four vaccine constructs (V1, V2, V3, and V4).

The results of the allergenicity and antigenicity analyses identified that all four vaccines were significantly non-allergenic and antigenic. The physicochemical properties evaluation showed that among the four constructs, three (V1, V2, and V4) were stable and one (V3) was unstable, which was removed from further analysis. Solubility and toxicity were checked by SOLpro and Toxinpred, respectively, which indicated that all three constructs were soluble and non-toxin (Table 4).

3.9. Prediction of 2D and 3D structures

The 2D structures of the designed vaccines were generated by the PDBsum and SOPMA servers. The resulted 2D structures consisted of 14.73 % alpha helix, 20.93 % extended strand, 61.24 %

random coil in V1, 48.78 % alpha helix, 9.27 % extended strand, 37.61 37.07 % random coil in V2, and 60.08 % alpha helix, 4.53 % extended strand, and 33.33 % random coil in V4, respectively (Fig. 4). SWISS-MODEL, a commonly used structure modeling server, was used to generate the tertiary structures of the vaccine constructs.
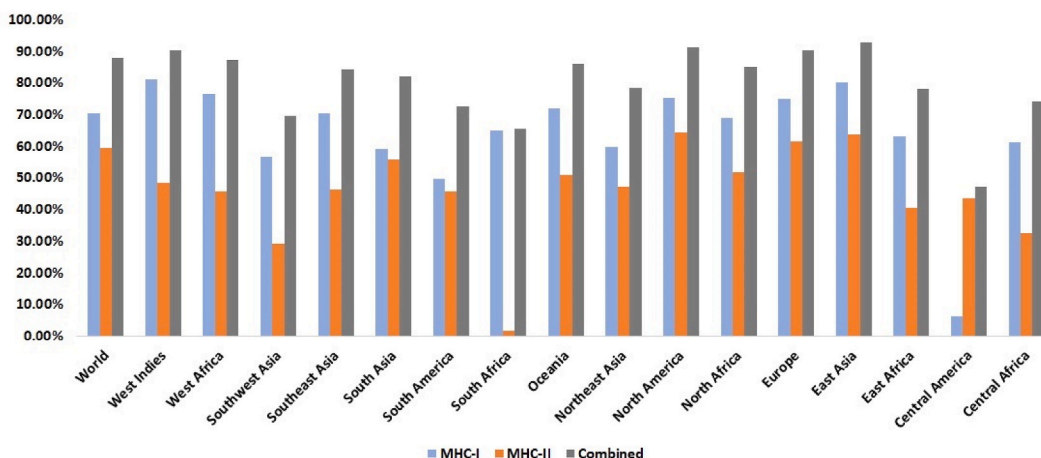
3.10. Refinement and validation of the designed vaccine 3D structure

The output models of the SWISS-MODEL were refined using the Galaxy Refine server to improve their quality and conformations. The efficacy of the improved structures was assessed using the ERRAT plot and the Ramachandran plot created by the SAVES server. The ERRAT quality factor values for V1, V2, and V4 were 86.2745, 97, and 95.1807, respectively. The Ramachandran plot validated all the designed 3D structures, as the majority of vaccine residues were present in its most favored regions. The vaccine-1 had 90.3 % amino acid residues, V2 had 99.1 %, and V4 had 100 % residues in the Rama favored regions. Both parameters ensured that all three vaccine constructs were of high quality (Fig. 5).

Table 3

Analysis of the physicochemical, antigenic, and allergenic properties of target proteins.

Sr. No.	Protein ID	Protein Name	No. of residues	Topology value	Mol. weight	Theoretical Pi	Aliphatic Index	GRAVY value	Antigenicity	Allergenicity	Instability Index
1	XP_045280810.1	Superoxide dismutase	154	0	15972.5	5.41	68.38	−0.44	0.8833 (Antigen)	Non-allergen	32.88 (stable)
2	XP_045276441.1	Aldehyde dehydrogenase	496	0	54033.6	5.99	89.92	−0.074	0.6976 (Antigen)	Allergen	20.79 (stable)

Fig. 2. Population coverage chart of the T-cell epitopes selected for vaccine construction.

HBHA Adjuvant
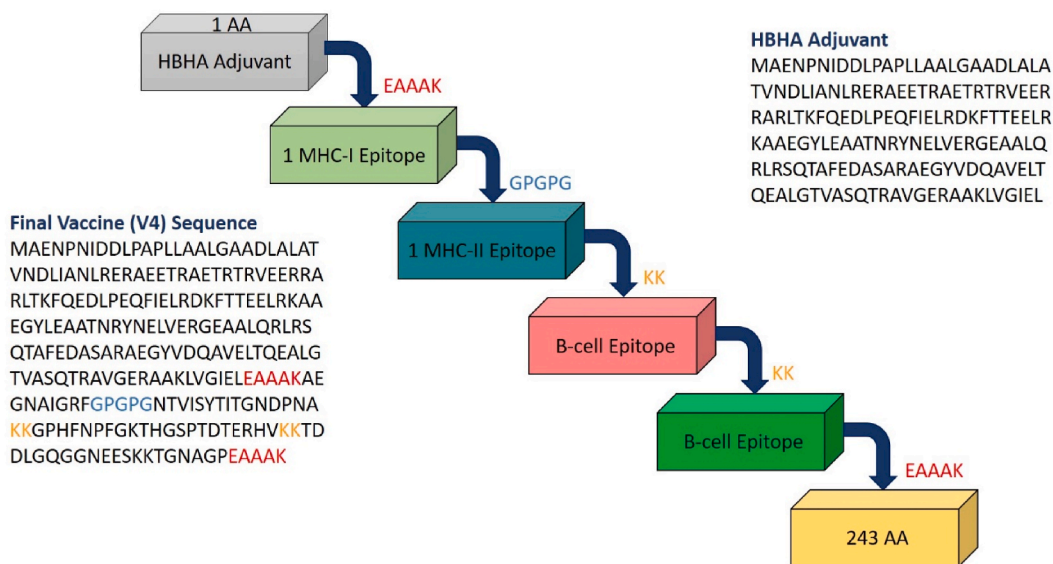MAENPNIDDLPAPLLAALGAADLALA
TVNDLIANLRERAEETRAETRTRVEER
RARLTKFQEDLPEQFIELRDKFTTEELR
KAAEGYLEAATNRYNELVERGEAALQ
RLRSQTAFEDASARAEGYVDQAVELT
QEALGTVASQTRAVGERAAKLVGIEL

Final Vaccine (V4) Sequence
MAENPNIDDLPAPLLAALGAADLALAT
VNDLIANLRERAEETRAETRTRVEERRA
RLTKFQEDLPEQFIELRDKFTTEELRKAA
EGYLEAATNRYNELVERGEAALQRLRS
QTAFEDASARAEGYVDQAVELTQEALG
TVASQTRAVGERAAKLVGIELEAAAKAE
GNAIGRFGPGPGNTVISYTITGNDPNA
KKGPHFNPFGKTHGSPTDTERHVKKTD
DLGQGGNEESKKTGNAGPEAAAK

Fig. 3. Construction of a peptide vaccine by applying adjuvants and linkers.

3.11. Determination of conformational B cell epitopes

B-cell epitopes produce cytokinin and antibodies, leading to an immune response. The residues of the vaccine that contribute to the formulation of the discontinuous B-cell epitopes were determined by utilizing the Ellipro server. Seven conformational or discontinuous B cell epitopes with 5–37 residues were identified, with scores ranging from 0.552 to 0.969 (Table 5 and Fig. 6).

3.12. Molecular docking of the vaccine

A vaccine can interact with immune receptors, like Toll-like receptors, which is crucial for triggering effective immune responses. The ClusPro server 2.0 was utilized for the molecular docking analysis and to determine the affinity of designed vaccines with the human immune receptor TLR4. The software resulted in vaccine-receptor docked models and their affinities to bind with each other. The interactions and complex formation between vaccine constructs (V1, V2, and V4) and TLR4 were also visualized in the Pymol software, providing information regarding the design of the vaccine and immune response regulation. After analyzing the interactions among the docked complexes of the vaccine and receptor, significant interactions between the vaccines were found with some specific residues of TLR4. The V1 showed 16 hydrogen bond interactions with different residues of TLR4, with the lowest energy of −703.2 kJ/

Table 4

Screening of the antigenicity, allergenicity, solubility, toxicity, and physicochemical properties of the vaccine constructs.

Vaccine Construct	Adjuvants	Solpro	Anti-genicity	Aller-genicity	Mol. Wt. (KDa)	Theo-retical Pi	GRAVY value	Aliphatic Index	Instability Index	No. of amino acids	Topo-logy	Toxi-city	Solubility
V1	Beta-defensin	0.95	0.7317 (antigen)	Non-allergen	13.71	9.7 Basic	−0.947	47.75	28.80 (Stable)	129	0	Non-toxin	Soluble
V2	L7/L12 Ribosomal protein	0.88	0.7118 (antigen)	Non-allergen	21.04	5.15 Acidic	−0.431	79.45	20.10 (Stable)	205	0	Non-toxin	Soluble
V3	Granulocytemacrophage colony	0.63	0.7747 (antigen)	Non-allergen	2473.	5.89 acidic	−0.552	66.7	49.43 (Un-stable)	227	0	Non-toxin	Soluble
V4	HBHA	0.79	0.6000 (antigen)	Non-allergen	26.18	5.1 Acidic	−0.698	72.51	34.06 (Stable)	243	0	Non-toxin	Soluble
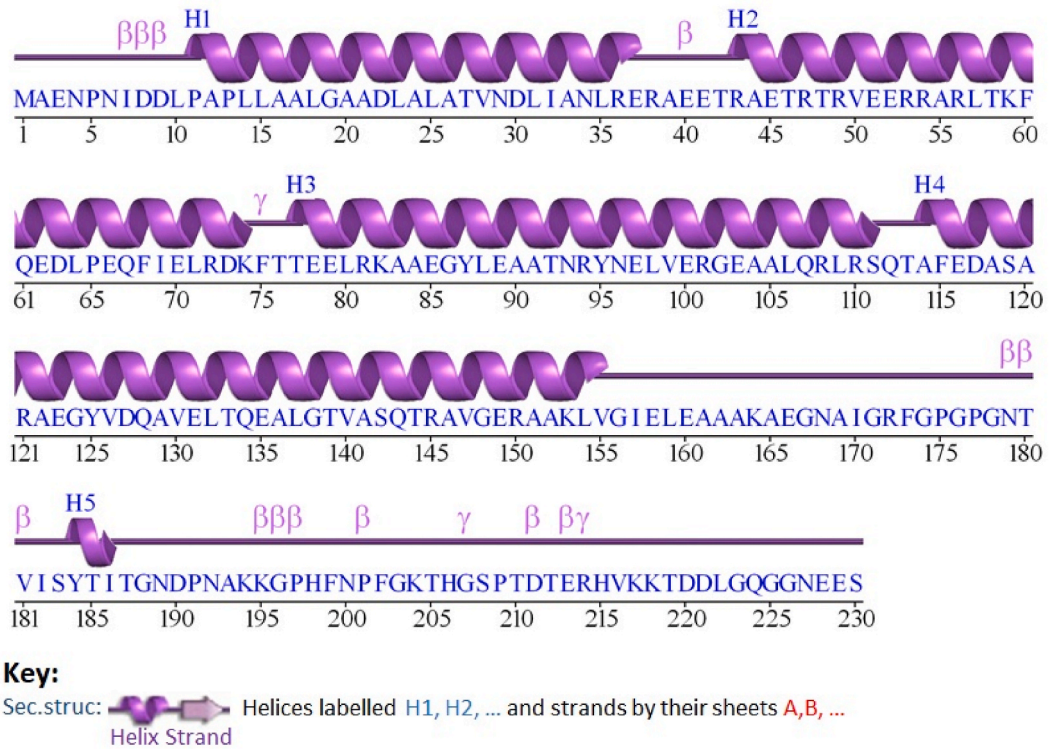
Fig. 4. Protein secondary structure indicates 60.08 % alpha helix, 4.53 % extended strand, and 33.33 % random coil.
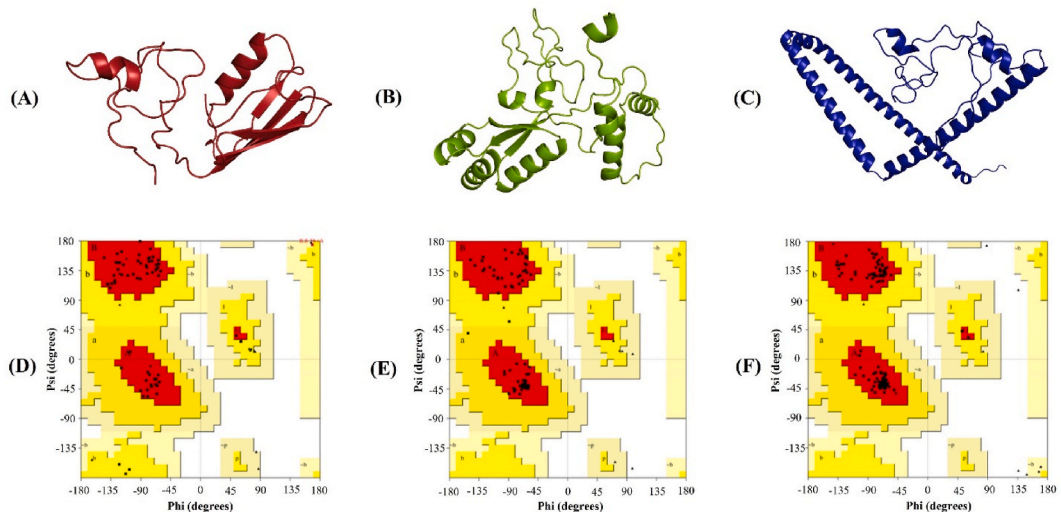

Fig. 5. (A–C) Visualization of the tertiary structures of the vaccines V1 (red), V2 (green), and V4 (blue) generated by the SWISS-MODEL server; (D–F) Ramachandran plot of the vaccines V1, V2, and V3 formed by the SAVES server. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mol. The V2 formed 12 H-bonds with the receptor with the lowest energy of −879.7 kJ/mol. However, the V4-receptor docked complex showed the lowest energy of −1225 and a total of 20 H-bond interactions. The findings of the docking analysis indicated that V4 has the highest affinity to bind with immune receptors as compared to the other two vaccines. The V4-TLR4 complex fulfilled the desired criteria of lowest binding energy and maximum number of interactions; therefore, the V4 was used for further analysis (Fig. 7).

Table 5
Residues of the prioritized vaccine construct involved in the formulation of conformational B-cell epitopes.

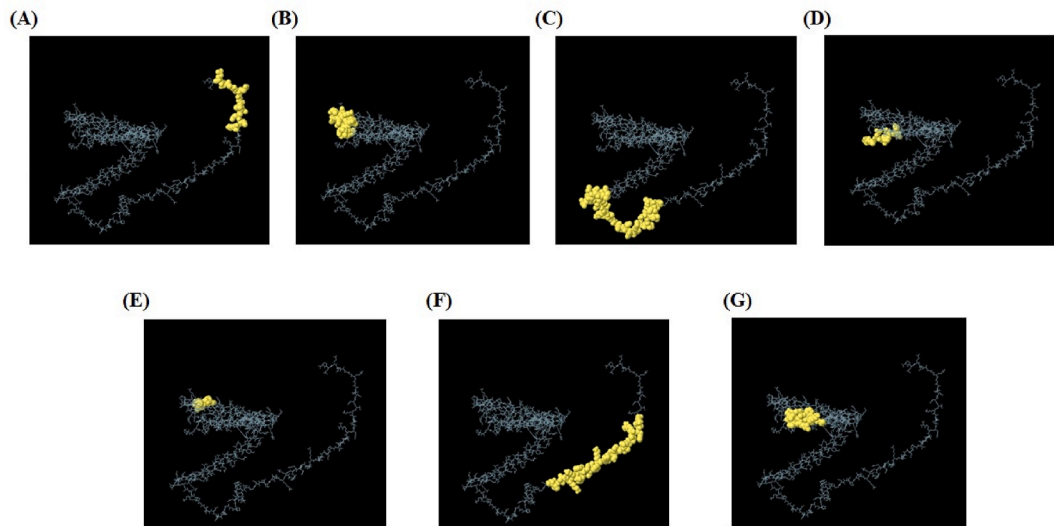| Sr. No.	Residues	Number of residues	Score
1.	A:K218, A:T219, A:D220, A:D221, A:L222, A:G223, A:Q224, A:G225, A:G226, A:N227, A:E228	11	0.969
2.	A:F68, A:I69, A:E70, A:L71, A:R72, A:D73, A:K74, A:F75, A:T76, A:T77, A:E78, A:E79, A:L80, A:R81	14	0.771
3.	A:D190, A:P191, A:N192, A:A193, A:K194, A:K195, A:G196, A:P197, A:H198, A:F199, A:N200, A:P201, A:F202, A:G203, A:K204, A:T205, A:H206, A:G207, A:S208, A:P209, A:T210, A:D211, A:T212, A:E213, A:R214, A:H215, A:V216, A:K217	28	0.75
4.	A:E149, A:A151, A:A152, A:K153, A:L154, A:V155, A:G156, A:I157, A:E158, A:L159, A:E160, A:A161, A:A162, A:A163, A:K164, A:A165, A:E166, A:G167, A:N168, A:A169, A:I170, A:G171, A:R172, A:F173, A:G174, A:P175, A:G176, A:P177, A:G178, A:N179, A:T180, A:V181, A:I182, A:S183, A:T185, A:I186, A:T187	37	0.715
5.	A:A55, A:K59, A:F60, A:E62, A:D63, A:L64, A:P65, A:E66, A:Q67	9	0.63
6.	A:M1, A:A2, A:E3, A:N4, A:P5, A:N6, A:I7, A:D8	8	0.599
7.	A:A83, A:A84, A:E85, A:G86, A:E89	5	0.552

Fig. 6. Conformational B-cell-forming residues of the prioritized vaccine are shown in yellow along with their scores **(A)** 0.969 **(B)** 0.771 **(C)** 0.715 **(D)** 0.599 **(E)** 0.552 **(F)** 0.75 **(G)** 0.63. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
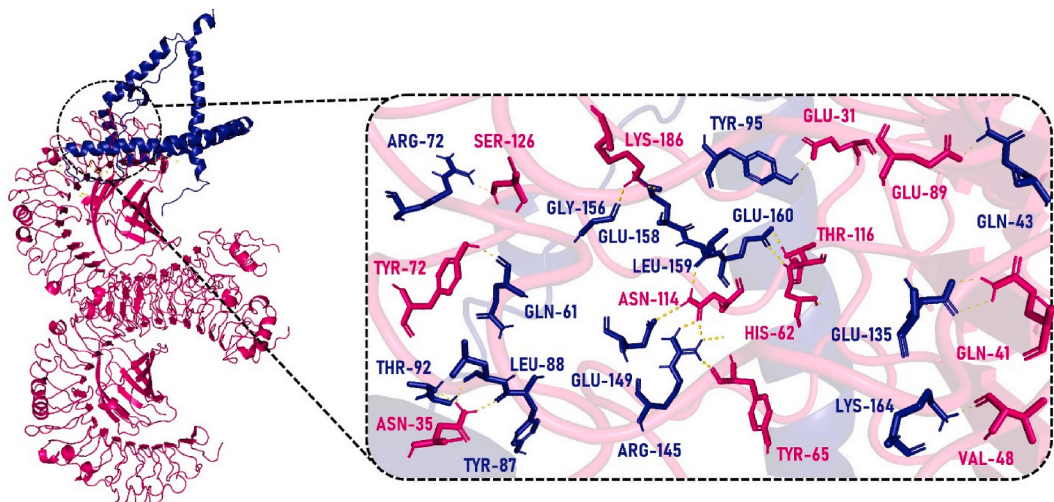

Fig. 7. Representation of the docked complex of the prioritized vaccine construct (blue) with TLR-4 (pink) in cartoon format and their interactive residues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.13. NMA evaluation

The IMODS server was utilized to perform the NMA to ensure V4 docked complex stability. The results indicated the eigenvalues, B-factor, covariance map, deformability, variance, and elastic network for the docked complex as graphs (Fig. S1). Deformability describes that a molecule is able to alter its residues, where the peaks serve as indicators (Fig. S1A). Higher peaks indicate greater deformability, and vice versa. B-factors in PDB files measure the movement of macromolecules, such as proteins. This server calculates B-factors by multiplying NMA mobility by 8 pi (Fig. S1B). Eigenvalue is a measure of motion stiffness that correlates with structure deformation. A lower eigenvalue indicates easier deformation of the macromolecule. The eigenvalue for complex movement rigidity was 4.687967e-08 (Fig. S1C). The eigenvalue and variance of each standard mode have an inverse relationship (Fig. S1D). The covariance matrix explains the relationships between complex residues (Fig. S1E). Stronger complexes have highly correlated residues. Residues with anti-correlations, no-correlations, or varied correlations were colored blue, white, and red, respectively. The elastic network model uses colored dots in graphs to represent the spring's stiffness that connects the pair of atoms. The stiffer the springs, the darker the dots (Fig. S1F).

3.14. Molecular dynamic simulations

In order to gain an understanding of the structural adjustment of V4 within the binding domains of the TLR4 receptor over the course of a 100-ns simulation period, statistical parameters such as root mean square fluctuation (RMSF) and root mean square deviation (RMSD) were evaluated (Fig. 8). The initial (0–370) residues in the RMSF graph showed significantly higher flexibility due to the presence of loop regions. After then, a decrease in the RMSF values was observed among the remaining residues, indicating that the majority of the docked complex was stable (Fig. 8B). It can also be seen that the residues (0–370) exhibited the biggest variations corresponding to loop areas, or the N- and C-terminal zones, indicating that this region was more adaptable or dynamic than the remaining parts of the complex. The RMSD values were found to be stable throughout the whole simulation period, indicating that the V4-TLR4 complex is stable. The average RMSD values of 8.2 Å $\pm$ 1 indicated the structural stability of the docked complex with no significant deviation over the whole period (Fig. 8A).

3.15. Immune simulation

The C-ImmSim server gave an immunological profile of V4. Within the immune simulations, the vaccine was given in three separate injections at intervals of 01, 84, and 168 h. Immunogenicity increased with each injection and reached its peak after the third dose. Due to the vaccine injections, an increase in activated Tc-cells, B-cells, TH-cells, and NK-cells was observed with each dose (Figs. S2A–D). Memory cells develop in the tertiary response, accompanied by high levels of B-cell activation, including IgG, IgG1, IgG2, and IgM antibodies (Fig. S2E). The vaccine injections effectively stimulate the immune system, as evidenced by high cytokine levels like IFN-γ and IL-2 (Fig. S2F). After vaccine injections, NK cells and dendritic cells resulted in the macrophages rapid reproduction, which is crucial for combating various diseases (Fig. S2G). Our vaccine also produces regulatory T cells, which maintain homeostasis by suppressing the immune response and preventing autoimmunity (Fig. S2H). All of these findings indicated that the preferred vaccine, V4, might be highly immunogenic.

3.16. Codon optimization and in-silico cloning of final vaccine

Before in silico cloning, the vaccine's codons were optimized using the Jcat server. The final construct's CAI and GC content values were 0.98 and 58.18, respectively. The vaccine was cloned by incorporating its optimized or improved sequence into the pET-28a (+) expression vector at the HPaI restriction site using the Snapgene tool, and its final length clone was 6098 bp. (Fig. S3).
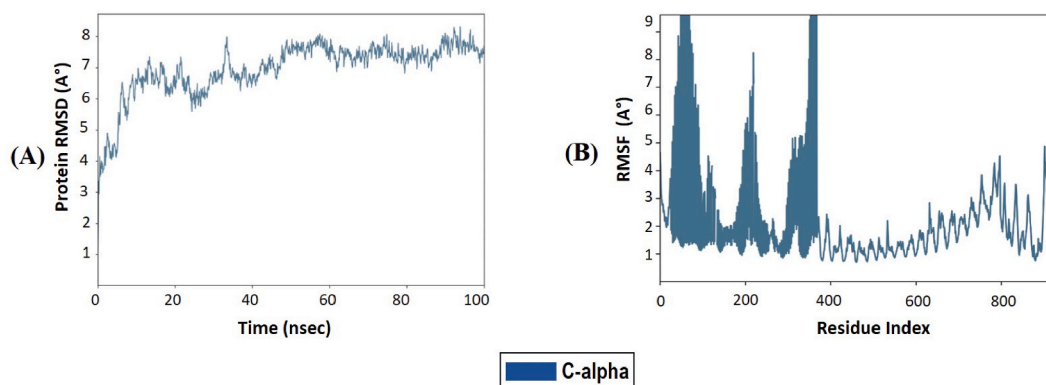

Fig. 8. Molecular dynamic simulation results evaluation by **(A)** RMSD **(B)** RMSF.

4. Discussion

Analyzing a pathogen's proteome enables complete investigation of proteins in biochemical and clinical pathways, leading to the discovery of novel therapeutic targets [16]. Identifying new therapeutic targets and developing commercial vaccines is crucial for combating *B. dermatitidis*-related blastomycosis. The high infection rate of this infection [1] highlights the requirement for a vaccine that is able to provoke an immune response against it [81]. New developments in immunoinformatics and reverse vaccinology strategies made it possible for researchers to develop potential vaccines for many diseases [82,83]. Immunoinformatics peptide-based vaccines give several benefits as compared to traditional vaccines [84]. They identify antigens unique to each pathogen that allow vaccines to target specific antigens, while traditional vaccines may not be as efficient in provoking an immune reaction due to a combination of antigens [54]. As compared to traditional vaccines, vaccines designed using immunoinformatics methods allow for faster identification of potential candidates because rapid response is crucial for arising infectious diseases that require immediate prevention. While identifying effective antigens and optimizing compositions may require several years with traditional vaccines. Therefore, the development of new vaccines is necessary for improved, safer activity [54].

B. dermatitidis can lead to fatal outcomes, but there is still no effective treatment or vaccine available for its treatment and prevention. This study offers a novel approach for providing potential therapeutic options against *B. dermatitidis* and also brings the attention of the scientific community to this deadly pathogen. This study used immunoinformatics approaches to filter the entire *B. dermatitidis* genome for the determination of potential new therapeutic targets and the formulation of a multi-epitope vaccine. Core genes are conserved across species, making the findings applicable to various infectious strains of a pathogen [85]. *B. dermatitidis* non-homologous essential proteins were investigated as potential therapeutic targets. Superoxide dismutase, which is involved in the peroxisome metabolic pathway, was selected for vaccine development. *B. dermatitidis*, like many other pathogenic fungi (*Canidida albicans, Cryptococcus neoformans,* and *Aspergillus fumigates*), is exposed to reactive oxygen and nitrogen species (ROS and RNS) during infections. ROS and RNS are generated by the host's immune cells as byproducts of cellular function and act as a defensive mechanism against invading pathogens [86,87]. Pathogenic fungi respond to host oxidative and nitrosative stress through transcriptional, post-translational, and enzymatic responses, including superoxide dismutase (SOD), catalase, thioreductase, and glutathione. These responses enable resistance to ROS/RNS toxicity and host adaptation [88]. SOD protein is crucial for fungal responses against reactive oxidant species, which are phagocytized by fungus and cause death. Fungal SODs may contribute to the pathogen's virulence by detoxifying superoxide anions into hydrogen peroxide (H_2O_2), which is further converted into water (H_2O) and oxygen through catalase. This ROS neutralization is critical for the fungus' survival in the hostile environment of the host [89]. That's why targeting the superoxide dismutase of *B. dermatitidis* can lead to fungus death because it is highly expressed during infection and is crucial for fungus pathogenic process, making it a valuable target for vaccine development.

This study used IEDB prediction techniques to find the epitopes of B- and T-lymphocytes that could cause humoral or cell-mediated responses. B-lymphocytes are primarily responsible for producing antibodies (also known as immunoglobulins) [90]. Each B-cell epitope has a linear or conformational shape. The linear B-cell structure is only fully present in the protein's primary structure. Protein folding brings together discontinuous or conformational B-cell epitopes, which is a crucial factor when developing vaccines [91,92]. B-lymphocytes use B-cell receptors (BCRs) to bind to antigenic epitopes on target cells, which are then processed and presented to T cells. HTLs with a T-cell receptor (TCR) recognize processed epitopes on B-cell surfaces alongside MHC class II [93]. Consequently, B cells develop into plasma cells, which produce the antibodies [94]. These antibodies are able to eliminate infections significantly [95]. This study identified two B-cell epitopes appropriate for designing vaccines.

T-lymphocytes are classified as helper T lymphocytes and cytotoxic T lymphocytes based on their ability to recognize and interact with Major Histocompatibility Complex (MHC) Class I and II molecules on Dendritic cells, macrophages, and B cells, respectively [96]. HTLs are triggered when antigen-presenting cells offer pathogen proteins along with MHC class II molecules. Many chemokines and cytokines, such as IL-4, IFN gamma, and IL-10, are secreted by HTLs, which play distinct roles in the immune system's reaction to foreign invaders [97]. In this investigation, a single HTL epitope was utilized to create the vaccine. Infected cells break down pathogen proteins, which are then expressed in CTLs along with MHC class I molecules. CTLs kill infected cells by emitting cytotoxic granules that detect protein-degraded fragments of pathogens known as epitopes [98]. One CTL epitope was selected for the development of the vaccine. A vaccine against B. dermatitidis was constructed by joining predicted peptides with various linkers and adjuvants [99].

The adjuvant's immunomodulatory properties make them popular for use in multi-epitope vaccines [100,101]. Four adjuvants: beta-defensin, L7/L12 ribosomal-protein adjuvant, granulocyte-macrophage colony, and HBHA-protein adjuvant, to design four vaccine constructs with different immune profiles. β-defensins are antimicrobial peptides that stimulate immune responses by acquiring immature DCs and naïve T cells via immune receptor complexes (e.g., CCR6 or TLRs) [102]. Previous research has shown that L7/L12 ribosomal protein, known as the TLR4 binder, is used as an adjuvant in diseases such as cancer for the activation of a strong immunological response [103].An adjuvant called granulocyte macrophage colony-stimulating factor (GM-CSF) was used to guide CD4$^+$ T cells toward the T-helper cell, resulting in stronger CTL responses [104]. HBHA basically increases the production of inflammatory cytokines and adhesion molecules. HBHA is an efficient immunized adjuvant that leads to the induction of a strong Th1 cell immune reaction, which makes it a significant tool for the design of potent vaccines for immunotherapy treatment, including tumor vaccines [102].

The 2D and 3D structures of the prioritized vaccines were formulated, refined, and justified. Understanding the interaction and complex formation between vaccines and host immune receptors is crucial for assessing their effectiveness. Prior studies have demonstrated the significance of human TLR receptors in identifying infectious pathogen peptides and initiating immune responses toward them [105,106]. As a member of the pattern recognition receptor family, TLR4 is essential to the human immune system because it reacts to invasive infections with extreme sensitivity and selectivity. TLR4 leads to the identification of pathogen-associated

molecular patterns (PAMPs), including lipo-oligosaccharide and lipopolysaccharide (LPS) from mycoplasmas, viruses, and fungi. Basically, TLR4 can activate due to tissue damage or inflammation, which leads to the production of specific endogenous ligands. The interaction between receptor and ligand initiates an intracellular signaling cascade, leading to a pro-inflammatory response. TLR4 is linked to several clinical disorders, making it a potential therapeutic target [107]. In this study, TLR-4 was used for vaccine-receptor docking, with which the V4 construct showed the greatest interaction having the least amount of binding energy. Then the NMA and MD simulations were performed, which gave information about the stability of the V4-TLR4 receptor complex. The findings of the immune simulation closely resembled the immune-stimulating responses [78]. Repetition of antigen exposure has led to the induction of a strong immunological reaction by the significant increase in T-cytotoxic and T-helper cell generation, as well as IgG and IgM antibodies, and the IFN-gamma and IL-2 levels, suggesting humoral responses to the vaccine. So, the top-ranked vaccine, V4, can activate the TLR receptor and trigger immune responses to B. dermatitidis. Moreover, in silico cloning indicated that the prioritized vaccine is capable of showing higher expression in host organisms (E. coli). In order to verify these research findings, it is suggested to express the potential vaccine in bacteria for additional research.

Studies using immunoinformatics have the potential to completely transform biomedical research by offering quick, affordable, and extremely accurate substitutes for conventional laboratory tests. These cutting-edge computational techniques have the potential to greatly speed up the creation of vaccines, improve our knowledge of immune responses, and open the door to personalized therapy, all of which will improve global health outcomes. In vitro immunological assays validate the vaccine, evaluate its immunogenicity, and prepare for a challenge-protection preclinical trial to confirm the effectiveness of the study's findings. To develop a safe and effective vaccine, pre-clinical trials must be conducted with caution to prevent severe side effects. Animal models (such as mice, rabbits, or non-human primates) can be used to assess the vaccine's safety, immunogenicity, and effective protection against pathogens, which is a new trend for clinical and pre-clinical studies [108,109].

Although the study used rigorous parameters to ensure accurate predictions, the approach has some limitations. Validating assumptions about MEV epitopes for MHC alleles and B cells is crucial, regardless of their proven reliability. Experimental research is needed to determine the optimal order of epitopes in MEVs for immunogenicity purposes and various pharmacological trials. Further, verifying the vaccine against blastomycosis infections in animal models is necessary to ensure its effectiveness in inducing an immune response and protecting against infection.

5. Conclusion

The most common cause of blastomycosis, a dangerous and deadly infection, is a fungus that is thermally dimorphic called *B. dermatitidis*. Despite its high mortality rate, there is no effective treatment or vaccine available to prevent it. So, there is a need to develop an effective vaccine against blastomycosis. In this study, a peptide-based multi-epitope vaccine was developed by applying immunoinformatics approaches that can be effective against B. dermatitidis. The study's findings showed that the designed vaccine has suitable physicochemical properties, a highly verified structure, and the ability to stimulate an immune reaction by activating both cell-mediated and humoral immunity. It might also have the ability to express successfully in a microbial expression system, allowing for a rapid increase in number in case of an outbreak to combat *B. dermatitidis*.However, these findings must be validated through experimental methods.

CRediT authorship contribution statement

Sawvara Mursaleen: Formal analysis, Investigation, Writing – original draft. **Asifa Sarfraz:** Formal analysis, Investigation, Writing – original draft. **Muhammad Shehroz:** Formal analysis, Validation, Visualization. **Aqal Zaman:** Data curation, Investigation, Writing – original draft. **Sheheryar Sheheryar:** Data curation, Formal analysis, Investigation, **Arlindo A. Moura:** Data curation, Methodology, Writing-review & editing. **Faiz U Rahman:** Investigation, Software. **Shahid Aziz:** Data curation, Formal analysis, Investigation. **Riaz Ullah:** Formal analysis, Writing – review & editing. **Zafar Iqbal:** Funding acquisition, Methodology, Resources, Writing – review & editing. **Umar Nishan:** Validation, Visualization, Writing – review & editing. **Mohibullah Shah:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Wenwen Sun:** Resources, Software, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

necessary infrastructure to perform this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36153.

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