In silico designing of a multitope vaccine against *Rhizopus* microspores

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Abstract

Purpose – Mucorales has been described to be widely distributed during the most recent COVID-19 pandemic, with a greater frequency of disease in India, particularly among those with immune deficiencies. This study aims to use computational tools to develop a vaccine.

Design/methodology/approach – The authors investigated at Mucorales proteins that had previously been associated to virulence factors. Recent research suggests that a vaccine based on high-level cytotoxic T lymphocyte (CTL), helper T lymphocyte (HTL) and B-cell lymphocyte (BCL) epitopes from diverse proteins might be developed. Furthermore, the vaccine assembly contains the targeted epitopes as well as PADRE peptides to induce an immune response. Computational approaches were used to analyze the immunological parameters used to build the suggested vaccine and validate its TLR-3 binding.

Findings – These studies show that the vaccination is capable of triggering a particular immune response. The authors offer a technique for developing and evaluating candidate vaccines using computational tools. To the best of their knowledge, this is the first immunoinformatic research of a prospective mucormycosis vaccine.

Originality/value – During this audit, a successful attempt was made to create a subunit MEV against black fungus. In the current study, MEV has been proposed as a suitable neutralizer candidate since it is immunogenic,

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secure, stable and interacts with human receptors. A stream study, on the other hand, is produced via a mixed vaccinosis approach. Following that, vaccinologists may perform more exploratory testing to evaluate whether the vaccine is effective.

Keywords Multi-epitope, Vaccine development, B-cells epitope, T-cells epitopes, Molecular docking
Paper type Research paper

1. Introduction

A fungus is a multicellular eukaryotic organism that includes yeasts, mold and more familiar mushrooms. Fungi are heterotrophic. Fungi reproduce by releasing spores into the environment, spreading through inhalation or direct contact. It is widespread, showing up in soil, air and even healthy people's noses and mucous. Fungi can take two forms: mold and yeast (Aguilera & González-Toril, 2019). Mold lives in a long, multiple-threaded cell 32 structure, whereas yeast lives in a single cell. The clinical term for black fungus is mucormycosis. Molds found in the environment, such as *Rhizopus arrhizus*, cause the resulting infection (belongs to the Mucoraceae family). According to the Centers for Disease Control and Prevention, mucormycosis is caused by a group of molds known as Mucormycetes (CDC). It most commonly affects the eyes, bones, nerves, brain, sinuses or lungs, and symptoms include fever, cough and shortness of breath if it gets to the lungs. And, if left untreated, proves fatal (Hadiyanto, Wilda, Cahyadi, & Adisuhanto, 2021). Mucormycosis emerged as another concerning infectious disease amid the life-threatening COVID-19 pandemic. There have been reports of it, mainly in India. This infection may primarily infect those with weakened immune systems who cannot fight off the infection (Fayemiwo & Adegboro, 2021). This infection is a significant concern for COVID-19 recovered patients because they took a lot of antibiotics and steroids to treat COVID-19, making them vulnerable to fungal infections due to their immunocompromised state (Roudbary *et al.*, 2021). More than 4,300 people are thought to have died in India due to this lethal "black fungus." According to some sources, approximately 45,374 cases of this deadly infection known as mucormycosis have been reported (Sahoo *et al.*, 2022). It typically strikes 12-18 days after recovery from Covid and attacks the eyes, nose and sometimes the brain. Antifungal medication is used to diagnose black fungus. Medication such as isavuconazole, posaconazole and amphotericin B can prevent the fungus from growing. Surgery is required in severe cases to remove the infected tissue. Medical Professionals are moving on to the principle of developing an effective vaccine that could limit the spread of the infection and lower the overall mortality rate. Vaccines are designed to stimulate to fight specific pathogens. They contain components such as weakened or inactivated viruses, bacterial proteins or genetic material that mimics the pathogen, and upon subsequent exposure to the same pathogen, the body is better able to respond quickly and strongly due to the earlier immunization. It provides a safe and effective way to protect the body from disease-causing pathogens, before exposure to them. Vaccines are a powerful tool for eliciting the production of specific antibodies, which are proteins produced by immune cells called B-cells. These antibodies are capable of recognizing and neutralizing the pathogens targeted by the vaccine, thus helping to prevent their entry into cells or enhancing their clearance from the body. This is an important mechanism for protecting against infection from the targeted pathogen and is one of the main reasons why vaccines are such an effective tool for disease prevention. By stimulating the production of antibodies, the vaccine helps to train the body's immune system to recognize and respond to the particular pathogen, providing a form of immunity even in the event of a later encounter. Vaccines are not just effective for producing antibodies, but can also activate cell-mediated immunity. This type of immunity is especially important for combatting intracellular pathogens, such as viruses, as it involves the activation of T-cells. These immune cells directly attack and destroy infected cells, or help coordinate the immune

response in other ways. Cell-mediated immunity is thus an important part of a comprehensive immune system, and vaccines can be incredibly useful in activating it. Vaccine design can be built using either traditional or modern technologies. Multi-epitope vaccines (MEVs) are more advantageous than conventional vaccines because they save time, are less expensive, are considered safe and effective, are specific and stable and do not require microbial culturing. Currently, immunoinformatics has paved the way for *in silico* vaccination computational vaccinology, in which scientific questions about vaccination are solved using computer-driven algorithms and various computational tools that aid in data analysis. The results have been promising in the majority of cases. Recent advancement in the field of vaccination is an *in silico* approach, which could combat various pathogens and take over the ability to elicit cellular and humoral responses in human hosts. To make a multi-epitope vaccination, we used an *in silico* technique (Kadam, Sasidharan, & Saudagar, 2020). We focused on identifying the target protein sequence derived from NCBI (National Center for Biotechnology Information), a leading source for databases, genomic data and computational biology research. Using various Internet applications, T-cell (MHC-Class I, MHC-Class II) and B-cell epitopes were later predicted. Its epitopes were chosen, and then allergenicity, immunogenicity, conservancy and antigenicity were tested. The vaccine was created using the obtained proteins and appropriate linkers cytotoxic T lymphocyte (CTL), helper T lymphocyte (HTL) and B-cell lymphocyte (Rakib *et al.*, 2020). The vaccine sequence is then inserted into a vector, converted into a DNA sequence, followed by molecular docking and immune stimulation. The current study aims to create a vaccine against black fungus using various web-based tools.

2. Methods

2.1 Protein sequence retrieval and analysis

The black fungus [*Rhizopus arrhizus*] proteome sequence was downloaded in FASTA format from the NCBI (Accession id = BAH03542.1) database (Abe, Asano, & Sone, 2009) and predicted for B- and T-cell epitopes, T-cell epitopes, located on the surface of APCs and delivered by MHC molecules to T-cell receptors. To anticipate epitopes from MHC classes I and II, the Net CTL 1.2 server and Net MHC 4.0 server were used to trace epitopes inside the specified sequence (Larsen *et al.*, 2007; Fadilah, Erlina, Paramita, & Istiadi, 2021) (Table 1, Table 2). As a result, T-cell-mediated immunity would be induced by these epitopes (Mahapatra *et al.*, 2022). There are two types of B-cell epitopes in the adaptive humoral immune system: linear (continuous) and conformational (discontinuous). The linear epitopes were predicted using the ABCPred server (Saha & Raghava, 2006) (Table 3, Table 4).

2.2 The development of a multiepitope vaccine

The derived epitopes were chosen for construction of a polytope vaccine. Adjuvants can be used to boost the immune response of the epitopes. The L7/L12 adjuvant is a ribosomal protein that was linked with the EAAAK linker and other linkers due to its capacity for inducing DC maturation; GGGGS was used to link CTL epitopes of MHC Class-I; GPGPG was used to link HTL epitopes of MHC Class-II; while KK linker was used to link B-Cell epitopes to preserve the immunogenic activities (Sami *et al.*, 2021). HTLs have a role in activating CTLs and other immune cells by releasing cytokines such as IFN-γ, interleukin-4 and interleukin-10. This shows that cytokine-inducing HTL epitopes are essential for vaccinations to work (Figure 1).

2.3 The succeeding sequence is the constructed vaccine

EAAAKMSITKDQIIEAVAAMSVMDVVELISAMEEKFGVSAAAAVAVAAGPVEA
AEEKTEFDVILKAAGANKVAVIKAVRGATGLGLKEAKDLVESAPAALKEGVSK

Residue No	Peptide sequence	MHC binding affinity	Rescale binding affinity	c-terminal cleavage affinity	Transport affinity	Prediction score
348	WTDDLQALF	0.6653	2.8249	0.8918	2.205	3.0689
540	CTAQVVPIY	0.6273	2.6636	0.9708	2.823	2.9504
691	LSPFLYSIY	0.4345	1.845	0.9068	2.963	2.1291
1153	LTSTVDSLF	0.4378	1.859	0.7615	2.567	2.1016
455	GSSLPSTRY	0.4094	1.7381	0.9783	2.836	2.0267
122	STLLVHCVY	0.3729	1.5834	0.976	3.021	1.8809
72	TSWQQYHTY	0.3349	1.422	0.9789	3.152	1.7264
1136	SIDTSGSLF	0.3496	1.4846	0.6219	2.594	1.7075
986	HVKQSVLAY	0.308	1.3077	0.9656	3.124	1.6087
412	LSDTTATIK	0.3385	1.4372	0.7634	0.188	1.5611
688	GSILSPFLY	0.2955	1.2546	0.9313	2.806	1.5346
135	LSDLEAMDI	0.2772	1.1769	0.504	0.457	1.2753
1154	TSTVDSLFF	0.2298	0.9757	0.8372	2.566	1.2296
199	QLAYGIPTY	0.1844	0.7831	0.9758	2.984	1.0787
69	RLMTSWQQY	0.1753	0.7443	0.9546	3.301	1.0526
279	LTEPDCLYV	0.1988	0.8439	0.8911	0.285	0.9918
278	RLTEPDCLY	0.1567	0.6654	0.8945	3.206	0.9599
904	LTSTPSMHV	0.1838	0.7806	0.9159	0.27	0.9314
304	YTSPTNSTA	0.2102	0.8924	0.3864	−0.571	0.9219
586	SLDPVQGGF	0.1515	0.6431	0.9667	2.286	0.9024
310	STAPDMDLL	0.1666	0.7072	0.7322	1.144	0.8742
43	TVDQTTNSL	0.1614	0.6855	0.9705	0.844	0.8733
719	YSSNPQREF	0.1509	0.6405	0.6302	2.499	0.86
322	LTSIIHSCL	0.146	0.62	0.9543	0.766	0.8014
442	ATTMANHLK	0.1468	0.6231	0.8251	0.705	0.7821
117	LSCQVSTLL	0.137	0.5816	0.9079	1.055	0.7705
96	LLVNPHCPY	0.1067	0.4531	0.9758	3.08	0.7535
34	WNANGCNRY	0.1109	0.4708	0.9414	2.81	0.7526

Source(s): Table by authors

Table 1. List of T-cell epitopes chosen based on C-terminal cleavage and TAP score generated from IEDB web server

Allele	Peptide	VaxiJen probability (threshold 0.5)
HLA-A0201	VLRKLFELCLQTTL	Antigen
HLA-A0201	SQALFSRWSLWWPV	Antigen

Source(s): Table by authors

Table 2. Helper T-cell (HTL) epitopes for *Rhizopus arrhizus* polyprotein using MHC II module (NetMHC −4.0-server)

DDAEALKKALEEAGAEVEVKEAAAKAKFVAAWTLKAAAGGGGSSTAPDMDLL GGGGSATTMANHLGPGPGVLRKLFELCLQTTLGPGPGSQALFSRWSLWWPVKK LLGDSRTTTRGIKLFSWIKKAKFVAAWTLKAAAGGGGS.

2.4 Determining the attributes of the polytope construct

The top-scoring epitopes from the predicted T and B-cell epitopes were chosen to evaluate their antigenic, allergic, toxicity and immunogenic aspects. AlgPred 2.0 is a web server for predicting allergenic regions in proteins, with a prediction threshold of 0.5 (Sharma *et al.*, 2021) (Table 5), (Table 6), (Table 3). Toxinpred, an InSilco web server, was used to aid in the prediction of least toxic epitopes (Table 7), (Table 8) and (Table 9) (Kalita, Padhi, Zhang, & Tripathi, 2020). Defined by calculating its fraction, the conservancy of the protein's epitopes was done utilizing the IEDB server (Azim *et al.*, 2019). Vaxijen 2.0 was used to

Rank	Sequence	Start position	Score
1	RWSLWWPVVCQVLLEIEQ	1107	0.93
2	TIFIRPRFEYGLCICTFL	854	0.92
3	VDRAIIWRALETYISPAL	637	0.9
4	TGGYTSVDDRLAHLSTDA	968	0.89
5	DRLAHLSTDAHVKQSVLA	976	0.88
5	SDTTATIKRIRRNRTMSP	413	0.88
6	ALYCRRRATWKQFCETLS	391	0.87
6	SIIHSCLDDSVGQKKPRG	324	0.87
7	LLFRLCWIWSAVPAAWCT	524	0.86
7	GPTVAATTMANHLKQVFS	437	0.86
7	CWNTQLAYGIPTYCAQGR	195	0.86
7	LLGDSRTTTRGIKLFSWI	171	0.86
8	NAWFWTDDLQALFDRREQ	344	0.85
8	TRGIKLFSWILENGMTCW	179	0.85
8	GPIHASRAHLLSCLRVAE	1055	0.85

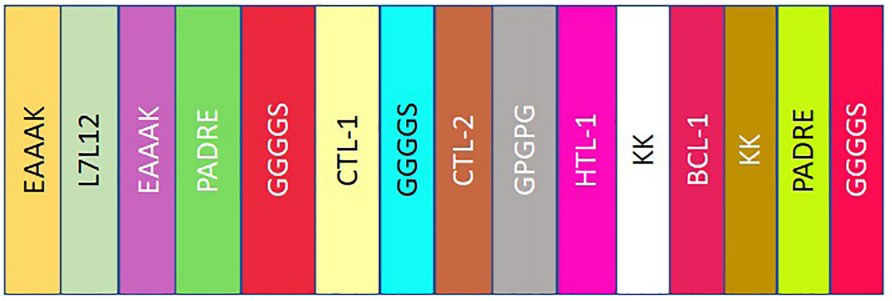
Source(s): Table by authors

Table 3. List of selected B-CELL epitopes by using ABCpred server

B-CELL epitopes	ML score	MERCI score	BLAST score	Hybrid score	Prediction
B-CELL 1	0.22	0	0	0.22	Non-allergen
B-CELL 2	0.28	0	0	0.28	Non-allergen
B-CELL 3	0.26	0	0	0.26	Non-allergen
B-CELL 4	0.3	0	0	0.3	Non-allergen
B-CELL 5	0.28	0	0	0.28	Non-allergen
B-CELL 6	0.29	0	0	0.29	Non-allergen
B-CELL 7	0.26	0	0	0.26	Non-allergen
B-CELL 8	0.25	0	0	0.25	Non-allergen

Source(s): Table by authors

Table 4. List of predicted B-CELL epitopes after allergenicity analysis



Source(s): Figure by authors

Figure 1. Visual representation of a sequence of epitopes and linkers used for polytope construct

anticipate the protective antigenicity of the protein's epitopes (Table 10), (Table 11), (Table 12) (Foroutan, Ghaffarifar, Sharifi, & Dalimi, 2020). Furthermore, immunogenicity was predicted using the IFN epitope web server (Table 13), (Table 14) and (Table 15).

2.5 Prediction of tertiary structure

The vaccine construct was built up of multiple epitopes, and the tertiary structure of a MEV was generated using Alphafold 2.0, an AI-powered platform with an algorithm for computationally predicting the 3-D structure of a protein. Chimera software (Figure 2) can be used to see this 3-D structure. The Z-score, QMEANDisCO and overall quality factor were calculated using ProSA and ERRAT2. To gain knowledge about the protein, qualitative analysis was performed using the Ramachandran plot with the help of the PDBsum web server (Figure 3). (Narang *et al.*, 2022).

2.6 Cloning

JCAT, a codon-optimized web server, was utilized to convert vaccine construct protein sequence to DNA sequence. The converted sequence was uploaded to SnapGene, a molecular design and cloning tool (Susithra Priyadarshni, Isaac Kirubakaran, & Harish, 2021; Gustiananda, Sulistyo, Agustriawan, & Andarini, 2021). PET 28a plasmid was used as a vector, and the resultant circular DNA was cloned using ECoR1 and BamH1 restriction enzymes (Figure 4).

Table 5.
List of predicted CTL epitopes after allergenicity analysis using AllerTOP web server

MHC Class I	Peptide sequence	ML score	MERCI sore	BLAST score	Hybrid Score	Prediction
CTL 1	STLLVHCVY	0.3	0	0	0.3	Non-allergen
CTL 2	GSILSPFLY	0.29	0	0	0.29	Non-allergen
CTL 3	LSDLEAMDI	0.28	0	0	0.28	Non-allergen
CTL 4	RLMTSWQQY	0.27	0	0	0.27	Non-allergen
CTL 5	LTSTPSMHV	0.29	0	0	0.29	Non-allergen
CTL 6	STAPDMDLL	0.3	0	0	0.3	Non-allergen
CTL 7	LTSIIHSCL	0.28	0	0	0.28	Non-allergen
CTL 8	ATTMANHL	0.3	0	0	0.3	Non-allergen

Source(s): Table by authors

Table 6.
List of predicted HTL epitopes after allergenicity analysis

MHC Class II	Peptide sequence	ML score	MERCI score	BLAST score	Hybrid score	Prediction
HTL 1	RGIKLFSWILENGM	0.2	0	0	0.2	Non-allergen
HTL 2	VLTLLFRLCWIWSA	0.21	0	0	0.21	Non-allergen
HTL 3	LTLLFRLCWIWSAV	0.21	0	0	0.21	Non-allergen
HTL 4	LLFRLCWIWSAVPA	0.29	0	0	0.29	Non-allergen
HTL 5	VLRKLFELCLQTTL	0.3	0	0	0.3	Non-allergen
HTL 6	ILSPFLYSIYINSL	0.29	0	0	0.29	Non-allergen
HTL 7	SPFLYSIYINSLPA	0.3	0	0	0.3	Non-allergen
HTL 8	FLYSIYINSLPALL	0.3	0	0	0.3	Non-allergen
HTL 9	SQALFSRWSLWWPV	0.3	0	0	0.3	Non-allergen
HTL 10	ALFSRWSLWWPVVC	0.29	0.29	0	0	Non-allergen
HTL 11	FSRWSLWWPVVCQV	0.29	0.29	0	0	Non-allergen
HTL 12	FLDKIRPMSLTSTV	0.2	0.2	0	0	Non-allergen

Source(s): Table by authors

MHC Class I	Peptide sequence	SVM score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge	Mol wt
CTL 1	STLLVHCVY	−0.84	Non-toxin	0.15	1.39	−1.17	0.5	1034.37
CTL 2	GSILSPFLY	−1.02	Non-toxin	0.22	1.11	−1.07	0	996.3
CTL 3	LSDLEAMDI	−1.28	Non-toxin	0	0.5	0.23	−3	1006.26
CTL 4	RLMTSWQQY	−1.44	Non-toxin	−0.27	−1.06	−0.61	1	1212.52
CTL 5	LTSTPSMHV	−1.04	Non-toxin	0	0.23	−0.59	0.5	972.25
CTL 6	STAPDMDLL	−1.16	Non-toxin	−0.04	0.13	0.06	−2	962.2
CTL 7	LTSIIHSCL	−0.68	Non-toxin	0.16	1.51	−0.94	0.5	986.33
CTL 8	ATTMANHL	−0.1	Non-toxin	−0.01	0.15	−0.65	0.5	858.09

Source(s): Table by authors

Table 7.
List of predicted CTL epitopes after toxicity analysis using ToxinPred web server

Table 8.
List of predicted HTL epitopes after toxicity analysis

MHC Class II	Peptide sequence	SVM score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge	Mol wt
HTL 1	RGIKLFSWILENGM	−0.85	Non-toxin	−0.02	0.24	−0.35	1	1664.22
HTL 2	VLTLLFRLCWIWSA	−0.81	Non-toxin	0.2	1.66	−1.31	1	1721.35
HTL 3	LTLLFRLCWIWSAV	−0.78	Non-toxin	0.2	1.66	−1.31	1	1721.35
HTL 4	LLFRLCWIWSAVPA	−0.76	Non-toxin	0.19	1.45	−1.19	1	1675.27
HTL 5	VLRKLFELCLQTTL	−0.75	Non-toxin	−0.05	0.84	−0.4	1	1677.3
HTL 6	ILSPFLYSIYINSL	−1.19	Non-toxin	0.21	1.26	−1.2	0	1643.17
HTL 7	SPFLYSIYINSLPA	−1.28	Non-toxin	0.13	0.68	−0.98	0	1585.03
HTL 8	FLYSIYINSLPA	−1.25	Non-toxin	0.23	1.39	−1.26	0	1627.17
HTL 9	SQALFSRWSLWWPV	−1.13	Non-toxin	0.02	0.12	−1.01	1	1763.23
HTL 10	ALFSRWSLWWPVVC	−0.92	Non-toxin	0.13	0.91	−1.23	1	1750.29
HTL 11	FSRWSLWWPVVCQV	−0.81	Non-toxin	0.06	0.56	−1.16	1	1793.32
HTL 12	FLDKIRPMSLTSTV	−0.95	Non-toxin	−0.09	0.32	−0.14	1	1608.13

Source(s): Table by authors

Table 9.
List of predicted B-CELL epitopes after toxicity analysis

B-CELL epitopes	Peptide sequence	SVM score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge	Mol wt
B-CELL 1	VDRAIIWRALETYISPAL	−0.53	Non-toxin	−0.02	0.52	−0.32	0	2087.71
B-CELL 2	DRLAHLSTDAHVKQSVLA	−1.05	Non-toxin	−0.17	−0.13	0.08	1	1961.47
B-CELL 3	SDTTATIKRIRRNRTMSP	−0.32	Non-toxin	−0.49	−1.23	0.66	4	2104.66
B-CELL 4	ALYCRRRATWKQFCETLS	−0.1	Non-toxin	−0.31	−0.54	−0.01	3	2232.85
B-CELL 5	LLFRLCWIWSAVPAAWCT	−0.27	Non-toxin	0.17	1.28	−1.22	1	2136.86
B-CELL 6	LLGDSRTTTRGIKLFSWI	−0.53	Non-toxin	−0.11	0.08	−0.19	2	2064.69
B-CELL 7	NAWFWTDDLQALFDRREQ	−0.83	Non-toxin	−0.28	−1.07	0.1	−2	2311.75
B-CELL 8	TRGIKLFSWILENGMTCW	−0.58	Non-toxin	−0.01	0.2	−0.56	1	2155.84

Source(s): Table by authors

2.7 Molecular docking

Cluspro, a web-based program, was utilized to conduct docking tests on the vaccine design and the human immune receptor for a vaccination based on numerous epitopes. The interaction of the vaccine design with the target immune cells of an organism can result in the production of an optimum immune response. Because of its well-known function in the development of antiviral immune responses, TLR3 was chosen as a target (Sudeshna Panda *et al.*, 2022). Chimera software can be used to visualize TLR-3 (Figure 5). For the intended

Table 10. List of predicted CTL epitopes after antigenicity analysis

MHC Class I	Epitope	Antigenicity
CTL 6	STAPDMDLL	Antigen
CTL 8	ATTMANHL	Antigen

Source(s): Table by authors

Table 11. List of predicted HTL epitopes after antigenicity analysis

MHC Class II	Epitope	Antigen
HTL 5	VLRKLFELCLQTTL	Antigen
HTL 9	SQALFSRWSLWWPV	Antigen

Source(s): Table by authors

Table 12. List of predicted B-CELL epitopes after antigenicity analysis

B-CELL epitopes	Epitope	Antigenicity
B-CELL 6	LLGDSRTTTRGIKLFSWI	Antigen

Source(s): Table by authors

Table 13. Final selection of CTL epitopes after IFN epitope analysis

MHC Class I	Peptides	Length	Score
CTL 6	STAPDMDLL	9	0.0971443
CTL 8	ATTMANHL	8	0.1735979

Source(s): Table by authors

Table 14. Final selection of HTL epitopes after IFN-epitope analysis

MHC Class II	Sequence	Method	Result	Score
HTL 5	VLRKLFELCLQTTL	SVM	NEGATIVE	−0.079391402
HTL 9	SQALFSRWSLWWPV	SVM	NEGATIVE	−0.066486387

Source(s): Table by authors

Table 15. List of predicted B-cell epitopes after IFN-epitope analysis

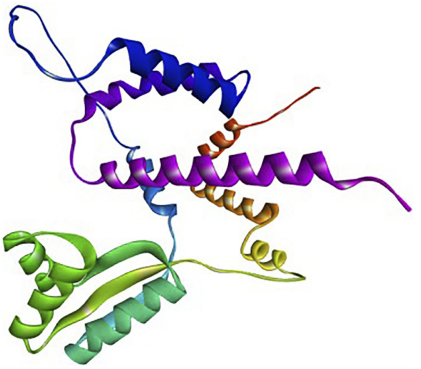
B-CELL epitopes	Epitope	Method	Result	Score
B-CELL 6	LLGDSRTTTRGIKLFSWI	SVM	POSITIVE	0.9737392

Source(s): Table by authors

immune response to be generated, ideal contacts between the receptor and the antigen are necessary. The docking findings indicated acceptable contacts between these two molecules with a weighted score of −1104.8 (Figure 6).

2.8 Analysis of vaccine construct and receptor complex using molecular dynamics simulation

The stability of the docked complex of vaccine construct and TLR3 was evaluated using a 50ns DESMOND simulation. Trajectories were saved every 2fs, and the root mean square deviation (RMSD), and root mean square fluctuation (RMSF) were computed using DESMOND (RMSF).



Source(s): Figure by authors

Figure 2.
3D Predicted structure of constructed polytope of black fungus (*Rhizopus arrhizus*) using AlphaFold Google Colab notebook

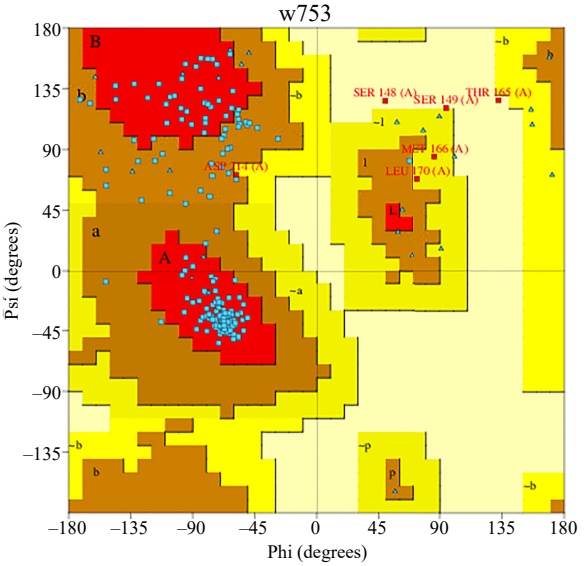


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Figure 3.
The Ramachandran plot of the modeled structure of black fungus generated using PDBSum web server indicates a predominant helix structure

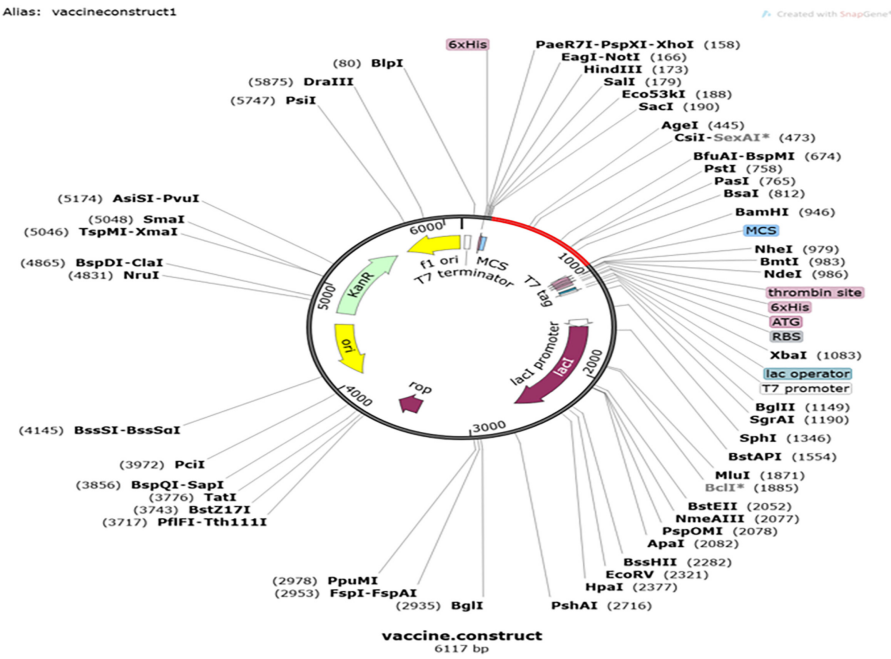

Figure 4.
Polytope construct ligated at *MCS* cloned in pET28a (+) vector at *Sac I* and *Bam HI* restriction sites using SnapGene tool (polytope construct highlighted in red)

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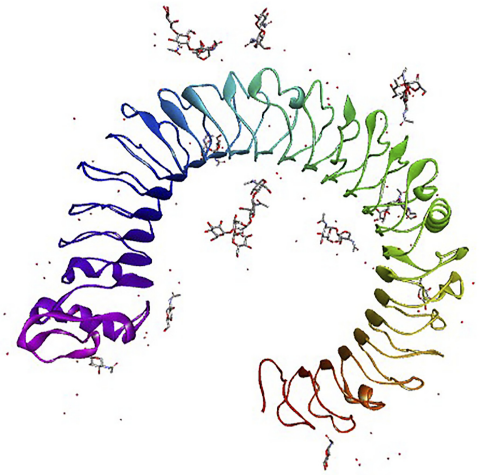

Figure 5.
TLR3 structure of *Homo Sapiens* derived from the PDB as a target for molecular docking

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3. Results and discussion

The amino acid sequences of Mucorales proteins previously associated with virulence factors were used in the current study to derive the epitopes in the IEDB software tool, which gave several numbers of combinatorial peptide sequences, of which the low adjusted rank peptides are reported to be good binders, and B-cell epitope conservation data indicated that 5 of the 8

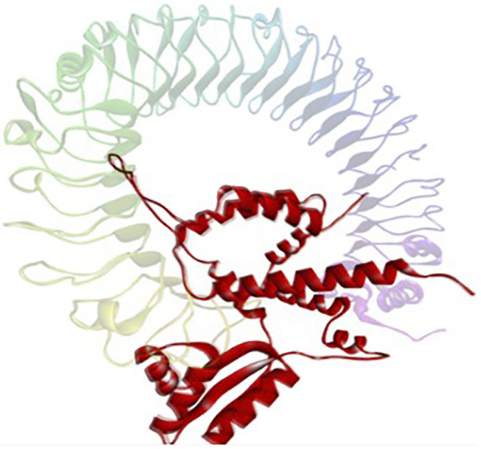

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Figure 6. The docked conformation of human TLR3 with the predicted AlphaFold structure of a black fungus (*Rhizopus arrhizus*) with a weighted score of −1104.8

epitopes were entirely conserved. Furthermore, in MHC-I, 5 of the 8 expected findings were completely retained, but in MHC-II, 6 of the 9 predicted results were intact. A total of IFN-generating epitopes were discovered in target proteins. All of the T- and B-cell epitopes chosen were projected to be non-toxic.

The final vaccine design was made up of four subunits (CTL-1, CTL-2, HTL-1 and BCL-1) joined by an EAAAK linker and GGGGS, and including NotI and BamHI restriction enzyme recognition sites in their coding DNA sequences. Because of its features in the formation of junctional epitopes, the GPGPG linker was included. These linkers help epitopes unite, and all other epitopes were added for their qualities in boosting immunization and epitope presentation. GPGPG, KK and linkers were utilized for CTL, HTL and BCL epitopes, respectively, to help in polytope conformation, and a polytope including 63 amino acids was created.

It was then tested for antigenic properties utilizing the Vaxijen server. The proteins were discovered to be highly antigenic. Both AllerTop V2 and ToxinPred tools yielded the outcome as non-allergic and non-toxin, respectively. After studying the data, Alphafold v2.0 was used to predict 3D models of the given epitopes by adding the appropriate linkers. The expected model was employed for further investigation.

The Z-score measures the total energy deviation of the predicted model with respect to an energy distribution derived from random conformations. The analysis from ProSA indicates a Z-score with overall model quality of −3.21 (Figure 7). The average per residue score and the provided error estimate based on global QMEANDisCo was given as 0.56, showing the good quality of the model. Analysis of non-bonded interactions between different atom types and overall quality factor using ERRAT2 shows score of 86.91, with most of the residues less than 95% error rate (Figure 8).

The Ramachandran plot reveals that the predicted model has 80% amino acids in the core area of the Ramachandran plot. It is widely assumed that if 90% of the residues fall within the permitted range, the model is deemed accurate and reliable. Figure 3 depicts the Ramachandran plot of the simulated structure. Secondary structure prediction is used to establish where beta strands and alpha helix are located within a protein family. PSIPRED and Alphafold were utilized to construct the secondary structure of the final vaccine. Figure 9 visually illustrates the secondary structural features. The proposed secondary structure is composed of 54.6% alpha-helix, 6.8% beta-strand and 38.5% coil.

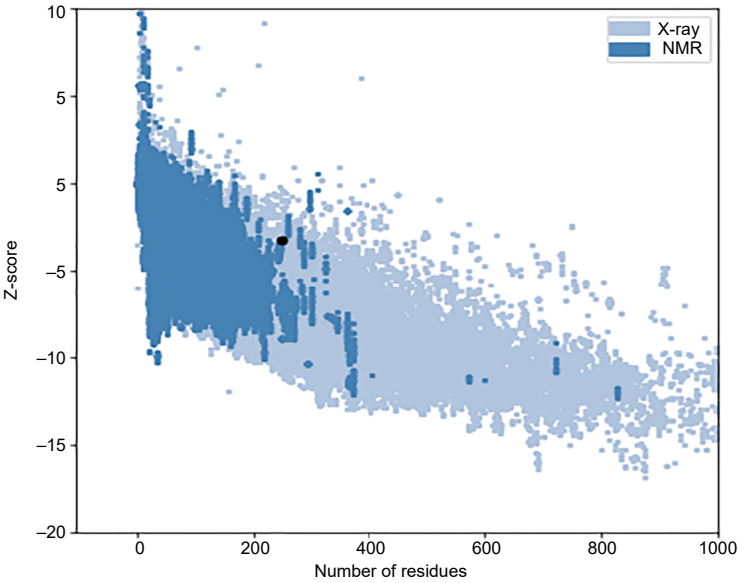

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Figure 7.
The ProSA Z-score of overall model quality for predicted protein given as −3.21 indicates a good model

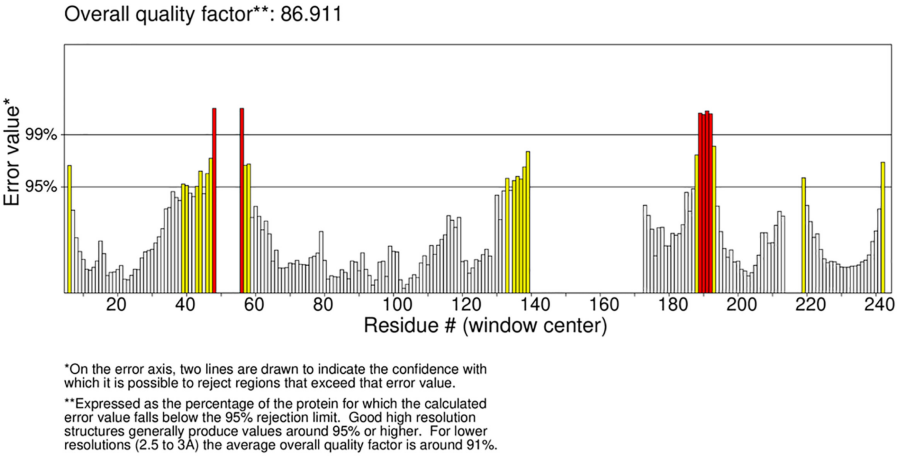

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Figure 8.
ERRAT predicted overall quality factor for structure analysis of the constructed model with a score of 86.911. The plot represents quality score along with error rates for the residues

To manufacture the vaccine candidate, the up-stream protocol requires a design of gene construct with a cloning vehicle. In Figure 4, the translated DNA of the polytope construct is *in silico* ligated in pET28(a) plasmid will enrich the quantity of polytope which upon down-streaming steps, formulating with TLR3 agonist, stabilizers and preservatives, vaccine for black fungus could be formulated for pre-clinical trials.

The docking results demonstrated appropriate contacts between these two molecules (Figure 6), with a weighted score of −1104.8.DESMOND used to perform MD analysis on the

docked complex of TLR3 and vaccine construct. As seen in the graph, the average RMSD and RMSF values obtained were 7A and 1A, respectively. The complex's C-alpha atoms were quite unstable at the start of the simulation, but they immediately regained stability at 22 n sec and remained stable throughout the 50-n sec simulation. During the simulation, the complex had an average RMSD of 7. To gain structural information about the complex, C-alpha atoms were aligned with the reference set. As a result, Figure 10 displays a

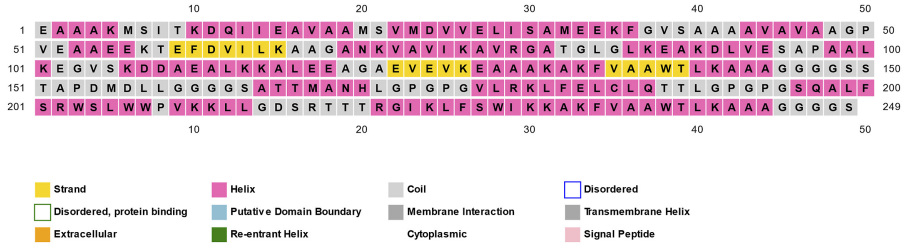

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Figure 9. Secondary structure of multiepitope vaccine (MEV). The alpha helix residues are pink, the coil residues are gray and the beta-strand is yellow

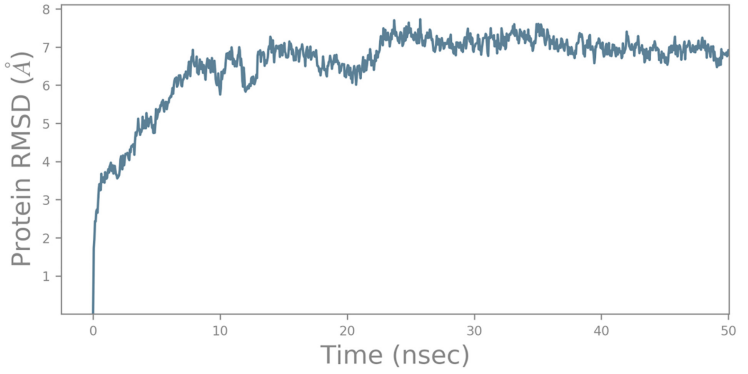

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Figure 10. RMSD analysis of TLR3 bound to vaccine construct (selected protein) indicating stability from 22ns to the end of the simulation, that is 50ns

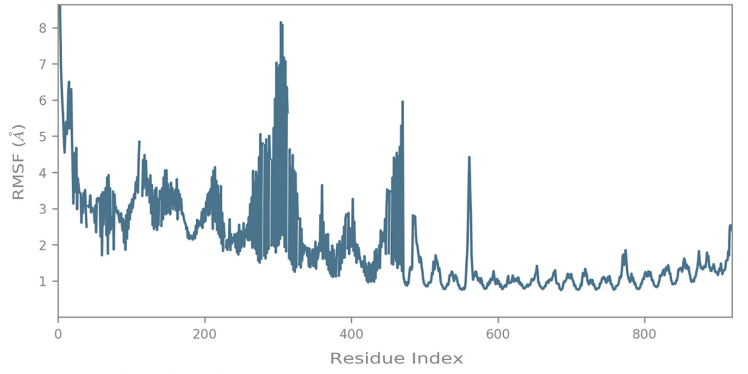

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Figure 11. RMSF analysis of TLR3 bound to a vaccine construct (selected protein) and stabilizing at the C-terminus, representing the fluctuations in the initial residues

well-equilibrated system experiencing substantial conformational changes over the simulation.

An RMSF study of a system provides the variations in a particular collection of atoms from their mean locations. The analysis was carried out on the system's C-alpha atoms using the DESMOND program (Narang *et al.*, 2021). The peaks in the graph indicate atom fluctuations; substantial variations in the graph were seen at the early residues with indices ranging from 200 to 400. The residues with indices 600 to 850 exhibited the lowest variations in the system of 919 residues, with an average RMSF of 1 (Figure 11). These findings support the docking interaction investigation by revealing that MEV can bind to immune receptors robustly enough to elicit an immune response against black fungus.

4. Conclusion

The recent black fungus outbreak resulted in the deaths of many people in India and caused the country's economy to collapse. There is no recognized treatment or immunization that is effective against black fungus. During this audit, a successful attempt was made to create a subunit MEV against black fungus. *In silico* immunoinformatic techniques were employed to produce a possibly safe MEV capable of eliciting three types of safe responses: humoral, natural and cell. In the current study, MEV has been proposed as a suitable neutralizer candidate since it is immunogenic, secure, stable and interacts with human receptors. A stream study, on the other hand, is produced via a mixed vaccination approach. The aforementioned attributes authenticate that the constructed polytope would be a vaccine candidate, which, upon formulation, will be used as a therapeutic candidate among the endemic group.

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