Immunoinformatic Aided Design and Evaluation of a Potential Multi-epitope Vaccine against Multidrug Resistant *Acinetobacter baumannii*

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(2021)

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A thesis submitted in partial fulfillment of the requirements for the degree of BS Applied Biosciences

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Declaration

We certify that this research work titled "Immunoinformatic Aided Design and Evaluation of a Potential Multi-epitope Vaccine Candidate against Multidrug Resistant *Acinetobacter baumannii*" is our own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred.

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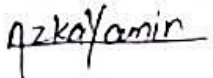

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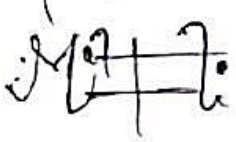

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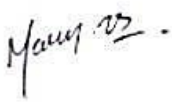

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To all those who have strived during these uncertain times

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List of Abbreviations and ACRONYMS

BPGA	Bacterial Pan Genome Analysis tool
CELLO	subCELlular LOcalization Prediction
CTB	Cholera toxin subunit B
DEG	Database of Essential Genes
ESBL	Extended Spectrum Beta Lactamases
ExPASy	Expert Protein Analysis System
ICUs	Intensive Care Units
IEDB	Immune Epitope Database (IEDB)
IFN	Interferon gamma
I-TASSER	Iterative Threading ASSEmbly Refinement
LPS	Lipopolysaccharide
MDR	Multi Drug Resistant
NCBI	National Center for Biotechnology Information
OmpA	Outer membrane protein A
OMPs	OMPs Outer Membrane Proteins
TLR	Toll-like Receptor
VFDB	Virulence Factor Database
WHO	World Health Organization
XDR	Extensive Drug Resistant

Abstract

Acinetobacter baumannii is an opportunistic, gram-negative pathogen which is particularly notorious for its extensive antimicrobial resistance profile. It causes a number of nosocomial infections, including bacteremia, ventilator-associated pneumonia, and meningitis. The clinical treatment of *Acinetobacter baumannii* infection has become increasingly difficult; a few pandrug resistant strains are thought to be unsusceptible to any current antibiotics. Infections caused by drug resistant strains have significantly higher mortality rates. Novel therapeutic agents, particularly prophylactic ones, are urgently required to save the lives of vulnerable individuals admitted in critical care. The aim of this study is to develop a novel potential multivalent vaccine against *A. baumannii*. In this study, pan-genomic and reverse vaccinology approaches have been applied for the identification of putative vaccine candidates for *A. baumannii*, using 208 publicly available complete genomes. Through a series of screening and analysis steps, to identify highly immunogenic and antigenic candidate proteins and the harboring epitopes, a total of 10 CTL epitopes and 4 HTL epitopes were screened in 8 prioritized vaccine candidates. These epitopes were then rationally linked together, and an adjuvant was added to form a multiepitope peptide vaccine. The designed polyvalent vaccine was modelled, refined, and simulated in the cellular environment to check its stability and flexibility. The vaccine construct showed encouraging results in interaction analysis with toll like receptors and in immune simulation experiments. In the future, we aim to express the multicomponent vaccine and evaluate it in vitro, followed by validation in the animal model. This will help determine the true immunological potential of the vaccine and further preclinical trials can be pursued.

Chapter 1

Introduction

Acinetobacter baumannii has emerged as one of the most successful and troublesome pathogens of the twenty-first century. It is responsible for a variety of nosocomial infections, particularly nosocomial pneumonia, often infecting those admitted in critical care units (Garnacho-Montero et al., 2019). The bacterium may also cause meningitis, infections of the skin and soft tissues, bacteremia, wound infections, urinary tract infections, and endocarditis. Moreover, it has developed a remarkable resistance to disinfectants and antiseptics (Bravo et al., 2019), and a wide range of mechanisms to evade the hosts' immune system. It thus thrives in a variety of environmental conditions. (McConnell et al., 2013)

The bacterium is notorious for its resistance to multiple classes of antibiotics and its extraordinary tendency to obtain resistance genes. It tops the World Health Organization's list of 'priority pathogens'- drug-resistant bacteria that currently pose the greatest threat to human health (Elhosseiny et al., 2018; Organization, 2017). A number of risk factors have been associated with colonization and infection. These include prolonged stays at the hospital, frequent visits to the intensive care, the insertion of the endotracheal tube during mechanical ventilation, colonization pressure (CP), the administration of antimicrobial therapeutic agents, freshly performed invasive surgical processes, and underlying sickness intensity (Dijkshoorn et al., 2007a).

With all the data available on genomes and proteomes, it is now possible to determine in silico vaccine candidates, and identify epitopes within them, employing a reverse vaccinology approach. Pioneered in the early twenty first century, reverse vaccinology is a modern in-silico method of vaccine design, which is less resource intensive then conventional approaches (Rappuoli, 2000). Reverse vaccinology has been widely used against *Acinetobacter baumannii*, however, the possibility of a multiepitope vaccine has hardly been explored. The task of creating a multiepitope vaccine involves the use of numerous bioinformatics and machine learning tools for pangenome analysis, vaccine candidate prioritization, epitope identification, epitope linking, characterization of the resulting multiepitope, molecular dynamics and immune simulations.

The resulting vaccine makes use of the latest genomic data available and is expected to be effective against all known strains of the pathogen to date. The vaccine is also rigorously tested in vitro before it is expressed in the lab or tested in vivo, thus conserving expended resources.

Our Contribution

The project has led to the design of a multiepitope vaccine effective against all existing strains of *Acinetobacter baumannii.* The pathogen's pan-core genome has been determined through pangenome analysis, and certain desirable protein vaccine candidates have been identified. Epitopes have been mapped within these vaccine candidates and filtered based on several stringent criteria to ensure that the resulting vaccine is safe, efficacious and its immunity is long-lasting. The epitopes have been linked together with a potent adjuvant via linkers to construct a chimeric vaccine, which has been subject to several characterizations and simulations.

This multi-epitope vaccine, once properly expressed, prepared, and administered, is predicted to work as a prophylactic vaccine which stimulates both humoral and cellular immunity in the patient. From our analysis, we predict that our proposed vaccine sequence is stable, flexible, soluble in water, non-allergenic, highly immunogenic, and high expression is experimentally possible.

1.1. Research Objectives

- Estimation of core genome employing all available *A. baumannii* strains and identification of vaccine candidates.
- Design, construction, characterization, and immune simulation of a multiepitope vaccine.

Chapter 2

Literature Review

2.1. Introduction: *Acinetobacter baumannii*

Acinetobacter baumannii is an aerobic, gram-negative coccobacillus responsible for a variety of nosocomial infections, particularly nosocomial pneumonia (Garnacho-Montero et al., 2010a). The *Acinetobacter* genus consists of 33 non-motile, non-spore forming, non-fermentative, catalase positive and oxidase negative bacterial species, of which only six have been named yet (Touchon et al., 2014)(Constantiniu et al., 2004).

A. baumannii has emerged as one of the most successful and troublesome pathogens of the twenty-first century due in part to its remarkable resistance to disinfectants and antiseptics (Bravo et al., 2019). The bacterium is notorious for its resistance to multiple classes of antibiotics; it tops the World Health Organization's list of 'priority pathogens'- drug-resistant bacteria that currently pose the greatest threat to human health (Organization, 2017). It is highly tolerant to desiccation and is able to grow at different temperatures and pH. It can be isolated on blood agar (Ajao et al., 2011) and grows well on standard lab media.

2.2. Human Infections and Transmission

A. baumannii is prone to infect various human body parts and the extent of infection varies from minor or asymptomatic infections to sepsis. Hospital-acquired pneumonia is the most frequent clinical presentation of infection from this pathogen (McConnell et al., 2013). Those receiving mechanical ventilation in the intensive care setting are usually infected most often. Because it persists on surfaces, researchers believe that ventilator-associated pneumonia develops from the colonization of the airway through environmental exposure (Dijkshoorn et al., 2007a; Morris et al., 2019)

Among its epidemiological features, experimental studies show that *A. baumannii* can remain active for extended periods on non-living surfaces in the surroundings of vulnerable hospital patients (Shamsizadeh et al., 2017). Using vascular catheters and other medical devices thus

becomes the most common source of infection (Skariyachan et al., 2019). Infectious outbreaks have been associated with respiratory-care gear, wound-care instruments, humidifiers, and healthcare equipment because the pathogen resists removal from non-living surfaces in the hospital premises (Maragakis et al., 2004; Marchaim et al., 2007). The bacterium is mostly transferred to the immunocompromised individuals through contaminated surfaces or via the hands of hospital staff (Wieland et al., 2018). The air may be contaminated by aerosol spray from the infected individual and this may be another mode of spread (Whitman et al., 2008).

Acinetobacter baumannii also frequently results in bacteremia in critical care settings (Wisplinghoff et al., 2004). In this case, intravascular devices or existing infections of the lower respiratory tract become the source of infection. The bacterium also colonizes open wounds and the urinary tract (Garnacho-Montero et al., 2010b).

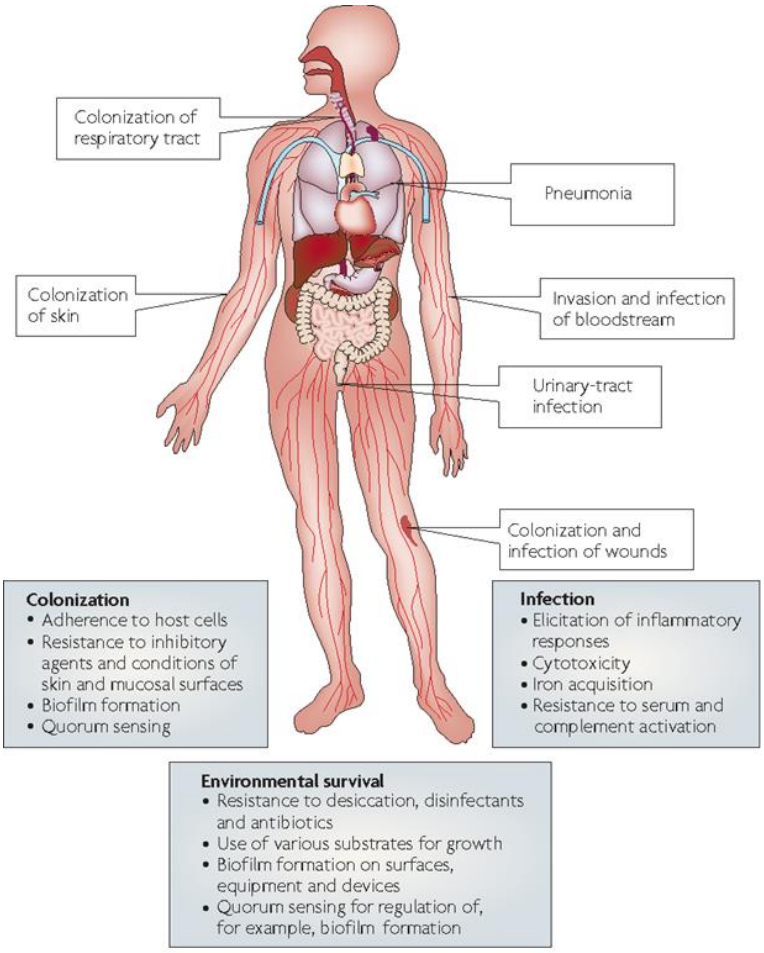


Figure 1: Factors involved in environmental persistence and infection and colonization of host by Acinetobacter baumannii. Adopted from (Dijkshoorn et al., 2007b)

2.3. Risk Factors

Patients who are deemed at risk of *A. baumannii* bloodstream infections are immunocompromised, dependent on the ventilator due to respiratory failure, have received antimicrobial therapy previously, and those undergoing invasive procedures. Surgical procedures of the brain and nervous system put patients at risk of meningitis. *A. baumannii* infection is also far more frequent in patients at risk of acquiring nosocomial soft tissue and skin infections, such as military personnel at risk of sustaining war-related trauma (Jung et al., 2010; Pachón et al., 2014).

2.4. Pathogenesis and Virulence Factors

A. Baumannii first adheres to human bronchial epithelial cells and erythrocytes using pilus-like structures (Harding et al., 2018). The strength of adherence varies among strains but is lower compared to other microorganisms (Asif et al., 2018; J. C. Lee et al., 2006).

The Outer Membrane Protein A(OmpA), which contributes to this adhesion, helps it to localize to the mitochondria. Once bound to the mitochondria, OmpA induces mitochondrial dysfunction and causes mitochondrial swelling. This is followed by the release of cytochrome c, which leads to the caspase dependent and independent pathway of apoptosis (Choi et al., 2005; Peleg et al., 2008). OmpA induces differentiation of $CD4^+$, activation and maturation of dendritic cells, and causes their premature apoptosis (J. S. Lee et al., 2010). The 33-36 Omp protein is another outer membrane porin which contributes significantly to the adherence and invasion of human lung epithelial cells, and cytotoxicity (Rumbo et al., 2014; Smani et al., 2013).

The human host environment is usually iron-deficient by the pathogen's standards. To thrive here, the bacterium secretes low molecular-mass ferric binding compounds, or siderophores (Peleg et al., 2008). At the infection site, the innate immune response is accelerated by the release of outer membrane vesicles containing several virulence-related proteins, including proteases, superoxide dismutase's and catalases. This ultimately leads to tissue damage (Asif et al., 2018; Nho et al., 2015).

The lipopolysaccharides (LPS) are immunoreactive molecules. They induce the release of tumor necrosis factor and interleukin 8 from macrophages using a Toll-like receptor 4 (TLR-4) mediated pathway (Erridge et al., 2007). The polysaccharide capsule of *Acinetobacter baumannii*

protects the bacteria against phagocytosis. Humoral immune responses may also be observed in *Acinetobacter* infection. Host antibodies target iron-repressible outer membrane proteins, and the O polysaccharide component of LPS (García-Patiño et al., 2017b).

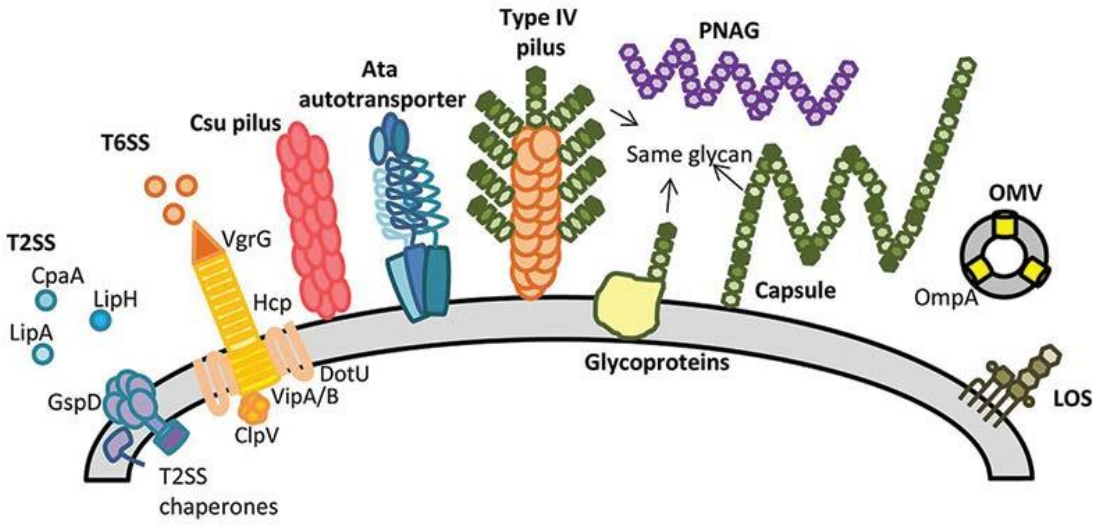


Figure 2: Virulence factors involved in Acinetobacter baumannii pathogenesis. Adopted from (Weber et al., 2016)

2.5. Global Prevalence

Outbreaks of multidrug resistant *A. baumannii* are generally pervasive and have been reported in several countries, including Brazil, Britain, America, India, Spain, China, Germany, Iran, Turkey and Iraq (Nasr, 2020). Additionally, the pathogen has been responsible for not just small-scale local epidemics, but international epidemics, such as those caused by the European clones I-III. Most of these clones are highly resistant to different classes of antibiotics (Dijkshoorn et al., 2007a).

A study conducted in 2007, in 75 different countries from five continents, found that *A. baumannii* was the fifth most common pathogen. Notably, researchers observed that patients who spent more time in Intensive Care Units(ICU) prior to the study had a higher rate of infection (Vincent et al., 2009).

2.6. Prevalence in Pakistan

The New Delhi metallo-beta-lactamase-1 (NDM-1) is a recent addition to the strains of *Acinetobacter baumannii* which display resistance to carbapenems. These are thought to originate in hospitals in the subcontinent, in India, Bangladesh and Pakistan. There is a documented high prevalence of multi-drug resistant *A. baumannii* isolates in healthcare facilities in Pakistan, with carbapenem resistance dominating (Hasan et al., 2013).

2.7. Treatment

Available treatment options are limited by the resistance mechanisms exhibited by the bacteria. The resistance capability of *Acinetobacter* species is attributed to the organism's relatively impermeable outer membrane and its exposure to a large pool of resistance genes in the environment (Bonomo et al., 2006). The antimicrobial of choice are carbapenems, but the majority of strains are already resistant to these (Hasan et al., 2013; Hawley et al., 2008; Maragakis et al., 2008).

Sulbactam, a potent β-lactamase inhibitor, can target *A. baumannii* by inhibiting penicillin-binding protein 2 (PBP2). Augmented therapy with ampicillin and sulbactam has emerged as an effective remedy for nosocomial bloodstream infections as well as respiratory and urinary tract infections. Even then MDR may render such treatment useless (Levin et al., 2003; Makris et al., 2018).

Usually, MDR strains are susceptible to polymyxins, but these pose toxicity concerns in human subjects (Bowers et al., 2015; Falagas et al., 2005; Levin et al., 2003). Polymyxin E, commonly known as Colistin, has manifested its clinical efficacy in treating bloodstream and urinary tract infection (Gounden et al., 2009). Colistin resistance too is a problem for the medical community (Pormohammad et al., 2020). Further research is required to set dosage, reduce associated toxicity and determine the better route of administration (Hawley et al., 2008).

Tigecycline, a minocycline derivate, has successfully combated skin intra peritoneal infections by eliminating MDR *A. baumannii* determinants. These success stories have encouraged the use of tigecycline against respiratory and bloodstream infections as well (Grosse, Babinchaket al. 2005). But in case of bacteremia, treatment with tigecycline may cause the disease to reoccur and lead to resistance against it (Gordon et al., 2009).

Combinations of synergistic drugs are administered when mono-therapeutic regimes fail. Rifampicin, minocycline, ceftazidime and imipenem were administered alongside colistin in certain in vitro studies and showed notable effects (Lertsrisatit et al., 2017; Principe et al., 2009). Similarly, tigecycline combined with various other therapeutic agents has shown antagonistic as well as agonistic effects in vitro (Principe et al., 2009). Still in vivo conformation is required.

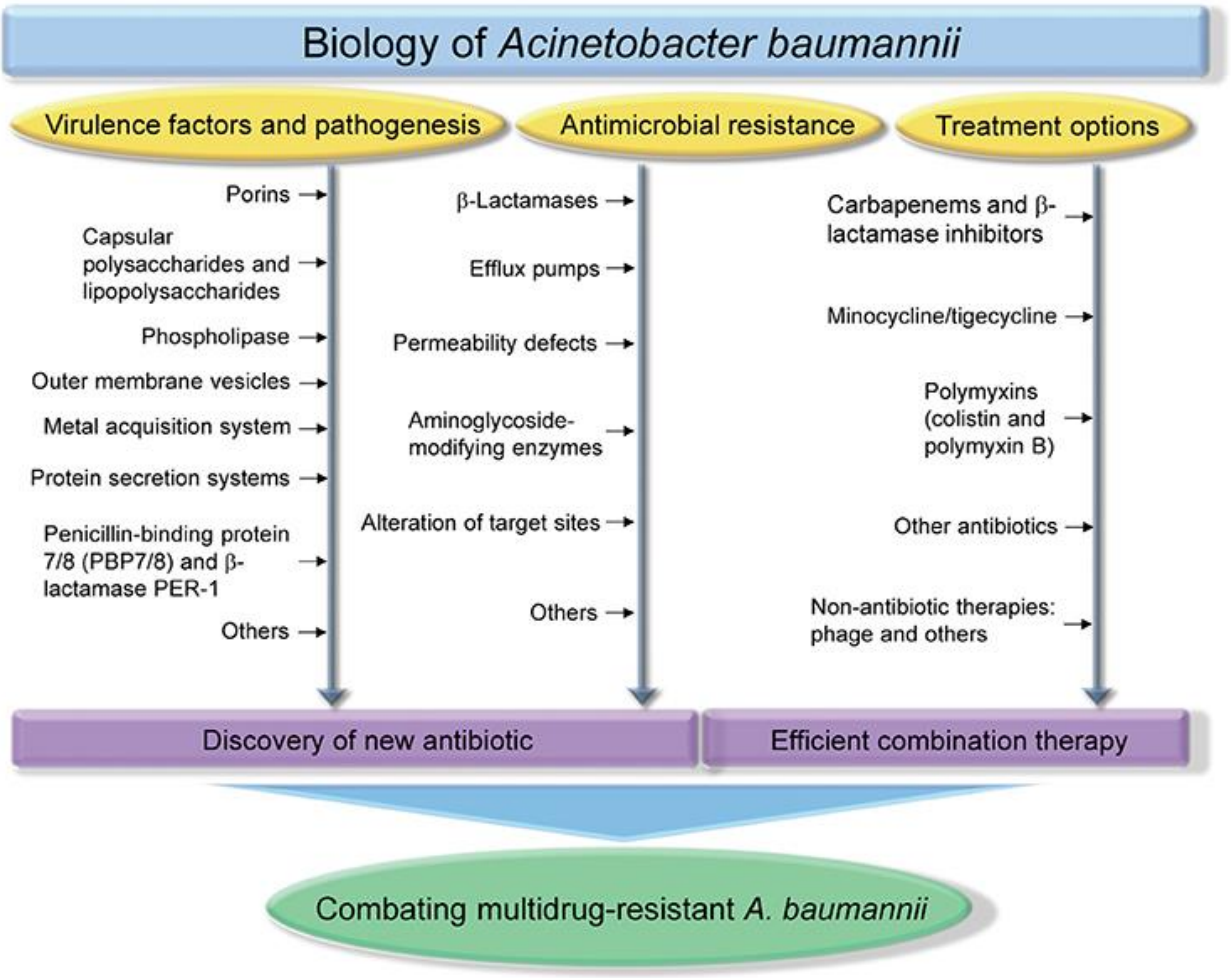


Figure 3: An understanding of virulence factors, pathogenesis, antibiotic resistance, and treatment alternatives of A. baumannii will allow researchers to develop efficient combination therapy. Adopted from (C.-R. Lee et al., 2017)

2.8. Drug Resistance

It is now becoming evident that pathogens like *Acinetobacter baumannii* possess an intrinsic resistome which contributes to their resistance mechanisms to counter antibiotics. Acinetobacter

species have been categorized into three levels depending upon the extent of resistance in the strain.

a. Multidrug resistant (MDR) *Acinetobacter* isolates are strains which are resistant to three classes of antibacterial drugs, namely all penicillin's, fluroquinolones, aminoglycosides or cephalosporins.
b. Extensively drug resistant (XDR) *Acinetobacter* isolates are MDR in addition to being resistant to carbapenems.
c. Pan drug resistant (PDR) *Acinetobacter* isolates are XDR and resistant to both polymyxins and tigecyclines.

MDR *A. baumannii* isolates have been found in hospitals in Europe, Korea, Hong Kong, Argentina, Brazil, Iraq, Afghanistan, China, Taiwan, Japan, Spain, Norway, North America, and certain isolated parts of the South Pacific. These can frequently cause outbreaks across cities, countries or even entire continents (Perez et al., 2007).

They have also been associated with increased mortality and prolonged hospital stays (Peters et al., 2019). The emergence of new multidrug resistant strains has been attributed to the overuse of antibiotics in the developed world (Jung et al., 2010).

2.8 Immunization against *A. baumannii*

Since clinicians now understand who is at risk of *A. baumannii* infection, they believe the active immunization of acutely at-risk patients would help reduce infection rates, and antibody-based immunotherapy will help improve infection outcomes. Scientists have looked into several strategies to harness the immune system against *A. baumannii*. One such example is of the immune system's recognition of the bacteria through PRRs (TLRs more specifically) which can lead to oxidative burst, cytokine and chemokine production or other bactericidal mechanisms in order to amplify the immune response (García-Patiño et al., 2017c).

Passive immunizations against *A. baumannii* have been studied for the past two decades and may enter the market soon. Active immunization has also been studied extensively in the past decade.

2.8.1 Active immunization

Immunogenic antigens have been widely tested as portions of vaccine formulation against MDR *A. baumannii*.

- **Whole-cell vaccines**

Vaccines containing *A. baumannii* cells inactivated with formalin and combined with aluminum phosphate adjuvant are administered as intramuscular injections. These produce rapid antibody titers in immunized mice. Bacterial burdens are reduced, and blood indicators of sepsis are also reduced (McConnell et al., 2010b). This approach although feasible and cost effective, still creates concerns regarding the safety and toxicity of the final formulation in humans.

Alternatively, the outer membrane protein complexes (OMCs) are also potential adjuvants that have been used as adjuvants in vaccine formulation. These reduce the chances of post-infection septic shock by limiting the production of pro-inflammatory cytokine. They do not present as much of a safety concern. This formulation has been effective in animal models so far against sepsis and pneumonia (Pulido et al., 2018).

- **Pure-protein-based vaccines**

The pure protein-based vaccines are made of favorable protein vaccine candidates in a suitable formulation. In a study, rational screening mechanism identified OmpA as the primary target of humoral immune response at the time of an A. *baumannii* infection and it was also found to be highly conserved in mice among several isolates. Scientists were able to successfully clone and confirm expression of the OmpA gene of *A. baumannii* into the pBudCe4.1 expression vector (eukaryotic). Therefore, the OmpA gene is a suggested DNA vaccine candidate against *A. baumannii* (Mahmoudi et al., 2020). A combination of purified OMPs and fimbriae were registered as a possible effective therapeutic candidate in 2011. These proteins conjugated with aluminum hydroxide in experiments have been proved much effective. (T. A. Ahmad et al., 2016) There may be however some difficulty in administration as purer preparations tend to be insoluble as vaccines.

NlpA is an antigenic factor in the composition of outer membrane vesicles(OMV) based vaccines against A Baumannii. In a study, scientists have successfully cloned and expressed NlpA gene in cells of eukaryotes. Evaluation of the levels of immune response following the injection of the formula as DNA vaccine in BALB/c mice were also carried out (Ansari et al., 2088).

2.8.2 Passive Immunization

Researchers have been exploring the option of inducing passive immunity by preparing antisera consisting of components of *A. Baumannii* cell to neutralize or eliminate it.

- **Antisera against "whole cells**"

Multi-antigen formulations have been used to raise a polyclonal immune response. In one study, mice were injected with attenuated whole cells of *A. Baumannii* along with a potent adjuvant which induced immunization and the raised antisera, causing the bacterial infection to reduce up to 1000 times (S. Li et al., 2020).

Carrying out immunization passively through specific antibodies' administration, such as IgY is an attractive and practical solution. In a study, inactivated whole cell of *A. baumannii* was used along with OmpA and Omp34 in a vaccine to raise specific IgY which have proved to induce immunity against pneumonia in the murine model. Among these, IgY raised against OmpA have produced a higher protective effect against *A. Baumannii*. Hence, against A. *baumannii* caused pneumonia, IgY can be used as a safe natural product (Jahangiri et al., 2018).

- **Polysaccharide antisera**

Protein-polysaccharide conjugate antigens can produce low tires of serum antibodies in infants and can be used in booster doses. In a study conducted in 2021, an O-linked glycosylation system was introduced into a host strain to construct a conjugate vaccine against *A. baumannii*. The vaccine demonstrated the ability to induce Th1 and Th2 immune responses, proving safe in mice models. Post-infection bacterial load in the tissues were evaluated which showed bactericidal activity in-vitro and prophylactic effects and suppressed levels of serum pro-inflammatory cytokine were observed. This proves that the conjugate vaccine elicited efficient immunity and can protect against infection in murine sepsis models (X. Li et al., 2021).

Poly-β-(1→6)-N-acetylglucosamine (PNAG), which is a virulence factor produced in various pathogens is considered a promising target for a potent vaccine due to the key role of de-N-acetylated glucosamine units in triggering immunity. Synthetic penta- (5GlcNH2) and nona-β-(1→6)-d-glucosamines (9GlcNH2) conjugates showed potential in passive and active immunization against various infectious agents. However due to plenty of PNAG-producing pathogens, a number of trials will be needed (X. Li et al., 2021).

Antisera based immunization with outer membrane proteins (OMPs) as targets has also induced protection against *A. Baumannii* in mice. Results show a significant role of a capsule polysaccharide in shielding OmpA and hence blocking anti-OmpA monoclonal anti-OMP antibodies from binding to the isolates. Therefore, the potential therapeutic properties of antisera against OmpA have not been explored (Wang-Lin et al., 2017).

- **Protein based antisera**

Biofilm associated protein is the cell surface protein of *A. baumannii* which is involved in biofilm formation and plays a major role in infections by aiding adherence. Bap706–1076 region of 371 residues has been used in a chimeric vaccine loaded on chitosan-adjuvant. When administered intranasally, the vaccine raised IgA and IgG antibodies in the serum, lung and fecal extractions in mice. Evaluation of mucosal and systemic immune response (passive) showed a higher survival rate (Darzi Eslam et al., 2020).

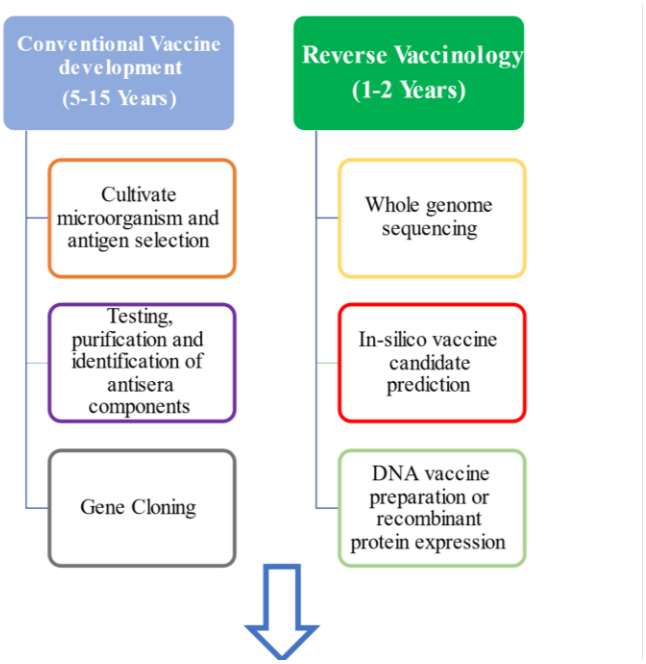
In in-silico analysis, BamA which is a highly conserved outer membrane β-barrel assembly protein, was identified a**s** a favorable target for antibody-based immunization against *A. baumannii* infections. Immunization with this protein caused the production of a significant concentration of macrophage dependent opsonophagocytic antibodies and provided 80% immunity by decreasing the concentrations of bacteria in the lungs and levels of pro-inflammatory cytokine in sera and tissue homogenate (Singh et al., 2017).

2.9 Reverse vaccinology

Pioneered by Rino Rappuoli in 2000, reverse vaccinology is a novel approach to finding vaccine candidates against a pathogen. The genomic sequences of the organism under study are screened based on the researcher's choice of desirable attributes. Once identified, the genes are synthetically expressed and screened in animal models of the infection. Although reverse

vaccinology takes far less time to identify and screen vaccine candidates than do its conventional counterparts, one limitation of this approach is that it can only identify proteins in the pathogen, and no other biomolecular targets such as polysaccharides.

From the potential vaccine candidates, one approach is to identify and link together favorable epitopes to form a multiepitope vaccine. Today, several reverse vaccinology pipelines are available, including ReVac, VacSol, PanRV and Vacceed (D'Mello et al., 2019, Rizwan et al., 2017, Naz et al., 2019, Goodswen et al., 2014). A few software's are even available in their web-server versions. These include Vaxign and Jenner-Predict (He et al., 2010, Jaiswal et al., 2013).



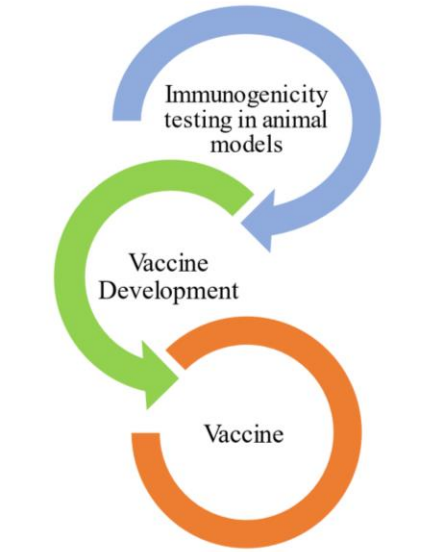


Figure 4: Vaccine development process by employing conventional approach and reverse vaccinology. Adapted from (Rappuoli, 2000

Chapter 3

Methodology

3.1 Strain Selection

A total of 222 complete genomes of *A. baumannii* were acquired from NCBI database (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/) on 30th August 2020. 13 genomes were excluded as they were taken from non-human hosts and environmental samples.

3.2 Pangenome Analysis

To obtain highly conserved regions of the genome, all strains were subjected to pan-genome analysis using the 'Bacterial Pan-Genome Analysis Tool' (BPGA) (Chaudhari et al., 2016). Sequence identity was set at 90% as our focus was on obtaining core genome with minimum variations. In addition to computing core, pan genome, BPGA also provides details about the newly added genes by each succeeding genome. A gene family distribution plot is also generated that describes core, pan and accessory genes.

3.3 Reverse Vaccinology and Epitope Prediction

Antigenic proteins were found from the core proteome using reverse vaccinology approach. The RV module of PanRV was used for protein prioritization using many filtration steps (Naz et al., 2019). The reverse vaccinology strategy used is outlined in the fig 5.

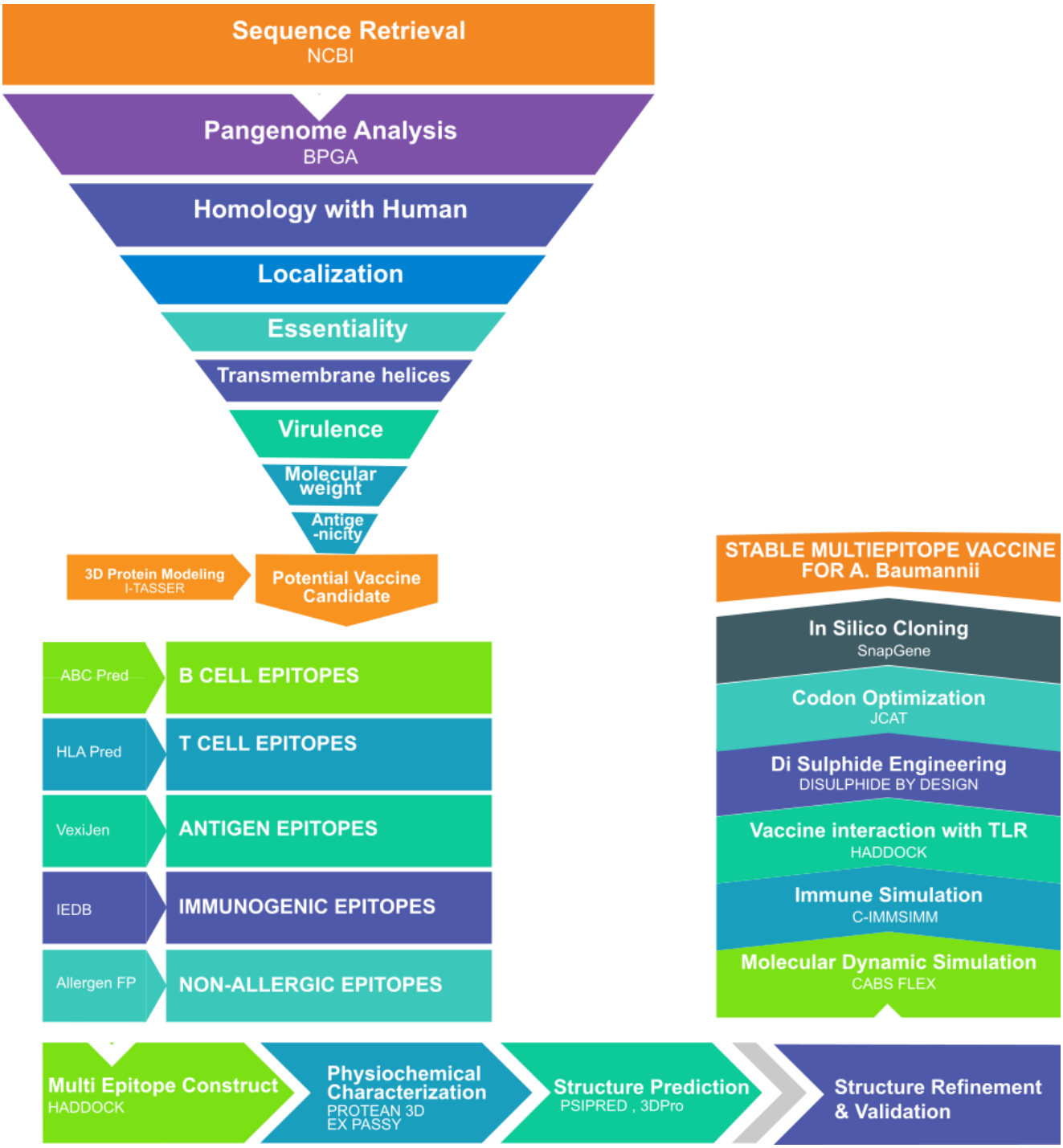


Figure 5. A schematic representation of the methodology followed for the in-silico design of multiepitope vaccine against Acinetobacter baumannii.

3.3.1 Non-homologous protein identification

The human body has its own gut flora, and many similar proteins are found in both humans and bacteria. In order to reduce the chances of any autoimmune reaction, human and gut homologs were excluded from the core proteome. BLASTp was used to check for human and gut homolog (Gish et al., 1993). The exclusion criteria for this study were set at: E-value <1.0 E^{-5}, percentage identity>35%, and Bit score >100.

3.3.2 Subcellular Localization

The proteins obtained after the first step are further screened on the basis of their localization in the cell using version 2.0 PSORTb inside PanRV (Gardy et al., 2005). Proteins located on the outer membrane or in the extracellular space were retained as previous studies have reported their usage as potential vaccine candidates. Cello and Cello2Go were used to further confirm the localization results generated by PSORTb (Yu et al., 2006, 2014). CELLO is a multiclass SVM classification program used for the prediction of bacterial protein subcellular localization (Yu et al., 2014). CELLO determines localization by studying four key features: sequence composition based on physicochemical properties, the composition of di-peptides, amino acid composition, and partitioned amino acid composition. Final assignment is performed based on majority voting criteria.

3.3.3 Essentiality, Virulence, and Antigenicity check

The potentially essential proteins for *A. baumannii* survival were screened by running BLASTp on the Database of Essential Genes (DEG) on the localization filtered proteins (Zhang et al., 2009). This database has been integrated into PanRV. The essentiality filter is a crucial step as it identifies proteins that perform functions vital for the bacteria and are potential therapeutic targets. Bacterial virulence factors play a vital role in pathogenesis and can be targeted for vaccine development. In order to find such proteins, BLASTp on the selected proteins was performed against the Database of Virulence Factor (VFDB) and the Database of Microbial Virulence (MvirDB). (Liu et al., 2019; Zhou et al., 2007)

Antigenic potential of the essential proteins was analyzed using version 2.0 of VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) that utilizes an alignment-independent method for prediction of protective antigens (Doytchinova et al., 2007). Proteins possessing antigenicity scores greater than 0.5 were considered antigenic.

3.3.4 Trans-membrane helices Assessment

HMMTOP version 2.0 was used for further filtration of the prioritized proteins for the presence of transmembrane helices (Also in PanRV) (Tusnady et al., 2001). A relaxed transmembrane helix filter criterion was used.

3.3.5 Molecular Weight Assessment

ProtParam tool of ExPASy (https://web.expasy.org/protparam/) was used to compute the molecular weights of the potential antigens (Gasteiger et al., 2005). Proteins having molecular weight < 110 were preferred as this is the recommended threshold for protein purification in wet lab experiments (Baseer et al., 2017).

3.3.6 Sequence-based annotation of candidate proteins

The functional annotation of prioritized proteins was done by uploading protein fasta sequences on BLASTP (Gish et al., 1993).

3.3.7 Structure prediction and epitope visualization of candidate proteins

The 3D structure of the candidate proteins was predicted using I-TASSER (Iterative Threading ASSEmbly Refinement) (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) which uses multiple threading and iterative template-based fragment assembly simulations for construction of protein models (Yang et al., 2015). ITASER constructs 5 models for each protein and quality check is performed as per C-score. C-score varies from -5 to 2, and a higher C-score suggests a better quality model. Epitope mapping on the modeled protein was carried out by using UCSF Chimera version 1.15.

3.4 Subtractive Epitope Screening

3.4.1 In silico prediction of B and T cells epitopes

The prioritized proteins were submitted on ABCpred for B-cell epitope prediction (Saha et al., 2006). ABCpred predicts epitopes by utilizing an artificial neural network-based algorithm. The B cell epitopes were further screened for CD8+ and CD4+ T cell epitopes using HLApred (Saha et al., 2007).

3.4.2 MHC binding alleles

Epitopes binding to higher number of HLA alleles were retained for vaccine development. Predicted MHCII and I epitopes binding to 5 or more than 5 alleles were retained.

3.4.3 Antigenicity Check

VaxiJen v2.0 was used to screen out probable protective antigenic epitopes. B cell derived T cell epitopes having score greater than 0.5 were selected as antigenic (Doytchinova et al., 2007).

3.4.4 MHC-I Immunogenicity Check

The immunogenic potential of the MHC-I binding T cell epitopes was determined using Immune Epitope Database (IEDB) (https://www.iedb.org/) (Vita et al., 2018). 9mer peptides having positive scores were selected as they have immunogenic potential.

3.4.5 MHC II epitopes IFN Induction Potential

The interferon gamma inducing potential of MHC-II binding T cell epitopes was determined through IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope/). The epitopes with positive score were selected as they possessed IFN inducing potential.

3.4.6 Allergenicity Check

All the epitopes were further filtered for probable allergenic potential using AllergenFP and AllerTOP v. 2.0 (Dimitrov et al., 2014). All the non-allergen epitopes were retained for the construction of a multiepitope vaccine.

3.5 Multi-epitope Vaccine Construction

3.5.1 Docking of Prioritized Epitopes

The prioritized epitopes were linked via linkers and docked together to make different combinations to find the best possible sequence for vaccine using HADDOCK Guru webserver (Zuiderweg et al., 2000). All these combinations were then refined via HADDOCK Refinement webserver. Linker's selection was made after extensive literature review. A flexible GPGPG linker was used to link the prioritized epitopes together to efficiently separate them and to avoid the formation of junctional epitopes.

3.5.2 Adjuvant Addition

In order to further increase the vaccine immunogenicity cholera toxin B (CTB), a potent, non-toxic adjuvant, was added to the N-terminal of the vaccine construct via EAAAK linker.

3.5.3 Immunological and Physiochemical Assessment of Vaccine Constructs

The final vaccine construct was evaluated for its physicochemical and immunological characteristics. Antigenicity of the multiepitope vaccine was determined through VaxiJen v2.0

and ANTIGENpro (Doytchinova & Flower, 2007b; C. N. Magnan et al., 2010). Probable allergenicity of the vaccine construct was evaluated by submitting the construct sequence to 1.0 version of AllergenFP and 2.0 version of AllerTOP (Dimitrov et al., 2014; Saha et al., 2006).SOLpro and cc SOL were used to determine the solubility potential of designed vaccine construct in E.coli (Agostini et al., 2014; Magnan et al., 2009). The vaccine construct sequence physicochemical characterization including half- life, molecular weight, instability index, GRAVY, and isoelectric point etc was carried out through Protean 3D and ExPASy portal (Gasteiger et al., 2005). Finally, the human homology of vaccine construct was also checked by performing BLASTp to confirm the vaccine's safety for use in humans. Moreover, potential signal peptides in the vaccine were predicted using SignalIP.

3.5.4 Secondary Structure Prediction

PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) and CLC genomics workbench were used for prediction of secondary structure of the vaccine construct (McGuffin et al., 2000). PSIPRED uses neural networks-based model on the iterative blast results of the protein sequences submitted to predict the secondary structure.

3.5.5 3D Structure Modelling, Refinement and Validation

High quality 3D structures of the vaccine were modeled through I-TASSER (Iterative Threading ASSEmbly Refinement) (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) (Yang et al., 2015). The best model from ITASSER was refined using Galaxy Refine server that subjects the model to repeated perturbations and relaxations by employing molecular dynamic simulations (Ko et al., 2012). The best model from the five resulting models was finalized on the basis of poor rotamers, GDT-HA, RMSD, MolProbity score, etc. The best model was further evaluated by its ERRAT score, Ramachandran plot analyses and ProSA generated model quality score and Z-score (Dym et al., 2012; Wiederstein et al., 2007).

3.5.6 Energy Minimization of Multi-Epitope Vaccine

The flexibility and stability of the refined vaccine structure was determined by Molecular dynamic simulation studies. The molecular recognition and function of a protein is determined by its structural flexibility so, CABS-flex 2.0 (http://biocomp.chem.uw.edu.pl/CABSflex2) was used for coarse grained simulations of the vaccine construct (Kuriata et al., 2018). Default distance restrained parameters and temperature was used.

3.5.7 Interaction of multiepitope vaccine with Toll-Like Receptors

To confirm our multiepitope agonistic behavior against the Toll-like receptors, we performed an interactional analysis between our multiepitope vaccine and TLR2 and TLR4. The PDB structures of the two receptors were obtained from the PDB database (the IDs were 2Z7X and 3FXI). The structures were submitted to the HADDOCK Guru interface, and default parameters were used. The resulting interactions were selected based on the lowest scores and refined using the HADDOCK Refinement interface (Zuiderweg et al., 2000).

Active and passive residues in the interaction were predicted using CPORT (Consensus Prediction of Interface Residues in Transient Complexes). (de Vries et al., 2011) The structures were visualized using UCSF Chimera v. 1.14 (Pettersen et al., 2004) and specific interacting residues were predicted using PDBsum.(Laskowski et al., 2018)

3.5.8 Disulphide Engineering

Disulphide engineering allows the identification of potential disulphides in the proposed construct. These are highly likely to form and improve the thermal stability of the multiepitope. The refined structure of the construct was submitted to the web-based Disulphide by Design (DbD) v2.13 (http://cptweb.cpt.wayne.edu/DbD2/), which predicted the locations of potential disulphide bonds which could be introduced into the sequence (Craig et al., 2013).

3.5.9 Codon Optimization and In-silico Cloning

For high expression level in *E. coli*, codon optimization and reverse translation of the multi-epitope vaccine were carried out using JCAT (JAVA Codon Adaptation Tool) online server. (http://www.jcat.de/). Salient cDNA features such as GC content and Codon Adaptation Index (CAI) were analyzed to assess the probable protein expression levels (Grote et al., 2005). SnapGene® software was used for in silico cloning of optimized multiepitope vaccine into pET-28a (+) vector.

3.5.10 Immune Simulation

Immune simulations were carried out using C-IMMSIM server (https://kraken.iac.rm.cnr.it/C-IMMSIM/) to further characterize the immunogenicity of the multivalent vaccine and its immune response profile. C-IMMSIM is an agent-based simulator that uses machine learning and position-specific scoring matrices (PSSM) for the prediction of epitopes and immune interactions. It simulates three major components of the mammalian immune system: i) A

tertiary lymphoid organ like lymph nodes, ii) bone marrow, where stem cells are stimulated to produce myeloid and lymphoid cells and iii) thymus, where T cells mature. The minimum recommended time interval between dose 1 and dose 2 for vaccines is 4 weeks for children while 8 weeks to 12 weeks for adults (Beysolow, 2012; Castiglione et al., 2012). Two vaccine injections were given 8 weeks apart at time steps 1 and 168 (each time step is 8 hours and time step 1 is injection at time = 0). Then another vaccine dose was injected at time step 1100 which is about 12 months after the simulation starts in order to check the vaccine efficacy.

Chapter 4

Results

4.1 Strain selection

A total 209 completely sequenced genomes of pathogenic bacterium *Acinetobacter baumannii* were retrieved out of the total 222 genomes from NCBI data base on 30th August 2020. The average genome size was approximately 4.038 Mb, and the genomes contained a GC content of about 39% per genome. Similarly, 209 proteomes of the completely sequenced genomes were obtained with an average of 3700 proteins per isolate.

4.2 Pangenome analysis

Through BPGA analysis a total of 839 core genes (conserved among 99% strains), 4449 unique genes (present in only one strain) and 11062 shell or accessory genes (occurring in two or more than two strains) and a total 16350 pan genes are calculated.

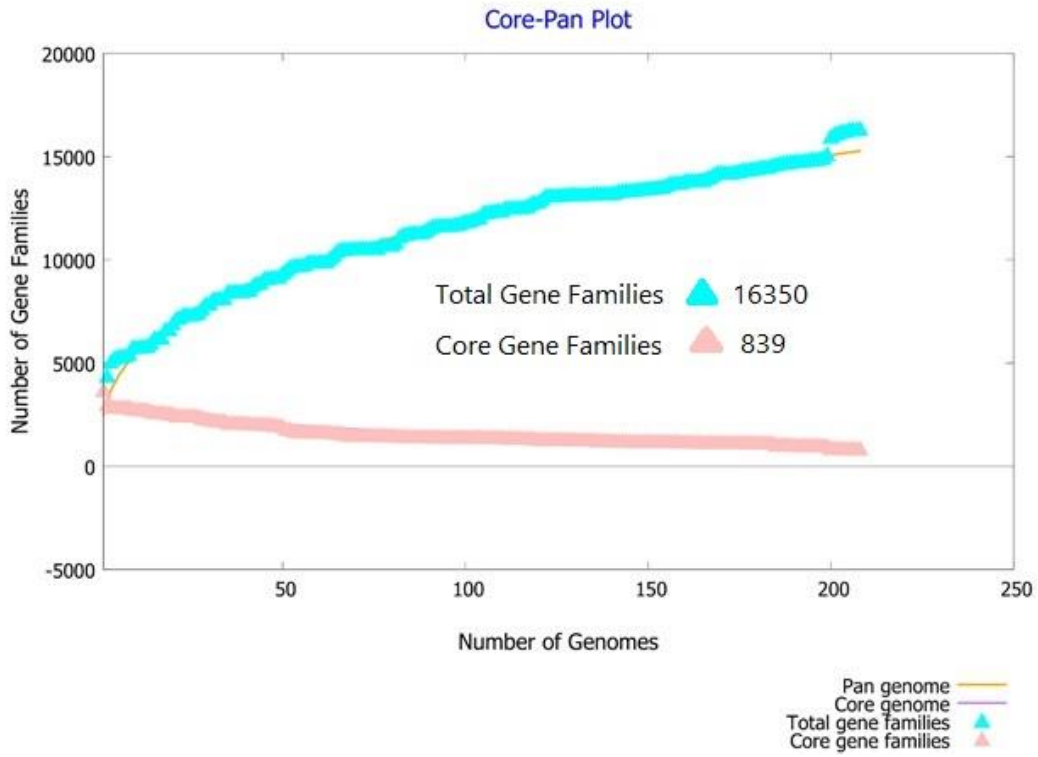


Figure 6: Pan-core analysis plot by BPGA. X-axis shows the number of genomes included in the study while Y-axis depicts the number of gene families.

4.3 Identification of core vaccine targets

Firstly, homology of the core proteins with humans were checked using BLASTp which showed that a total of 647 proteins had less than 35% similarity to human proteins. These proteins were further filtered for their subcellular localization as most potential vaccine targets for gram negative bacteria lie in the vicinity of the periplasm, outer membrane or are extracellular. 62 proteins were found in the extracellular, periplasm, or outer membrane location. The Database of Essential Genes (DEG) was employed to predict essential proteins in the core proteome. Results showed that 14 of the screened proteins were essential. The proteins were further filtered for virulence and all 14 proteins were found to be virulent.

Uniprot and InterProScan analysis revealed two proteins to be cytoplasmic, therefore, they were removed from further consideration. These proteins were further evaluated for their antigenicity and 8 proteins were found to be probable antigens. Hence, 8 putative vaccine candidates were shortlisted namely, LPS-assembly protein LptD, Periplasmic serine protease (ClpP class), D-alanyl-D-alanine endopeptidase, Iron-sulfur cluster insertion protein ErpA, Endolytic peptidoglycan transglycosylase RlpA, LPS export ABC transporter periplasmic protein LptC, and a hypothetical protein. All these targets are present in the outer membrane, extracellular or periplasmic location and are antigenic as well, thus their use as potential vaccine candidates for *A. baumannii* are strongly justified.

4.4 Predicted biological and molecular function of prioritized vaccine candidates.

LPS-assembly protein LptD was predicted by Interpro and CELLO2Go to have molecular functions associated with outer membrane organization, response to substances and Gram-negative bacterium outer membrane assembly (GO:0043165). As expected, the protein was projected to be involved in lipopolysaccharide transport biological process. According to Interpro and CELLO2Go results, Periplasmic serine protease (ClpP class) was found to have molecular function of peptidase activity (GO:0008233). The periplasmic protein was found to be involved in the biological processes of proteolysis (GO:0006508). D-alanyl-D-alanine endopeptidase was reported to be involved in various biological processes like cell wall organization or biogenesis and biosynthetic processes in the cell by CELLO2GO. Interpro indicated that the endopeptidase is likely to be involved in biological process of proteolysis

(GO:0006508) and molecular function of serine-type D-Ala-D-Ala carboxypeptidase activity (GO:0009002). According to CELLO2GO the transporter protein is involved in biological process of transport. Endolytic peptidoglycan transglycosylase RlpA was found to have molecular function of lyase activity (GO:0016829) and lytic endotransglycosylase activity (GO:0008932). The extracellular protein was predicted to have role in biological processes like peptidoglycan metabolic process (GO:0000270) and cell wall organization (GO:0071555). According to Interpro, LPS export ABC transporter periplasmic protein LptC is predicted to have molecular functions of lipopolysaccharide transmembrane transporter activity (GO:0015221) and is involved in biological process of lipopolysaccharide transport (GO:0015920). Iron-sulfur cluster insertion protein ErpA was found to have molecular function of iron-sulfur cluster binding (GO:0051536) and to be involved in process of iron-sulfur cluster assembly (GO:0016226) by interpro analysis.

Table 1: Prioritized putative vaccine candidates with their location and molecular functions

Vaccine Candidate	Associated Pathway	Location
LPS-assembly protein LptD	lipopolysaccharide transport, outer-membrane organization	Outer membrane
Periplasmic serine protease (ClpP class)	Proteolysis	Extracellular
D-alanyl-D-alanine endopeptidase	Proteolysis, Carboxypeptidase activity	Extracellular
Endolytic peptidoglycan transglycosylase RlpA	lyase activity, peptidoglycan metabolic process, cell wall organization	Outermembrane
LPS export ABC transporter periplasmic protein LptC	lipopolysaccharide transport	Periplasmic
Iron-sulfur cluster insertion protein ErpA	iron-sulfur cluster binding	Extracellular
Transporter protein	Transport activity	Outer membrane
Hypothetical Protein	No function determined	Outer membrane

4.5 Epitope prioritization

ABC Pred predicted a total of 236 B-cell epitopes among our proteins. Among these, we predicted B cell-derived T cell epitopes. There was a total of 252 unique MHC I and 104 MHC II epitopes.

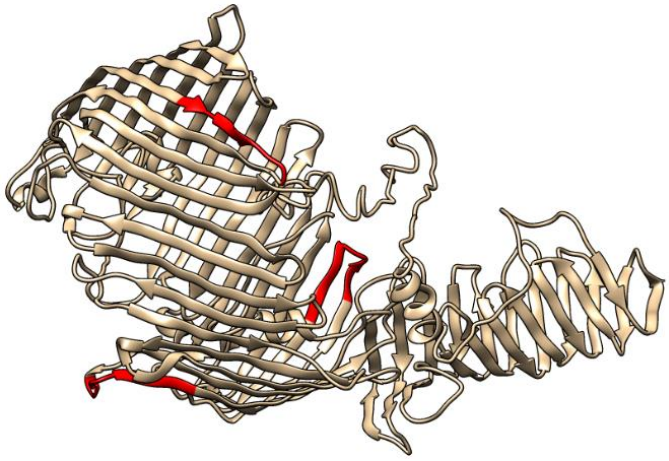
Since this number was too high, the epitopes were prioritized based on their antigenicity, immunogenicity, allergenicity and their ability to induce the release of interferon. These prioritized epitopes were found to bind to more than 5 HLA alleles. All of them had an antigenicity score greater than 0.5. The prioritized MHC I epitopes all had positive immunogenic scores, and the MHC II epitopes possessed an IFN inducing potential. All epitopes with an allergenic potential were removed. Finally, we determined 14 B cell-derived T cell epitopes, of which 4 were MHC II epitopes and the rest were MHC I epitopes.

Table 2: B-cells and their derived T-cell epitopes in the filtered protein candidates and their characteristics.

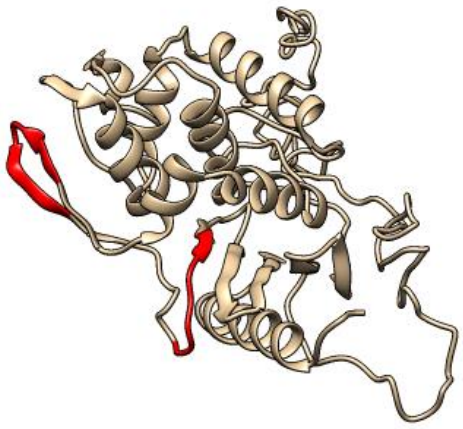
Proteins	B cell epitopes	T cell epitopes	Class	Allele count	Antigenecity score	Immunogenecity score
LPS-assembly protein LptD	PIYLNLAPNYDATITP	NLAPNYDAT	MHC I	6	0.9317	0.03334
	LDTGLNFEREGKYLQT	GLNFEREGK	MHC I	8	0.9051	0.36622
	DPKSPNVNEKRAIMAE	PKSPNVNEK	MHC I	8	0.8055	0.02074
D-alanyl-D-alanine endopeptidase	TPTYDFNLGYRVLKSN	FNLGYRVLK	MHC I	6	0.662	0.10398
	NGGWNINLSKTGYINE	GGWNINLSK	MHC I	7	1.8574	0.08288
Periplasmic serine protease	QKLGIEDRTLTAGTNK	QKLGIEDRT	MHC I	7	0.8876	0.30331
	SEMGKGLSESVAERLQ	LSESVAERL	MHC I	6	0.76	0.0578

	IYLLFIIVLMGKGCST	LLFIIVLMG	MHC II	41	1.3832	-----
Lipoprotein-34 precursor	TGLADGLELQLGWQ GP	GLELQLGWQ	MHC I	6	2.1125	0.05612
	RMRIFQILTFALTAVA	FQILTFALT	MHC I	6	1.3012	0.20658
Endolytic peptidoglycan trans-glycosylase	SLKYILALTASLSMAP	YILALTASL	MHC II	29	0.5563	-----
Transporter protein	EVVIRYYPISPDRAET	VVIRYYPIS	MHC II	24	1.4911	-----
LPS export ABC transporter periplasmic protein	AVSGGYYYYSGKAK KL	YYYSGKAKK	MHC II	16	0.8518	-----
Iron-sulfur cluster insertion protein	DSEGNDDLMLRVYV TG	DLMLRVYVT	MHC I	6	0.9919	0.0421

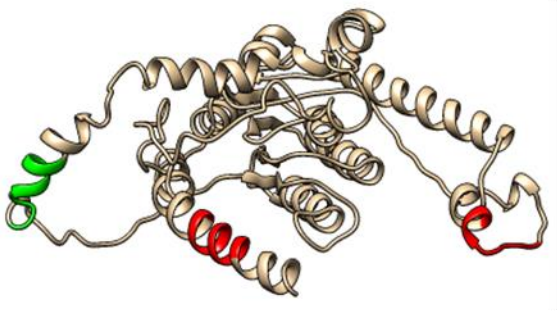
4.6 Epitope Mapping



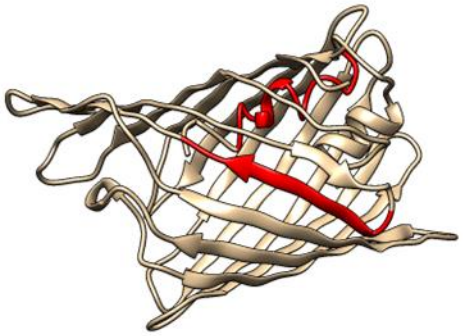
LPS-assembly protein LptD



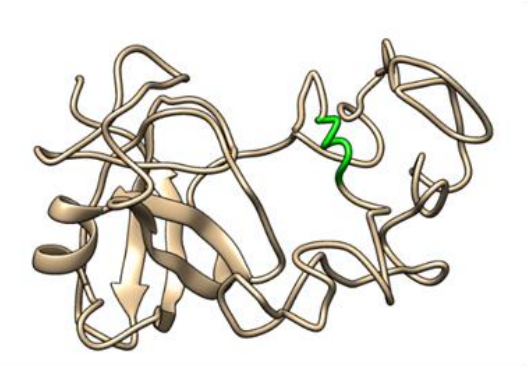
D-alanyl-D-alanine endopeptidase



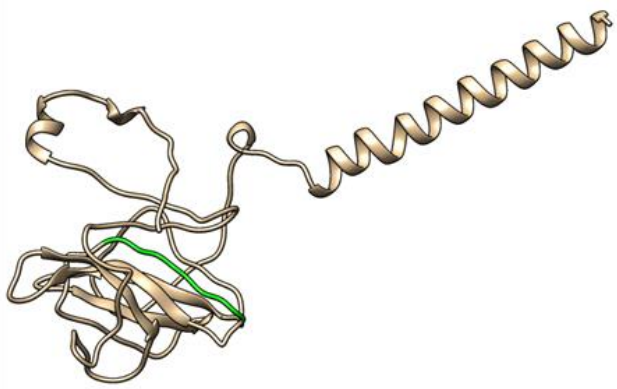
Periplasmic serine protease



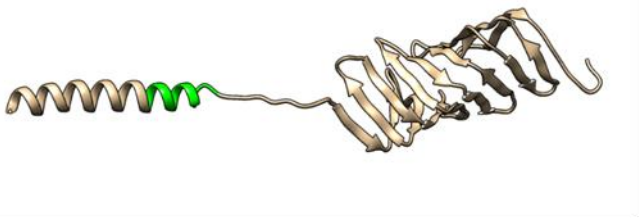
Hypothetical Protein



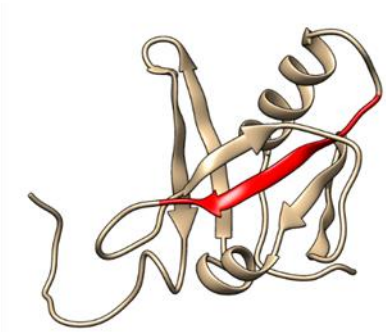
Endolytic peptidoglycan transglycosylase



Transporter protein



LPS export ABC transporter protein



Iron-sulfur cluster insertion protein

Figure 7: Tertiary Structure of prioritized proteins with CTL epitopes marked by red color while HTL epitopes are marked by green color showing their surface positions.

4.7 Multiepitope vaccine design

The prioritized epitopes were docked together using the Guru Interface of HADDOCK, and then refined using the Refinement Interface of HADDOCK. In the first round of docking, 45 different combinations of two epitopes were possible between the 10 unique MHC I epitopes. The combination which showed the lowest docking score was selected for the next round. They were then linked using the GPGPG linker and docked with the 8 remaining epitopes in the next round.

	NLAPNYDAT	GLNFEREGK	PKSPNVNEK	FNLGYRVLK	GGWNINLSK	QKLGIEDRT	LSESVAERL	GLELQLGWQ	FQILTFALT	DLMLRVYVT
NLAPNYDAT		-46.5 +/- 1.0	-50.1 +/- 3.2	-57.4 +/- 2.6	-50.5 +/- 0.4	-43.8 +/- 3.1	-32.5 +/- 1.6	-50.3 +/- 2.2	-40.2 +/- 2.9	-55.6 +/- 1.8
GLNFEREGK	-46.5 +/- 1.0		-61.2 +/- 8.6	-63.1 +/- 2.7	-64.4 +/- 4.8	-57.6 +/- 1.6	-38.5 +/- 5.0	-62.2 +/- 1.3	-49.9 +/- 2.6	-80.7 +/- 2.8
PKSPNVNEK	-50.1 +/- 3.2	-61.2 +/- 8.6		-49.8 +/- 2.3	-56.0 +/- 1.5	-52.6 +/- 1.3	-59.1 +/- 3.7	-64.8 +/- 2.9	-53.5 +/- 2.8	-57.9 +/- 1.8
FNLGYRVLK	-57.4 +/- 2.6	-63.1 +/- 2.7	-49.8 +/- 2.3		-57.1 +/- 4.7	-56.8 +/- 4.1	-50.5 +/- 6.5	-67.0 +/- 1.5	-53.3 +/- 1.0	-60.7 +/- 3.2
GGWNINLSK	-50.5 +/- 0.4	-64.4 +/- 4.8	-56.0 +/- 1.5	-57.1 +/- 4.7		-48.7 +/- 3.1	-46.9 +/- 3.8	-66.5 +/- 7.6	-58.7 +/- 3.1	-70.2 +/- 4.1
QKLGIEDRT	-43.8 +/- 3.1	-57.6 +/- 1.6	-52.6 +/- 1.3	-56.8 +/- 4.1	-48.7 +/- 3.1		-48.5 +/- 2.5	-51.4 +/- 4.7	-52.6 +/- 2.4	-52.7 +/- 1.3
LSESVAERL	-32.5 +/- 1.6	-38.5 +/- 5.0	-59.1 +/- 3.7	-50.5 +/- 6.5	-46.9 +/- 3.8	-48.5 +/- 2.5		-47.5 +/- 0.7	-42.7 +/- 2.6	-45.3 +/- 1.9
GLELQLGWQ	-50.3 +/- 2.2	-62.2 +/- 1.3	-64.8 +/- 2.9	-67.0 +/- 1.5	-66.5 +/- 7.6	-51.4 +/- 4.7	-47.5 +/- 0.7		-61.1 +/- 4.	-68.9 +/- 3.
FQILTFALT	-40.2 +/- 2.9	-49.9 +/- 2.6	-53.5 +/- 2.8	-53.3 +/- 1.0	-58.7 +/- 3.1	-52.6 +/- 2.4	-42.7 +/- 2.6	-61.1 +/- 4.		-52.6 +/- 2.0
DLMLRVYVT	-55.6 +/- 1.8	-80.7 +/- 2.8	-57.9 +/- 1.8	-60.7 +/- 3.2	-70.2 +/- 4.1	-52.7 +/- 1.3	-45.3 +/- 1.9	-68.9 +/- 3.	-52.6 +/- 2.0	

Figure 8: First stage docking scores of epitopes before downstream processing. GLNFEREGK was linked with DLMLRVYVT via GPGPG linker as they had the highest docking score

After all the MHC I epitopes had been linked together, they were linked to the four MHC II epitopes, again using the GPGPG linker and using the same approach. The final multiepitope bound to the adjuvant had the following sequence:

MTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKNGATFQVEVPGSQHIDS
QKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMANEAAAKGLNFEREGKG
PGPGDLMLRVYVTGPGPGGGWNINLSKGPGPGGLELQLGWQGPGPGFNLGYRVLKGP
GPGPKSPNVNEKGPGPGQKLGIEDRTGPGPGFQILTFALTGPGPGNLAPNYDATGPGPGL
SESVAERLGPGPGVVIRYYPISGPGPGYYYSGKAKKGPGPGLLFIIVLMGGPGPGYILALT
ASL

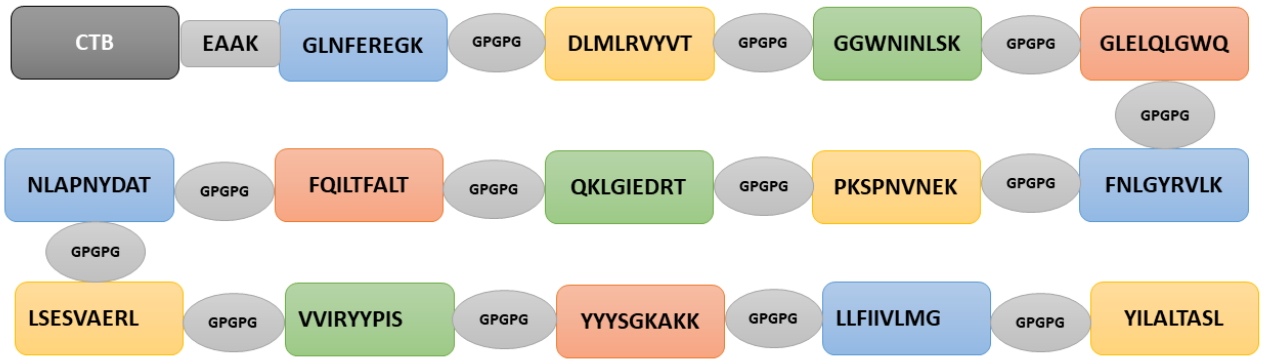


Figure 9: Multiepitope vaccine construct showing different epitopes in different colors. The CTL and HTL epitopes have been linked using the GPGPG linker. CTB adjuvant is attached to the free terminus using the EAAAK linker.

4.8 Characterization of multiepitope vaccine

The final vaccine construct is antigenic, non-allergenic, and soluble in water. According to AntigenPro and Vaxijen, the antigenicity scores were 0.95 and 0.98, respectively. Both AllerTOP and AllergenFP predicted that the construct was a probable non-allergen. Solpro gave a high probability (0.96) that the construct was water soluble, while ccSol gave a 100% probability prediction.

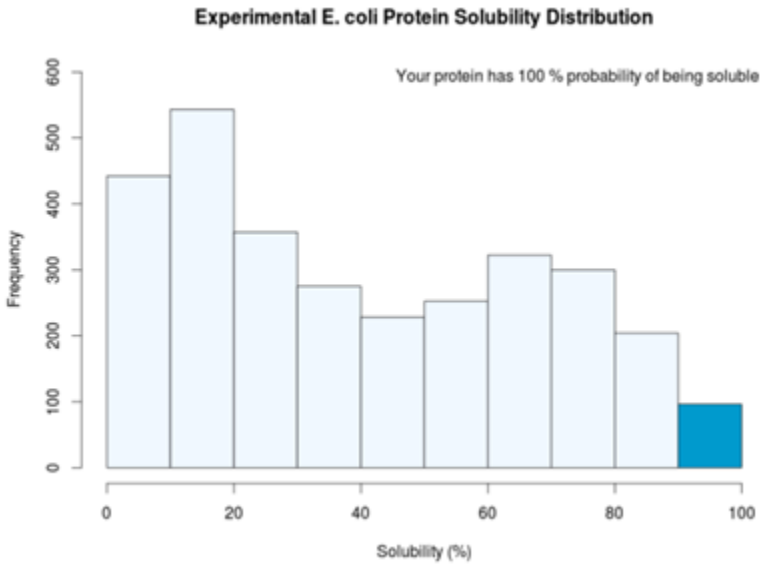


Figure 10: Figure by ccSol showing the vaccine's solubility (in deep blue) on an experimental E.coli protein solublility distribution plot

The construct's length was 300 amino acids; its molecular weight was 31310.85 g/mol. Estimated half-life was 30 hours in mammalian reticulocytes in vitro, more than 20 hours in yeast in vivo, and more than 10 hours in Escherichia coli in vivo. The construct had a net charge of 6.25 at neutral pH (7). Its pI was 9.15 and the grand average of hydropathy (GRAVY) was -0.302 which shows that the vaccine is hydrophilic in nature. The aliphatic index and instability index were 77.1 and 28.75, respectively. An instability index less than 40 indicates that the construct is stable.

4.9 Secondary structure prediction

The secondary structure prediction shows that our vaccine construct has a sheet containing 5 helices and 40 beta turns.

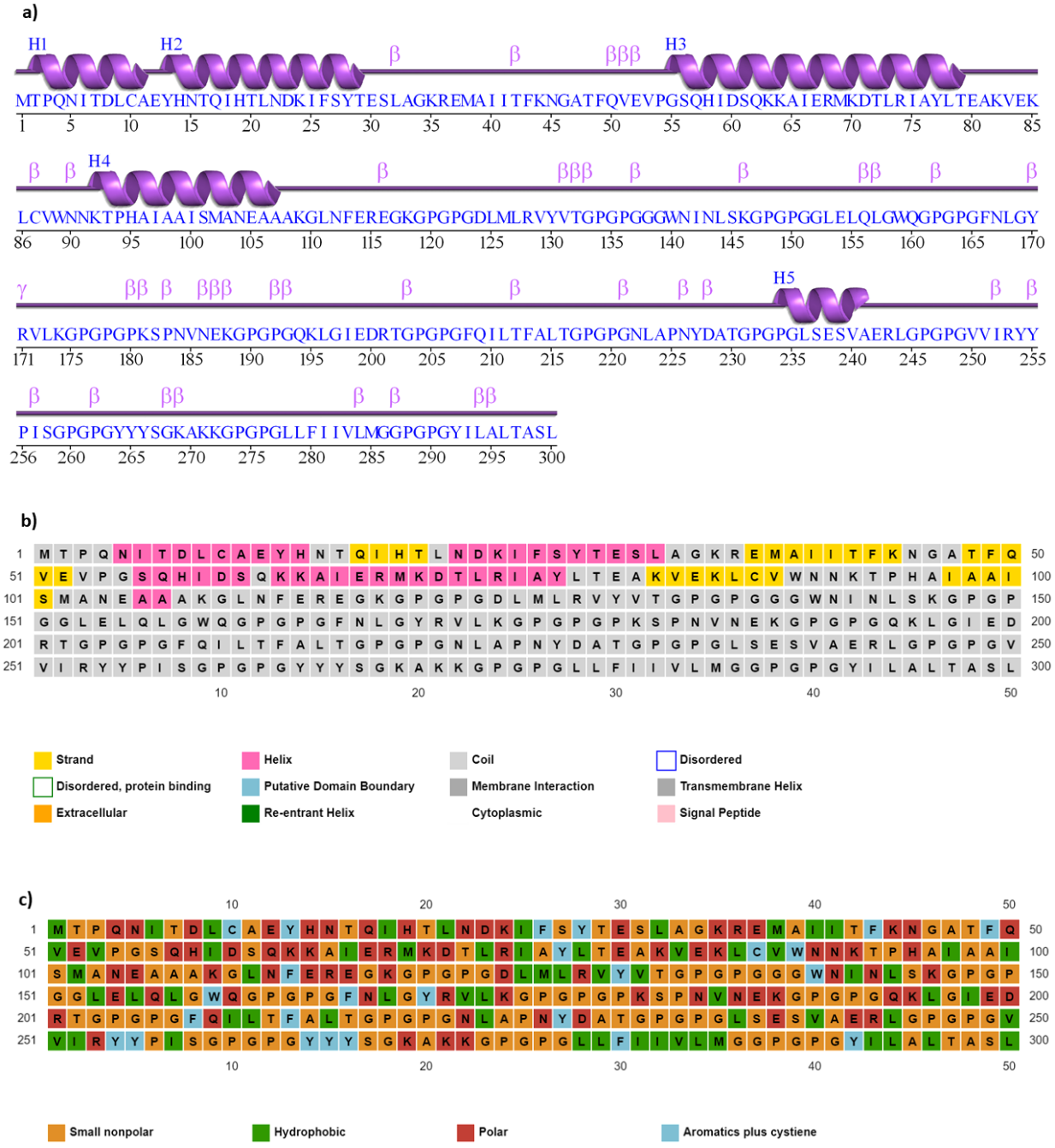


Figure 11: Secondary structure prediction. (a) The figure from PSIPRED transmembrane helix prediction function shows that our vaccine construct does not contain any hair-pin loops and only one gamma turn. It is composed of helices and strands. (b)The PDBSum figure shows that our vaccine construct consists only of strands and helices. It does not contain any disordered protein binding, extracellular residues, putative domain boundaries, re-entrant helices, cytoplasmic membrane interaction, disordered residues, transmembrane helices,

and signal peptide. (c) Figure showing amino acid types that the vaccine construct contains. Aromatic and hydrophobic residues are relatively rare, whereas polar and small non-polar residues are abundant.

4.10 Tertiary structure modeling

ITASER was used to predict the 3D structure of the vaccine construct shown below.

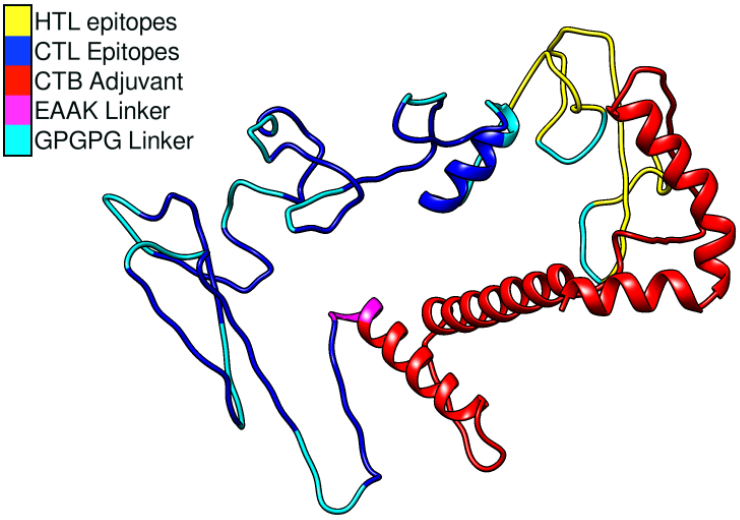


Figure 12: Three-dimensional Structure of protein model obtained by ITASER

GalaxyRefine generated 5 protein models, from which model-1 was selected because of least poor rotamers 0.4. According to ProSa evaluation, the Z-score of the refined model was –3.57. (Figure 12)

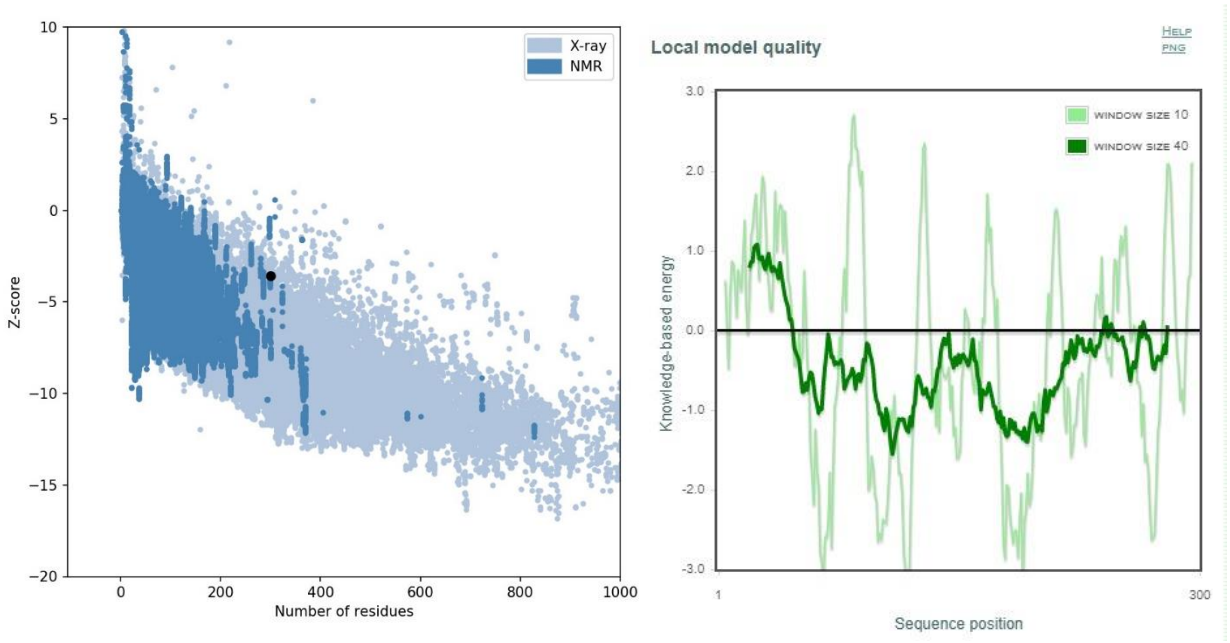


Figure 13: Evaluation of the refined modeled structure of multi-epitope vaccine using ProSA. The placement of model in the vicinity of experimentally derived structures confirms the high-quality nature of designed vaccine.

Ramachandran plot analysis by PROCHECK depicted that 90.1% residues in favored region, 9.4 % residues in the additional allowed region, 0.5% in the generously allowed region and 0.0% residues and in the disallowed region. ERRAT server gave quality factor 70.8108.

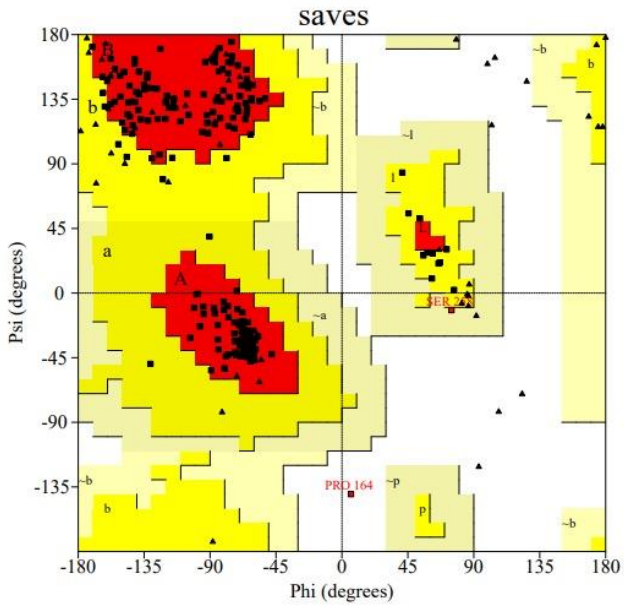


Figure 14: The Ramachandran plot of refined vaccine. The plot clearly indicates that maximum residues of designed vaccine reside in the favored and allowed regions while only few residues lie in the generously allowed regions.

4.11 Molecular dynamic simulation of vaccine construct

The trajectory of 10 models was generated after fast simulation. The Root Mean Square Fluctuation (RMSF) of the structure was observed to be 0.281 to 7.177 Å. The minimum fluctuation was at residue 73 while the maximum fluctuation was observed at residue 191.

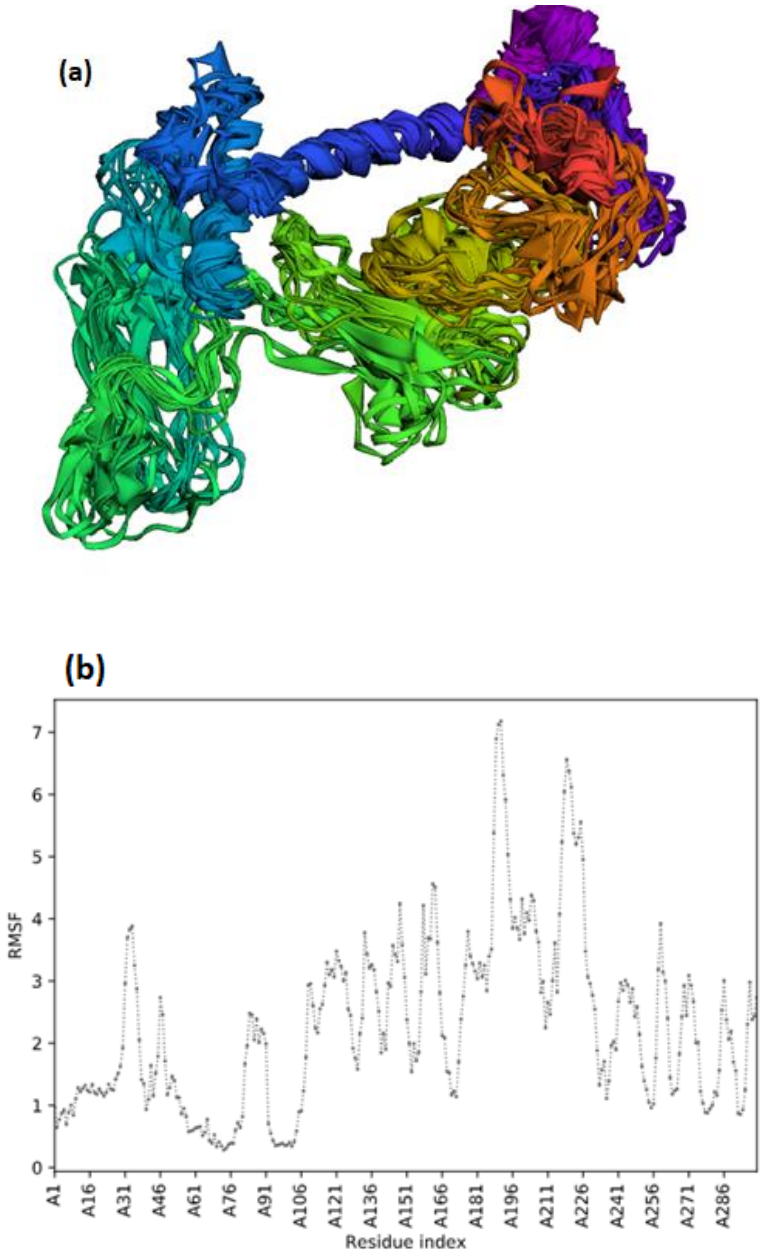


Figure 15: Coarse Grained MD simulations of Vaccine construct. (a) Vaccine model obtained after simulation showing multiepitope trajectory, (b) Fluctuation plot of the multiepitope vaccine showing residue-wise fluctuations recorded during the whole simulation course

4.12 Molecular docking analyses of vaccine with Toll-like receptor 4 (TLR4)

Molecular docking of the vaccine construct with Toll-like receptors 2 and 4 was done by HADDOCK webserver. In case of docking with TLR 2, HADDOCK clustered 122 structures into 14 clusters, which represents 61.0 % of the water-refined models HADDOCK generated. The top ranked cluster in HADDOCK is the most reliable and had score 57.7 +/- 18.7. Lower HADDOCK values (negative ones) signify high protein interactions. Then from this top ranked cluster, one representative structural model was subjected to refinement via HADDOCK Refinement interface. Refinement clustered 20 structures in 1 cluster representing 100% of the water-refined models generated by HADDOCK. The HADDOCK score of Vaccine-TLR2 complex improved significantly to about -255.9 +/- 4.0, after undergoing refinement. The statistics and details of refined interactions are given in table 3. The vaccine-TLR4 complex HADDOCK score also improved significantly after refinement. In case of docking, HADDOCK clustered 132 structures in 13 clusters, which represents 66.0 % of the water-refined models HADDOCK generated and the top cluster had a score of 60.2 +/- 42.0. However, the HADDOCK score was -276.8 +/- 3.2 after refinement of the complex in which HADDOCK clustered the 20 resulting structures into one cluster that represented 100% of the refined water models generated. The statistics and details of refined interactions are given in table 4.

Table 3:Statistics of refinement of top TLR2-vaccine cluster.

HADDOCK score	-255.9 +/- 4.0
Cluster size	20
RMSD from the overall lowest-energy structure	0.3 +/- 0.2
Van der Waals energy	-105.4 +/- 3.2
Electrostatic energy	-562.3 +/- 36.5
Desolvation energy	59.8 +/- 4.6
Restraint's violation energy	0.0 +/- 0.00
Buried Surface Area	3549.5 +/- 64.3
Z-Score	0.0

Table 4: Statistics of refinement of top TLR4-vaccine cluster.

HADDOCK score	-276.8 +/- 3.2
Cluster size	20
RMSD from the overall lowest-energy structure	0.3 +/- 0.2
Van der Waals energy	-116.3 +/- 1.6
Electrostatic energy	-380.7 +/- 9.9
Desolvation energy	-4.0 +/- 4.4
Restraint's violation energy	0.0 +/- 0.00
Buried Surface Area	3087.7 +/- 49.9
Z-Score	0.0

The vaccine construct docking with TLR2 and TLR4 gave good results. The refined models were then submitted to PDBSUM for detailed analysis on interaction and binding forces between molecules of docked complexes.

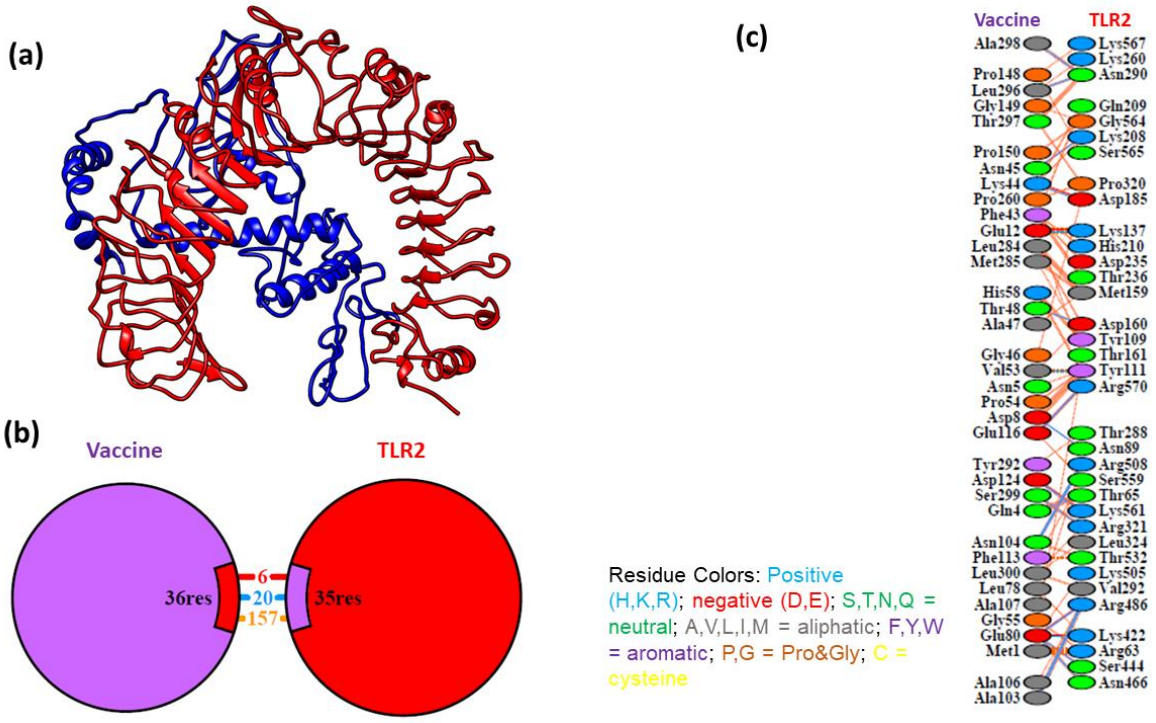


Figure 16: Protein-protein interaction analysis of refined vaccine construct and TLR2 (a) 3D interactional model of vaccine construct and TLR2 (blue color depicting our vaccine construct while red color shows TLR2), (b) 36

interacting amino acid residues of the vaccine construct and 35 interacting residues of TLR2 depicted showing 6 salt bridges, 20 hydrogen bonds and 157 non-bonded contacts. (c) List of all residues of both interacting proteins.

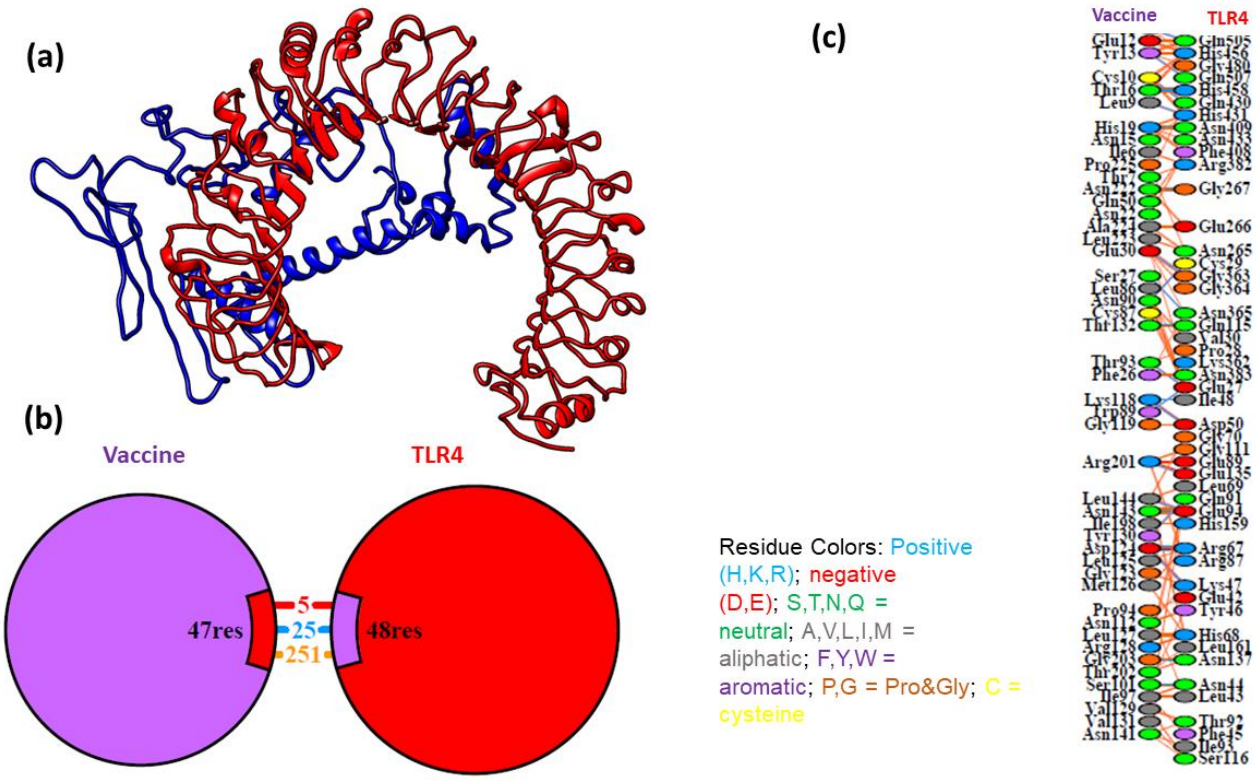


Figure 17: Protein-protein interaction analysis of refined vaccine construct and TLR4 (a) 3D interactional model of vaccine construct and TLR4 (blue color depicting our vaccine construct while red color shows TLR4), (b) 47 interacting amino acid residues of the vaccine construct and 48 interacting residues of TLR4 depicted showing 5 salt bridges, 25 hydrogen bonds and 251 non-bonded contacts. (c) List of all residues of both interacting proteins.

4.13 Disulphide Engineering

A total number of 13 unique amino acid residue pairs were predicted by the DbD2 webserver to have the potential to form disulfide bonds. These residues, including GLU-12 and ASN-15, ILE-41 and LYS-44, PHE-49 and GLU-52, ALA-76 and LYS-82, TYR-77 and MET-102, LEU-86 and TRP-89, ARG-128 and GLY-151 etc. can be mutated to cysteine to increase the thermal stability of our construct.

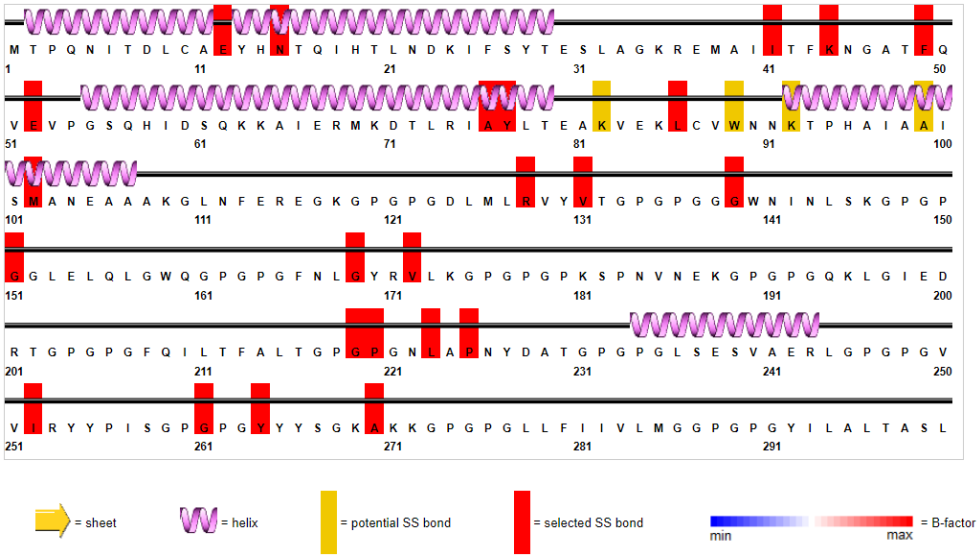


Figure 18: Disulphide engineering of multi-epitope vaccine. a) Locations of potential disulphide bonds shown in red. These bonds are predicted to increase thermal stability of the construct.
b) Three-dimensional model of the multiepitope with potential disulfide bonds shown in red

4.14 Codon optimization of the final multiepitope vaccine construct

Reverse translation and codon optimization were performed using JCat server for the expression analysis and cloning of the vaccine construct. cDNA was generated by reverse translation with an optimized expression in Escherichia coli strain K12. JCat server optimizes the sequence on the basis of GC content and CAI calculation of the cDNA sequence. Higher the CAI value, higher the gene expression. The length of the optimized codon sequence was 900 nucleotides.

Our construct has the CAI value of 1.0 and GC content of 55.222222222222214. The GC content of Escherichia coli strain K12 is 50.7340272413779. The DNA sequence of restriction sites for XhoI and EcoRI restriction enzyme was added at 5′ and 3′ ends of the adapted vaccine codon, respectively. At last, the recombinant plasmid was designed by inserting the adapted codon into pET 28a(+) vector.

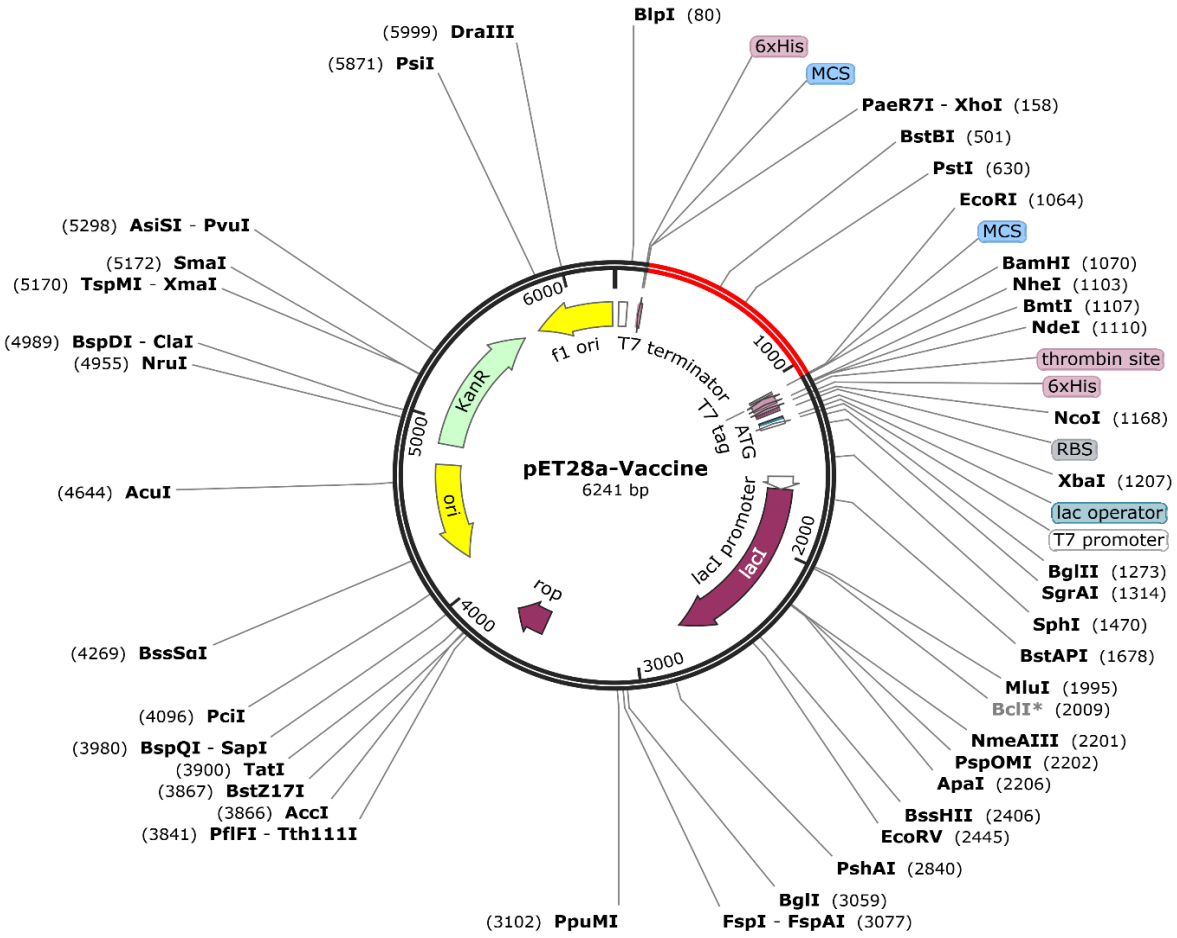
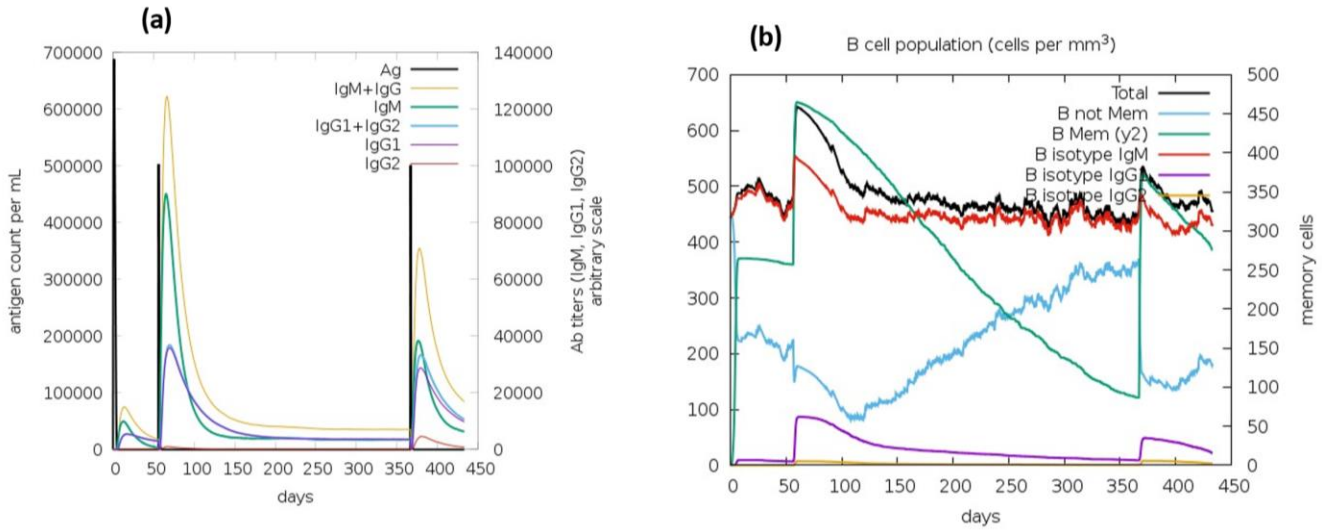


Figure 19: In silico restriction cloning of vaccine construct into pET 28a (+) vector. The red colored portion represents the adapted DNA sequence of the multi-epitope while the black color indicates the vector DNA.

4.15 Immune simulation

In order to assess the immunogenic profile of the multi-epitope vaccine, in-silico immune response was generated using C-IMMSIMM server. The immune response generated after second exposure to the vaccine was significantly higher than the primary response. High levels

of IgM characterized the primary response, while a marked increase of B cell populations, immunoglobulin activity (IgG, IgG1, IgM and IgG + IgM antibodies) and decrease in antigenic concentration was seen in secondary and tertiary response. This immune profile indicates immune memory formation and possible isotype switching leading to increased clearance and immune response to the vaccine on subsequent exposure. The TC (cytotoxic) and TH (helper) cell populations also show a similar higher response after first vaccination dose. High levels of IFN-g and IL-2 production also shows a good immune response generation. After the two vaccination doses, another dose was injected at around day 366, after one year of the first dose to check the vaccine efficacy. The antigen and immunoglobulin graph (Fig.19) shows that after the vaccination, when the third dose is given after 1 year, the antigenic surge is far less, which indicates an effective immune response due to the protective action of high concentration of specific antibodies. However, the immune response generated after the 3rd dose is of less intensity as compared to the 2nd immune response, but this can be attributed to the long gap between the third and 2nd dose.



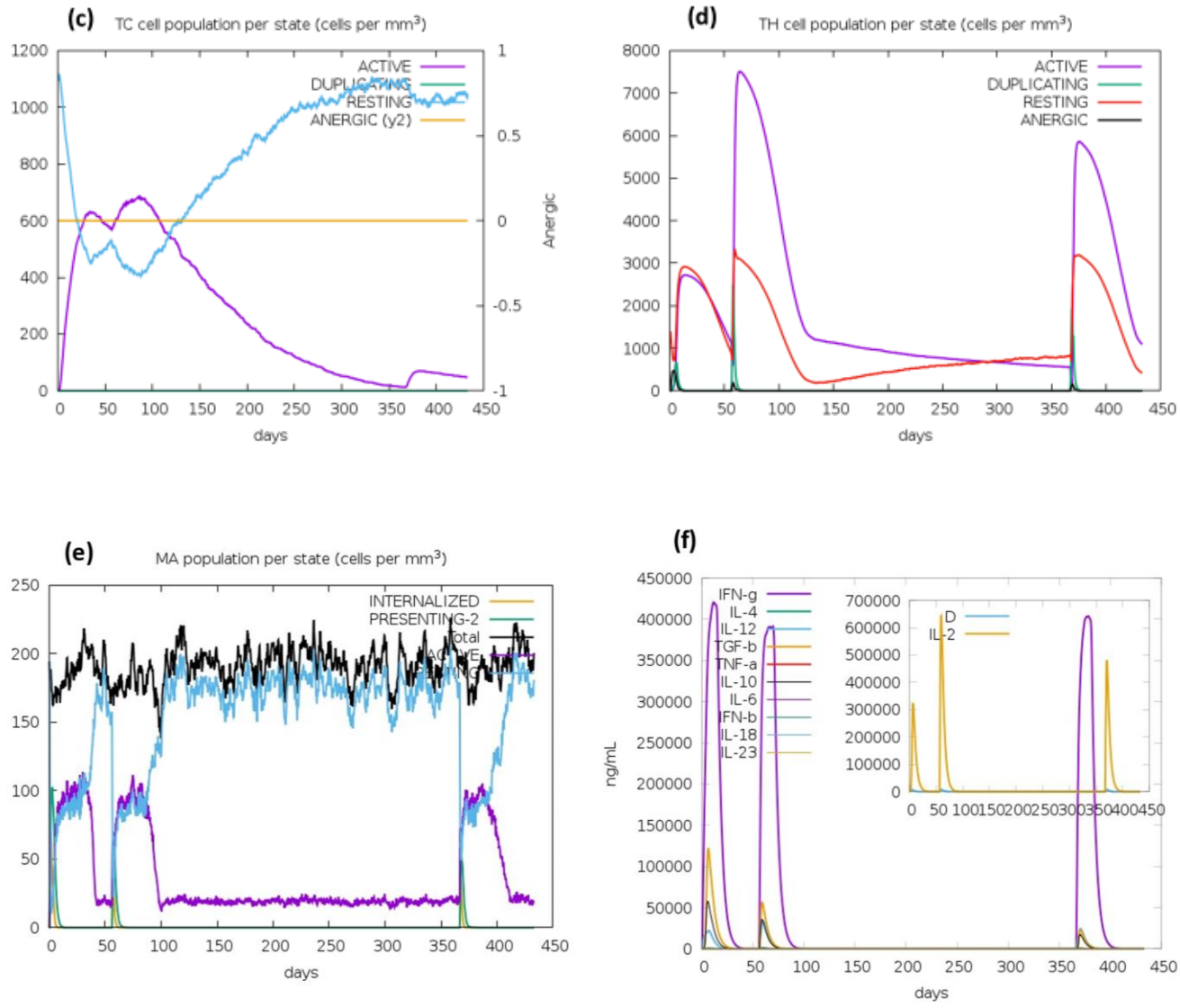


Figure 20: C-ImmSim generated in silico immune response profile of the multi-epitope vaccine. (a) Immunoglobulin production in response to the injected antigen (black vertical lines). (b) Corresponding evolution of B cell population after vaccine injection (c-d) the activity (shown as Active, duplicating, and resting) of cytotoxic T cells and helper T cells respectively. (e) Activity of macrophages (detailed as Total, internalized, presenting, Active and resting) (f) Shows the cytokine level during the whole simulation period. The insert plot shows IL-2 levels with the Simpson index, D that measures diversity (indicated by the blue line). Increase in D shows emergence of different epitope-specific T-cell clones.

Chapter 5

Discussions

Acinetobacter baumannii is placed first in the critical priority group in the WHO's list of ESKAPE pathogens, a list of highly virulent and antibiotic resistant pathogens. Drugs against these ESKAPE pathogens should be a ripe frontier in research because previous treatment options are failing fast. Vaccination of populations which are at high risk of infection from *A. baumanni* may prove a viable solution against infection. Drug resistance in *Acinetobacter baumannii* is currently inflicting enormous costs on healthcare systems worldwide and causing a significant increase in patient mortality. Globally, carbapenem resistance in *Acinetobacter baumannii* alone costs healthcare systems an excess of $742 million and 15,000 deaths per annum. (Spellberg et al., 2013)

Back in 2001 in India, the first trial for passive immunization against *A. baumannii* appeared, when researchers induced the production of monoclonal antibodies against IROMP (Iron Regulated Outer Membrane Proteins) in mice (Goel et al., 2001). In 2010, many groups began extensive work on developing active and passive immunization against the increasingly drug-resistant pathogen, in Spain (McConnell et al., 2010a), in America (Bentancor, O'Malley, et al., 2012; Bentancor, Routray, et al., 2012), in Iran (Fattahian et al., 2011), in Australia (Moriel et al., 2013), and in China (Chiang et al., 2015).

A number of vaccine candidates against *Acinetobacter baumannii* are currently in the early stages of development. These include whole inactivated vaccines and subunit vaccines, including pure protein (F. Badmasti et al., 2015; Farzad Badmasti et al., 2015; Bentancor, Routray, et al., 2012) and polysaccharide-based vaccines (Skurnik et al., 2016). None of these vaccine candidates have yet progressed beyond the preclinical stage. Efforts for the development of passive immunization are one step ahead; one preparation of monoclonal antibodies against PNAG, a basic component of *A. baumannii* biofilm, is currently in phase II clinical trials.(T. A. Ahmad et al., 2016) It can be hoped that within a few years, with the increasing prevalence of drug resistance in *A. baumannii*, the development of vaccines against it will be further accelerated.

Previous vaccines designed against *Acinetobacter baumannii* have exploited 14 (Chiang et al., 2015) and 33 fully sequenced genomes (Fereshteh et al., 2020) and 1191 (Elhosseiny et al., 2018) and 52 proteomes (Solanki et al., 2018) of the said bacterium. In the current study, we have analyzed 208 complete *A. baumannii* genomes by employing pangenome analysis combined with reverse vaccinology. The pan-genome analysis and graph of the genomes showed that the pangenome is still open (Imperi et al., 2011). It may become closed after the addition of a few more genomes, and the design of a vaccine against global strains of the virus may become possible. Our results showed that there was a total of 16350 proteins (pan-genome) of which 839 were core proteins. The majority of the pangenome comprises of accessory and unique genes which have been acquired by the bacteria via horizontal gene transfer. This demonstrates that bacteria possess the ability to gain and lose genes in order to survive in varying environments and during evolution.

We conducted pangenome analysis of all 208 genomes to obtain the core genome and to identify potential vaccine candidates against all *A. baumannii* strains available at the time. A reverse vaccinology approach was used for the identification of potential vaccine candidates to form a multi-component vaccine. Since its inception at the turn of the century, reverse vaccinology and multiepitope vaccines created using an immunoinformatic approach, have gained global recognition. These prophylactic strategies require far less time and resource than their conventional counterparts. They also require in vivo testing and animal model experiments much later in the vaccine development process.

Employing a pangenome and reverse vaccinology approach has enabled us to identify vaccine candidates which would be able to induce immunity against various strains of the bacterium. This approach has resulted in a successful multicomponent vaccine called 4CMenB for meningococcus diseases that is caused by the gram-negative bacterium Neisseria meningitidis. The vaccine was designed by the Rappuoli research group and consists of 3 antigens of Neisseria and OMV derived from a Neisseria meningitidis serogroup B (MenB) outbreak strain. 4CMenB is the only meningococcal vaccine that is authorized for use in all age groups as it provides protective immunity against majority of the meningococcus strains. The data obtained since authorization of the vaccine has shown that it is highly effective in prevention of serogroup B meningococcal disease in both infants and adults. In UK, where the first infant vaccination

campaign was initiated, an overall effectiveness of 82.9% was observed after two doses of the vaccine.(Rappuoli et al., 2018) The success of 4CMenB shows that multicomponent vaccines designed through the reverse vaccinology approach are promising candidates and can induce protective immunity in humans.

Only recently has the possibility of a multiepitope vaccine against *A. baumannii* been studied. (S. Ahmad et al., 2019; Du et al., 2021; Ren et al., 2019) Several factors need to be considered when designing an effective vaccine against *A. baumannii.* Firstly, the vaccine should specifically impact only the said pathogen and should not affect the human gut microbiota. Secondly, the vaccine targets should be easily accessible and recognizable to the human immune system, therefore only surface and extracellular proteins should be considered. Thirdly, the vaccine targets should be essential for bacterial survival and antigenic so they may elicit an immune response in the host. Subtractive proteomics approach was used to filter out putative vaccine candidates from the core genome. Out of 839 core genes from 208 genomes, only 8 proteins passed through all the filters in the RV module of PanRV. These 8 PVCs were selected as good vaccine candidates because of their sub-cellular localization and antigenicity scores.

A few of our proposed candidates have also been predicted by other researchers, as preferred candidates against other pathogens. The LptD and LptC proteins are essential outer membrane proteins; they mediate the transport of lipopolysaccharides to the outer leaflet of the bacterium. LptD has been previously identified as a vaccine candidate against *Vibrio* species infection owing to its strong ability to induce an antibody response (Zha et al., 2016). The ErpA protein plays a role in iron-sulfur cluster binding. Several members of the Erp protein family have been found to be favorable vaccine candidates against *Borrelia burgdorferi.* (Bencurova et al., 2018)

Both MHC I and MHC II epitopes were used in vaccine construction. 10 CTL and 4 HTL epitopes derived from B cell epitopes were shortlisted based on their allele count, antigenicity, immunogenicity and IFN-g induction potential. These epitopes are responsible for the induction of both humoral and cell mediated immunity in the host. The prioritized epitopes were linked together based on their docking scores to determine the best possible vaccine sequence. Glycine rich, flexible GPGPG linkers were used to avoid junctional epitope formation and to reduce the chances of interaction between vaccine subunits. Linkers also facilitate vaccine flexibility and

solubility inside the human body (Pandey et al., 2018). In order to improve vaccine immunogenicity, non-toxic CTB was added to the start of the vaccine via EAAK linker.

The multiepitope vaccine is 300 amino acids long with a molecular mass of 31.31 kDa. The protein is predicted to be basic in nature according to its theoretical PI while the GRAVY index of our vaccine suggested it to be hydrophilic in nature. The instability index and aliphatic scores indicate that the vaccine construct is stable and can prove to be thermostable in a test tube if tested experimentally. The vaccine construct was found to be soluble, antigenic, non-allergic and immunogenic. Thus, it is proposed that it will elicit a strong immune response without causing an allergic reaction.

Garcia et al demonstrated that *A. baumannnii* lipopolysaccharide, peptidoglycan and other virulent proteins are recognized by TLR4 and TLR2 in the human body (García-Patiño et al., 2017a). Moreover, Knapp et al showed that during *A. baumannii* infection TLR4 and CD14 play a crucial role in recognition and resolution of the infection. This was further proved by pulmonary infection model experiments in CD14 and TLR 4 deficient mice as they had higher bacterial burden and infection rate as compared to normal mice (Knapp et al., 2006). Moreover, in order to elicit a strong immune response, it is necessary that the vaccine shows strong binding with the immune receptors. Thus, the designed vaccine was assessed for its interaction and association with TLR 2 and TLR 4 via molecular docking. The scores obtained from docking suggest that the binding affinity of our multi-epitope vaccine for TLR 2 and 4 is good, which also predicts the efficacy of the vaccine. Immune simulation studies also confirmed that our multiepitope vaccine construct was able to elicit immune responses necessary for antigen clearance upon secondary exposure. A general increase in the generated immune response was observed after second dose which indicates the development of memory B and T cells lasting several months.

It is important to check the stability and flexibility of vaccine constructs in cellular environments. Molecular dynamic simulations are performed to check the behavior and stability of proteins by simulating them in a cellular environment. But these simulations are computationally expensive and time intensive, therefore, fast, dynamic simulations were conducted. CABS-flex analysis was conducted as it is fast and gives results similar to those performed by MD simulations at 10ns. Moreover, the RMSF obtained from CABS-Flex analysis

is somewhat similar to the RMSF of NMR ensembles (Jamroz et al., 2014). Cabs flex analysis showed that our protein remained stable throughout the simulation period and the average RMSF was found to be 2.27.

In-silico cloning analysis showed that the expression of our vaccine construct in E. coli K-12 is experimentally possible with high expression. The CAI value obtained suggests that translational efficacy of our multi-epitope vaccine construct is compatible with the host system. The vaccine construct was also inserted inside the pET-28a expression vector to facilitate vaccine expression in bacterial systems. The in silico cloned multiepitope vaccine can be expressed and used for experimental validation of these results. Computational studies like ours possess several advantages over conventional approaches, but they are based on predictive models and experimental verification of their results is highly necessary.

Our current approach did not identify certain important and well-studied potential vaccine candidates, including the Outer Membrane Protein A (OmpA) (F. Badmasti et al., 2015), the Acinetobacter trimeric autotransporter (Ata) (Bentancor, Routray, et al., 2012) and the Biofilm Associated Protein (Bap) (F et al., 2015), which could play a critical role in *A. baumannii* pathogenesis. Moreover, we have identified several challenges in the development and administration of a vaccine against *Acinetobacter baumannii*. As the pathogen does not exhibit typical communal spread, it is difficult to identify a target population for vaccination. We have already discussed the risk factors which may warrant the use of a vaccine, however, in practice, it may be difficult to confidently predict the incidence of wounds, injuries, surgeries and hospital admissions. The target population may also be predominantly immunocompromised and hence unfit for immunization. It has also been suggested that vaccination may not significantly reduce antibiotic use, and that the development of a vaccine against *Acinetobacter baumannii* may not be commercially attractive to pharmaceutical companies and governments (Rosini et al., 2020).

Chapter 6

Conclusion and Future Prospects

Acinetobacter baumannii is an increasingly drug resistant pathogen, which at the turn of the twenty first century, poses a serious threat to human health. In the present study, we employed a pangenome approach in combination with subtractive proteomics and reverse vaccinology. We identified 8 vaccine candidates (including ErpA, LptC, LptD, RlpA, ClpP and others) and constructed a multiepitope vaccine containing 14 epitopes (NLAPNYDAT, GLNFEREGK, PKSPNVNEK, FNLGYRVLK, GGWNINLSK, QKLGIEDRT, LSESVAERL, LLFIIVLMG, GLELQLGWQ, FQILTFALT, YILALTASL, VVIRYYPIS, YYYSGKAKK and DLMLRVYVT). The prioritized novel vaccine candidates and their selected epitopes possess several noteworthy features which makes them suitable for use in a vaccine. Our proposed multiepitope has shown great promise in inducing immunity against *A. baumannii* in extensive *in-silico* analysis, including characterizations and simulations. The multi-epitope vaccine designed in this study can be used as a genome-based preventive treatment for *A. baumannii* infections in the future.

The stability of the proposed chimeric vaccine should be evaluated in vitro before further validation in the animal model to determine whether our *in-silico* predictions are accurate. The sequence of the vaccine construct has also been submitted to the NCBI database, and patent filing is underway. Finally, when a vaccine formulation is prepared, the authors suggest the use of nanoparticles as an adjuvant and mechanism of delivery for the vaccine as this may improve the immunogenicity and delivery of the vaccine to the target site.

Chapter 7

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