Subtractive proteomics and reverse vaccinology were employed to design a chimeric multi-epitope vaccine against melioidosis, a severe infectious disease caused by the multidrug-resistant bacterium, *Burkholderia pseudomallei*. From 21 non-redundant pathogen proteomes, four proteins were selected as vaccine candidates based on essentiality, virulence, non-homology to humans, structural availability, subcellular localization, and antigenicity. The major histocompatibility complex class I, II, and B-cell epitopes were predicted and evaluated for immunogenicity, solubility, allergenicity, and hydrophilicity. A chimeric vaccine was constructed using the epitopes, adjuvants, linkers, and PADRE sequences. Docking and molecular dynamics simulations confirmed interactions between HLA alleles and TLR4, indicating the potential to elicit an immune response, demonstrating the utility of computational tools in developing vaccines against *B. pseudomallei*.

Dr. Mohammad Kalim Ahmad Khan, Professor of Bioinformatics at Integral University, Lucknow, India, has over 16 years of teaching experience and 85+ publications and has successfully guided six PhD scholars. His research interests include PAH-induced carcinogenesis, lifestyle diseases, and chimeric vaccine design using subtractive proteomics.

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Chimeric Vaccine Design Against Melioidosis via Subtractive Proteomics

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LIST OF SYMBOLS AND ABBREVIATIONS

COPD	Chronic Obstructive Pulmonary Disease
TLR	Toll like receptor
IFN	Interferon
IL	Interleukin
TID	Target Identification
CD-HIT	Cluster Database at High Identity with Tolerance
DEG	Database of Essential Genes
CEG	Cluster of Essential Genes
BLAST	Basic Local Alignment Search Tool
RefSeq	Reference Sequence
POCKDRUG	Pocket Druggability Prediction
SVM	Support vector machines
ACC	Auto cross covariance
QSAR	Quantitative structure-activity relationship
CTL	Cytotoxic T lymphocytes
TAP	Transporter associated with antigen processing
MHC	Major Histocompatibility complex
UNIPROT	Universal Protein knowledgebase
PDB	Protein data bank
RV	Reverse vaccinology
OMP	Outer Membrane Protein
HBHA	Heparin-Binding Hemagglutinin Adhesin
GRAVY	Grand Average of Hydropathy
RCSB-PDB	Research Collaboratory for Structural Bioinformatics Protein Data Bank
HLA	Human Leucocyte antigen
FIREDOCK	Fast Interaction REfinement in molecular DOCKing.
GROMACS	GROningen MAchine for Chemical Simulations
CAI	Codon Adaptation Index
RMSD	Root Mean Square Deviation

ABSTRACT

Multidrug-resistant *Burkholderia pseudomallei* is a significant global health concern associated with high morbidity and mortality rates. To address this issue, a chimeric multi-epitope vaccine against *B. pseudomallei* was designed using subtractive proteomics and reverse vaccinology. A subtractive proteomics strategy was applied to the 21 non-redundant proteomes of the pathogen. From these, proteins that were non-homologous to humans, essential for survival, and virulent were identified. BLASTp analysis against the PDB database and druggability assessments revealed nine proteins with available three-dimensional structures, of which four were deemed potential vaccine candidates based on subcellular localization and antigenicity predictions.

Utilizing online tools, MHC class I, II, and B-cell epitopes were predicted and screened for toxicity, solubility, allergenicity, and hydrophilicity. Immunogenic epitopes were selected to construct a chimeric multi-epitope vaccine incorporating adjuvant, linker, and PADRE sequences. Molecular docking and dynamics simulations demonstrated strong interactions between the vaccine construct and HLA alleles and TLR4, indicating its potential to elicit a robust immune response. The availability of the *B. pseudomallei* proteome facilitated this study, demonstrating the effectiveness of insilico tools for identifying vaccine targets and designing chimeric vaccines using reverse vaccinology and immunoinformatics methodologies.

Chapter 1: Review of Literature

1.1 INTRODUCTION

Melioidosis is a tropical bacterial infection caused by Burkholderia pseudomallei, a bacterium found in the environment (Wiersinga et al., 2012). The disease can manifest in different ways, ranging from acute septicemia, which can be rapidly fatal, to a chronic condition resembling other common infections like tuberculosis (Dance, 2002). Melioidosis is primarily found in Southeast Asia and Northern Australia, with increased cases during monsoon seasons (Cheng & Currie, 2005). People contract the infection through inhalation, inoculation, or consumption of the bacteria (Currie et al., 2010).

Whitmore (1913) first identified the organism in 1911. It can infect humans and many animal species. It is commonly found in the rhizosphere, which is the layer of soil surrounding plant roots that contains microorganisms (Kaestli et al., 2011). It is also present in surface groundwater in most subtropical and tropical regions (Limmathurotsakul et al., 2016; Wieringa et al., 2012).

1.2 REVIEW OF LITERATURE

1.2.1 BURKHOLDERIA PSEUDOMALLEI AND MELIOIDOSIS

1.2.1.1 MICROBIOLOGY AND TAXONOMY

Proteobacteria: This major group of gram-negative bacteria contains many disease-causing genera like Escherichia, Salmonella, Vibrio, Helicobacter, and more.

Bacteria: Bacteria are single-celled microorganisms, the highest level of classification. They can live independently, cooperatively with other organisms, or parasitically by causing disease.

Burkholderiaceae: This Proteobacteria family includes the genera Burkholderia and Ralstonia.

Burkholderiales: Among the harmful bacteria in this order of Proteobacteria are species of Bordetella and Burkholderia.

Burkholderia: This genus of Proteobacteria includes certain pathogenic species, such as B. cepacia, B. pseudomallei, and B. mallei.

Burkholderia pseudomallei: This specific bacterium causes melioidosis in humans and animals. It can be found in contaminated water and soil.

1.2.1.2 GLOBAL DISTRIBUTION

The worldwide burden of melioidosis is estimated to be over 4.6 million disability-adjusted life years (DALYs). This burden is higher than that of other tropical diseases such as intestinal worm infections, leptospirosis, schistosomiasis and dengue (Birnie et al., 2019). Globally, there are thought to be 89,000 melioidosis fatalities and 165,000 instances of the disease per year. The actual mortality rates range from 9% to 70% in different regions (Gassiep et al., 2020). Melioidosis is greatly underreported in at least 45 countries where it is endemic, including Bangladesh and Sri Lanka. Nearly 99% of the DALY burden was due to deaths from the disease. An estimated 165,000 cases and 89,000 melioidosis deaths globally were projected for 2015. These estimates exclude critical data from 45 countries where melioidosis is known to be endemic but severely underreported (Limmathurotsakul et al., 2016). Global warming along with changes in soil conditions, hot and humid weather, and uncontrolled urbanization may lead to an increased problem and spread of melioidosis (Chai & Fisher, 2018). The B. pseudomallei and B. mallei, which were employed for bioterrorism during World War I, are categorised as category B priority agents, the second highest priority, by the Centres for Disease Control and Prevention (CDC) in the United States. In tropical nations like India,

melioidosis is far more common than previously believed to be diagnosed. The main tactic, like with any infectious condition, is still infection prevention.

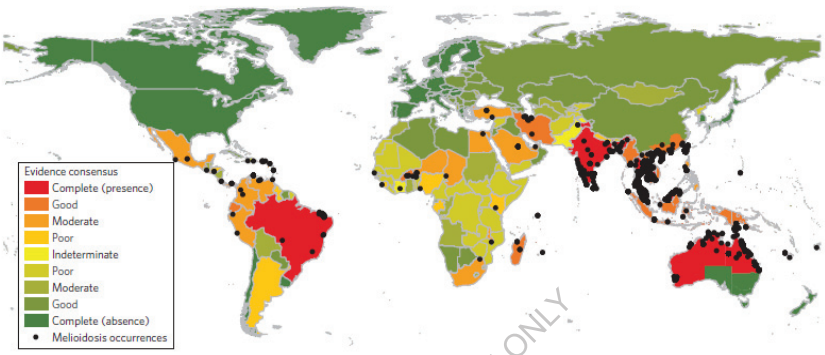

Figure 1.1. Global evidence consensus and geographic locations of occurrence data from 1910 to 2014 ((Limmathurotsakul et al., 2016)

1.2.1.3 DISTRIBUTION IN INDIA

This study shows the rise of melioidosis as a very big health issue in India. Due to a referral bias, two thirds of the patients in our research were from eastern and northeastern India. . The disease frequently affected middle-aged working men, indicating potential occupational exposure. In certain regions, the association between acute melioidosis and the rainy season is consistent with earlier studies that demonstrate a connection between precipitation and the incidence of disease (Vidyalakshmi et al., 2012). The high proportion of diabetes patients (82.3%) versus other studies (39%-60%) may reflect India's growing diabetes epidemic (Suputtamongkol et al., 1999).

The high incidence of long-lasting chronic melioidosis in this study differs with other research and may be partly due to referrals of undiagnosed patients from relatively arid areas. Patients with acute disease often lived near our hospital (Vidyalakshmi et al., 2012) (Chetchotisakd et al., 2014). Acute flare-ups in chronic illness patients are linked to unfavourable consequences. Given their similar risk factors and immunological responses, co-infection between tuberculosis and melioidosis is not surprising (Koh et al., 2013).

Most patients responded well to intense therapy followed by eradicative therapy. Melioidosis mortality was 14.9%, decreasing over the study. A prior study here found 17% mortality 7, lower than Thailand's rate (≈40%) (Limmathurotsakul & Peacock, 2011). Poor outcomes were shown to be predicted by bacteremia, lung involvement, low albumin, and high SOFA scores. Roughly 6 percent of the isolates of B. pseudomallei were resistant to trimethoprim/sulfamethoxazole. Given that the susceptibility testing was altered during the trial, this might represent a small overestimate. In this instance, susceptibility should be the guiding factor for eradication, notwithstanding a trial's conclusion that combination therapy using trimethoprim and sulfamethoxazole was not inferior (Chetchotisakd et al., 2014).

Public health officials as well as Indian practitioners should take melioidosis seriously. Organ involvement might be helpful in situations with limited resources as it can signal acute or chronic disease. Early treatment and a high degree of suspicion are crucial given the significant fatality rate.

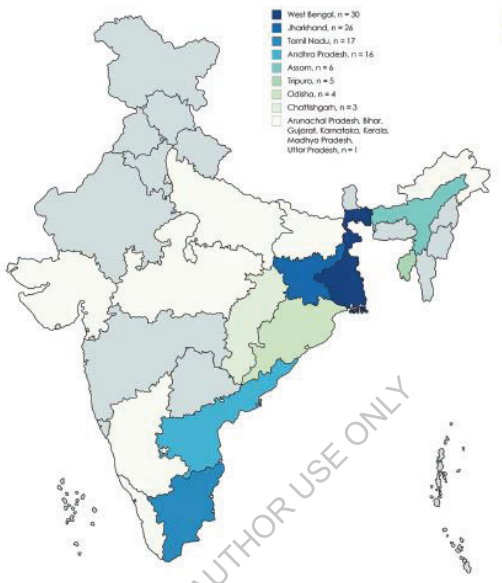

Figure 1.2. Distribution of melioidosis in India ((Koshy et al., 2019)

1.2.1.4 CLINICAL MANIFESTATION

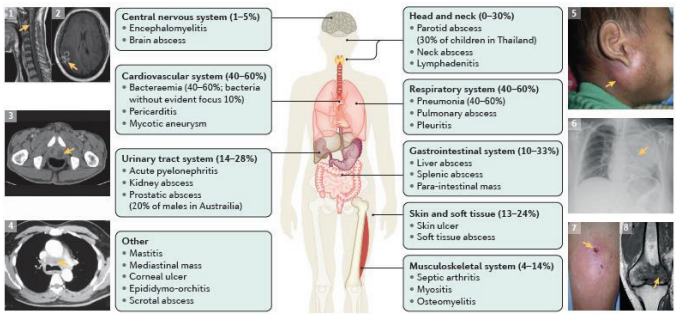

Figure1.3: Clinical manifestations of melioidosis (Wiersinga et al., 2018)

The clinical presentation of melioidosis is varied, so it is helpful for doctors to categorize it into four types (Kerr, 2005), (Kanai & Dejsirilert, 1988). The acute septicemic form has a short incubation period of a few days. It presents as a sudden, community-acquired sepsis resembling other diseases like, typhus, typhoid fever, malaria, leptospirosis, necrotizing fasciitis, gram-negative bacteremia, staphylococcal bacteremia, invasive pneumococcal disease, meningococcemia, plague, and Vibrio vulnificus infection. Clues include seasonal onset during the rainy season and exposure to water or soil (similar to leptospirosis), and rapid progression to death in outbreaks (like meningococcal sepsis and plague). Larger abscesses if the patient lives, or microscopic abscesses after death, may be discovered in the skin, soft tissues, lymph nodes, bones, lungs, liver, spleen, kidneys, and brain due to the bacteria's bloodstream spread.

Jaundice, pneumonia, gastroenteritis or meningitis may predominate. Mortality used to be as high as 90% but can now be reduced to around 12-40% with early antibiotics and intensive care. (Cheng & Currie, 2005),2 The subacute form has a longer, variable incubation period over weeks to months. It causes milder sepsis and multiple abscesses in the lungs, liver, spleen, psoas muscle, bones, brain, eyes and soft tissues. Without treatment, the abscesses may become chronic. The chronic form may occur by itself or follow the subacute form, either directly or through recurrence after treatment. It progresses slowly over months to years, mimicking tuberculosis, fungal infections or cancer. The latent or asymptomatic form is found incidentally in those with past exposure, even years later. It can reactivate with immunosuppression like trauma, burns, steroids, cancer, diabetes or other infections. Furthermore, certain regions exhibit elevated prevalence of symptoms, such as parotitis in Thailand, encephalomyelitis in Australia, and genitourinary and prostate abscesses (Cheng & Currie, 2005).

1.2.1.5 DIAGNOSIS

Melioidosis continues to be severely underdiagnosed globally. This is mostly due to the lack of diagnostic microbiology labs serving low-income rural communities, which are at the highest risk of contracting the disease. It is also because medical professionals and lab staff are not well-informed on melioidosis. B. pseudomallei may first be written off as a contaminant or non-pathogenic environmental bacteria, even in well-equipped microbiology labs, especially in areas where the disease is not common (Doker et al., 2014).

1.2.1.5.1 CLINICAL DIAGNOSIS

According to Currie et al. (2000), the incubation period for acute melioidosis infections ranges from 1 to 21 days, with an average of 9 days. On the other hand, a more severe form of the disease with a shorter incubation period may arise from breathing or aspirating contaminated fresh water (Chierakul et al., 2005). The clinical presentation, severity, and prognosis of patients with melioidosis are influenced by various factors such as the existence or lack of risk factors, the infection route, the bacterial load and strain, and the presence or lack of specific B. pseudomallei virulence genes (Currie, 2015; Sarovich et al., 2014). The illness might range from sepsis and death to localised cutaneous symptoms at the site of bacterial penetration without systemic repercussions. 20% of melioidosis patients experience septic shock at admission, and 40–60% of patients have bacteremia. In over 50% of patients, pneumonia is the first ailment. Internal organs are frequently affected by the bacteria, with the spleen, prostate, liver, and kidneys being particularly vulnerable.

LIMITATIONS OF CLINICAL DIAGNOSIS

Patients with clinically suggestive characteristics (e.g., diabetes, exposure to certain substances at work or during certain seasons) should be treated empirically with antibiotics that target B.

pseudomallei in known endemic areas, despite the fact that diagnosing melioidosis solely based on clinical features is extremely difficult. Melioidosis should be considered for any fever in endemic and possibly endemic areas (Limmathurotsakul et al., 2016), particularly if the patient has pneumonia or an abscess (especially in the liver, spleen, prostate, or parotid). Additionally, melioidosis might manifest as vague clinical symptoms. However, as not all patients have risk factors, laboratory testing is required to confirm a diagnosis of melioidosis (Hoffmaster et al., 2015).

1.2.1.5.2 MICROBIOLOGICAL DIAGNOSIS

Culture

Culture remains the cornerstone for the diagnosis of melioidosis. B. pseudomallei may grow on most common laboratory media, but it may be overlooked and dismissed as a contaminant. It can also be confused for other bacteria, like Pseudomonas and Bacillus species, if lab staff members are unfamiliar with its appearance (Hoffmaster et al., 2015).

B. pseudomallei should be diagnosed with melioidosis since it is never present in the normal human flora and its isolation from any clinical sample supports this theory. Since B. pseudomallei is classified as a hazard category 3 pathogen and tier 1 select agent (Federal Select Agent Programme, n.d.), physicians should notify the hospital laboratory of any suspected melioidosis in a patient who has been hospitalised. Appropriate safety precautions can be taken by doing this. All employees working in microbiology laboratories in areas where melioidosis is endemic should receive regular, sufficient training and adhere to local safety laws.

Direct detection in clinical samples

Treatment shouldn't be put off while awaiting culture results because melioidosis might have serious or even deadly effects. Therefore, Burkholderia pseudomallei could be directly detected in clinical samples to expeditiously confirm the diagnosis. B. pseudomallei looks like a safety pin-shaped, Gram-negative rod under a light microscope due to its bipolar staining; however, the sensitivity and specificity of microscopy are low (Sheridan et al., 2007). Compared to culture, immunofluorescent microscopy has less than 50% sensitivity but nearly 100% specificity (Tandhavanant et al., 2013). Other approaches, such as antigen detection or nucleic acid amplification, have also been tried. A lateral flow immunoassay for detecting extracellular capsular polysaccharides was developed by Houghton et al. (2014), although it was not extensively tested. Particularly when it comes to blood, it seems to have poor sensitivity, but it has respectable specificity (Robertson et al., 2015). Many specific PCR tests for B. pseudomallei have been developed and tested in short-term clinical studies since the 1990s. The most promising focuses on the T3SS gene cluster; nonetheless, blood sensitivity requires a substantial number of bacteria (Kaestli et al., 2012; Richardson et al., 2012). These PCR assays are not commonly used for diagnosis in endemic areas, not even in high-income countries. Apart from its sensitivity issues, the tests do not provide the timely confirmation needed to make economical treatment decisions.

1.2.1.6 ANTIMICROBIAL THERAPY

There is an intensive phase at the beginning of antimicrobial therapy and an eradication phase at the end.

Intensive therapy

At least 10 to 14 days should elapse during the first rigorous therapy cycle. Either ceftazidime (2 g intravenously every 6 hours in wards; or 50 mg/kg up to 2 g in children under 15 years old) or meropenem (1 g intravenously every 8 hours in critical care units; or 25 mg/kg up to 1 g in children) should be included. Meropenem is the first-choice intravenous treatment for melioidosis that affects the central nervous system. In situations such as these with neurological melioidosis, the dose of meropenem ought to be increased.

Consider initiating trimethoprim-sulfamethoxazole at the onset of vigorous therapy in cases of neurological melioidosis, osteomyelitis, septic arthritis, skin and soft tissue infections, and genitourinary infections, including prostatic abscesses. Trimethoprim-sulfamethoxazole has high tissue penetration.

Prolonged intravenous therapy (four to eight weeks or longer) is recommended in cases of neurological melioidosis, septic arthritis, deep-seated infections (including prostatic abscesses), and acute pneumonia.

Eradication therapy

The infection-eradication therapy should be continued for a minimum of three months following the conclusion of the first, intensive treatment. It has 320 + 1600 mg given orally every 12 hours to those weighing more than 60 kg. It comprises trimethoprim-sulfamethoxazole at doses of 6 + 30 mg per kilogramme up to 240 + 1200 mg for adults weighing 40–60 kg and 240 + 1200 mg for children. Additionally, it entails taking 5 milligrams of folic acid orally every day (or 0.1 mg per kilogramme up to 5 mg in infants).

Additional therapy for three months or more is recommended to fully remove the condition.

1.2.1.7 RISK FACTORS

People may be more susceptible to melioidosis due to specific risk factors and underlying medical disorders. Diabetes is a major risk factor; it is estimated that people with diabetes account for around 50% of instances of melioidosis. Melioidosis was three times more common in persons with diabetes mellitus than in those without the disease. Melioidosis risk is higher in those with renal impairment. People who suffer from long-term lung conditions, like chronic obstructive pulmonary disease (COPD), are more vulnerable. Melioidosis is more common in those with HIV infection, cancer, and immunosuppressive medications.

Melioidosis has been observed to exhibit seasonal patterns in certain endemic regions. In Northern Australia, cases tend to peak during the wet season, characterized by heavy rainfall and increased soil and water exposure (Radzi et al., 2019) (Cheng & Currie, 2005). It's important to note that the epidemiology of melioidosis is dynamic, and the distribution and incidence can change over time. Surveillance systems and improved diagnostic capabilities are essential for accurate monitoring and reporting of cases.

1.2.1.8 IMMUNE RESPONSE IN MELIOIDOSIS

1.2.1.8.1 INNATE IMMUNE RESPONSE

Early innate immune response has a vital role in identifying Burkholderia pseudomallei, the melioidosis-causing pathogen, and instigating an inflammatory reaction when it enters the body. The host innate immune response to B. pseudomallei, the causative agent of melioidosis, involves both pro- and anti-inflammatory responses that are dysregulated, allowing the bacteria to evade elimination. It was found that genes involved in immune response, stress response, cell cycle regulation, proteasomal degradation, cellular metabolism and signal transduction pathways were differentially regulated in mice with acute melioidosis (Chin et al., 2010).

While TLR2 and inflammatory responses were upregulated initially, cell death pathways were also activated, and the complement system was only activated after 24 hours, allowing uncontrolled bacterial spread. It was shown that B. pseudomallei inhibits NF-κB and IFN pathways through the virulence factor TssM, suppressing the host inflammatory response (Tan et al., 2010). The early innate response is characterized by production of pro-inflammatory cytokines like IL-18, as Wiersinga 2007 found IL-18 deficient mice were more susceptible to acute melioidosis. Genome-wide analysis of host responses during early infection in Chin 2010 revealed activation of inflammatory pathways and cell death mechanisms within 24 hours of infection, although these responses became suppressed by 42 hours, allowing uncontrolled bacterial growth. However, the complement system was not activated until 24 hours, suggesting a delay that may contribute to disease severity.

It was found that IL-18, which stimulates IFN-γ, was elevated in melioidosis patients and improved survival in mice, indicating its importance in host defense (Wiersinga et al., 2006). However, it was showed that IFN-mediated signaling, while dominant in the host responses to both melioidosis and TB, did not distinguish between these diseases (Koh et al., 2013). Few researchers found dysregulation of TNF-α and IFN-γ in chronically infected mice across multiple B. pseudomallei strains, suggesting these could serve as biomarkers. (Amemiya et al., 2020, Krishnananthasivam et al., 2017) showed downregulation of immune response genes and epigenetic regulators in melioidosis patients compared to sepsis patients, which could also indicate disease and distinguish it from other infections.

(Aschenbroich et al., 2016) summarized that B. pseudomallei and the related B. mallei modulate the innate immune system to evade intracellular killing, but the specific mechanisms

remain unclear. A better understanding of how these pathogens dysregulate host immune responses could identify new vaccine and therapeutic targets.

Burkholderia pseudomallei, elicits a strong innate immune response in humans that helps determine the outcome of infection. Multiple studies have found that melioidosis patients and individuals with subclinical exposure to B. pseudomallei generate robust antibody (Rongkard et al., 2020, Vasu et al., 2003) and cell-mediated (Ketheesan et al., 2002, Barnes et al., 2004) immune responses to the pathogen. Specifically, melioidosis patients produce IgG, IgA and IgM antibodies against B. pseudomallei antigens throughout infection, with IgG1 and IgG2 being the predominant IgG subclasses (Vasu et al., 2003). Although all patients generate these antibodies, IgG and IgG1/IgG2 levels correlate well with clinical outcomes, suggesting they may be useful for monitoring infection status and treatment (Vasu et al., 2003). Individuals with subclinical melioidosis also mount strong cell-mediated responses, including lymphocyte proliferation and interferon-gamma production, which may protect against disease progression (Barnes et al., 2004, Wiersinga et al., 2007)

Cytokines like interleukin-18 (IL-18) and tumor necrosis factor-alpha (TNF-α) are crucial components of the early innate response to B. pseudomallei. IL-18 levels are elevated in melioidosis patients and promote interferon-gamma production, which is essential for controlling infection (Wiersinga et al., 2007). Mice lacking IL-18 experience accelerated mortality and increased bacterial burdens upon B. pseudomallei challenge, indicating IL-18 is protective (Wiersinga et al., 2007). TNF-α is also upregulated in melioidosis, and dysregulation of TNF-α and interferon-gamma is a common host response across different B. pseudomallei strains (Amemiya et al., 2020).

The innate response to melioidosis differs between diabetic and non-diabetic individuals. Diabetic patients rely more on antibodies and double-negative T cells for survival, whereas non-diabetic patients depend more on NK cells, CD8+ T cells and granzyme B (Kronsteiner et al., 2019). IL-15, IL-18 and CX3CR1 are also linked to outcome in melioidosis, with excessive IL-15 and IL-18BP reducing survival, and CX3CR1 expression on lymphocytes improving survival (Kronsteiner et al., 2019).

In summary, the host innate immune response to melioidosis involves a mix of inflammatory and anti-inflammatory responses that are improperly regulated, allowing the bacteria to thrive intracellularly. Certain cytokines like IFN-γ and TNF-α, as well as other immune regulators, are dysregulated across multiple studies, suggesting they could serve as biomarkers or therapeutic targets. However, the specific mechanisms by which B. pseudomallei modulates the host immune response remain to be fully elucidated.

1.2.1.8.2 ADAPTIVE IMMUNE RESPONSE

The adaptive immune response to melioidosis, caused by the bacterium Burkholderia pseudomallei, involves both humoral and cell-mediated immunity. Several studies found that melioidosis patients and individuals exposed to B. pseudomallei develop antibodies against the bacterium, including IgG, IgA and IgM (Vasu et al., 2003, Rongkard et al., 2020). IgG1 and IgG2 were the predominant IgG subclasses (Vasu et al., 2003). Although antibody levels were high in melioidosis patients, they did not always correlate with disease severity or outcome (Vasu et al., 2003).

Cell-mediated immunity also plays an important role in the adaptive immune response to B. pseudomallei. T cells, especially CD4+ and CD8+ T cells, proliferate in response to B. pseudomallei antigens in melioidosis patients and exposed individuals (Barnes et al., 2004,

Ketheesan et al., 2002, Rongkard et al., 2020). This T cell proliferation was accompanied by production of interferon-gamma (IFN-γ), a key cytokine in the immune response to intracellular pathogens (Barnes et al., 2004, Ketheesan et al., 2002). Mice deficient in IFN-γ were more susceptible to B. pseudomallei infection, demonstrating its importance in controlling the infection (Healey 2005).

IL-18, a cytokine that stimulates IFN-γ production, was also shown to be important in the immune response to B. pseudomallei. Plasma IL-18 levels and monocyte IL-18 mRNA levels were elevated in melioidosis patients (Wiersinga et al., 2007). Mice deficient in IL-18 were more susceptible to lethal B. pseudomallei infection, with higher bacterial burdens and more severe organ damage (Wiersinga et al., 2007). This indicates that IL-18 helps improve the early antimicrobial host response in melioidosis.

The host immune response to the bacterial infection melioidosis, caused by Burkholderia pseudomallei, involves both innate and adaptive immunity. (Ketheesan et al., 2002) found that patients who survived melioidosis developed cell-mediated immunity against B. pseudomallei, indicating an adaptive T cell response is important for protection. Supporting this, (Barnes et al., 2004) found that individuals with subclinical melioidosis who did not develop disease had stronger T cell responses to B. pseudomallei compared to those with clinical melioidosis.

Both type 1 and type 2 interferon responses are elicited in melioidosis, according (Koh et al., 2013), who found interferon-mediated signaling dominated host responses to both melioidosis and tuberculosis. They also found an 86-gene signature thought to be specific for tuberculosis was also present in melioidosis patients, indicating some similarity in host responses. However, found some differences in gene expression between melioidosis patients and those with sepsis from other causes. Certain immune response genes like IL8 and epigenetic regulators were

downregulated in melioidosis patients compared to other sepsis patients, suggesting they could serve as diagnostic biomarkers (Krishnananthasivam et al., 2017).

In summary, the host adaptive immune response to melioidosis involves T cell activation and production of interferons, although the early innate response is also crucial for controlling infection. A delay in complement activation and suppression of some inflammatory responses may contribute to disease severity. Differences in gene expression profiles could help distinguish melioidosis from other causes of sepsis. A combination of innate and adaptive immune responses, as well as rapid diagnosis and treatment, are required for effective host defense against this potentially deadly disease.

So, both humoral and cell-mediated immunity are required to control B. pseudomallei infection. Antibodies, CD4+ and CD8+ T cells, IFN-γ, and IL-18 all contribute to the adaptive immune response in melioidosis. A deficiency or dysregulation in any of these immune components can lead to severe, life-threatening disease. Continued research on the immune response to B. pseudomallei may identify new prevention and treatment strategies for this neglected tropical disease**.**

1.2.1.8.3 IMMUNE EVASION MECHANISM

Melioidosis, caused by the bacterium Burkholderia pseudomallei, is a potentially fatal disease endemic to Southeast Asia and northern Australia. B. pseudomallei is adept at evading the host immune system through various mechanisms, allowing it to persist intracellularly and chronically infect its host.

Several studies found elevated levels of cytokines and other immune markers in melioidosis patients, indicating activation of the immune system. However, this immune activation is ineffective at clearing the infection (Barnes & Ketheesan, 2007, Ketheesan et al., 2002) found

melioidosis patients demonstrate cell-mediated immune responses and lymphocyte proliferation against B. pseudomallei antigens. Yet, (Barnes et al., 2004) found individuals with subclinical melioidosis actually had stronger cell-mediated responses, suggesting the immune system may play a role in determining disease outcome. Iliukhin (1980) found laboratory animals developed immunity against reinfection with B. pseudomallei, but golden hamsters showed no such response, indicating sensitivity to infection depends on individual immunity levels.

B. pseudomallei is adept at modulating the host immune response to its advantage. (Wiersinga et al., 2007) found interleukin-18 (IL-18), a cytokine important for inducing interferon-gamma (IFN-γ) production, was elevated in melioidosis patients. However, IL-18 knockout mice were more susceptible to B. pseudomallei infection, suggesting IL-18 plays a protective role, and B. pseudomallei may suppress its effects. Brown (1991) found IFN-γ and soluble IL-2 receptors were elevated in melioidosis patients, indicating immune cell activation, but soluble CD8, a marker of cytotoxic T cell activation, was unchanged, suggesting B. pseudomallei may selectively activate certain immune pathways.

Aschenbroich (2016) proposes that B. pseudomallei exploits host immune signaling pathways to thrive intracellularly, evade killing, and establish chronic infection. A better understanding of how these pathogens manipulate the host immune system may reveal new vaccine targets to counter these effects and prevent melioidosis.

While the host immune system responds to B. pseudomallei infection, the pathogen is adept at selectively modulating the immune response to its advantage, allowing it to persist in the host and cause chronic or fatal disease. A vaccine that can counteract B. pseudomallei's immune evasion mechanisms may be key to preventing melioidosis.

B. pseudomallei expresses many proteins on the surface of infected erythrocytes that help the pathogen evade detection. For example, it was found that the B. pseudomallei protein RIFIN binds to inhibitory receptors on immune cells, suppressing their activation. Additionally, B. pseudomallei flagellin proteins trigger host pathogen recognition receptors like TLR5 and NLRC4, but the pathogen has developed ways to avoid the immune responses activated by these receptors. (West et al., 2014) showed that while TLR5 and NLRC4 help control B. pseudomallei infection in the lungs, mice deficient in both receptors were not more susceptible, indicating the pathogen can evade each individual receptor.

B. pseudomallei also directly inhibits immune responses. For instance, (Stone et al., 2014) reviewed how B. pseudomallei secretes factors that manipulate host cell processes and disable parts of the immune system. Specifically, B. pseudomallei uses a type III secretion system to inject effector proteins into host cells that suppress phagocytosis and the production of reactive oxygen species. The pathogen also interferes with antigen presentation to avoid detection by T cells.

However, the host is still able to mount some immune responses against B. pseudomallei. Krishnananthasivam et al., 2017 found that melioidosis patients had different expression of certain immune response genes compared to patients with other infections, indicating the host can recognize B. pseudomallei as a distinct pathogen. A study also showed that guinea pigs and mice developed immunity against B. pseudomallei after infection, exhibiting signs of allergic responses, antibody production, and increased phagocytosis. Still, golden hamsters remained highly susceptible, highlighting how B. pseudomallei potently evades immunity in some hosts.

In summary, B. pseudomallei has developed mechanisms to evade host immunity by expressing surface proteins that inhibit immune cells, secreting effectors that suppress immune responses, and interfering with antigen presentation. However, some hosts are still able to mount adaptive immune responses against B. pseudomallei, though susceptibility varies significantly between host species. A greater understanding of how B. pseudomallei so effectively evades and suppresses the host immune system may help identify new therapeutic targets for this deadly disease.

1.2.1.9 VACCINE DEVELOPMENT STRATEGY

There are several promising vaccine candidates for melioidosis currently in development. Multiple research groups are pursuing structure-based epitope design to identify immunogenic B. pseudomallei antigens for vaccine formulations (Gori et al., 2017). A 2014 meeting of melioidosis experts recommended testing all available epitopes in animal models and combining multiple epitopes onto a single scaffold to stimulate both arms of the immune system (Limmathurotsakul et al., 2015).

Live attenuated and subunit vaccines have shown promise in animal studies but have not yet demonstrated long-term survival after lethal challenge (Patel et al., 2011, Johnson & Ainslie, 2017). An early Russian study found live attenuated B. pseudomallei strains provided statistically significant protection in moderately susceptible animals but not highly susceptible ones; a F. tularensis-based bivalent vaccine also showed promise.

A recent study found that a subunit vaccine combining B. pseudomallei capsular polysaccharide and recombinant proteins (CPS-CRM197 and AhpCC57G) stimulated high antibody and T cell responses and provided 70% survival in mice after high-dose inhalational challenge (Schmidt et al., 2022). CPS-CRM197 alone also stimulated high anti-CPS antibody

responses (Burtnick et al., 2018b). Another group found the proteins Hcp1 and TssM stimulated robust T cell responses and, when combined with CPS-CRM197, provided 100% survival and sterilizing immunity in mice after inhalational challenge (Burtnick et al., 2018b). A cost-effectiveness analysis found melioidosis vaccines could be cost-effective, especially in high-risk groups like diabetics, even with only partial immunity (Peacock et al., 2012). Diabetic and respiratory challenge models will be key to evaluating melioidosis vaccine candidates, as these reflect common routes of natural infection (Peacock et al., 2012).

The development of an effective vaccine against melioidosis has been an ongoing challenge, but recent progress provides hope. Multiple vaccine strategies have been explored, including live attenuated, whole cell killed, subunit, plasmid DNA, and dendritic cell vaccines (Peacock et al., 2012, Warawa & Woods, 2002). Live attenuated vaccines, while the most immunogenic in animal models, are unlikely to be suitable for humans due to safety concerns (Sarkar-Tyson & Titball, 2010). Killed and subunit vaccines have shown promise and continue to be refined (Peacock et al., 2012, Warawa & Woods, 2002).

A cost-benefit analysis found that even a partially effective vaccine could be cost-effective, especially if targeted at high-risk groups like diabetics in endemic areas (Peacock et al., 2012). The ideal vaccine would provide both humoral and cell-mediated immunity, as melioidosis can be acquired through multiple routes of exposure (Peacock et al., 2012). While vaccine research has largely focused on biodefense, vaccines could have significant public health impact in highly endemic areas like Thailand and northern Australia (Peacock et al., 2012, (Limmathurotsakul et al., 2015)

Multiple studies have evaluated vaccine candidates in mouse models, but few have used models that accurately reflect natural infection in humans, including diabetic or inhalational

models (Peacock et al., 2012). Heterologous vaccines, like one using Francisella tularensis, have shown promise, as have certain attenuated B. pseudomallei mutants, but more research is needed, especially in models that better reflect human disease (Sarkar-Tyson & Titball, 2010). Ongoing research continues to identify new potential antigens for subunit vaccines.

Classification of melioidosis into distinct clinical categories will help in evaluating new treatments and vaccine efficacy (Leelarasamee, 2004). Recent discoveries about B. pseudomallei's mechanisms of virulence and intracellular survival may aid in developing new therapies and vaccines (Leelarasamee, 2004). New selective media and molecular techniques improve diagnosis and distinguish B. pseudomallei from near neighbors (Leelarasamee, 2004)

Table 1.1 Various reported vaccines developed to regulate the *B. pseudomallei* infection.

Vaccine Type	Antigens	Response	Reference
Nanoparticle based vaccines	The B. pseudomallei-derived OMVs (M9 OMVs) include proteins linked to intracellular survival but are not harmful to live cells.	Mice that have been immunized show high resistance to lung infection, comparable to that seen with a live attenuated vaccine, and this resistance is accompanied by an increase in IgG, CD4+, and CD8+ T cells.	(Baker et al., 2021)
Nanoglycoconjugate Vaccines	The lipopolysaccharide (LPS) from Burkholderia thailandensis served as an additional antigen	This sophisticated multicomponent glycoconjugate vaccine formulation can protect against	(Tapia et al., 2021)

	and was covalently linked to several nanoglycoconjugates using predicted immunogenic protein candidates, Hcp1, FlgL, OpcP, OpcP1, OmpW, and hemagglutinin.	deadly B. pseudomallei infection by inducing both humoral and cell-mediated responses.	
Nanoglycoconjugate Vaccines	Hcp1 was joined to lipopolysaccharide (LPS), and two more new proteins were added to the surface of a gold nanoparticle (AuNP).	Animals given AuNP glycoconjugate vaccinations produced significant antibody titers against specific proteins and polysaccharides. Importantly, following a fatal challenge with B. pseudomallei, immunized rats receiving the AuNP-FlgL-LPS alone or in combination showed up to 100% survival and decreased lung colonisation.	(Muruato et al., 2017)
Manno-Heptopyranose Hexasaccharide Glycoconjugate	The innocuous Hc domain of the tetanus toxin was connected to the homopolymer of unbranched 1- 3 linked	Developed natural capsule-specific IgM and IgG responses and were resistant to infection by B.	(Scott et al., 2016)

	2-O-acetyl-6-deoxy-d-manno-heptopyranose that was created.	pseudomallei strain K96243 at concentrations more than 120 LD50.	
Glycoconjugated vaccines	CPS-CRM197 was created by covalently attaching the 6-deoxyheptan capsular polysaccharide (CPS) from B. pseudomallei to the recombinant CRM197 diphtheria toxin mutant (CRM197).	High-titer IgG and opsonizing antibody responses were obtained after immunising against CPS-CRM197.	(Burtnick et al., 2018a)
Vectored	Alternative subcellular targeting flagellin DNA vaccines	Compared to the empty vector, C57BL/6 vaccine-treated mice demonstrated a 10-fold reduction in bacterial burdens in the lungs and other distant organs.	(Lankelma et al., 2017)
Bacterial subunits	Combination of BPSL2765, a protein known to trigger immunological responses, and three genes from E. coli, BPSL1897,	Mice that were immunised with the combination of the chronic stage antigens displayed improved protection against experimental disease in mice as compared	(Champion et al., 2016)

	BPSL3369, and BPSL2287.	to animals who were only immunized with capsular polysaccharide or LolC protein.	

In summary, while no licensed vaccine yet exists, multiple promising strategies are being explored. Continued research into virulence mechanisms, clinical classification, and new treatments will all support vaccine development. Melioidosis vaccine research is an active area, with multiple promising subunit candidates in development. Future work should focus on combining epitopes to stimulate both B and T cell responses, improving models to better reflect natural infection, and progressing candidates to clinical trials, especially in endemic areas where a vaccine could have substantial public health impact. Targeting high-risk groups in endemic areas could make even a partially effective vaccine cost-beneficial. The ideal vaccine will provide both humoral and cell-mediated immunity to this challenging disease.

1.2.2 SUBTRACTIVE PROTEOMICS FOR IDENTIFICATION OF DRUG TARGETS AND VACCINE CANDIDATES

Subtractive proteomics involves comparing the proteome of a pathogen with that of its host to identify unique proteins or antigens that could serve as potential drug targets or vaccine candidates. This method makes it easier for researchers to identify chemicals that are vital to the pathogen's survival, which makes them desirable targets for medication development.

Over the last five years, the concept of subtractive proteomics has significantly progressed, with improvements in technology and methods leading to expanded uses and greater precision. Here are some key developments:

1. Integration with Other Omics Techniques: Data from transcriptomics, metabolomics, and genomes are increasingly being coupled with subtractive proteomics. This multidisciplinary approach has led to the identification of novel therapeutic targets and vaccination candidates while facilitating a more comprehensive understanding of biological processes.

2. In Silico Methods: The application of computational subtractive proteomics has become more widespread. Software tools and algorithms are used to predict potential drug targets and vaccine antigens, reducing extensive lab work and speeding up the discovery process.

3. Immunoinformatics: Subtractive proteomics is now often paired with immunoinformatics techniques. This helps identify immunogenic proteins that can be targeted for vaccine development, advancing vaccine design.

4. Pathogen Research: Subtractive proteomics has been exhaustively used in pathogen research to pinpoint essential, non-homologous proteins as possible drug targets. This is especially relevant for emerging infectious diseases and antibiotic resistance.

5. Increased Automation: Automated computer algorithms and software have been developed to streamline the analysis of mass spectra of proteins and peptides in subtractive proteomics studies, improving efficiency and accuracy.

6. Reverse Vaccinology: Subtractive proteomics is often used with reverse vaccinology. This combination helps identify potential vaccine candidates by subtracting pathogen-specific genes and selecting immunogenic proteins for vaccine development.

In summary, over the past five years, subtractive proteomics has evolved through integrating omics data, in silico methods, immunoinformatics, and increased automation. These advancements have expanded its applications in various areas, from drug discovery to vaccine

development, and have sped up the potential therapeutic targets identification in pathogens and other biological systems.

Several studies have leveraged comparative proteomics to elucidate immunogenic proteins in Burkholderia pseudomallei, the bacterium responsible for melioidosis. Research by Burtnick et al., 2017 focused on analysing the proteome of the B. pseudomallei type III secretion system. Their work identified proteins with high immunogenic potential, providing insights into the pathogen's virulence and possible therapeutic targets (Burtnick et al., 2014).

Vander Broek et al. conducted a quantitative proteomic analysis, generating one of the most comprehensive cores secretomes of B. pseudomallei published to date. Their study identified 26 putative Bsa-dependent secreted proteins, illuminating the intricate bacterial secretion mechanisms and potential vaccine candidates (Vander Broek et al., 2015).

Notable research by Al-Maleki et al. examined alterations in the proteome of B. pseudomallei colony variants after exposure. This study offered crucial understanding of the link between morphological and phenotypic traits, establishing a basis for comprehending the adaptable nature of B. pseudomallei and its response to environmental factors (Al-Maleki et al., 2015).

Furthermore, Harding et al. identified the immunogenic surface proteins of B. pseudomallei using proteomic techniques. They helped identify potential vaccination targets by using parallel approaches, underscoring the significance of surface proteins in host-pathogen interactions (Harding and others, 2007).

Felgner et al., 2009 utilized a protein microarray approach, probing a diverse array of patient sera against 1,205 proteins. Their study identified 49 antigens, demonstrating the extensive

utility of proteomic techniques for uncovering immunogenic proteins for diagnostic and therapeutic purposes (Felgner et al., 2009).

Additionally, Mariappan et al. mapped and identified differently expressed proteins in host-adapted B. pseudomallei using gel-based comparative proteomic profiling. According to Mariappan et al. (2017), this method provided insights into bacterial adaptability and possible treatment targets.

In summary, these studies collectively demonstrate the power of comparative proteomics in elucidating the immunogenic proteome of Burkholderia pseudomallei, contributing to the development of diagnostic tools and prospective vaccine candidates for melioidosis.

1.2.2.1 BIOINFORMATICS TOOLS AND DATABSES FOR SUBTRACTIVE PROTEOMICS

1.2.2.1.1 TID TOOL

TID is a stand-alone program based on the premise that for a protein to be a potential drug target, it must be crucial for the pathogen's existence and have no homologs in the host. It takes a bacterial proteome as input, removes duplicate proteins, selects essential ones, and excludes those similar to host proteins. The proposed targets exhibit no homology with at least 40 of 84 common gut microbes, implying they would be harmless to humans. TID groups the targets as established, new, or pathogenic. Users can perform pathway analysis, choke point analysis, interactome analysis, predict subcellular localization, and assign functions by accessing linked web servers. TID's identified targets for Listeria monocytogenes, Bacillus anthracis and Pseudomonas aeruginosa significantly overlap with those from prior studies. It takes TID about 2 hours to scan potential targets from a ~5000 protein bacterial proteome. Therefore, we propose TID as a useful tool for rational drug design. TID is available at http://bmicnip.in/TiD/.

1.2.2.1.2 CD-HIT FOR PARALOGOUS SEQUENCE REMOVAL

CD-HIT is a most extensively used program for clustering together similar gene and protein sequences to reduce redundancy during target identification (Sarangi et al., 2015, Sarangi et al., 2015, Rahman et al., 2014). TID includes a Python script that utilizes the CD-HIT program with a sequence identity threshold set to greater than 60%. This takes out duplicate, paralogous sequences from a given bacterial proteome (Sarangi et al., 2015)

1.2.2.1.3 ESSENTIALITY ANALYSIS USING DEG AND CEG IN TID TOOL

DEG is a database that includes key genetic components like protein-coding genes and non-coding RNAs from bacteria, archaea and eukaryotes (Luo et al., 2013). CEG stores essential genes in orthologous groups, so essential genes in different bacteria that share the same function are part of one CEG cluster (Ye et al., 2013). The bacterial essential genes from DEG and the essential gene groupings from CEG are combined with the BLAST+ software to build an essential gene analysis tool. In the essential protein screening section of TID, adjustable settings for defining user-specified e-value and bit score thresholds are made available through drop-down menus.

1.2.2.1.4 NON-HOMOLOGY ANALYSIS USING BLAST IN TID TOOL

The RefSeq database provides a non-redundant, well-annotated, complete, and integrated set of reference sequences comprising genomes, transcripts, and proteomes. The full proteomes of Human, Laboratory Mouse, Cow, Rhesus Macaque, Guinea Pig and Domestic Dog were taken from the RefSeq database and combined with the BLAST+ program to create the Host Non-Homology Analysis feature. This feature helps identify user-specified proteins from pathogenic bacteria that lack homology to host proteins, based on specified e-value and bit score thresholds. A gut microbiota proteome dissimilarity analysis tab was constructed

utilizing the BLAST+ program and 84 gut microbiome protein sequence datasets. (Shanmugham & Pan, 2013, Jadhav et al., 2014).

1.2.2.1.5 POCKET DRUGGABILITY PREDICTION USING POCKDRUG

Determining if a protein's binding site can bind to drug-like molecules with high affinity (called druggability) is crucial for identifying drug targets early in drug development. Thus, investigating the druggability of a binding pocket is a key step in creating new drugs. Currently, computer models that predict druggability rely on a single method for estimating the pocket, despite uncertainties around pocket estimation. In this work, we introduce "PockDrug-Server" which predicts pocket druggability robustly for both pockets extracted near a bound ligand in a protein structure's holo form using various distance thresholds, and unguided pockets based on surface amino acids forming potential binding sites. PockDrug- server consistently yields druggability findings for various pocket estimating techniques. It performs effectively even for difficult apo pockets without a bound ligand because it is resilient to errors in pocket borders and estimating techniques. It outperforms recent models for apo pockets and clearly separates druggable from less druggable pockets using different estimation approaches. It can be applied to any user-defined pocket or to individual or sets of apo/holo proteins utilising the different pocket estimation techniques our website offers. (Hussein et al.,2015)

1.2.3 DEVELOPMENT OF VACCINES USING REVERSE VACCINOLOGY

Reverse vaccinology is an approach used to develop vaccines by studying the pathogen's genetic code and predicting possible antigens that could be used for vaccine creation. It enables scientists to make vaccines capable of provoking a robust immune reaction against the pathogen.

Regarding Burkholderia pseudomallei, these techniques are used to identify new drug targets and antigens for a hybrid vaccine. This tactic combines various components from different pathogens to generate a powerful vaccine against melioidosis.

The integration of subtractive proteomics and reverse vaccinology in studies can result in the invention of new strategies for fighting infectious diseases caused by challenging pathogens such as Burkholderia pseudomallei.

Over the past five years, the technique of reverse vaccinology has significantly progressed and advanced in the field of vaccine development. Some major advancements in reverse vaccinology during this time include:

Explosion of Genomic Data: The availability of genomic data has greatly increased, allowing researchers to study a wider range of pathogens and identify potential vaccine targets more efficiently. This has sped up vaccine development for many infectious diseases.

Improvements in Bioinformatics: More accurate bioinformatics tools and algorithms have been created, enabling better prediction and selection of vaccine candidates. These tools simplify the analysis of genomic and proteomic information, making reverse vaccinology more accessible and effective.

Integration with Omics Technologies: Reverse vaccinology has been mixed with transcriptomics and proteomics, two more omics techniques. This multidisciplinary approach enhances the discovery of antigens for vaccine production and offers a thorough grasp of the biology of the disease.

Faster Clinical Trials: The power of reverse vaccinology 2.0 has permitted rapid progress in vaccine development. Clinical trials for vaccines designed via reverse vaccinology have started quickly, showing the practicality and efficacy of this method.

Next-Generation Vaccine Development: Researchers have explored next-generation vaccine development utilizing reverse vaccinology. This approach focuses on developing safe and effective vaccines using the latest genomics and proteomics techniques and insights.

1.2.3.1 BIOINFORMATICS TOOL FOR REVERSE VACCINOLOGY

1.2.3.1.1 SUBCELLULAR LOCALIZATION PREDICTION

1.2.3.1.1.1 PSORTb

Since its initial release in 2003, PSORTb has proven to be the most accurate method for determining the subcellular localization of bacterial proteins. It still has to get better at recalling all the relevant proteins, though. Furthermore, there are yet no reliable methods for identifying significant sub-categories of localization, such as proteins that target bacterial organelles or host cells, or for predicting subcellular localization in archaea.

Improved recall, increased prediction coverage over entire proteomes, and newly developed subcellular localization subcategories are features of PSORTb version 3.0. It is the first technology designed specifically to predict the location of any prokaryote, including bacteria, archaea, and species with unusual cell membranes or walls. A new batch results mechanism is added to the standalone programme to enhance its web interface. Using 5-fold cross-validation and an independent proteomics study, we assessed the most accurate subcellular localization prediction tools. The results showed that PSORTb 3.0 is the most accurate tool, however it can be useful to supplement its predictions with those from Proteome Analyst (Yu et al., 2010).

1.2.3.1.1.2 CELLO v2.5

While pSORTb is a great tool for protein subcellular localization prediction, many other powerful prediction programs have emerged over the years. One example is CELLO, first introduced in 2004 and later upgraded to CELLO II with enhanced algorithms (Yu et al., 2006).

CELLO stands out because it utilizes support vector machines, which are not affected by sequence similarity. The updated CELLO II uses a two-layer SVM framework to determine subcellular location. The first layer has multiple SVM classifiers that operate on sequence-derived characteristics. The second layer SVM processes the outputs of the first layer and calculates the probability distribution across subcellular compartments (Yu et al., 2006).

To elaborate, the SVMs in the first layer each generate a probability profile for the subcellular localization based on the sequence encoding. The second layer SVM then combines all these probabilities to produce a final probability result. Finally, the location with the highest probability is predicted as the subcellular site (Yu et al., 2006). Compared to other tools, CELLO II demonstrated exceptional accuracy at 90% versus 82.6% for PSORTb 2.0 (Yu, 2006). Although the newer pSORTb 3.0 is more accurate, its high rate of "unknown" predictions makes it less useful than CELLO II. Thus, CELLO II can serve as an alternative for analyzing proteins that PSORTb cannot categorize. (Yu et al., 2006)

1.2.3.1.2 Antigenicity prediction by VAXIJEN server

Genomics research has revolutionized vaccine development. The sequencing of whole genomes has enabled scientists to use computational methods to screen for the most promising antigen candidates to test as vaccines before performing confirmation experiments. This technique is called "reverse vaccinology." Sequence alignment is the method most algorithms employ to identify antigens. This strategy does have some drawbacks, though. Despite not sharing a sequence, certain proteins have comparable structures and functions because of divergent or convergent evolution. Techniques based on alignment could produce unclear outcomes in certain situations. The development of alignment-independent methods for antigen prediction based on auto cross covariance (ACC) transformation of protein sequences

into vector representations of equal length was prompted by the limitations of alignment-dependent sequence similarity approaches. Protein categorization and quantitative structure-activity relationship (QSAR) investigations of peptides are conducted using the ACC protein sequence mining technique, which was created by Wold in 1993.

The first service for alignment-independent protective antigen prediction is called VaxiJen. It permits the classification of antigens without the need for sequence alignment based on the physicochemical characteristics of proteins. The server can be utilised with alignment-based prediction techniques or on its own. Online at http://www.jenner.ac.uk/VaxiJen, it is freely available. (Flower & Doytchinova, 2007)

1.2.3.1.3 NetCTL server

Accurate prediction of Cytotoxic T lymphocyte (CTL) epitopes is critical for rational vaccine development. Most importantly, it can reduce the experimental effort required to identify epitopes. NetCTL is a web-based tool developed to predict human CTL epitopes in any protein. It does this by combining predictions of proteasome cleavage, TAP transport efficiency, and MHC class I binding affinity.

The NetCTL server assists in pinpointing potential cytotoxic T lymphocyte (CTL) epitopes, which are key to understanding immune system responses.

NetCTL 1.2 and NetCTLpan 1.1 are two versions of the server, with NetCTLpan 1.1 being an upgraded version that allows prediction of CTL epitopes restricted to specific major histocompatibility complex (MHC) molecules. These tools contribute to large-scale validation of methods for predicting human CTL epitopes in different proteins.

Scientists use the NetCTL server for its ability to analyze protein sequences and predict potential CTL epitopes, facilitating research related to immunology, vaccine development, and understanding cellular immune responses (Larsen et al., 2007)

1.2.3.1.4 BCPRED server

B-cell epitope characterization and identification are crucial for the development of vaccines, immunodiagnostic procedures, and antibody synthesis. Consequently, there is a strong need for computational methods that can predict linear B-cell epitopes with high accuracy. BCPred is a novel subsequence kernel-based approach for linear B-cell epitope prediction. Eleven SVM-based classifiers developed and tested in the trials are outperformed predictively by BCPred (AUC = 0.758) and AAP (AUC = 0.7), a recently disclosed method for predicting linear B-cell epitopes using amino acid pair antigenicity. Additionally, a comparison of BCPred with AAP and ABCPred—a recurrent neural network-based approach—using two datasets of distinct B-cell epitopes that were previously used to assess ABCPred shows that BCPred performed better than ABCPred and AAP (EL-Manzalawy et al., 2008).

1.3 HYPOTHESIS

We hypothesized that analyzing the proteomes of different disease-causing Burkholderia pseudomallei could pinpoint shared or core antigens. We could then screen the immunogenic proteins using reverse vaccinology to find potential antigen candidates. Construction of chimeric vaccine and then its insilico validation will help us understand that it is possible to construct vaccine insilico.

1.4 AIMS AND OBJECTIVES

The study is divided and completed by four objectives:

- Comparative and subtractive proteomics approach for selection of protein for design of chimeric vaccine.
- Reverse vaccinology approach for identification of epitopes.
- Design and construction of chimeric vaccine
- In silico validation of vaccine construct.

1.5 SIGNIFICANCE

Burkholderia pseudomallei is a tier 3 select agent and causes disease melioidosis. These is no vaccine for the disease. Also, the organism is multidrug resistant and hence a study on chimeric vaccine development becomes mandatory.

Chapter 2: Comparative and Subtractive Proteomics Approach for Selection of Protein for Design of Chimeric Vaccine

2.1 INTRODUCTION

The relentless pursuit of fighting infectious diseases has brought about innovative approaches to speed up vaccine development using modern biotechnology. Among these approaches, comparative and subtractive proteomics stand out as powerful tools for selecting proteins to design chimeric vaccines. These methods take advantage of the wealth of genomic and proteomic data available for pathogens, allowing researchers to identify and prioritize immunogenic proteins that play critical roles in the pathogen's biology and ability to cause disease.

Comparative proteomics involves systematically comparing the proteomes of different strains or species of a pathogen. This approach helps identify proteins unique to the pathogen of interest and not present in closely related non-pathogenic strains. Such distinct proteins are often ideal candidates for inclusion in chimeric vaccines because of their potential to provoke a strong immune response.

Subtractive proteomics takes the comparison further by subtracting the proteomic data of non-pathogenic strains or related organisms from that of the pathogen. This subtraction process narrows down the selection to proteins not only specific to the pathogen but also vital for its survival and ability to cause disease. These proteins become prime targets for chimeric vaccine design.

In this examination of comparative and subtractive proteomics, we explore how these methods are used to select immunogenic proteins for developing chimeric vaccines. By identifying and prioritizing these key protein targets, researchers are advancing the field of vaccinology and paving the way for more effective strategies to fight infectious diseases.

The comparative genomics offers the means to investigate ideal targets through the examination of biological processes and cellular activities. Additionally, selective screening of the genomic content is crucial for the survival and maintenance of bacteria due to the abundance of freely available bioinformatics tools and software. Numerous biological "omics" data available in public sources provide credence to the idea of finding new targets for diseases that pose a hazard to human and animal life. Genomic strains that have undergone complete sequencing provide the basis for identifying, validating, characterising, and evaluating potential therapeutic targets. The default settings of specificity and sensitivity are taken into account while doing selective screening.

To find the non-homologs of the query pathogen with regard to humans, subtractive screening is applied to the whole genome data of strains that have undergone sequencing. In order to prevent produced medications from having adverse effects, this analysis was done. The core genome content of the entire microbial lineage consists of genes that are defined as necessary for information processing, transcriptional apparatus, metabolic machinery, etc. Evolutionary changes cannot cause inactivation of such fundamental genomic content by mutation. Microorganisms become fatal when the core genome is inactivated. Additionally, cytotoxicity assessment helps to prevent expensive dead ends during the drug and vaccine development process.

Duplicate and pseudo coding sequences have been eliminated to eliminate redundancy in the original text. A database of essential genes facilitates the analysis of a pathogen's important genes. Proteome content that has no effect on the regular physiology of gut microbes is identified by non-homology analysis performed against the gut flora proteome content. Selective "differential genome analysis" in an integrated manner classifies vital genes into

unique (pathogen-specific) and shared (host and pathogen) pathways. In order to find and develop new drugs, this characterisation aids in identifying pathogen-specific pathways to target. Additionally, qualitative characterization through subcellular localization, virulence analysis, physicochemical characterization, broad spectrum analysis, functional annotation, and druggability analysis is used to identify potential therapeutic targets of life-threatening diseases in humans and animals.

2.2 MATERIALS AND METHODS

2.2.1 Collection of Proteome data

The UNIPROT service provided a list of all strains of Burkholderia pseudomallei that were available. The reference strain is Burkholderia pseudomallei (strain K96243). In the investigation, twenty more non-redundant proteomes were collected. According to Huang et al. (2010), shared proteins across proteomes and reference proteomes were discovered using CD-hit-2D, which is accessible on the CD-HIT suite. After that, every common protein was assembled into a single fasta file and extracted for additional examination.

2.2.2 Identification and removal of duplicate proteins

Utilising CD-HIT (Huang et al., 2010), subtractive protein analysis was carried out in order to find the duplicate proteins utilising clustering approaches. The sequence identity cut-off was selected at 0.6 or 60% identity since sequences with more than 60% identity showed similarities in their structures and functions (Huang et al., 2010). To align the amino acids, the Global Sequence Identity technique was employed. Alignment coverage was achieved by selecting a bandwidth of 20 amino acids and the default parameters.

2.2.3 Screening of essential proteins using TID tool

Essential protein-coding genes that are identified by genome-wide gene essentiality analysis are included in the database of essential genes (DEG) (Luo et al., 2013). By utilising the TID programme to BLASTp non-redundant sequences against the DEG database, essential proteins were filtered out (Gupta et al., 2017). With a bit score of 100, the e value that was used was 10^{-5}.

2.2.4 Screening of virulence factors using TID tool.

Under adhesion, colonisation, and invasion, virulence components of bacteria modify and impair the host defence mechanism. With an e value of 10^-3, a BLASTp of the CdHIT result was performed using TID (Gupta et al., 2017) and the VFDB database (Chen, 2004).

2.2.5 Screening of proteins which are non-homologous to humans

A combined list of proteins was produced by the aforementioned three separate searches. Using the TID programme (Gupta et al., 2017), the BLASTp search of the proteins was conducted against the non-redundant protein sequence (nr) database of the host Homo sapiens (taxid:9606), with a bit score of 100 and a cut-off value of 10^{-3}. To identify non-human homologous pathogen proteins, proteins were compared to those of the human host. The development of treatments targeted at certain pathogens will benefit from this work.

2.2.6 Identification of proteins with PDB structure which are non-homologous to humans

Non-homologous proteins to humans were BLASTp against the Burkholderia pseudomallei PDB database (strain K96243).

2.2.7 Identification of proteins with druggable pockets

Borrel et al. (2015) used the PockDrug web servers (http://pockdrug.rpbs.univ-paris-diderot.fr) to predict possible gaps in the protein structure, prioritising those that are located near or on their Pleckstrin Homology domain (PH).

2.2.8 Prediction of biological location of proteins

Using consensus location, which was predicted online by servers like PSORTb v3.0 (Yu et al., 2010) and CELLO v2.5 (Yu et al., 2014), subcellular localization prediction of proteins was performed to determine biological location of druggable proteins.

2.3 RESULTS

2.3.1 Shortlisted B. pseudomallei strains

We only extracted 20 non-redundant proteomes from UNIPROT, which includes the reference proteome of Burkholderia pseudomallei (strain K96243). UNIPROT contains many strains of selected organisms. We were able to identify the common protein between the proteome and reference proteome by using CD-HIT 2d. By using CD-HIT analysis, we eliminated repetitive sequences, leaving only non-redundant sequences.

2.3.2 Identification of essential proteins of B. pseudomallei

The DEG result showed that 2027 proteins of B pseudomallei were critical, therefore we retained them and threw away the remaining ones. This set of 2027 critical proteins is necessary for Burkholderia pseudomallei to survive. By inhibiting these bacterial proteins, this microbe will be eliminated.

2.3.3 Identification of virulence factors of B. pseudomallei

According to the results of the virulence factor database (VFDB), 1796 proteins were linked to B. pseudomallei's pathogenicity. Targeting these proteins is crucial for preventing the

development of B. pseudomallei. The quest for new virulence factors (VFs) is crucial since VFs investigate the role of pathogens in a range of illnesses. These newly discovered protein targets will be increasingly crucial for drug discovery and for screening well-known antibiotics.

2.3.4 Identification of non-human homologous proteins in B. pseudomallei

In all, 1446 VF proteins—that is, non-human homologous proteins—and 1411 essential proteins were found in this analysis. The remaining 616 essentials and 350 VFs screened out because they had protein similarities to human proteins. Non-human homologous protein detection aids in the identification of candidate proteins, which is beneficial for medication development and screening.

2.3.5 BLASTp with PDB database

Following the study of the BLAST structure database, we were able to extract the structures of seven virulence proteins and five essential proteins from PDB. At least three proteins (4RLH, 4CFI, and 5X9Q) are shared by both virulence and essential proteins, according to a comparison of the manual analysis.

2.3.6 Pocket druggability prediction

Table 2.1 displays the results of the pocket druggability prediction, which indicated that all nine proteins (2XBL, 4RLH, 4CFI, 5X9Q, 5WNN, 4JGB, 4HCN, 4UTI, AND 4USM) were druggable and had druggability scores above 0.5.

Table 2.1 Pock drug results for shortlisted nine druggable proteins

PDB ID	NUMBER OF POCKETS	NUMBER OF DRUGGABLE POCKETS	BEST DRUGGABLE POCKET SCORE
2XBL	27	7	0.99
4RLH	34	27	1.0
4CFI	4	4	0.88
5X9Q	24	13	0.99
5WNN	23	13	1.0
4JGB	12	9	0.99
4HCN	12	2	0.81
4UT1	38	14	0.99
4USM	19	10	0.8

2.3.7 Subcellular localization prediction and antigenicity prediction

Understanding the biological location of the druggable protein required accurate subcellular localization prediction. Proteins found in the extracellular, periplasmic, and outer membranes were thought to be potential vaccination candidates. 4CFI, 5WNN, 4HCN, and 4UTI were determined to be likely candidates for vaccination. The VaxiJen server's antigenicity prediction, which showed that all four had antigenicity values greater than 0.5, further supported this. For the creation of chimeric vaccines, these four proteins have been chosen. The outcomes are displayed in Tables 2.1 and 2.2.

Table 2.2 Subcellular localization prediction of druggable proteins

PDB ID	PSORTb	CELLO
2XBL	Cytoplasmic	Cytoplasmic
4RLH	Cytoplasmic membrane	Cytoplasmic
4CFI	Extracellular	extracellular
5X9Q	Cytoplasmic	Cytoplasmic
5WNN	Periplasmic	Periplasmic
4JGB	Unknown	Cytoplasmic
4HCN	Cytoplasmic	Outer membrane

4UT1	Extracellular	Extracellular
4USM	cytoplasmic	cytoplasmic

2.4 DISCUSSION

Advanced proteomics techniques such as subtractive and comparative proteomics are essential in the selection of proteins for chimaera vaccine formulation. By using these methods, researchers can discover and rank particular proteins that are crucial to the pathogen's survival and virulence by utilising the enormous quantity of genomic and proteomic data that is available for infections. In this section, we go over the importance of subtractive and comparative proteomics in choosing proteins for chimeric vaccine design:

Identification of Immunogenic Proteins: Comparative proteomics allows for the systematic comparison of pathogenic strains or species with closely related non-pathogenic counterparts. Proteins that are specific to the pathogen and probably immunogenic are revealed by this comparison, which makes them good candidates for the creation of vaccines.

Focus on Essential and Non-Homologous Proteins: Subtractive proteomics takes the selection process a step further by subtracting proteomic data of non-pathogenic strains. This narrows down the candidates to essential, non-homologous proteins that are specific to the pathogen. Such proteins are attractive targets for chimeric vaccines as they are more likely to induce a strong immune response.

Prioritization of Targets: The prioritisation of vaccination targets according to their significance in the biology of the pathogen is made possible by these proteomic techniques. Proteins that play crucial roles in virulence mechanisms become prime candidates for chimeric vaccine inclusion.

Enhancing Vaccine Efficacy: By selecting proteins that are integral to the pathogen's survival and virulence, comparative and subtractive proteomics increase the likelihood of designing chimeric vaccines that are highly effective in preventing infection.

Personalized Vaccine Development: These techniques can also be tailored to identify patient-specific immunodominant proteins, allowing for the development of personalized vaccines, as seen in some studies.

2.5 CONCLUSION

In conclusion, comparative and subtractive proteomics are invaluable tools in the rational design of chimeric vaccines. They enable the selection of proteins that are crucial for the pathogen's biology, improving the chances of developing vaccines with enhanced efficacy and specificity, ultimately contributing to the fight against infectious diseases.

Chapter 3: Reverse Vaccinology Approach for Identification of Epitopes

3.1 INTRODUCTION

Current developments in immunology, structural biology, and computational biology underpin the development of vaccines. Reverse vaccinology (RV) is the term for these methods (Donati, 2013). Regardless of an antigen's quantity, time of expression, or capacity to elicit an immune response, RV can currently be utilised more extensively to detect all putative antigens encoded in a genome (Pizza, 2000; Seib, 2012; Masignani, 2002). In addition to being more effective against illnesses like leptospires that grow slowly in vitro, reverse vaccinology also dramatically reduces the cost of making vaccinations (Guimares, 2015). Numerous pathogens, including as RV, pathogenic Escherichia coli, serogroup B Neisseria meningitidis, Streptococcus agalactiae, and Streptococcus pyogenes, have all been successfully treated (Masignani, 2019; Serruto, 2012).

Candidate proteins are chosen for RV methods using structural characteristics such as integrated transmembrane, β-barrel configurations, transmembrane alpha-helices, lipoproteins, secreted proteins, signal peptides, and B- and T-cell epitopes. These candidates are then developed further into a subunit or recombinant protein vaccine. But with the advent of numerous additional pathogenic genomes and the most recent bioinformatics and machine learning methods, RV has changed and improved in accuracy.

In this study, the four outer membrane, periplasmic and extracellular proteins were taken for further analysis These proteins were further analyzed for B-cell epitopes, T-cell epitopes, and B cell epitope prediction. Further several analyses led to selection of few epitopes which can be a part of the chimeric vaccine.

3.2 MATERIALS AND METHODS

3.2.1 Prediction of antigenic protein

We can identify extracellular, periplasmic, and outer membrane proteins with promise for vaccine development by using the subtractive proteomics technique. The VaxiJen web server was utilised to interpret the antigenic characteristics of the chosen proteins (Doytchinova & Flower, 2007). A protein with a threshold value of 0.5 was deemed to be a strong antigenic. These powerful antigenic proteins were taken for additional examination.

3.2.2 T-Cell MHC Class I Epitope Prediction

The NetCTL server predicted the MHC class I epitopes of a subset of proteins (Larsen et al., 2007). We selected epitopes with a high combinatorial score overall, and we established a 0.75 prediction threshold value for epitope identification. Numerous prediction techniques, such as proteasomal cleavage, MHC I affinity predictor, and TAP transporter efficiency with antigen processing, have been combined. After combining the scores from each approach, an epitope composite score was generated.

3.2.3 MHC I binding prediction

The Immune epitope database analysis resource (IEDB AR) was used to predict MHC I binding for epitopes (Kim et al., 2012). The consensus approach, which consists of ANN (Nielsen et al., 2003), SMM (Peters et al., 2005), CombLib (Sidney et al., 2008), and NetMHCpan (Lundegaard et al., 2008), is used as the default prediction technique, which is IEDB endorsed.

To choose the identified T cell epitope with HLA alleles, IC50 value and percentile rank were employed. A greater contact between the peptide epitope and the MHC molecule is indicated by a lower percentile rank. The IEDB server was utilised to predict the MHC I binding of the predicted T cell epitope. For the purpose of predicting Class I immunogenicity, the projected epitope with a stronger affinity (IC<200 nm) and percentile rank (<=0.2) was selected.

3.2.4 Class I immunogenicity prediction

MHC complexes or epitopes are thought to be able to initiate an immunological response. As a result, the IEDB server-based MHCI immunogenicity prediction tool (Calis et al., 2013) was employed. Predictions of immunogenicity were done using the default settings. Following the immunogenicity prediction, epitopes with a positive value were selected for additional analysis

3.2.5 Analysis of Antigenicity, conservancy, and toxicity of predicted epitopes

The list of promiscuous epitopes produced by the immunogenicity tool was examined further for antigenicity using a threshold of 0.5 by the VaxiJen version 2.0 server. The conservancy degree of epitopes within genotype sequences was evaluated using IEDB conservancy analysis (Bui et al., 2007). For this, the default sequence identity parameters were employed. The goal of this research is to determine how conserved an epitope is within a protein sequence (Dash et al., 2017). To estimate the toxicity level, the physicochemical properties of epitopes were examined using the ToxinPred web server programme. In order to verify that the host cell immune response will only target bacteria and not host tissue, this analysis is conducted using default parameters (Gupta et al., 2013).

3.2.6 T cell MHC class II epitope predictions

The IEDB-AR server was utilised to forecast the binding of T-cell epitopes to class II MHC molecules. T-cell epitopes were calculated using the consensus technique (Wang et al. 2008,

Wang et al. 2010). Furthermore, this consensus prediction method combined the usage of the stabilisation matrix alignment method and the average relative binding matrix method.

3.2.7 Analysis of antigenic and toxicity behavior of predicted epitopes

The MHC class I and class II epitopes predicted above underwent antigenicity and toxicity investigation. ToxinPred (Gupta et al., 2013) and VaxiJen version 2.044 (Doytchinova & Flower, 2007) were the tools utilised for the toxicity and antigenicity analyses, respectively. VaxiJen (threshold >0.5) predicts its antigenic behaviour by utilising the physiochemical behaviour of epitopes. The ToxinPred tool examined whether the host cells or solely the bacterial cells would be the target of the triggered particular immune response.

3.2.8 B cell epitope prediction

It was possible to predict the linear B cell epitope for proteins. The BEPIPRED servers (Jespersen et al., 2017), FBCPREDS (EL-Manzalawy et al., 2008), and BCPREDS (EL-Manzalawy et al., 2008) all predicted epitopes. Next, we used the VaxiJen server to forecast antigenicity. For analysis, the epitope with a greater antigenicity value (>0.5) was chosen.

3.2.9 Comparative prediction of MHC I, MHC II, and B cell epitopes along with physiochemical analysis

After comparing MHC-I, MHC-II, and B cell epitopes, these epitopes were used to develop the final vaccine construct. All epitopes used in chimeric vaccines must be hydrophilic in order for the human cells to respond to the epitopes by mounting an immune response. A GRAVY score was discovered with the PROTPARAM tool. A protein with a positive GRAVY score is thought to be hydrophobic, whereas a protein with a negative score is thought to be hydrophilic. All values with negative values were chosen

3.3 RESULTS

Table 3.1 Antigenicity prediction of shortlisted membrane, periplasmic and extracellular proteins

PDB ID	NAMES	ANTIGENICITY
4CFI	3D structure of FliC from Burkholderia pseudomallei	0.6542 antigen
5WNN	Crystal structure of Phosphate-binding protein PstS protein from Burkholderia pseudomallei	0.7290 antigen
4HCN	Crystal structure of Burkholderia pseudomallei effector protein CHBP in complex with ubiquitin	0.5191 antigen
4UTI	The structure of the flagellar hook junction protein FlgK from Burkholderia pseudomallei	0.5822 antigen

3.3.1 Selection of potent T-cell MHC-I epitopes

The NetCTL server predicted the best T-cell epitopes for four proteins that were shortlisted based on a high combinatorial score. We have 0.75 as the prediction threshold value. Seven epitopes in 4CFI, eight in 4HCN, 33 in 4UTI, and seven in 5WNN were found by the software.

The IEDB server was used to anticipate all the T cell epitopes' MHC-I binding. For predicting class I immunogenicity, we chose epitopes with high affinity (IC<200 nm) and percentile rank (<=0.2). For additional analysis, 2 out of 7 in 4CFI, 4 out of 8 in 4HCN, 15 out of 33 in UTI, and 1 out of 7 in 5WNN were chosen.

3.3.2 Class I immunogenicity prediction

We used IEDB immunogenicity prediction to test the previously chosen epitopes. The immunogenicity score of the epitope varied from -0.44395 to 0.18585. A high immunogenicity score indicates a strong capacity for inducing cellular immunity and stimulating naïve T cells. Positive scores were found for 2 out of 2 in 4CFI, 2 out of 4 in 4HCN, 6 out of 15 in 4UTI,

and 0 out of 1 in 5WNN; hence, we chose these (2+2+6+0=10) epitopes for additional examination.

Table 3.2. Predicted MHC I epitopes, HLA alleles interaction, and class I immunogenicity analysis using the IEDB server

Protein PDB ID	Peptide	Interacting MHC I allele	Class I immunogenicity
5WNN	YAKKNNMVY	HLA-B*15:01 (0.8) HLA-B*35:01 (1.5)	-0.42347
4CFI	**LSSTAVTAV**	HLA-A*68:02 (1.1)	0.11794
	SSTAVTAVF	HLA-B*58:01 (0.5) HLA-B*15:01 (0.7)	0.18585
4HCN	**LTQEPRTAY**	HLA-B*15:01 (1.0)	0.15669
	SLDELNQLL	HLA-A*02:01 1.2	-0.01318
	KLRFASHEY	HLA-B*15:01 (0.5) HLA-A*30:01 (0.8)	0.10277
	TLDSHKNYV	HLA-A*02:01 (1.0)	-0.33839
4UTI	TTSDYALSY	HLA-B*15:01 (2.0)	-0.10417
	TSATTPVPY	HLA-B*35:01 (1.1)	0.10748
	ISNAATPGY	HLA-B*58:01 (0.7) HLA-B*15:01 (0.9) HLA-B*35:01 (1.1)	0.12235
	LLDQRDLAV	HLA-B*08:01 (0.8) HLA-A*02:01 (2.0)	-0.02458

	SLSTYYTLV	HLA-A*02:01 (0.5)	0.00456
	SAQPGPTQY	HLA-B*35:01 (1.7)	-0.06112
	SSAAQTALV	HLA-A*68:02 (0.5)	0.00235
	QLVAAGQQY	HLA-B*15:01 (1.0)	-0.04267
	ALDGFSLAI	HLA-A*32:01 (0.5) HLA-A*02:01 (1.3)	0.01307
	FAVGAPAVY	HLA-B*35:01 (0.1) HLA-B*53:01 (1.2) HLA-B*15:01 (1.2)	0.1323
	QSNGNYSVF	HLA-B*15:01 (1.0) HLA-B*58:01 (1.2)	-0.09328
	NTGSATLSV	HLA-A*68:02 (0.48)	-0.18685
	GSATLSVSF	HLA-A*32:01 (0.7) HLA-B*58:01 (0.9)	-0.17659
	SQGSVSAGY	HLA-B*15:01 (0.22)	-0.21818
	TQGSSLSTY	HLA-B*15:01 (0.5)	-0.44395

3.3.3 Toxicity, conservancy, and antigenicity prediction

To find out if the epitopes were non-toxic, we employed the ToxinPred tool. Every one of the ten epitopes was confirmed to be non-toxic. Using the IEDB conservancy analysis tool, we also conducted a conservancy analysis on a subset of epitopes. For additional examination, we chose epitopes with more than 50% conservation. Every epitope was 100% conserved, as we discovered. We used the VaxiJen server to predict the antigenicity of epitopes. The findings

indicated that three epitopes of 4UTI (SSAAQTALV, ALDGFSLAI, and FAVGAPAVY), two epitopes of 4HCN (LTQEPRTAY and KLRFASHEY), and two epitopes of 4CFI (LSSTAVTAV and SSTAVTAVF) all had antigenicity scores above 0.5, indicating that they were highly antigenic. Next, for additional hydrophobicity analysis, we chose one epitope from each protein that had the greatest antigenicity score. The chosen candidates were ALDGFSLAI of 4UTI, KLRFASHEY of 4HCN, and SSTAVTAVF of 4CFI. Table 3.3 displays the outcome.

Table 3.3 . Predicted MHC I epitope, toxicity, antigenicity, and conservancy analysis

Protein name	Peptide epitopes	Toxicity (SVM score)	Antigenicity	Conservancy
4CFI	**LSSTAVTAV**	Non-toxin (-1.02)	0.7342 antigen	100%
	SSTAVTAVF	Non toxin (-1.08)	0.7876 antigen	100%
4HCN	**LTQEPRTAY**	Non toxin(-1.45)	0.5702 antigen	100%
	KLRFASHEY	Non toxin (-1.06)	0.7290 antigen	100%
4UTI	TSATTPVPY	Non toxin (-1.04)	0.2237 non-antigen	100%
	ISNAATPGY	Non toxin (-0.87)	0.2937 non-antigen	100%
	SLSTYYTLV	Non-toxin (-1.09)	0.3229 non-antigen	100%
	SSAAQTALV	Non toxin(-0.56)	0.6819 antigen	100%
	ALDGFSLAI	Non toxin (-0.97)	1.579 antigen	100%
	FAVGAPAVY	Non-toxin (-1.28)	0.7139 antigen	100%

3.3.4 MHC II epitope prediction

The IEDB server was utilised to anticipate the MHC II epitopes for the four proteins that were chosen. Following that, 9 epitopes in 4CFI, 14 in 4HCN, 14 in 5WNN, and 13 in 4UTI were shortlisted as antigenic and non-toxic based on antigenicity and toxicity study. Following that, one epitope from each protein that had the highest antigenicity score was chosen for additional hydrophobicity testing. The chosen ones were QSVNSQLTDTVTQIN in 4UTI, KVDIKKLHLDGKLRF in 4HCN, EPKTETFKAAAAGAN in 5WNN, and QINVVSDGKGGFTFT in 4CFI. Findings in Tables 3.4,3.5,3.6, 3.7.

Table 3.4. Predicted class II epitopes of 4CFI by IEDB server, antigenicity, and toxicity analysis

Allele	Toxicity (SVM score)	Antigenicity	Start	End	Peptide	Percentile rank
HLA-DRB1*03:01	-0.93 Non-toxin	0.9294 (Probable ANTIGEN)	202	216	**ETTQINVVSDGKGGF**	0.03
HLA-DRB1*03:01	-0.94 Non-Toxin	1.1150 (Probable ANTIGEN).	203	217	**TTQINVVSDGKGGFT**	0.03
HLA-DRB1*03:01	-1.14 Non-Toxin	1.1178 (Probable ANTIGEN).	204	218	**TQINVVSDGKGGFTF**	0.03
HLA-DRB1*03:01	-1.02 Non-Toxin	1.2455 (Probable ANTIGEN).	205	219	**QINVVSDGKGGFTFT**	0.03
HLA-DRB1*03:01	-1.04 Non-Toxin	0.8924 (Probable ANTIGEN).	206	220	**INVVSDGKGGFTFTD**	0.03
HLA-DQA1*01:02/DQB1*06:02	-0.40 Non-Toxin	0.6162 (Probable ANTIGEN).	265	279	**ATDQANATAMVAQIN**	0.04
HLA-DQA1*01:02/DQB1*06:02	-0.28 Non-Toxin	0.6597 (probable ANTIGEN)	266	280	**TDQANATAMVAQINA**	0.04
HLA-DQA1*01:02/DQB1*06:02	-0.50 Non-Toxin	0.6322 (Probable ANTIGEN).	264	278	**SATDQANATAMVAQI**	0.06

HLA-DRB4*01:01	-1.29 Non Toxin	0.1580 (Probable NON-ANTIGEN).	85	99	TNSLQRIRQLAVQAS	0.06
HLA-DRB4*01:01	-1.30 Non Toxin	0.0761 (Probable NON-ANTIGEN)	86	100	NSLQRIRQLAVQASN	0.06
HLA-DRB4*01:01	-1.36 Non Toxin	0.4018 (probable NON-ANTIGEN)	87	101	SLQRIRQLAVQASNG	0.08
HLA-DQA1*01:02/DQB1*06:02	-0.39 Non Toxin	0.4076 (probable NON-ANTIGEN)	267	281	DQANATAMVAQINAV	0.09
HLA-DRB4*01:01	-1.33 Non Toxin	-0.0658 (Probable NON-ANTIGEN)	84	98	LTNSLQRIRQLAVQA	0.11
HLA-DRB4*01:01	-1.23 Non Toxin	-0.0302 (probable NON-ANTIGEN)	83	97	SLTNSLQRIRQLAVQ	0.13
HLA-DRB4*01:01	-1.30 Non Toxin	0.2310 (Probable NON-ANTIGEN).	88	102	LQRIRQLAVQASNGP	0.14
HLA-DRB1*07:01	-1.05 Non Toxin	0.3738 (Probable NON-ANTIGEN)	68	82	NDGVSILQTASSGLT	0.18
HLA-DRB1*07:01	-0.88 Non Toxin	0.2619 (probable NON-ANTIGEN)	67	81	ANDGVSILQTASSGL	0.2
HLA-DRB1*07:01	-1.33 Non Toxin	0.3539 (Probable NON-ANTIGEN).	70	84	GVSILQTASSGLTSL	0.2
HLA-DQA1*01:02/DQB1*06:02	-0.41 Non-Toxin	0.5635 (probable ANTIGEN)			**QANATAMVAQINAVN**	0.2
HLA-DRB1*08:02	-1.26 Non Toxin	0.3494 (probable	323	337	QNRFTAIATTQQAGS	0.2

		NON-ANTIGEN)				

Table 3.5. Predicted class II epitopes of 4HCN by IEDB server, antigenicity, and toxicity analysis

Allele	Toxicity (SVM score)	Antigenicity (score)	Start	End	Peptide	Percentile rank
HLA-DRB1*03:01	-1.31Non-Toxin	0.5212 (Probable ANTIGEN)	296	310	**IKKLHLDGKLRFASH**	0.01
HLA-DRB1*03:01	-1.07Non-Toxin	1.3367 (Probable ANTIGEN)	293	307	**KVDIKKLHLDGKLRF**	0.02
HLA-DRB1*03:01	-1.13Non-Toxin	1.1233 (Probable ANTIGEN).	294	308	**VDIKKLHLDGKLRFA**	0.02
HLA-DRB1*03:01	-1.24Non-Toxin	0.7486 (Probable ANTIGEN).	295	309	**DIKKLHLDGKLRFAS**	0.02
HLA-DRB1*03:01	-1.22Non-Toxin	0.5526 (Probable ANTIGEN).	297	311	**KKLHLDGKLRFASHE**	0.02
HLA-DRB1*03:01	-1.02Non-Toxin	0.7110 (Probable ANTIGEN)	298	312	**KLHLDGKLRFASHEY**	0.03
HLA-DRB1*03:01	-0.93Non-Toxin	0.6783 (Probable ANTIGEN).	299	313	**LHLDGKLRFASHEYD**	0.03

HLA-DRB1*11:01	-1.35Non-Toxin	0.6535 (Probable ANTIGEN	273	287	**PDDVQMRLLASILQI**	0.09
HLA-DRB1*11:01	-1.37Non-Toxin	0.8034 (Probable ANTIGEN).	274	288	**DDVQMRLLASILQID**	0.09
HLA-DRB1*11:01	-1.26 Non Toxin	0.3477 (Probable NON-ANTIGEN).	275	289	DVQMRLLASILQIDK	0.09
HLA-DRB1*11:01	-1.43 Non toxin	0.1986 (Probable NON-ANTIGEN).	276	290	VQMRLLASILQIDKD	0.09
HLA-DRB1*03:01	-1.12 Non Toxin	0.0178 (Probable NON-ANTIGEN).	197	211	HKNYVVIVNDGRLGH	0.09
HLA-DRB1*03:01	-1.12 Non Toxin	0.3948 (Probable NON-ANTIGEN).	198	212	KNYVVIVNDGRLGHK	0.09
HLA-DRB1*03:01	-0.94Non-Toxin	0.6503 (Probable ANTIGEN).	199	213	**NYVVIVNDGRLGHKF**	0.09
HLA-DRB1*03:01	-0.92Non-Toxin	0.6535 (Probable ANTIGEN).	200	214	**YVVIVNDGRLGHKFL**	0.09
HLA-DRB1*03:01	-0.93Non-Toxin	0.7029 (Probable ANTIGEN).	201	215	**VVIVNDGRLGHKFLI**	0.09
HLA-DRB1*11:01	-1.76 Non Toxin	0.4055 (Probable NON-ANTIGEN)	272	286	MPDDVQMRLLASILQ	0.1

HLA-DRB1*03:01	-0.81Non-Toxin	0.8333 (Probable ANTIGEN)	202	216	**VIVNDGRLGHKFLID**	0.14
HLA-DRB1*03:01	-0.88Non-Toxin	1.0533 (Probable ANTIGEN)	203	217	**IVNDGRLGHKFLIDL**	0.15

Table 3.6. Predicted class II epitopes of 4UTI by IEDB server, antigenicity and toxicity analysis

Allele	Toxicity (SVM score)	Antigenicity (score)	Start	End	Peptide	Percentile_ rank
HLA-DRB1*09:01	-1.66 Non-Toxin	0.4175 (Probable NON-ANTIGEN).	62	76	VTVERQYNQYLSNQL	0.05
HLA-DRB1*09:01	-1.62 Non-Toxin	0.5096 (Probable ANTIGEN)	63	77	**TVERQYNQYLSNQLN**	0.05
HLA-DRB1*09:01	-1.50 Non Toxin	0.4736 (Probable NON-ANTIGEN)	64	78	VERQYNQYLSNQLNA	0.05
HLA-DRB1*09:01	-1.49 Non-Toxin	0.5434 (Probable ANTIGEN).	65	79	**ERQYNQYLSNQLNAA**	0.05
HLA-DRB1*09:01	-1.32 Non-Toxin	0.7859 (Probable ANTIGEN)	433	447	**ANGSAIAAASPVLAA**	0.08
HLA-DRB1*09:01	-1.31 Non-Toxin	0.5757 (Probable ANTIGEN).	434	448	**NGSAIAAASPVLAAG**	0.08
HLA-DRB1*09:01	-1.46Non-Toxin	0.5611 (Probable ANTIGEN).	435	449	**GSAIAAASPVLAAGV**	0.08

HLA-DRB1*09:01	-1.51 Non Toxin	0.4833 (Probable NON-ANTIGEN).	436	450	SAIAAASPVLAAGVA	0.08
HLA-DQA1*01:02/DQB1*06:02	-1.17 Non Toxin	0.3981 (Probable NON-ANTIGEN).	430	444	LAIANGSAIAAASPV	0.08
HLA-DQA1*01:02/DQB1*06:02	-1.11 Non Toxin	0.4440 (Probable NON-ANTIGEN).	429	443	SLAIANGSAIAAASP	0.1
HLA-DRB3*01:01	-1.14Non-Toxin	0.6805 (Probable ANTIGEN).	156	170	**RQSVNSQLTDTVTQI**	0.11
HLA-DRB3*01:01	-1.23Non-Toxin	0.8551 (Probable ANTIGEN).	157	171	**QSVNSQLTDTVTQIN**	0.11
HLA-DRB3*01:01	-1.07Non-Toxin	0.7808 (Probable ANTIGEN).	158	172	**SVNSQLTDTVTQINS**	0.11
HLA-DRB3*01:01	-1.13Non-Toxin	0.5874 (Probable ANTIGEN).	159	173	**VNSQLTDTVTQINSY**	0.11
HLA-DRB3*01:01	-1.21 Non Toxin	0.4986 (Probable NON-ANTIGEN)	160	174	NSQLTDTVTQINSYT	0.11
HLA-DQA1*05:01/DQB1*03:01	-1.18 Non Toxin	0.4397 (Probable NON-ANTIGEN)	431	445	AIANGSAIAAASPVL	0.11
HLA-DQA1*05:01/DQB1*03:01	-1.17 Non Toxin	0.3981 (Probable NON-ANTIGEN).	430	444	LAIANGSAIAAASPV	0.12
HLA-DQA1*05:01/DQB1*03:01	-1.24 Non Toxin	0.4904 (Probable	432	446	IANGSAIAAASPVLA	0.12

		NON-ANTIGEN).				
HLA-DQA1*05:01/DQB1*03:01	-1.32 Non-Toxin	0.7859 (Probable ANTIGEN).	433	447	**ANGSAIAAASPVLAA**	0.12
HLA-DQA1*01:02/DQB1*06:02	-1.18 Non Toxin	0.4397 (Probable NON-ANTIGEN).	431	445	AIANGSAIAAASPVL	0.15
HLA-DRB4*01:01	0.95 Non Toxin	0.0697 (Probable NON-ANTIGEN).	634	648	EAANLMQYQQLYQAN	0.15
HLA-DQA1*01:02/DQB1*06:02	-1.31Non-Toxin	0.6585 (Probable ANTIGEN).	428	442	**FSLAIANGSAIAAAS**	0.16
HLA-DQA1*05:01/DQB1*03:01	-1.11 Non Toxin	0.4440 (Probable NON-ANTIGEN)	429	443	SLAIANGSAIAAASP	0.16
HLA-DRB1*09:01	-1.31 Non Toxin	0.3079 (probable NON-ANTIGEN)	474	488	TTLAYNAASKTLSGF	0.17
HLA-DRB1*09:01	-1.11Non-Toxin	0.5803 (Probable ANTIGEN).	472	486	**GTTTLAYNAASKTLS**	0.18
HLA-DRB1*09:01	-1.25 Non Toxin	0.4118 (Probable NON-ANTIGEN).	475	489	TLAYNAASKTLSGFP	0.18
HLA-DRB1*09:01	-1.20Non-Toxin	0.6347 (Probable ANTIGEN).	473	487	**TTTLAYNAASKTLSG**	0.19

Table 3.7 Predicted class II epitopes of 5WNN by IEDB server, antigenicity, and toxicity analysis

Allele	Toxicity (SVM score)	Antigenicity (score)	Start	End	Peptide	Percentile rank
HLA-DPA1*01/DPB1*04:01	-1.23Non-Toxin	0.6013 (Probable ANTIGEN).	266	280	**GKEAWPVVGATFVLL**	0.01
HLA-DPA1*01/DPB1*04:01	-1.30Non-Toxin	0.5675 (Probable ANTIGEN)	267	281	**KEAWPVVGATFVLLH**	0.01
HLA-DPA1*01/DPB1*04:01	-1.34Non-Toxin	0.5568 (Probable ANTIGEN)	268	282	**EAWPVVGATFVLLHA**	0.01
HLA-DPA1*01/DPB1*04:01	-1.08Non-Toxin	0.6616 (Probable ANTIGEN).	269	283	**AWPVVGATFVLLHAK**	0.01
HLA-DPA1*01/DPB1*04:01	-1.02Non-Toxin	0.5595 (Probable ANTIGEN).	270	284	**WPVVGATFVLLHAKQ**	0.01
HLA-DRB1*09:01	-1.27Non-Toxin	0.8929 (Probable ANTIGEN).	238	252	**EPKTETFKAAAAGAN**	0.07
HLA-DPA1*01/DPB1*04:01	-1.35Non-Toxin	0.5532 (Probable ANTIGEN).	271	285	**PVVGATFVLLHAKQD**	0.07

HLA-DPA1*01/DPB1*04:01	-1.17Non-Toxin	0.6022 (Probable ANTIGEN).	272	286	**VVGATFVLLHAKQDK**	0.07
HLA-DQA1*04:01/DQB1*04:02	-0.78Non-Toxin	0.6989 (Probable ANTIGEN).	9	23	**AGLAGALFAVAAHAD**	0.07
HLA-DRB1*09:01	-1.22Non-Toxin	0.5219 (Probable ANTIGEN).	239	253	**PKTETFKAAAAGANW**	0.08
HLA-DRB1*09:01	-1.22Non-Toxin	0.6325 (Probable ANTIGEN)	240	254	**KTETFKAAAAGANWS**	0.08
HLA-DRB1*09:01	-1.20 Non Toxin	0.4477 (Probable NON-ANTIGEN).	241	255	TETFKAAAAGANWSK	0.08
HLA-DRB1*01:01	-1.13 Non Toxin	0.2367 (Probable NON-ANTIGEN).	5	19	QTAFAGLAGALFAVA	0.09
HLA-DQA1*04:01/DQB1*04:02	-0.89Non-Toxin	0.7895 (Probable ANTIGEN).	10	24	**GLAGALFAVAAHADI**	0.1
HLA-DQA1*04:01/DQB1*04:02	-1.05Non-Toxin	0.7103 (Probable ANTIGEN)	11	25	LAGALFAVAAHADIT	0.1
HLA-DQA1*05:01/DQB1*03:01	-1.20 Non Toxin	0.4477 (Probable NON-ANTIGEN).	241	255	TETFKAAAAGANWSK	0.11

HLA-DPA1*01:03/DPB1*02:01	-0.82 Non Toxin	0.4440 (Probable NON-ANTIGEN).	158	172	GSGTSFIWTNYLSKV	0.12
HLA-DQA1*05:01/DQB1*03:01	-1.21 Non Toxin	0.3770 (Probable NON-ANTIGEN).	242	256	ETFKAAAAGANWSKS	0.12
HLA-DQA1*05:01/DQB1*03:01	-1.19 Non Toxin	0.3860 (Probable NON-ANTIGEN).	243	257	TFKAAAAGANWSKSF	0.12
HLA-DRB1*04:05	-1.50 Non Toxin	0.1557 (Probable NON-ANTIGEN).	252	266	NWSKSFYQILTNQPG	0.12
HLA-DRB1*04:05	-1.42 Non Toxin	0.3352 (Probable NON-ANTIGEN)	253	267	WSKSFYQILTNQPGK	0.12
HLA-DRB1*04:05	-1.27Non-Toxin	0.6026 (Probable ANTIGEN).	254	268	**SKSFYQILTNQPGKE**	0.12
HLA-DRB1*04:05	-1.37Non-Toxin	0.6240 (Probable ANTIGEN).	255	269	**KSFYQILTNQPGKEA**	0.12
HLA-DPA1*01:03/DPB1*02:01	-0.93 Non Toxin	0.2636 (Probable NON-ANTIGEN)	159	173	SGTSFIWTNYLSKVN	0.14

HLA-DPA1*01/DPB1*04:01	-0.93 Non Toxin	0.2636 (Probable NON-ANTIGEN)	159	173	SGTSFIWTNYLSKVN	0.14
HLA-DPA1*01/DPB1*04:01	-0.91 Non Toxin	0.2126 (Probable NON-ANTIGEN).	160	174	GTSFIWTNYLSKVND	0.14
HLA-DQA1*05:01/DQB1*03:01	-1.22Non-Toxin	0.6325 (Probable ANTIGEN).	240	254	**KTETFKAAAAGANWS**	0.14
HLA-DQA1*04:01/DQB1*04:02	-0.92 Non Toxin	0.4504 (Probable NON-ANTIGEN).	8	22	FAGLAGALFAVAAHA	0.14
HLA-DPA1*01/DPB1*04:01	-0.82 Non Toxin	0.4440 (Probable NON-ANTIGEN).	158	172	GSGTSFIWTNYLSKV	0.16
HLA-DPA1*01:03/DPB1*02:01	--0.56 Non-Toxin	0.8813 (Probable ANTIGEN).	157	171	DGSGTSFIWTNYLSK	0.18
HLA-DPA1*01:03/DPB1*02:01	-0.91 Non Toxin	0.2126 (Probable NON-ANTIGEN).	160	174	GTSFIWTNYLSKVND	0.18
HLA-DRB1*01:01	-1.38 Non Toxin	0.3206 (Probable NON-ANTIGEN).	4	18	MQTAFAGLAGALFAV	0.19

HLA-DQA1*04:01/DQB1*04:02	-0.99Non-Toxin	0.8033 (Probable ANTIGEN)	12	26	AGALFAVAAHADITG	0.19

3.3.5 B-cell epitope prediction

B-lymphocytes must locate an epitope in order to trigger humoral immunity. Different servers, such as BCPREDS, FBCPREDS, and Bepipred server, produced predictions on B cell epitopes. Several servers predicted various B cell epitopes; for example, three servers' predictions of the same epitope were chosen. Following our antigenicity prediction, epitopes having an antigenicity value greater than 0.5 were chosen for additional hydrophobicity study. Table 3.8 displays the results.

3.3.6 Comparative prediction of epitopes and further hydropathy analysis

The final chimeric vaccine sequence was created following a manual comparison study of MHC I, MHC II, and B-cell epitopes. After that, epitopes were subjected to a GRAVY score analysis using the Protparam tool. A protein is hydrophobic if its GRAVY score is positive; hydrophilic proteins have a negative value. In order for epitopes to trigger an immune response in the host cell, they must be hydrophilic or present on the surface. as shown in Table 3.9.

Table 3.8. Prediction of B cell epitopes using BCPRED, FBCPred, and IEDB server and their antigenicity analysis

protein	BCPRED	start	antigenicity	FBCPred	start	Antigenicity	BEPIPRED	start	antigenicity
4CFI	DPCGTDASAPGGAKSVSIVQ	396	**0.9791 (probable ANTIGEN)**	EDPCGTDASAPGGA	395	**1.2901 (probable ANTIGEN)**	AFDEDPCGTDASAPGGAKS	392	**1.2053 (probable ANTIGEN)**

4CFI	VFGSSTAGTGTAASPSFQTL	234	**1.1854 (probable ANTIGEN)**	GSSTAGTGTAASPS	236	**1.9710 (probable ANTIGEN)**	GSSTAGTGTAASPSF	236	**1.9410 (probable ANTIGEN)**
4HCN	SSAATSPAGPLGGLPARSSS	36	**0.5827 (probable ANTIGEN)**	AATSPAGPLGGLPA	38	**0.1331 (probable ANTIGEN)**	INNVGKTGQAGGETERIPSTEPLGSSAATSPAGPLGGLPARSSSISNTNRTGENPM	12	**0.8203 (probable ANTIGEN)**
	SNTNRTGENPMITPIISSNL	57	**1.0913 (probable ANTIGEN)**	SNTNRTGENPMITP	57	**1.4813 (probable ANTIGEN)**	INNVGKTGQAGGETERIPSTEPLGSSAATSPAGPLGGLPARSSSISNTNRTGENPM	12	**0.8203 (probable ANTIGEN)**
	DVPIDPTSIEYLENTSFAEH	171	**0.2266 (probable NON-ANTIGEN)**	TEKDVPIDPTSIEY	168	**0.6727 (probable ANTIGEN)**	DVPIDPTSIE	171	**1.0831 (probable ANTIGEN)**
	QSLSGESSNRVMWNDRYDTL	94	**0.7454 (probable ANTIGEN)**	GESSNRVMWNDRYD	98	**0.8637 (probable ANTIGEN)**	SGESSN	97	**2.7352 (probable ANTIGEN)**

4UTI	NITSATTPVPYDPSKGASMT	505	**0.4465 (probable NON-ANTIGEN)**	TSATTPVPYDPSKG	507	**0.1948 (probable NON-ANTIGEN)**	VTIAGTPPTSINITSATTPVPYDPSKGASMTISSTTQPAPSGVM	494	**0.5384 (probable ANTIGEN)**
	SKGVAGSAQPGPTQYLPDVS	258	**0.8982 (probable ANTIGEN)**	GVAGSAQPGPTQYL	260	**1.0484 (probable ANTIGEN)**	VAGSAQPGPTQYLP	261	**0.9731 (probable ANTIGEN)**
	WGLTTTGQNISNAATPGYSV	18	**0.7917 (probable ANTIGEN)**	WGLTTTGQNISNAATPGYSV	18	**0.7917 (probable ANTIGEN)**	TTGQNISNAATPGYSVERPVYAEASGQYTSSGYLPQGVSTV	22	**0.6516 (probable ANTIGEN)**
	GTPADGDQFTIGANKGTNDG	546	**1.4694 (probable ANTIGEN)**	GTPADGDQFTIGAN	546	**1.4373 (probable ANTIGEN)**	SLSGTPADGDQFTIGANKGTNDGRN	543	**1.4773 (probable ANTIGEN)**
	AVGAPAVYANQNNTGSATLS	332	**0.9549 (probable ANTIGEN)**	AVYANQNNTGSATL	337	**1.0837 (probable ANTIGEN)**	AVYANQNNTGSAT	337	**1.2373 (probable ANTIGEN)**

	TVANNAADPSARQTAMSNAQ	120	**0.6727 (probable ANTIGEN)**	TVANNAADPSARQT	120	**0.8115 (probable ANTIGEN)**	VANNAADPSARQTAMS	121	**0.5948 (probable ANTIGEN)**
	DGTQPTTSDYALSYDGAKYT	356	**1.0344 (probable ANTIGEN)**	QPTTSDYALSYDGA	359	**0.8989 (probable ANTIGEN)**	VDGTQPTTSDYALSY	355	**1.1391 (probable ANTIGEN)**
5WNN	EGTTVNWPTGTGGKGNDGVA	182	**1.8714 (probable ANTIGEN)**	TTVNWPTGTGGKGN	184	**1.7655 (probable ANTIGEN)**	NDEWKSKVGEGTTVNWPTGTGGKGNDGV	173	**1.7688 (probable ANTIGEN)**

Table 3.9. Comparative prediction of MHC I, MHC II and B cell epitopes and physiochemical analysis.

Protein	**MHC 1 epitopes**	**hydrophobicity**	**MHC II epitopes**	**hydrophobicity**	**B cell Epitopes**	**hydrophobicity**
4CFI	SSTAVTAVF	1.311	QINVVSDGKGGFTFT	0.047	GSSTAGTGTAASPS	-0.193
4HC	KLRF	-0.978	KVDIKKLH	-0.520	SGESSN	-1.633

N	ASHEY		LDGKLRF			
4UTI	ALDGFSLAI	1.533	QSVNSQLTDTVTQIN	-0.533	SLSGTPADGDQFTIGANKGTNDGRN	-1.020
5WNN	-none	-none	EPKTETFKAAAAGAN	-0.660	EGTTVNWPTGTGGKGNDGVA	-0.770

3.4 DISCUSSION

Even with the abundance of immunoinformatics techniques available, preventing melioidosis is still a challenging task. To find more effective vaccine options to combat this infectious disease, research on soft-core or core bacterial genes may be helpful (Aslam, 2020). The traditional method of developing vaccines is time- and money-consuming, labour-intensive, and less successful. By comparison, the design of vaccines by innovative immunoinformatics is less expensive in terms of time and money, and it is more stable, safe, specific, and efficacious (Sette, 2010).

We continue from chapter 2, where we identified four proteins based on their subcellular localization as potential candidates for vaccines. After determining that all four were antigenic by antigenicity prediction, we proceeded with epitope prediction. We performed the B cell, MHC-I, and MHC-II predictions. Following thorough study, we decided to focus primarily on 1 MHC-I epitope, 2 MHC-II epitopes, and 4 B cell epitopes because the GRAVY score indicated that these epitopes were hydrophilic and may therefore elicit an immunological response.

3.5 CONCLUSION

We were able to shortlist 1 MHC I epitope, 3 MHC II epitope and 4 B cell epitopes by various prediction and filtering methods. These will be taken further for vaccine construction as we want our vaccine to illicit both cell and humoral immunity.

Chapter 4: Design and Construction of Chimeric Vaccine

4.1 INTRODUCTION

The use of computational tools to create chimeric vaccines targeting multiple epitopes of bacterial pathogens is an innovative in silico approach for vaccine design. Scientists utilize immunoinformatics techniques to identify and analyze potential antigen targets, enabling the development of chimeric vaccines with enhanced immunogenicity. This strategy aims to more comprehensively combat bacterial infections by incorporating epitopes that stimulate both antibody and cell-mediated immune responses.

Studies like Andongma et al.'s focus on the computational design of promiscuous chimeric multi-epitope vaccines against tuberculosis bacteria specifically. These vaccines are intended to provoke immune responses across multiple strains or variants of the bacteria.

Additionally, Al-Megrin et al. emphasize extensive use of computational tools to design multi-epitope chimeric vaccines, demonstrated through their work on Enterobacter cloacae bacteria. This highlights the versatility of the in silico approach across different bacterial species.

Furthermore, Keshri et al.'s research explores the development of multi-epitope chimeric vaccines against Taenia solium bacteria, which causes certain infections. Their computational approach shows the potential of these vaccines to generate robust immune responses, representing a promising strategy against bacterial diseases.

To summarise, the utilisation of computational methods to develop vaccines with broad efficacy across strains is a promising and sophisticated strategy to vaccine development: in silico multiepitope chimeric vaccine design against bacteria.

4.2 MATERIALS AND METHODS

4.2.1 Construction of multiepitope chimeric vaccine

All of the chosen OMP epitopes—HTL, CTL, and B cell epitopes—were linked together using amino acid linkers (HEYGAEALERAG and GGGS linkers) in order to create a chimeric vaccine. Utilising 'EAAAK' linkers at both termini (N and C), various adjuvants were combined to improve the immunogenicity of the constructs. Adjuvants included beta-defensin (Mei et al., 2012), HBHA protein (M. tuberculosis, accession number. AGV15514.1), HBHA conserved sequence (Rana & Akhter, 2016), and 50 s ribosomal L7/L12 protein (Lee et al., 2014). In order to increase the vaccine's potency and effectiveness, adjuvants were additionally added to a non-natural pan DR (PADRE) sequence. This PADRE stimulates CD4+ T-cells and consists of a 13 amino acid epitope (AKVAAWTLKAAAC).

4.3 RESULTS

4.3.1 Construction of Chimeric vaccine

All of the anticipated B cell, MHC I, and MHC II epitopes were connected by amino acid linkers (HEYGAEALERAG and GGGS) in order to create a chimeric vaccine. To increase the construct's immunogenicity, adjuvants were attached to it utilising EAAAK linkers at both the N and C termini. Adjuvants included beta-defensin, HBHA protein (M. tuberculosis, accession number AGV15514.1), 50s ribosomal L7/L12 protein, and HBHA conserved sequence. By utilising HEYGAEALERAG and GGGS linkers, a non-natural pan DR (PADRE) sequence is also inserted in order to boost vaccine efficacy and potency and address the issue caused by polymorphism of HLA-DR molecules in the global population. PADRE has the amino acid

sequence AKVAAWTLKAAA. As shown in table 12, four vaccine constructs in total were created.

Table 4.1 Vaccine constructs with different adjuvants.

VACCINE	CONSTRUCT SEQUENCES
Vaccine Construct 1 with HBHA adjuvant (V1)	EAAAKMAENPNIDDLPAPLLAALGAADLALATVNDLIANLRE RAEETRAETRTRVEERRARLTKFQEDLPEQFIELRDKFTTEELR KAAEGYLEAATNRYNELVERGEAALQRLRSQTAFEDASARAE GYVDQAVELTQEALGTVASQTRAVGERAAKLVGIELEAAAK AKFVAAWTLKAAAHEYGAEALERAGKLRFASHEYGGGSKVD IKKLHLDGKLRFGGGSQSVNSQLTDTVTQINGGGSEPKTETFK AAAAGANGGGSGSSTAGTGTAASPSGGGSSGESSNGGGSSLS GTPADGDQFTIGANKGTNDGRNGGGSEGTTVNWPTGTGGKG NDGVAHEYGAEALERAGAKFVAAWTLKAAAHEYGAEALER AG
Vaccine construct 2 with HBHA conserved adjuvant (V2)	EAAAKMAENSNIDDIKAPLLAALGAADLALATVNELITNLRER AEETRRSRVEESRARLTKLQEDLPEQLTELREKFTAEELRKAA EGYLEAATSELVERGEAALERLRSQQSFEEVSARAEGYVDQA VELTQEALGTVASQVEGRAAKLVGIELEAAAKAKFVAAWTL KAAAHEYGAEALERAGKLRFASHEYGGGSKVDIKKLHLDGK LRFGGGSQSVNSQLTDTVTQINGGGSEPKTETFKAAAAGANG GGSGSSTAGTGTAASPSGGGSSGESSNGGGSSLSGTPADGDQF TIGANKGTNDGRNGGGSEGTTVNWPTGTGGKGNDGVAHEYG AEALERAGAKFVAAWTLKAAAHEYGAEALERAG

Vaccine construct 3 with BETA DEFENSIN adjuvant (V3	EAAAKGIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGR KCCRRKKEAAAKAKFVAAWTLKAAAHEYGAEALERAGKLRF ASHEYGGGSKVDIKKLHLDGKLRFGGGSQSVNSQLTDTVTQI NGGGSEPKTETFKAAAAGANGGGSGSSTAGTGTAASPSGGGS SGESSNGGGSSLSGTPADGDQFTIGANKGTNDGRNGGGSEGTT VNWPTGTGGKGNDGVAHEYGAEALERAGAKFVAAWTLKAA A HEYGAEALERAG
Vaccine construct 4 with 50s ribosomal L7/L12 adjuvant (V4)	EAAAKMAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAA APVAVAAAGAAPAGAAVEAAEEQSEFDVILEAAGDKKIGVIK VVREIVSGLGLKEAKDLVDGAPKPLLEKVAKEAADEAKAKLE AAGATVTVKEAAAKAKFVAAWTLKAAA HEYGAEALERAGKLRFASHEYGGGSKVDIKKLHLDGKLRFGG GS QSVNSQLTDTVTQINGGGSEPKTETFKAAAAGANGGGSGSST AGTGTAASPSGGGSSGESSNGGGS SLSGTPADGDQFTIGANKGTNDGRNGGGS EGTTVNWPTGTGGKGNDGVAHEYGAEALERAGAKFVAAWT LKAAA HEYGAEALERAG

4.4 DISCUSSIONS

The development of vaccines has been instrumental in preventing and controlling infectious diseases. Traditionally, vaccines were made using weakened or killed pathogens to prompt an

immune response. However, recent advances in biotechnology have led to a more sophisticated method: designing and making chimeric vaccine constructs.

Chimeric vaccines represent an innovative strategy in vaccinology. These vaccines are engineered by combining genetic components from multiple pathogens or strains to create a single, all-encompassing vaccine candidate. This approach has several key benefits, including the ability to target multiple diseases at once, enhance the immune response, and overcome the limitations of conventional vaccines.

Designing and constructing chimeric vaccine constructs relies on a thorough understanding of the target pathogens, their antigenic parts, and immunology principles. Researchers use various techniques, like genomics, proteomics, and bioinformatics, to identify and select the most immunogenic and conserved epitopes from different pathogens. These epitopes are then fused together to create a single, multifaceted vaccine construct.

Chimeric vaccines have shown great potential in providing protection against a wide variety of infectious diseases, including viruses, bacteria, and parasites. Their ability to address emerging and reemerging threats in a rapidly changing global landscape has made them a focus of research and development.

In this article, we explore the fascinating world of chimeric vaccine design and construction. We examine the methodologies, successes, and challenges associated with these innovative vaccines, and how they represent a powerful tool in our ongoing fight against infectious diseases.

MHC-I, MHC II, and B cell epitopes would all be present in an ideal chimeric vaccination in order to elicit a robust immune response. Both humoral and cell-mediated immunity will be induced by it. Four B cell epitopes, three MHC-II, and one MHC-I epitope were chosen to

create chimeric sequences. An adjuvant is a component of a vaccine that boosts the body's immune system. Various adjuvants were chosen for the vaccination designs in order to examine their impact on antigenicity and allergenicity. A PADRE sequence was inserted in addition to the adjuvants to address problems brought on by global variations in the HLA-DR molecule. In comparison to vaccines lacking the T helper epitope, PADRE demonstrated 100 times greater potency than universal T helper epitopes, while PADRE-containing vaccines demonstrated improved CTL responses and protection. There were eight prepared and examined vaccine prototypes. Using an EAAAK linker, adjuvants were applied to each design. HEYGAEALERAG and GGGS linkers were used to join MHC-I, MHC-II, B-cell epitopes, and the PADRE sequence; these linkers do not change the conformation of the construct. These two linkers were used to join the epitopes.

4.5 CONCLUSION

We constructed 4 set of chimeric vaccines using the shortlisted B cell and T cell epitopes. We not only included the epitope sequences but also adjuvant and PADRE sequences. These were knit together using the linker sequences. These four constructs will go for validation further.

Chapter 5: In silico Validation of Vaccine Construct

5.1 INTRODUCTION

The development of vaccines has played a critical part in fighting infectious diseases and safeguarding public health for a long time. Traditionally, designing and testing vaccines involved a lot of laboratory work and clinical trials, which usually take a long time, cost a lot, and sometimes produce uncertain results.

Using computational methodologies, vaccine development has undergone a significant change in recent years. In silico validation, a term that comes from the Latin phrase "in silicon" (referring to computer simulations), has emerged as a powerful and innovative way to assess the potential of vaccine designs, especially chimeric vaccines.

Chimeric vaccines are a type of vaccine made by combining parts from different pathogens or strains to improve their effectiveness. Before vaccine candidates are evaluated in the lab or in clinical trials, in silico validation simulates, analyses, and improves them using technologies from computational biology, immunoinformatics, and bioinformatics.

This approach has several benefits. It greatly reduces the time and cost of traditional vaccine development, allows researchers to quickly assess a wide range of candidate designs, and provides insights into their safety and effectiveness. In silico validation also plays a key role in epitope-based vaccine design, ensuring the selected epitopes can provoke a strong immune response.

In this context, this chapter elucidates the world of *in silico* validation of chimeric vaccine designs, looking at the methods, successes, and challenges of this innovative approach. By leveraging the power of computational tools and predictive modeling, researchers are

revolutionizing vaccine development, offering hope for more efficient and effective solutions to combat infectious diseases.

5.2 MATERIALS AND METHODS

5.2.1 Allergenicity, antigenicity and solubility prediction of vaccine construct

Four vaccine constructs (VT1, VT2, VT3, and VT4) were made. To determine the optimal vaccination, methodologies for predicting solubility, allergenicity, and antigenicity were used to vaccine designs. The AlgPred server was used to analyse allergenicity (Saha & Raghava, 2006). However, two servers-ANTIGENpro (Magnan et al., 2010) and VaxiJen 2.044 server-were utilised to predict antigenicity. The solubility of the vaccine designs and the likelihood (≥0.5) were also predicted using the SOLpro service (Cheng et al., 2005).

5.2.2 Prediction of various physiochemical properties of vaccine constructs using PROTPARAM tool.

Utilising the ExPASy Protparam server, the physiochemical characteristics of vaccine formulations were assessed. The physicochemical parameters of the vaccine constructs were characterised using the ExPASy Protparam tool. The Protparam website provides data on PI values, quantity of amino acids, aliphatic index, instability index, molecular weight, and GRAVY score. The protein's instability index (<40) predicts the stability of the protein. A protein's hydrophilic and hydrophobic properties are explained by GRAVY values, and its thermostability is explained by the aliphatic index.

5.2.3 Prediction of Secondary structure of vaccine construct

Using the PSIPRED v3.3 programme, vaccine constructions (V1, V2, and V3) were used to predict the components of its secondary structure (Buchan et al., 2013). Its accuracy in predicting structure is 81.6%. Alpha and beta helices as well as coils can be components.

5.2.4 Molecular docking and molecular dynamics simulation

All four vaccine constructs were modelled using the Phyre2 online tool (Kelley et al., 2015). Using RCSB-PDB, the PDB IDs of every HLA allele were obtained. The HLA alleles 1A6A, 1XRS, 2Q6W, and 6J1W were employed. Using PatchDock (Schneidman-Duhovny et al., 2003), the HLA alleles were molecularly docked with four vaccine constructions to demonstrate HLA-peptide interactions. The Fire Dock (Fast Interaction Refinement in Molecular Docking) server was used to improve and rescore the rigid body molecular docking score. The top ten final refining solutions are offered by FireDock. The global binding energy and binding score provided the basis for the enhanced models. Furthermore, using the ClusPro server, the vaccine construct (V1) was docked with the TLR4/MD2 complex (PDB ID 2z65) (Kozakov et al., 2017). GROMACS was used to perform molecular dynamics simulations of the V1-TLR4 complex.

5.2.5 Codon optimization and insilico cloning of vaccine constructs

To adapt vaccine codon usage to the E. Coli host strain, the codon adaptation tool (JCAT) was used (Grote et al., 2005). The amino acid sequence of the vaccine was changed to work with codons in E. coli by converting it from reverse to DNA sequence. An algorithm was used to compute the CAI values, which served as the basis for the adaptation. Our research did not include restriction enzyme cleavage sites, rho-independent transcription terminators, or prokaryotic ribosome binding sites. The modified gene sequence of the final vaccine design was cloned using the SnapGene tool (Adames et al., 2015) in the E. coli pET28a vector to ensure vaccine expression.

5.3 RESULTS

5.3.1 Allergenicity, antigenicity, and solubility prediction of designed vaccine construct

ANTIGENPRO, VaxiJen, SOLpro server, AlgPred, and VaxiJen were used to further analyse the four vaccine constructs, V1, V2, V3, and V4. V4, which was discovered to be allergic and was subsequently removed from the analysis, is non-allergic according to the anticipated vaccine construct score for V1, V2, and V3. ANTIGENpro and VAXIJEN server were used to further predict the antigenicity of V1, V2, V3, and V4. With a predicted antigenicity value of greater than 0.90 and.75 in VaxiJen, the four vaccine constructs were shown to have good antigenicity. The solubility scores of all four were greater than 0.7, indicating that the vaccine design will be highly soluble when it is expressed heterologously in E. coli. Table 5.1 contains all of the results.

Table 5.1. Characterization of the construct with different adjuvant.

VACCINE CONSTRUCT	ALGPRED	ANTIGENpro	VAXIJEN	SOLpro
V1	Non-allergen	0.859882	1.1665	0.974576
V2	Non allergen	0.884183	1.1826	0.980100
V3	Non allergen	0.919282	1.4418	0.964183
V4	allergen	0.926492	1.1456	0.982857

5.3.2 Physiochemical analysis of vaccine construct

The Protparam server was utilised to analyse the vaccine construct's physiochemical characteristics. It had a molecular weight of 26–39 kDa. It was discovered that the GRAVY values were negative, indicating that the vaccine design is hydrophilic. High aliphatic index range (58 to 71.29) indicates vaccine construct stability at varying temperatures. Additionally, the instability scores for V1, V2, and V3 were all less than 40, indicating that the proteins are well-stabilized to elicit immunogenic responses. The table 5.2 also included the total amount of positive and negative amino acids.

Table 5.2. Characteristic properties of vaccine construct by protparam server.

VACCINE CONSTRUCT	MOL WT.	PI	GRAVY	ALIPHATIC INDEX	INSTABILITY INDEX	NEGATIVE AMINO ACID	POSITIVE AMINO ACID
VI	39001.67 (378 AA)	5.15	-0.512	68.44	28.03	54	42
V2	37883.45 (369 AA)	5.05	-0.486	70.87	32.08	54	40
V3	26534.22 (264 AA)	9.26	-0.554	54.58	21.30	25	34
V4	34813.51 (349 AA)	5.15	-0.262	71.29	71.29	48	37

5.3.3 Structure prediction of selected vaccine construct

The PSIPRED service was used to anticipate the secondary structures of the last three vaccine constructions (V1, V2, and V3). In Figures 5.1,5.2,5.3 the secondary structure is displayed. Every vaccine construct has a helix, strand, and coil in its structure.

Using the Phyre2 tool, the 3-D models of V1, V2, and V3 were created, and Ramachandran plot analysis was used to validate the models. The figure displays the modelled structure of the Ramachandran and V1 plots. Based on Figure 5.4 and 5.5, 83.64% of residues are located in the permitted zone.

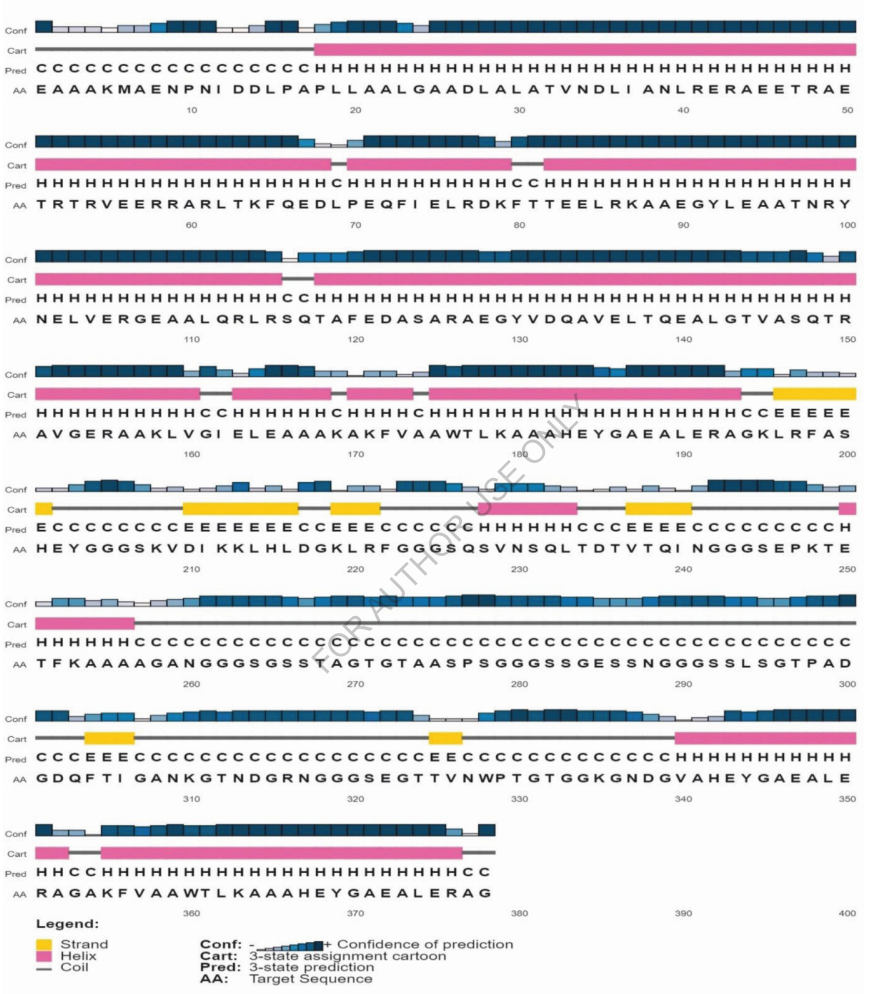

Figure 5.1. Secondary structure of vaccine constructs V1 using PSIPRED server

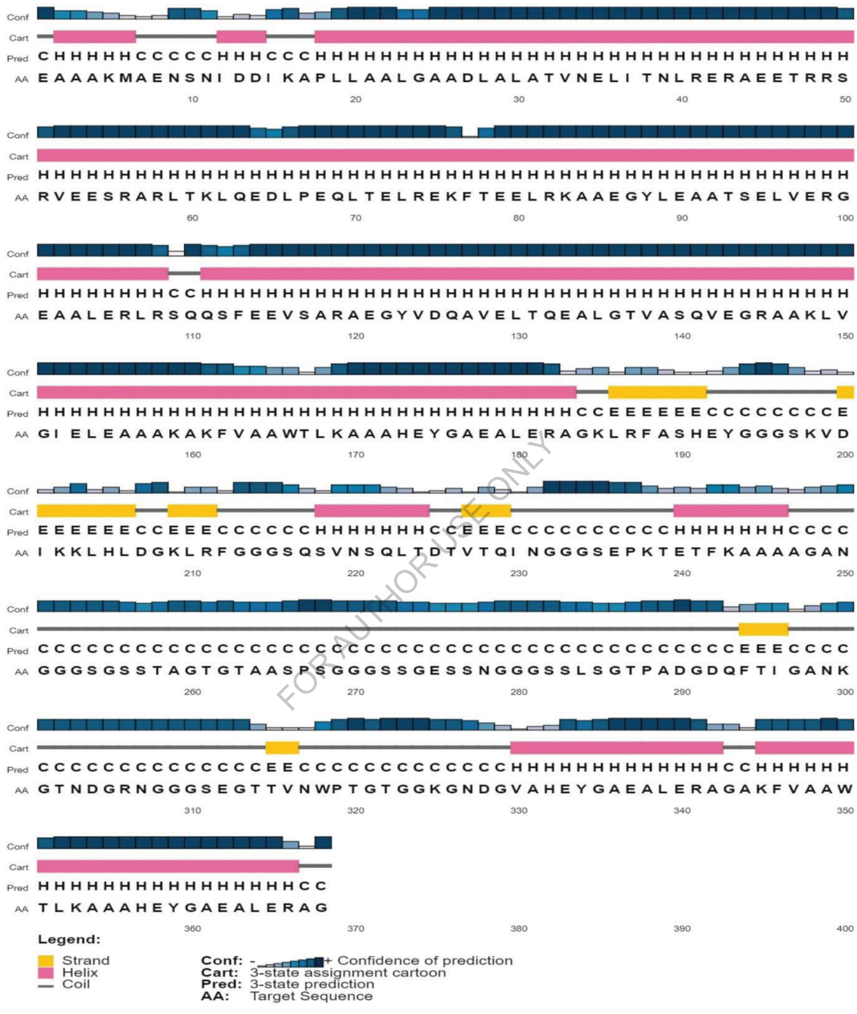

Figure 5.2. Secondary structure of vaccine constructs V2 using PSIPRED server

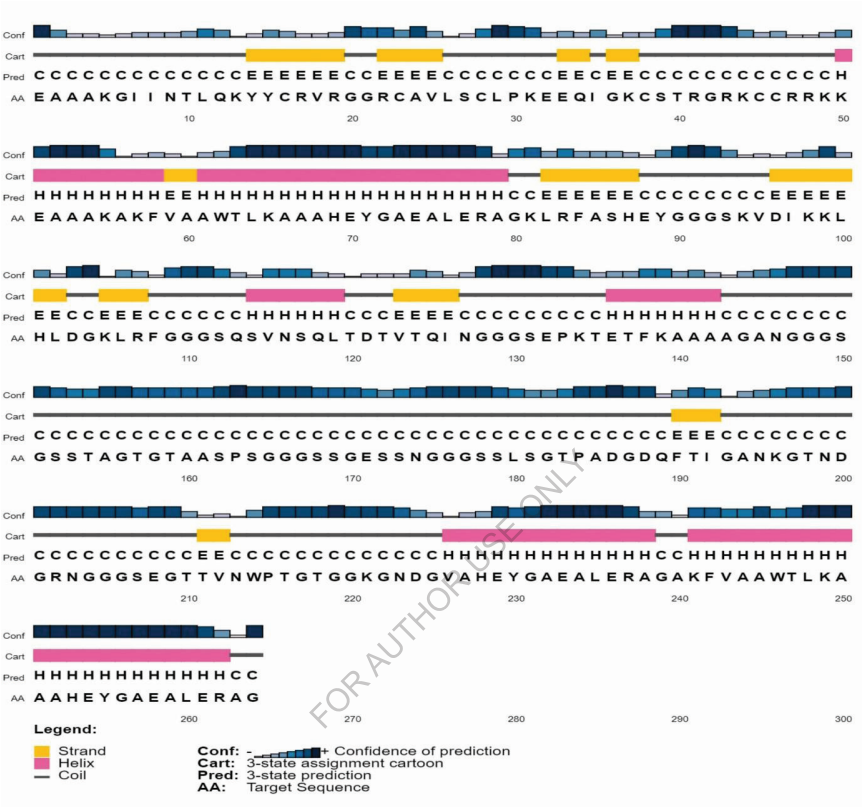

Figure 5.3. Secondary structure of vaccine constructs V3 in PSIPRED server.

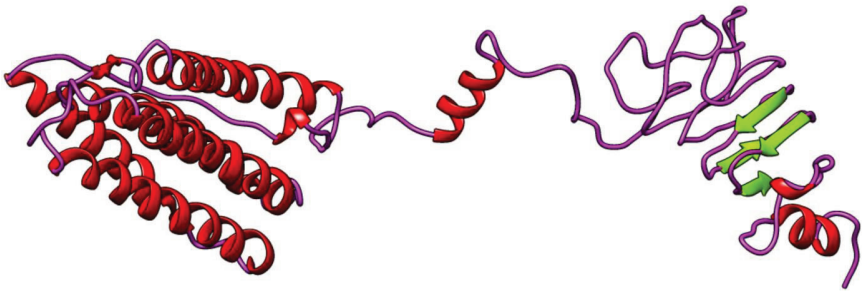

Figure 5.4. Tertiary structure prediction of vaccine V1 using Phyre2 server.

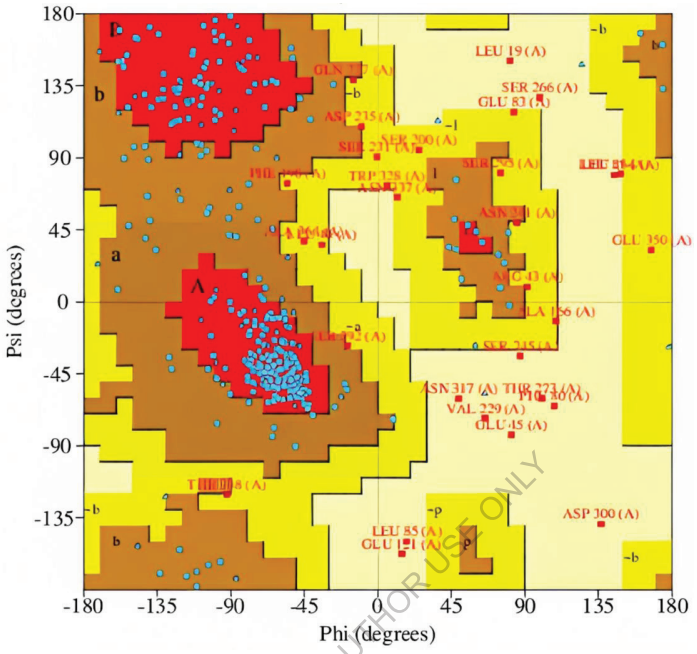

Figure 5.5. Stereochemical arrangement of V1 residues using Ramachandran plot, in which 83.64% residues are in allowed region.

5.3.4 Docking of V1, V2, and V3 with HLA alleles protein

Vaccines and the human population's HLA allele interact. We have docked V1, V2, and V3 with 4 distinct alleles-1A6A, 1XR8, 2Q6W, and 6J1W-in order to investigate this. With respect to the various alleles, as indicated in Table 5.3, V1 has the lowest global binding energy value: 1A6A (HLA-DR B1*03:01); -11.45, 1XR8 (HLA-B*15:01); -12.88, 2Q6W (HLA-DR B3*01:01); -12.88 and 6J1W (HLA-A*30:01); -5.26. After examining the three distinct constructs, we have determined that the V1 appropriate can be used to control Burkholderia pseudomallei.

Table 5.3 . Docking score of different vaccine construct with different HLA alleles.

Vaccine constructs	HLA allele PDB ID	score	area	Hydrogen bond energy	Global energy	ACE
V1	1A6A	19756	3090.10	-2.40	-11.45	5.07
	1XR8	16426	2784.50	-2.24	-12.88	6.28
	2Q6W	17566	3001.90	-3.06	-12.88	5.55
	6J1W	17780	2307.30	-4.18	-5.26	7.95
V2	1A6A	16338	1865.0	-5.14	-53.53	7.80
	1XR8	18626	3338.0	0.00	8.66	0.68
	2Q6W	17640	2423.2	-1.97	9.56	11.82
	6J1W	21846	3122.7	-1.47	7.13	5.76
V3	1A6A	14968	2066.20	-2.21	-3.94	4.69
	1XR8	15660	2149.10	-2.72	3.62	11.70
	2A6A	15008	2220.40	-1.28	-8.10	0.88
	6J1W	15972	2260.90	-1.42	-14.36	10.28

5.3.5 Docking and molecular dynamics simulation of V1 with TLR4

Adjuvant interacts with TLR4 when it is affixed to a vaccination design. As a result, we investigated the interaction between V1 and the TLR4/MD2 Complex (2Z65). The PatchDock result showed a binding energy that is negative (-2.03), indicating a positive interaction between the V1 and TLR/MD2 complex. ClusPro was also utilised to investigate this connection. With a minimum energy score of -1136.1, the best-modelled ClusPro complex—which is depicted in the figure—explains how V1 interacts with the TLR/MD2 complex. Molecular dynamics simulation using GROMACS was used to further validate the best docked complex interaction. As seen in Figures 5.6, 5.7, the RMSD graph demonstrates that the complex stabilises after 20 ns.

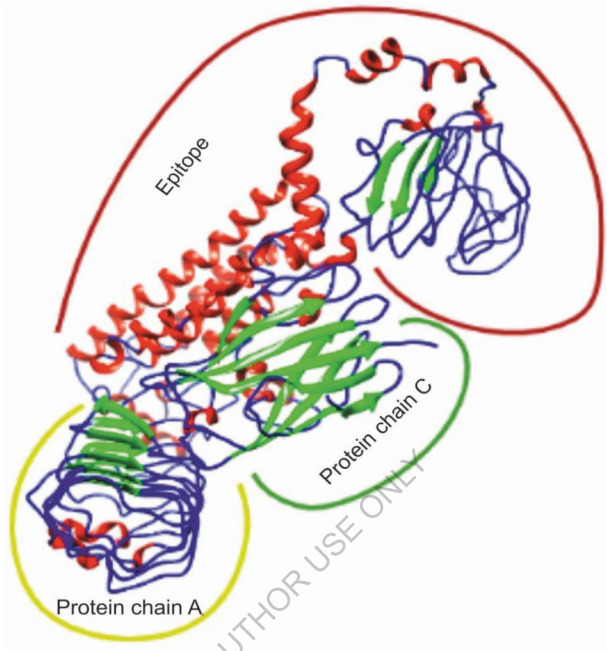

Figure 5.6. A docked complex of vaccine construct V1 with A and C chain of human TLR4

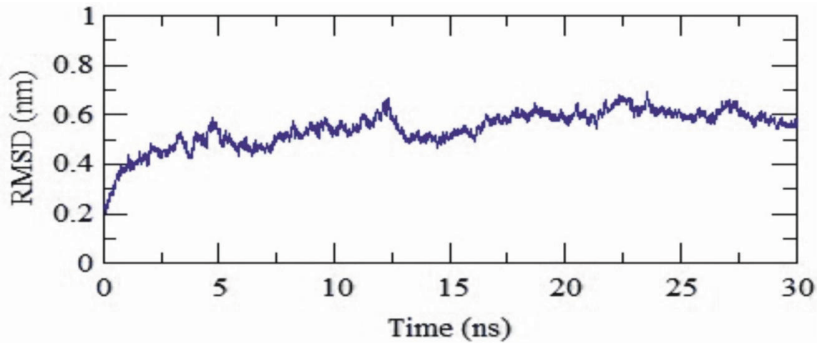

Figure 5.7. Molecular Dynamics Simulation of V1-TLR4 complex

5.3.6 In silico cloning of chimeric vaccine construct V1 for its heterologous expression in E. coli

The cloning of the chimeric multivalent vaccination and its expression within the expression vector pET28a were analysed using the Java Codon Adaptation Tool. In silico cloning, a cDNA sequence generated through reverse translation is employed. V1's codon optimisation study showed that the construct had a 53.88% GC content. V1's CAI value of 0.988 indicates the heterologous expression of a selected gene that will be strongly expressed in E. coli cells. The DNA sequences of EcoR1 and BMT1 were supplied at their 5' and 3' ends, respectively. V1 was insilico cloned into the pET28a vector for heterologous expression in E. coli using the restriction enzymes EcoR1 and BMT1.

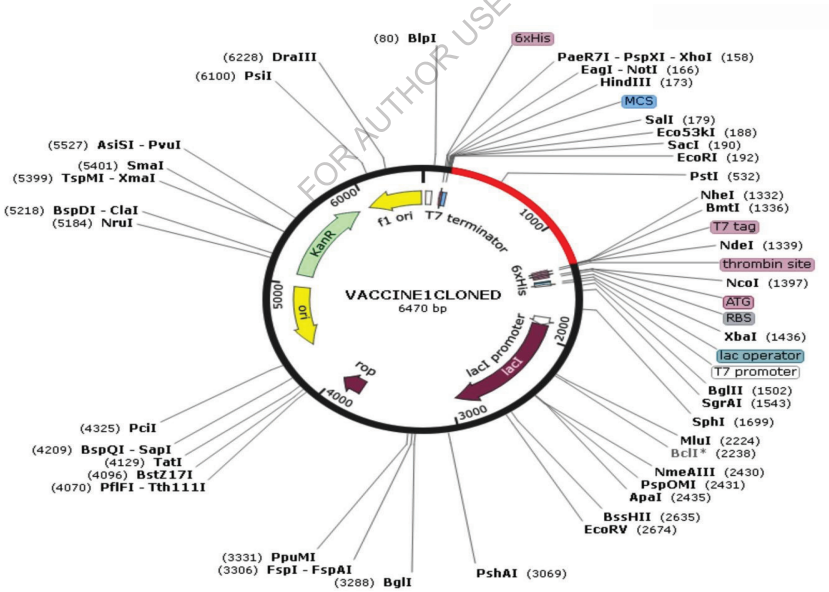

Figure 5.8. Insilico restriction cloning of final vaccine construct V1 into pET28a expression vector using EcoR1 and BMT1 restriction enzymes

5.4 DISCUSSION

The results showed that there would probably be no problems with the generated vaccine construct V1's potency, stability, or expression during the experimental stages. As previously indicated, the suggested vaccine construct V1 was demonstrated to be a suitable non-allergen that is highly antigenic and immunodominant, demonstrating the vaccine's capacity to stimulate strong immune responses while lowering the risk of allergies. This illustrates the basic properties of V1, which in principle can form stable connections at pH levels that are physiological. The estimated aliphatic and instability indices demonstrated the thermostatability and stability of V1, but a negative GRAVY score indicated hydrophilicity, which was supported by significant interactions with water molecules.

The significant Z-score suggested exceptional model quality, and the majority of amino acid residues were located in the preferred Ramachandran plot region. Due to its strong ability to bind MHC molecules, V1 can initiate immunological responses and then promote adaptive immunity by exposing epitopic antigens. Solid connections between V1 and TLR4 and MHC II complexes as well as other receptors were validated by molecular docking and MD modelling. During docking, a large number of H-bonds were seen with just slight variations from MD simulations, indicating that V1 has a high affinity for immunological receptors.

A appropriate vector is used to clone and overexpress the multi-epitope candidate, therefore developing it. Understanding viral biology provides important growth aspects that are useful in the design of vaccines. Here, in silico cloning verified that the pET-28a vector translated and expressed vaccine designs. T cell and B cell epitopes were included in the design, which are essential for inducing immunological responses by stimulating immune cells via intricate signalling. One drawback is that computational analysis relies on preliminary experimental

data without any experimental validation. Forecast efficiency is determined by assessing computational approach and input data quality; experimental research can fully unlock the potential of the chimeric vaccine V1 against Burkholderia pseudomallei.

5.5 CONCLUSION

During the in silico validation vaccine V1 was found to be the most suitable vaccine candidate. It is a chimera of MHC I, MHC II and B cell epitopes sequences. It is multiepitope chimeric vaccine, capable of eliciting humoral and cell mediated immune response.

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