TEKNOFEST

AEROSPACE AND TEKNOLOGY FESTIVAL

BIOTECNOLOGY INNOVATION COMPETITION

PROJECT DETAIL REPORT

IDEA CATEGORY FOR THE BACHELOR DEGREE AND UPPER-LEVEL

TEAM NAME

Dengue Chimeric Vaccine – Unpad

PROJECT NAME

Development of Chimeric Virus-Like Particle Dengue Vaccine from Recombinant Domain III Protein of Indonesian Strain without Pre-membrane Protein Produced in Pichia pastoris is Capable of Eliciting Specific Domain III-Directed Netralizing Antibodies

APPLICATION ID

447770

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1. Project Summary (Project Description) (5 points)

To date, Dengue disease remains a serious worldwide issue. A viral epidemic caused by Dengue virus 1-4, transmitted by Aedes mosquitoes. In 2016, Sanofi launched Dengvaxia, a Live Attenuated Virus (LAV) vaccine to overcome the Dengue problem. However, the LAV vaccine may be associated with Antibody-Dependent Enhancement (ADE) risk despite their robust immunity against all four DENV serotypes. This research aims to develop a chimeric Virus-like Particle (VLP) vaccine consisting of envelope of DenV 3 and 4 without pre-Membrane (prM) protein that contributes to the ADE. The VLP produced in Pichia pastoris elicit predominantly serotype-specific DenV neutralizing antibodies and avoid ADE phenomena. In this study, the chimeric bivalent VLP Dengue was used as a model for efficient Dengue protein production that can later be used for tetravalent vaccines. We have already designed sequence DIII DENV-3 and DENV-4 and constructed pAO815_Den3Den4_2 copy for producing chimeric $Den3Den4_{bv}$ VLP.

2. Problem (5 points)

Dengue disease caused by dengue virus serotypes (DENV 1–4) is still the major concern among mosquito-borne diseases (Harapan et al., 2019). The Dengue infection has been a global health issue as 40% of world's population living in dengue-endemic regions. More than 70% population in the Asia-Pacific region contributes the most to global Dengue cases. According to WHO, in the last 20 years, the number of Dengue cases has increased to 4.2 million cases in 2020 (Sindi, 2021). As a tropical country, Indonesia have a relative high Dengue cases per year. Based on the data from Ministry of Health of Indonesia, the Dengue cases at 16th week of 2022 is 36,596 cases with 377 mortality cases (Figure 1). Reflecting on the data, it is important to control the Dengue infection especially in dengue-endemic regions like in Indonesia.

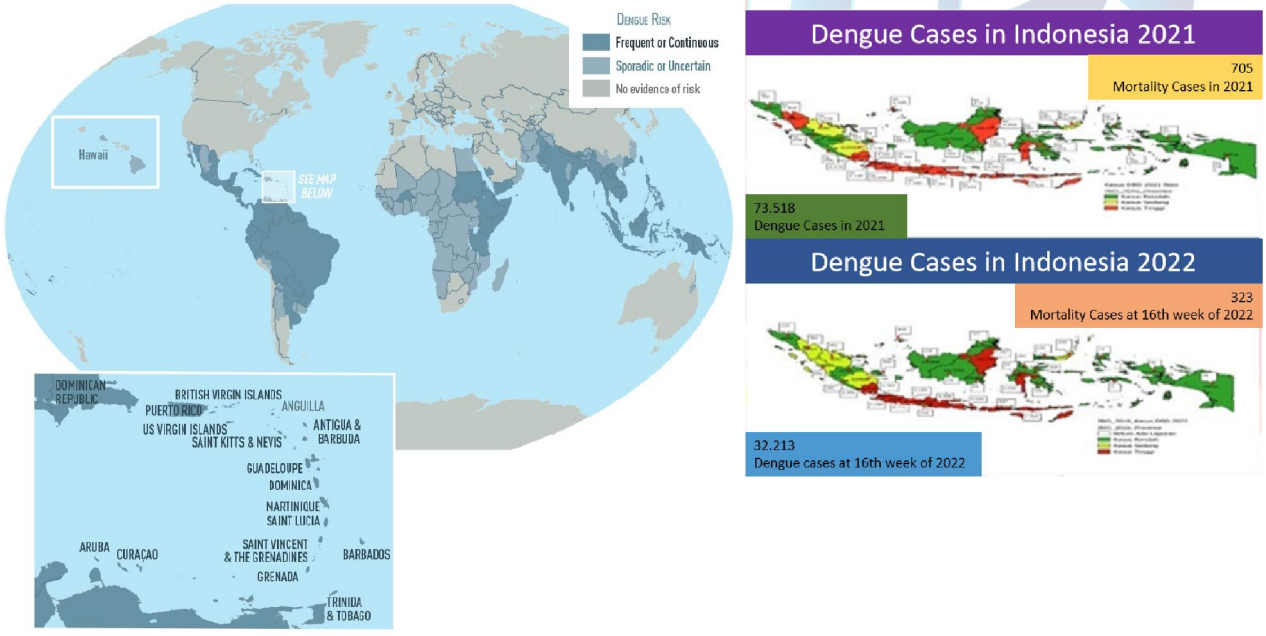

Figure 1. Dengue cases in worldwide and Indonesia (CDC, 2022 and Kemenkes 2022)

Development of Dengue vaccine was challenging due to fact that Dengue virus has four infectious serotype (DENV-1, DENV-2, DENV-3, DENV-4). A Tetravalent immune response is required to achieve optimum immune protection against all serotypes. However, this closely related serotype

are potentially inducing disease enhancement (Wilder-Smith, 2020). In April 2016, Sanofi launched the tetravalent dengue vaccine, Dengvaxia, which composed of live attenuated vaccine (LAV) from four dengue virus serotypes (DENV) 1–4. Nowadays, it becomes the only one available Dengue vaccine in market. The LAV vaccine is known to produces specific antibodies against prM and FLE of DENV. However, the presence of cross-reactive anti-DENV antibody against prM and FLE in the LAV vaccine potentially results in Antibody-Dependent Enhancement (ADE) (Figure 2A). Antibody-antigen complexes attach to the Fcγ receptor in macrophages, increasing viral load and immunological response. Consequently, dengue fever can shift into more severe symptoms, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) (Shukla et al., 2018) (Figure 2B). Therefore, it is crucial to develop a safe dengue vaccine that can elicit specific antibodies against different DENV serotypes and have not induce ADE.

The potential results of Dengue disease prevention by Dengvaxia vaccine. During the primary infection, an unvaccinated person will experience mild symptoms followed (yellow) by severe manifestation on the secondary infection (red), later no manifestation was observed during post-secondary infection (green). For a vaccinated person without primary infection will experience severe manifestation (red) as the vaccination behaves like a second natural infection on unvaccinated person. In seropositive individuals, no manifestation was observed (green) because vaccination behaves as a post-secondary infection. The box color represents the level of disease risk, green – no clinical manifestation, yellow – mild symptoms, and red – severe symptoms (DHF) (Figure 2A).

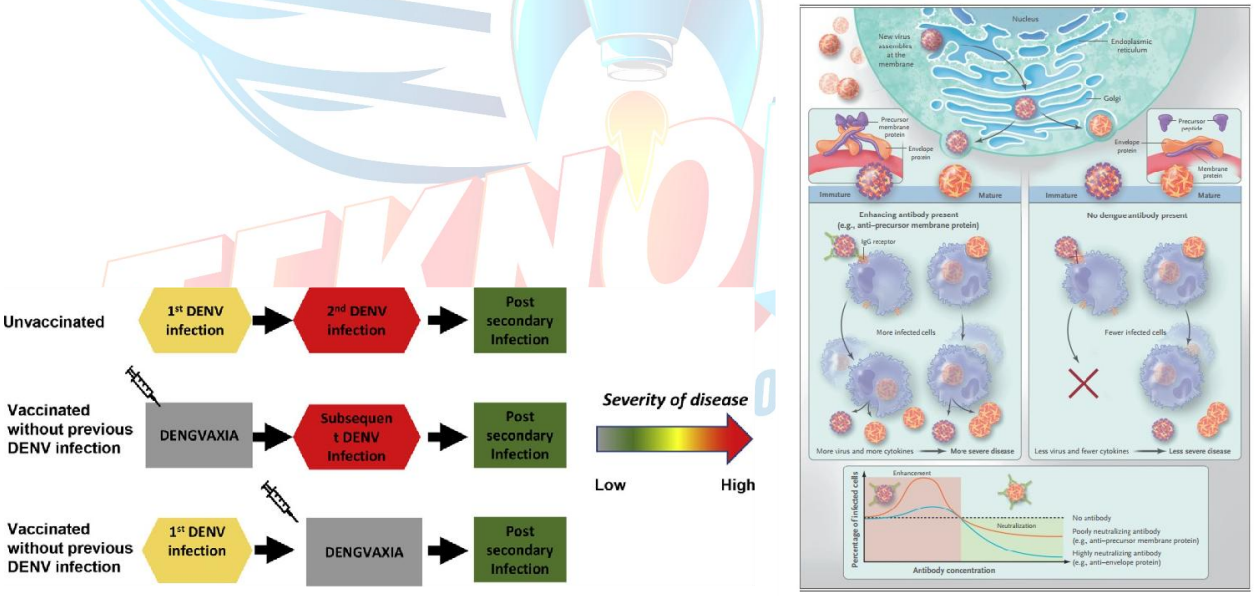

Figure 2. (A) Potential outcome of Dengvaxia vaccine on Dengue infection. (B) Mechanism of antibody dependent enhancement (Schmidt, 2010; Wang et al., 2020).

3. Solution (20 points)

The ADE potency remains the main problem of the dengue vaccine development. The presence of cross-reactive antibody against co-circulating dengue serotype can mediated virus enhancement, leading to severe disease symptoms (Figure 3). Thus, tetravalent immune response is required to achieve optimum immune protection against all serotypes. It could be achieved by administration a mixture of four dengue serotypes antigen in a tetravalent vaccine. Therefore, it is important to

develop a tetravalent dengue vaccine that provided durable immunity and also lack of ADE potency. This research aims to produce chimeric bivalent of DENV-3 and DENV-4 VLP from an Indonesian strain in *P. pastoris* GS115, which is expected to be a candidate active dengue vaccine that does not induce ADE. The envelope protein is essential for virus infection, thus it was the major target of host immune response. At particular, the envelope domain III (EDIII) was the region that mediated the recoginition function of envelope protein and also containing potent virus-neutralization epitope (Fahimi et al., 2018). Therefore, the EDIII was the potential antigen target for dengue vaccine development. The prM and FLE was excluded from the E protein to eliminate the ADE potency (Rajpoot et al., 2018). Later, this design was produced as a sub-unit protein through the recombinant protein approach, which was later assembled into VLP. The VLP vaccine platform was been choosen to overcome the low-immunogenicity of sub-unit vaccine. The VLP vaccine has icosahedral with repetition of antigen structure on the surface, thus the morphology resembles a virus structure but without the genetic material, therefore it is non-infectious (Cimica & Galarza, 2017). It is an advantageous platform because it can induce humoral and cellular responses without the addition of adjuvant (Middelberg et al., 2011).

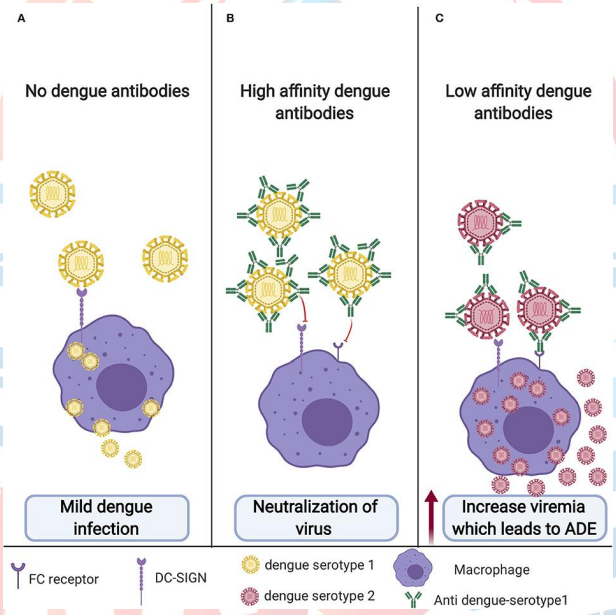

Figure 3 Antibody dependent enhancement causes the increase of viremia during secondary heterotypic infection due to the present of pre-existing cross-reactive antibody (Izmirly et al., 2020).

Dengue VLP vaccine has been developed extensively in *Pichia pastoris* as monovalent, also chimeric bivalent and tetravalent (Figure 3). The E protein with addition of prM-derived signal peptide sequence at N-terminal could form VLP when expressed in *P. pastoris*. This signal peptide can mediated self-assembly of EDIII into highly immunogenic VLP (Tripathi et al., 2015). The production of VLP vaccine for each serotype has been successfully performed but it would be more effective if produce it as chimeric form in *P. pastoris*. Co-expression of E protein from two different DENV serotype will produce chimeric VLP which has two different E protein in its surface. The development of chimeric bivalent E VLP is a more cost-effective approach compared to formulation of monovalent E VLP (Figure 4). Since, it is potentially reducing cost for production and purification process (Rajpoot et al., 2018). In order to increase the expression rate of E protein during production step, we used multi-cassette expression. This strategy potentially increase the yield of E thus lowering cost production and make the vaccine more cost-effective.

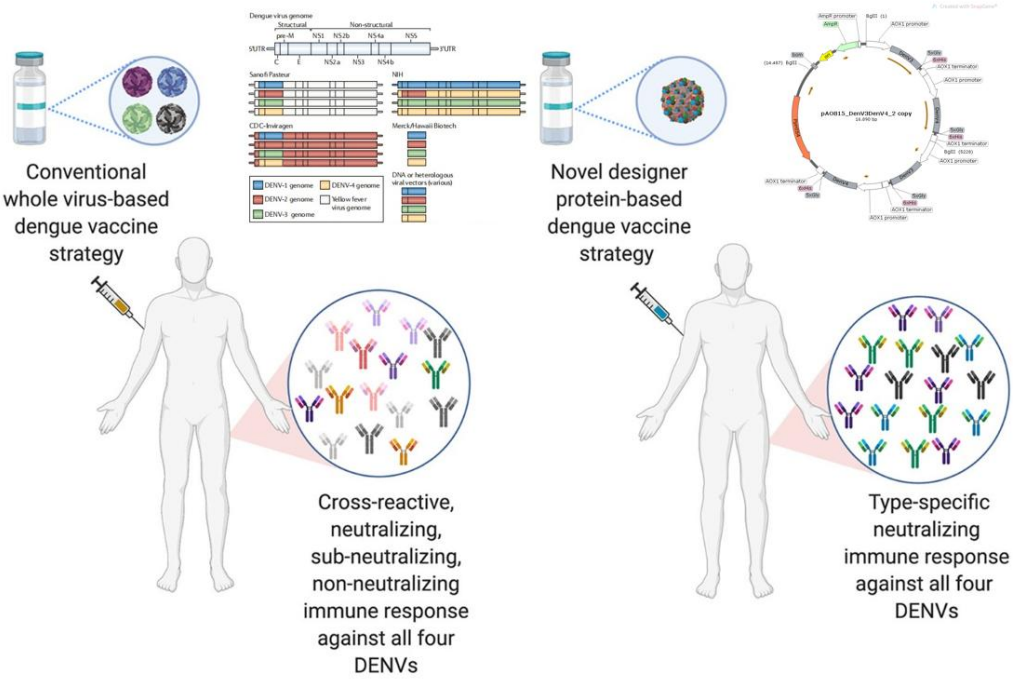

Figure 4.Comparison of conventional DENV vaccine strategy vs. Chimeric VLP Dengue (modified from Shukla *et.al.,* 2020).

4. Method (20 points)

4.1 Bioinformatics Study of Dengue Virus 3 and 4 Envelope Protein from Indonesian strain

The DENV-3 E and DENV-4 E from Indonesian strain was obtained from NCBI (https://www.ncbi.nlm.nih.gov/protein/). To generate the consensus sequence, the sequences were alligned using UGENE program against reference sequence, JX292266 (DENV-3) and JX292267 (DENV-4) (Rajpoot et al., 2018). Later, obtained consensus sequence was constructed into following expression cassette: 5'–start codon–34 AA pRM–DENV-3/4 E-5X Gly–6X His-stop codon–3'. The DNA sequences were optimized using Genesmart codon optimization server (https://www.genscript.com/tools/gensmart-codon-optimization) using *P. pastoris* codon table. The Kozak sequence, protease cleavage sites, and restriction enzyme anlysis from optimized gene sequence was evaluated. The nucleotide changes were introduced to avoid double Kozak sequence. The protease cleavage sites and restriction enzyme analysis were performed using Snapgene software. The alteration of amino acid residue of consensus sequences was investigated using BIOVIA Discovery Studio Visualizer. Later, the sequences were rationally modelled using Swiss Model server, the available structure reference with the highest homology and identity was choosen as model. The modelled DENV-3 E and DENV-4 E structure was evaluated according to local geometry assesment (Laskowski et al., 2018), the presence of key amino acid residues, identification of antigenic region, and predictive quality of cumulative resolution (http://molprobity.biochem.duke.edu/). The epitope region of DENV-3 E and DENV-4 E was predicted using Ellipro tool from IEDB server (https://www.iedb.org). To predict the interaction between antigen and antibody, the DENV-3 E and DENV-4 E design was docked against 1A1D-2 (PDB ID 2R69) and 4E11 (PDB ID 3UZQ) antibody. The molecular docking was performed using PATCHDOCK and FIREDOCK. The optimum antigen-antibody pose was used to investigate the interaction between antigen and antibody.

4.2 Generation of *P. pastoris* GS115/pAO815_Den3Den4_2 copy clone expressing recombinant chimeric bivalent DENV-3 and -4 E (Den3Den4$_{bv}$)

The following expression cassette of DENV-3 E and DENV-4 E: 5'AOX1–start codon–34 AA pRM-DENV E–5x Gly–6x His–Stop codon–3'AOX1 was synthesized, then sub-clonned into pAO815 plasmid between *BamHI/BglII*. The pAO815-Den3Den4 was introduced into *E. coli* TOP10' by using electroshock method. The multicopy expression cassette plasmid was generated *in vitro* using cloning strategy. The pAO815-Den3Den4 was digested by *BamHI/BglII* to result expression cassette of Den3Den4 (also contained AOX promoter and terminator), then it was re-inserted into *BamH1*-linearized pAO815-Den3Den4 to generate pAO815_Den3Den4_2 copy. *BamH1* and *BgIII* have different restriction site but have similar overhangs end, CTAG/GATC. The successfull ligation of similar overhangs end will re-create a new restriction site so the *BamH1*/*BgIII* restriction site will not be recognized again (Cost & Cozzarelli, 2007). The success of ligation was characterized by restriction analysis, digested pAO815_Den3Den4_2 copy will produced two fragments, the pAO815 backbone and Den3Den4_2 copy insert (the size was twice compared to single copy of cassette expression). The *BgIII*-linearized pAO815_Den3Den4_2 copy was introduced into *P. pastoris* GS115 by using electroshock method. The clone was verified by PCR using universal AOX primer, then the amplicon was sequenced using specific primer for DENV-3 E and DENV-2 E. The expression rate of *P. pastoris* GS115/pAO815_Den3Den4_2 copy clone was evaluated by RT-PCR, *P. pastoris* GS115/pAO815_Den3Den4 was used as control. Furthermore, the ratio of mRNA amount from each expression cassette of DENV-3 E and DENV-4 was also evaluated by RT-PCR. To generate cell bank, a verified *P. pastoris* GS115/pAO815_Den3Den4_2 copy was cultured overnight buffered minimal glycerol (BMG) then mix with glycerol (final concentration of 15%). The mixture was aliquoted 1 mL per cryovial tube, then stored at -80^{o}C.

4.3 Production, Purification, and Characterization of Recombinant Chimeric Den3Den4$_{bv}$

The recombinant chimeric Den3Den4$_{bv}$ was produced by methanol induction 5-L bioreactor. The *P. pastoris* GS115/pAO815_Den3Den4_2 copy clone was cultured in YNB medium for 48 h, then inoculated into fermetation medium (95.2 g/L glycerol, 9.4 g/L potassium dihydrogen phosphate, 1.14 g/L Yeast Trace Metal (YTM), 15.7 g/L ammonium sulphate, 1.83 g/L magnesium sulphate heptahydrate, 0.28 g/L calcium chloride dihydrate, and 0.4 g/L L-biotin. The YTM solution consisted of 207.5 mg/L potassium iodide, 760.6 mg/L magnesium sulphate, 484 mg/L di-Sodium molybdate, 46.3 mg/L boric acid, 5.032 g/L Zinc sulphae heptahydrate, 12.9 g/L Iron (III) chloride hexahydrate, and 9.2 g/L sulfuric acid. The protein expression was induced with methanol solution (98% w/w Metanol dan 1.4% w/w YTM) at 6 g/L for 72 h. The protein expression parameters were monitored every 24 h (OD_{600}, wet cell weight (WCW), dry cell weight (DCW), pH, microscopic, total protein concentration, and SDS-PAGE) (Gurramkonda et al., 2009).

The induced cell pellet resuspended in Cell Suspension Buffer (CSB) (50mM Tris-Cl, 500 mM NaCl, pH 8.5) and disrupted using high-pressured homogenizer for 10 cycles. The membrane-enriched fraction (P) was separated and solubilized in 2:1 ratio of membrane extraction buffer (MEB) (CSB supplemented with 8 M urea and 30 mM imidazole). Afterward, the supernatant was purified by Ni-NTA affinity purification using AKTA system. The protein was eluted using a step-wise gradient of imidazole. The purified protein-enriched fractions were pooled and dialyzed against assembly buffer (20 mM Tris-Cl, 50 mM NaCl, pH 8.5). The recombinant Den3Den4$_{bv}$ in P fraction and purified protein were characterized by SDS-PAGE and Western Blot (using the anti-His tag mAb and anti-Dengue envelope mAb). The chimeric Den3Den4$_{bv}$ VLP formation was examined under Transmission Electron Microscopy (TEM).

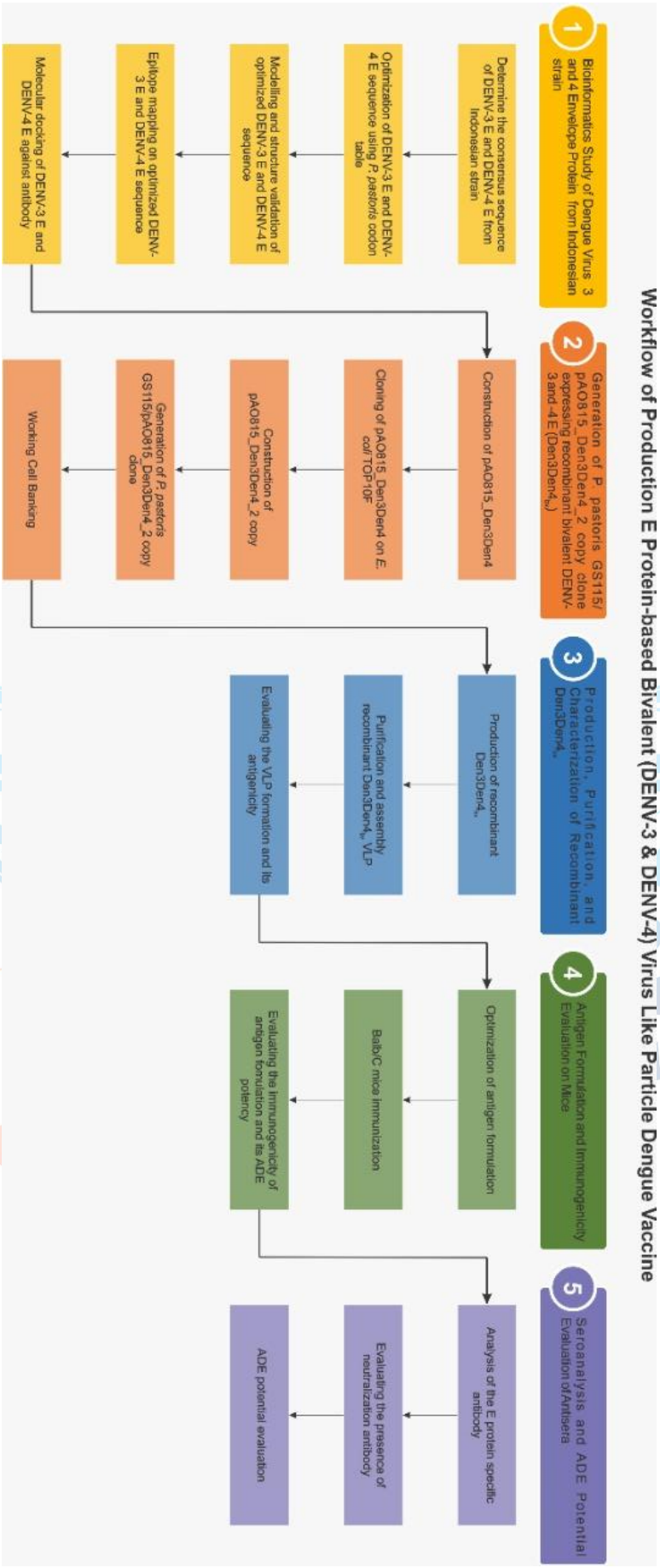

Figure 5. Development Chimera Virus Like Particle (VLP) Dengue Vaccine Work Flow

4.4 Antigen Formulation and Mice Immunization

The formulation of chimeric Den3Den4$_{bv}$ VLP was prepared for evaluation on 10 groups of mice (eight treatment groups and two control groups). The formula was optimized prior to imunization by using three different independent variables: antigen concentration, ajuvant type (CFA, MPL, and Alum), and adjuvant concentration (Mani et al., 2013; Poddar et al., 2016; Shukla et al., 2018). The groups of Balb/C mice (6–8 weeks old, n = 6) was immunized three times intra-peritoneally (i.p) at 0, 30th, and 90th day. The sera were collected after 7-10 days of immunization.

4.5 Seroanalysis, and ADE Potential Evaluation

The specific interaction of elicited antibody against Den3Den4$_{bv}$ VLP was evaluated and quantified by Western Blot (WB) and Enzyme-linked Immunosorbent Assay (ELISA), respectively. The present of neutralizing antibody was measured by *in vitro* virus neutralization using Plaque Neutralization Test (PRNT) on Vero cell. This method was used as gold standard to assess the immunogenicity of vaccine candidate by characterizing and quantifying the circulating neutralization antibody. The ADE potential of anti-DENV antibody was evaluated *in vitro* on K562 cell line. This cell was widely used as model for ADE study on anti-DENV antibody. The infected cells were detected by using Flouresence-Activated Shorting Cell (FACS), the 4G2 Alexa 488 conjugated antibody was used as reporter (O'Donnell et al., 2019; Rajpoot et al., 2018). The Den3Den4$_{bv}$ VLP formula that capable to elicite specific neutralization antibody without ADE potency was the optimal Dengue vaccine candidate.

5. Innovative (Innovative) Year (15 points)

To date, the structural-based design aided by bioinformatics tools (i.e., molecular modeling, epitope prediction, and molecular docking) is important for rational vaccine design. It was helping in immunogenic antigen design by evaluating the structure of antigen, the presence of potential epitope and its accesibility, antigen-antibody interaction, etc. Eventually, it aims the effective antibody-inducing antigen design for vaccine candidate. Sequence of Denv3 and denv4 Indonesia share 80% homology. The envelope protein plays an important structure due to the location in the outside and interact with the receptor and antibody. The structure of envelope protein consists of three domain, domain I, II, and III. B-cell epitope prediction showed that almost part of domain III is epitope (Figure 6).

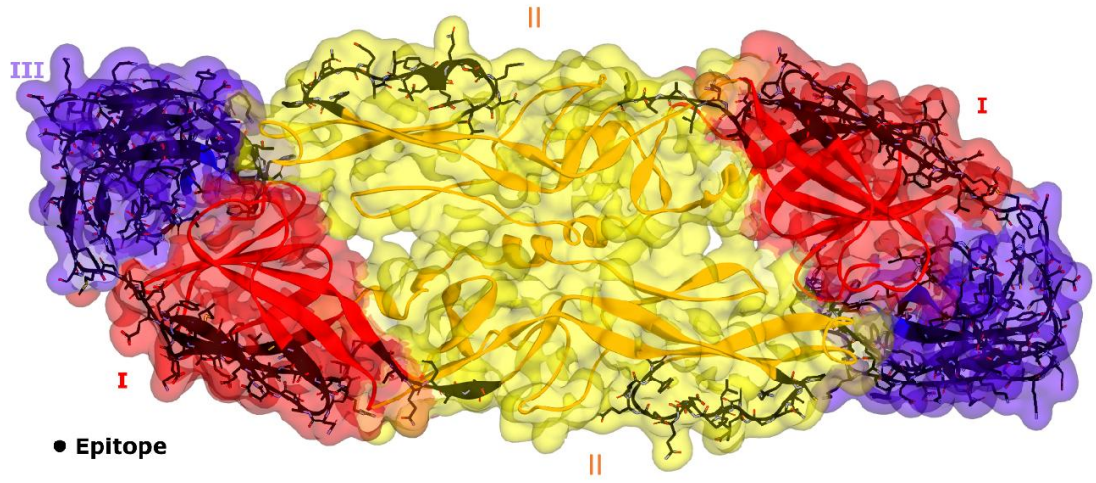

Figure 6. Structure of envelope denv in dimer. Domain I, II, and III are colored in red, yellow, and blue, respectively. The B-cell epitope is drawn in black ribbon.

The development of a Dengue vaccine candidates is vital to overcome the vaccine demand in the dengue-endemic region. Since, to-date there is only one available Dengue vaccine on the market.

The production vaccine in the *Pichia pastoris* expression system was the most feasible technology, especially for developing countries like Indonesia, because it provides affordable technology and cheap production cost. The VLP vaccine platform could increase the immunogenicity of antigen due to the similar structure to the virus thus not requiring a potent adjuvant. This platform has entered the market for HPV and Hepatitis vaccine. In Dengue case, the latest study show VLP vaccine has entered the pre-clinical study. Therefore, the development of VLP dengue vaccine is still necessary to obtain the active vaccine candidate. Chimeric Den3Den4$_{bv}$ VLP is an efficient technology platform for Dengue vaccine production. We could produce an immunospecific VLP against two different DENV serotype (DENV-3 and DENV-4) in one batch production. This approach is more cost-effective compared to formulation of two monovalent VLP (Figure 7). Furthermore, the two copies expression cassette (two copy E DENV-3 cassettes and two copy E DENV-4 cassettes) that being used could increase the yield of recombinant chimeric Den3Den4$_{bv}$. This strategy is potentially produce high amount of chimeric Den3Den4$_{bv}$ VLP with lower cost production, thus make the vaccine more affordable. Furthermore, this established chimeric vaccine technology could be applied for another universal vaccines such as Influenza chimeric vaccine or HPV multi-epitope vaccine.

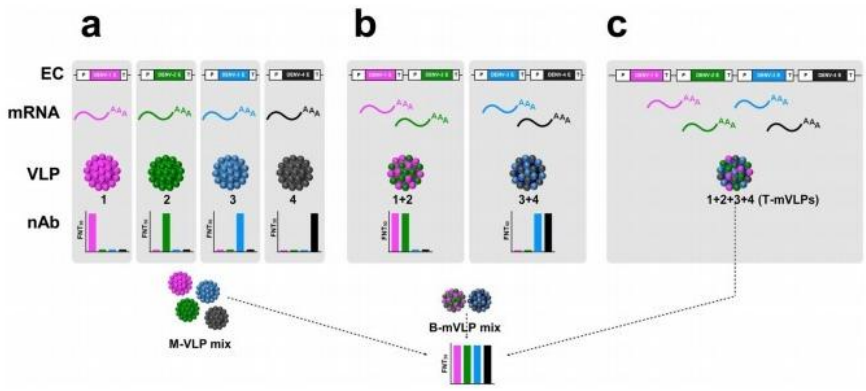

Figure 7. Schematic illustration of different P. pastrosi-expressed E protein-based tetravalent VLP dengue vaccine candidates (Rajpoot et al., 2018).

6. Applicability (10 points)

Vassileva has sucessfully produce Hepatitis B surface antigen (HBsAg) with high level expression in *P.pastoris.* Increasing number of cassete expression HBsAg has positive response to rHBsAg mRNA level . By increased 2 fold serial number of cassette expression, they are 2,4,and 8 increased mRNA level 2.32, 4,86, 9,38 fold stimulation over one copy. Furthermore, we aim to increase the expression level of E protein by increasing the expression cassette in plasmid construction. For example, the pAO815_DenV3DenV_2 copy plasmid has four expression cassettes, two cassettes of EDIII DenV3, and two cassettes of EDIII DenV4. It was well known that increasing copy number expression cassette will increase mRNA number and expression level of EDIII protein (Vassileva et al., 2001).

Rajpoot reported that ‘four-in-one’ chimeric dengue virus-like particles that produced in P. pastoris have equal mRNA level, high immune titer and do not induce ADE. Evaluation mRNA level using qPCR from the methanol-induced tetravalent P. pastoris clone using primer pairs (specific to each E genes of four dengue serotype) shown that the amplification profile and Ct value for any given E gene of the tetravalent clone was very similar and comparable to that of the corresponding monovalent clone. This phenomenon indicating that the tetravalent clone capable to

co-express all four E genes as efficiently as the monovalent clones. The chimeric VLP Dengue concept is confirmed by electron microscopy (EM) with size of ~40 nm after urea was removed through dialysis. Purified chimeric VLP Dengue has been formulated and used for immunogenicity test. The present of antibody in sera was evaluated by ELISA and dot-blot assay was used to evaluate the proportion of envelope protein of each serotype. Immune sera from the chimeric dengue VLP group shown very high titers of anti-E antibodies corresponding to DENV serotypes 1–3. Furthermore, ADE testing of antibody generated by chimeric VLP Dengue not enhance the infection of DENV-2 in AG129 mice while immune complex of cross-reactive antibody 4G2 and DENV-2 S221 could enhance the infection of DENV. The E-based tetravalent VLPs did not manifest capillary leakage or elevation in pro-inflammatory cytokine production in the intestinal tissue. The hypothesis of this phenomena is the elimination of prM in our VLP vaccines design leading to immune-specific response and ADE potency can be ruled out.

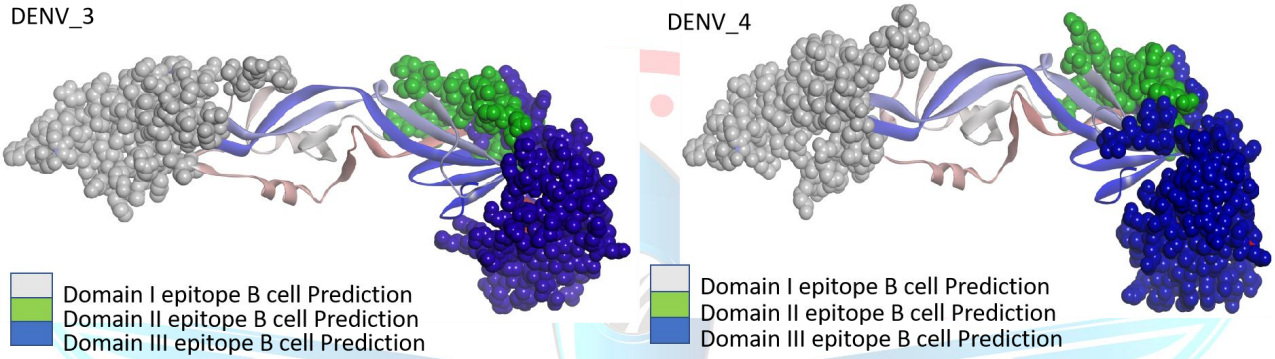

Figure 8. Structure and epitope B cell prediction of our Dengue 3 and Dengue 4 antigen design

The computational vaccinology was currently used by several research group during the development of novel vaccine candidate by combining bioinformatics approach and experimental study. The step-by-step vaccine design using in silico approach includes selection of antigenic target, prediction of B- and T-cell epitope, selection linker and adjuvant, construction of vaccine, prediction of antigenicity and toxicity, prediction of 3D structure, prediction of antigen-antibody interaction, and analysis of protein stability. The usage of bioinformatics tools could save time, money, and energy. (Arya and Bhatt, 2021). B cell epitopes is based on the interaction between antibodies and antigens. B cell epitopes become one of immunological research and application to help predict vaccine design based on subunit protein, mRNA vaccine, chimera, and many more. Th prediction of B cell epitopes is built from calculations of hydrophilicity, flexibility, Beta-turns, and surface accessibility (Kringelum *et.al.,* 2012). Structure and epitope B cell prediction of our Dengue 3 and Dengue 4 antigen design shown in figure 8.

If chimeric Den3Den4bv VLP was passing the immunogenicity and ADE potency evaluation, then this project results have the potential for production scale-up and later being commercialized. Indonesia has the leading vaccine manufacturer, PT. Biofarma which located in the same city as Universitas Padjadjaran in Bandung, West Java. PT. Biofarma is the only one manufacturer that produces s human vaccine in Indonesia and the largest vaccine manufacturer in Southeast Asia. PT. Biofarma has production capacity of more than 3.2 billion doses per year and has exported its product to more than 130 countries, of which 50 countries are members of the Organization of Islamic Cooperation (OIC). PT. Biofarma also has many certifications including CPOB (Good Manufacturing Practice) from the National Agency of Drugs and Food Control of the Republic of Indonesia (biofarma.co.id)

7. Estimated Cost and Project Scheduling (5 points)

The chimeric dengue VLP development project planned to be completed within 12 months with a budget of 1.064.351 ₺ (Table 1). The budget is divided into four aspects such as (a) production, characterization, and purification of chimeric Den3Den4bv VLP; (b) fermentation of recombinant chimeric Den3Den4bv at 5L bioreactor; (c) animal testing; and (d) quality control. It covers the general reagents for cultivation and purification, specific reagents for chimeric Den3Den4bv VLP characterization, animal testing, specific analysis for chimeric Den3Den4bv VLP, quality control reagents, and consumables. The schedule of project is divided into six aspects such as (a) bioinformatics study of dengue virus 3 and 4 envelope protein from Indonesian strain; (b) generation of P. pastoris GS115/pAO815_Den3Den4_2 copy clone expressing recombinant chimeric Den3Den4bv; (c) production, purification, and characterization of recombinant chimeric Den3Den4bv; (d) antigen Formulation and immunization in mice; (e) seroanalysis and (f) ADE evaluation of antisera. Each member is responsible for every aspect above based on their expertise and experience (Figure 9).

Table 1. Estimated Cost of Development of Chimeric Virus-Like Particle Dengue Vaccine

No	Item	Pack	Unit price	Unit	Amount	Total	Material Usage
Production, Characterization, and Purification of Dengue 3and 4 VLP							
1	Plasmid pAO815_Den1Den2 (Genesript)	1	20.520 ₺	Pcs	1	20.520 ₺	for generation of clone
2	*Eschericia coli*TOP10	1	11.902 ₺	Pcs	1	11.902 ₺	for generation of clone
3	*Pichia pastoris GS115*	1	10.927 ₺	Pcs	1	10.927 ₺	for generation of clone
4	Bacto Yeast Extract (BD 212750)	1	2.565 ₺	bottle	5	12.825 ₺	for growth medium
5	Bacto Tryptone (BD 5211705)	1	1.642 ₺	bottle	5	8.208 ₺	for growth medium
6	Bacto Agar (BD 214010)	1	13.338 ₺	bottle	5	66.690 ₺	for growth medium
7	Sodium Chloride (PT.Elo Karsa Utama (1.06404.1000))	1	7.285 ₺	bottle	2	14.569 ₺	for growth medium
8	Neutralized soay peptone (Oxoid LP0044)	1	1.847 ₺	bottle	5	9.234 ₺	for growth medium
9	Dextose	1	6.772 ₺	bottle	1	6.772 ₺	for growth medium
10	Gene Pulser Cuvette (Bio-Rad 165-2086)	1	308 ₺	bottle	24	7.387 ₺	for transformation
11	D-Sorbitol (Sigma Aldrich S3889-1KG)	1	1.539 ₺	bottle	1	1.539 ₺	for growth medium
12	Zeocin (Invitrogen R25001)	1	3.899 ₺	bottle	4	15.595 ₺	for gene selection
13	Ampicillin	1	2.257 ₺	bottle	4	9.029 ₺	for gene selection
14	EndoFree Plasmid Maxi Kit (Qiagen 12362)	1	5.438 ₺	kit	4	21.751 ₺	for DNA purification
15	QIAquick Gel Extraction Kit (Qiagen 28704)	1	2.052 ₺	kit	4	8.208 ₺	for DNA extraction
16	MasterPure DNA purification Kit (Epicenter MPY80200)	1	6.669 ₺	kit	1	6.669 ₺	for DNA purification
17	Qubit dsDNA BR Assay Kit (Invitrogen Q32850)	1	1.642 ₺	pcs	2	3.283 ₺	for DNA analysis
18	Restriction enzymes*EcoRI (Thermo #FD0274)*	1	1.129 ₺	pcs	2	2.257 ₺	for DNA characterization
19	Restriction Enzymes*SacI (NEB R0156S)*	1	1.231 ₺	pcs	4	4.925 ₺	for DNA characterization
20	Restriction Enzymes*BglII (NEB R0144S)*	1	1.026 ₺	pcs	4	4.104 ₺	for DNA characterization
21	Agarose LE, Analytical Grade (Promega V31250)	1	9.850 ₺	bottle	4	39.398 ₺	for DNA characterization
22	Ultra Pure 10x TBE Buffer (Invitrogen 15581-044)	1	923 ₺	bottle	4	3.694 ₺	for DNA characterization
23	SYBR safe DNA gel stain (Invitrogen S33102)	1	12.312 ₺	pcs	4	49.248 ₺	for DNA characterization
24	High DNA Mass Ladder (Invitrogen 10496016)	1	1.744 ₺	pcs	4	6.977 ₺	for Characterization of gene insert
25	Platinum taq DNA Polymerase High Fidelity (Invitrogen 11304-011)	1	3.078 ₺	pcs	4	12.312 ₺	for Characterization of gene insert
26	Deoxynucleotide (dNTP) Solution Mix (NEB N0447S)	1	1.436 ₺	pcs	2	2.873 ₺	for Characterization of gene insert
27	AOX1 Primer Forward	1	513 ₺	pcs	2	1.026 ₺	for Characterization of gene insert
28	AOX1 Primer Reverse	1	513 ₺	pcs	2	1.026 ₺	for Characterization of gene insert

No	Item	Pack	Unit price	Unit	Amount	Total	Material Usage
29	Den Primer Forward	1	513 ₺	pcs	2	1.026 ₺	for Characterization of gene insert
30	Den Primer Reverse	1	513 ₺	pcs	2	1.026 ₺	for Characterization of gene insert
31	Big Dye Terminator Ready Reaction Mix	1	513 ₺	kit	2	1.026 ₺	for DNA characterization
32	Big dye XTerminator Purification Kit (Applied biosystems 4376486)	1	4.207 ₺	kit	2	8.413 ₺	for DNA purification
33	Yeast Nitrogen Base (YNB) (Invitrogen Q300-07)	1	9.952 ₺	bottle	4	39.809 ₺	for growth medium
34	Potassium dihydrogen phosphate/KH2PO4 (Merck 1,04871,5000)	1	16.416 ₺	bottle	4	65.664 ₺	for growth medium
35	di-potassium hydrogen phosphate (Merck 1.05101.5000)	1	6.156 ₺	bottle	4	24.624 ₺	for growth medium
36	Glycerol 85% (Merck 1,04094.1000)	1	2.155 ₺	bottle	4	8.618 ₺	for growth medium
37	Methanol dried (Merck 1,06012,2500)	1	2.668 ₺	bottle	6	16.006 ₺	for growth medium
38	Biotin	1	2.052 ₺	bottle	2	4.104 ₺	for growth medium
Den3Den4bv recombinant protein fermentation 5L scale							
39	Glycerol (Himedia- MB060-500 mL)	1	790 ₺	bottle	5	3.950 ₺	for fermentation medium
40	Nitrogen (769061-1L)	1	1.719 ₺		3	5.156 ₺	for fermentation medium
41	Biotin (B4639-5G)	1	14.877 ₺	bottle	1	14.877 ₺	for fermentation medium
42	Potassium dihydrogen phosphate (P5655-1KG)	1	3.796 ₺	bottle	2	7.592 ₺	for fermentation medium
43	Ammonium sulfate (A4418-1KG)	1	3.078 ₺	bottle	3	9.234 ₺	for fermentation medium
44	Magnesium sulfate heptahydrate (13142-1KG)	1	1.693 ₺	bottle	3	5.079 ₺	for fermentation medium
45	Calcium chloride di-hydrate (202940-10G)	1	4.966 ₺	bottle	2	9.932 ₺	for fermentation medium
46	Potassium Iodide (221945-500G)	1	5.304 ₺	bottle	2	10.609 ₺	for fermentation medium
47	Magnesium sulfate (M2643-1KG)	1	5.079 ₺	bottle	2	10.157 ₺	for fermentation medium
48	di-Sodium molydate (M1003-1KG)	1	7.644 ₺	bottle	1	7.644 ₺	for fermentation medium
49	Boric acid (B6768-1KG)	1	2.360 ₺	bottle	2	4.720 ₺	for fermentation medium
50	Zinc sulfate heptahydrate (Z0251-500G)	1	2.647 ₺	bottle	2	5.294 ₺	for fermentation medium
51	Ferric chloride hexa-hydrate (157740-1KG)	1	1.231 ₺	bottle	2	2.462 ₺	for fermentation medium
52	Sulfuric acid (258105-2.5L-PC)	1	2.360 ₺	bottle	2	4.720 ₺	for fermentation medium
53	Antifoam (A5633-100G)	1	2.534 ₺	bottle	2	5.068 ₺	for fermentation medium
54	Ammonia (779423-1L)	1	12.391 ₺	bottle	1	12.391 ₺	for fermentation medium
55	Methanol (322415-2L)	1	2.514 ₺	bottle	3	7.541 ₺	for fermentation medium
56	Falcon centrifuge tubes 50 mL (NEST-602001)	1	487 ₺	ps rack	20	9.747 ₺	Disposable used for multi-purpose usage
57	Falcon centrifuge tubes 15 mL (NEST-601001)	1	385 ₺	ps rack	20	7.695 ₺	Disposable used for multi-purpose usage
58	Blue Tips, 100 - 1000 L, 1000/bag (Biologix 20-1000)	1	2.565 ₺	bag	5	12.825 ₺	Disposable used for multi-purpose usage
59	Tip 200 L	1	2.565 ₺	bag	5	12.825 ₺	Disposable used for multi-purpose usage
60	Tip 10 uL	1	2.565 ₺	bag	5	12.825 ₺	Disposable used for multi-purpose usage
Animal Test							
61	BALB/c . mice	1	616 ₺	tail	100	61.560 ₺	for animal immunization
62	feed	1	103 ₺	kg	10	1.026 ₺	for animal immunization
63	Cryotube 1.5 mL	1	513 ₺	crib	10	5.130 ₺	for animal immunization
64	Cryobox	1	513 ₺	Sir	10	5.130 ₺	for animal immunization
65	alumni	1	1.026 ₺	bottle	4	4.104 ₺	for animal immunization
66	vial	1	513 ₺	crib	10	5.130 ₺	for animal immunization
67	K562 . cell	1	15.390 ₺	Sir	1	15.390 ₺	for ADE potency evaluation
68	4G2 antibody	1	15.390 ₺	vials	2	30.780 ₺	for ADE potency evaluation

No	Item	Pack	Unit price	Unit	Amount	Total	Material Usage
69	Falcon centrifuge tubes 50 mL (NEST-602001)	1	487 ৳	ps rack	2	975 ৳	Disposable used for multi-purpose usage
70	Falcon centrifuge tubes 15 mL (NEST-601001)	1	385 ৳	ps rack	2	770 ৳	Disposable used for multi-purpose usage
71	Blue Tips, 100 - 1000 L, 1000/bag (Biologix 20-1000)	1	2.565 ৳	bag	2	5.130 ৳	Disposable used for multi-purpose usage
72	Tip 200 L	1	2.565 ৳	bag	2	5.130 ৳	Disposable used for multi-purpose usage
73	Tip 10 uL	1	2.565 ৳	bag	2	5.130 ৳	Disposable used for multi-purpose usage
74	Syringe	1	2.565 ৳	bottle	20	51.300 ৳	Disposable used for multi-purpose usage
75	PRNT test	1	103 ৳	test	100	10.260 ৳	for Neutralization antibody analysis
76	Flowcytometry Test	1	103 ৳	test	100	10.260 ৳	for ADE potency evaluation
Quality Control Process							
77	Pierce BCA Protein Assay Kit (Thermo 23225)	1	1.847 ৳	Kit	5	9.234 ৳	for protein quantification
78	Amicon Ultra-15 Centrifugal Filters Ultracel 10K (Merck UFC901024)	1	4.925 ৳	pk	2	9.850 ৳	for protein concentrate
79	12% Mini-Protean TGX precast protein gels, 12-well (Biorad #456-1045)	1	3.488 ৳	bottle	10	34.884 ৳	for SDS-PAGE analysis
80	Trans-Blot Turbo mini format 0.2 m PVDF, single application (Biorad 1704156)	1	1.949 ৳	bottle	5	9.747 ৳	for western blot analysis
81	SimplyBlue Safe stain (LC6065)	1	1.231 ৳	bottle	5	6.156 ৳	for western blot analysis
82	Glycine (Merck 1.04201.1000)	1	2.565 ৳	bottle	1	2.565 ৳	for western blot analysis
83	PageRuler Prestain Protein Ladder, 10to 180 kDa (ThermoFisher 26616)	1	3.488 ৳	pcs	1	3.488 ৳	for western blot analysis
84	Skim Milk (BD 232100)	1	923 ৳	bottle	5	4.617 ৳	for western blot analysis
85	Bovine Serum Albumin (Sigma A9647-100G)	1	6.361 ৳	crib	1	6.361 ৳	for western blot analysis
86	PBS Tablets (Merck 524650-1EA)	1	1.436 ৳	Sir	1	1.436 ৳	for western blot analysis
87	PBS-Tween Tablets (Merck 524653-1EA)	1	1.436 ৳	Sir	1	1.436 ৳	for western blot analysis
88	Tween 20 (Polysorbate) (Merck P1379-1L)	1	1.231 ৳	bottle	1	1.231 ৳	for western blot analysis
89	Costar Assay Plate 96 Well, No Lid For Protein Assay Procedures Vinyl (Costar 129)	1	4.412 ৳	crib	1	4.412 ৳	for western blot analysis
90	Novorex HRP Chromogenic Substrate ((TMB) (WP20004))	1	3.078 ৳	pcs	1	3.078 ৳	for western blot analysis
91	Anti-His (C-Term)-HRP Antibody (Invitrogen 46-0707)	1	4.822 ৳	pcs	1	4.822 ৳	for western blot analysis
92	Anti-envelope (Gentex Cat No. GTX629116)	1	8.109 ৳	pcs	1	8.109 ৳	for western blot analysis
93	Anti-DenV3	1	8.109 ৳	pcs	1	8.109 ৳	for western blot analysis
94	Anti-DenV4	1	8.926 ৳	pcs	1	8.926 ৳	for western blot analysis
95	Anti-Mouse IgG HRP	1	3.078 ৳	pcs	1	3.078 ৳	for western blot analysis
Total						**1.064.351 ৳**	

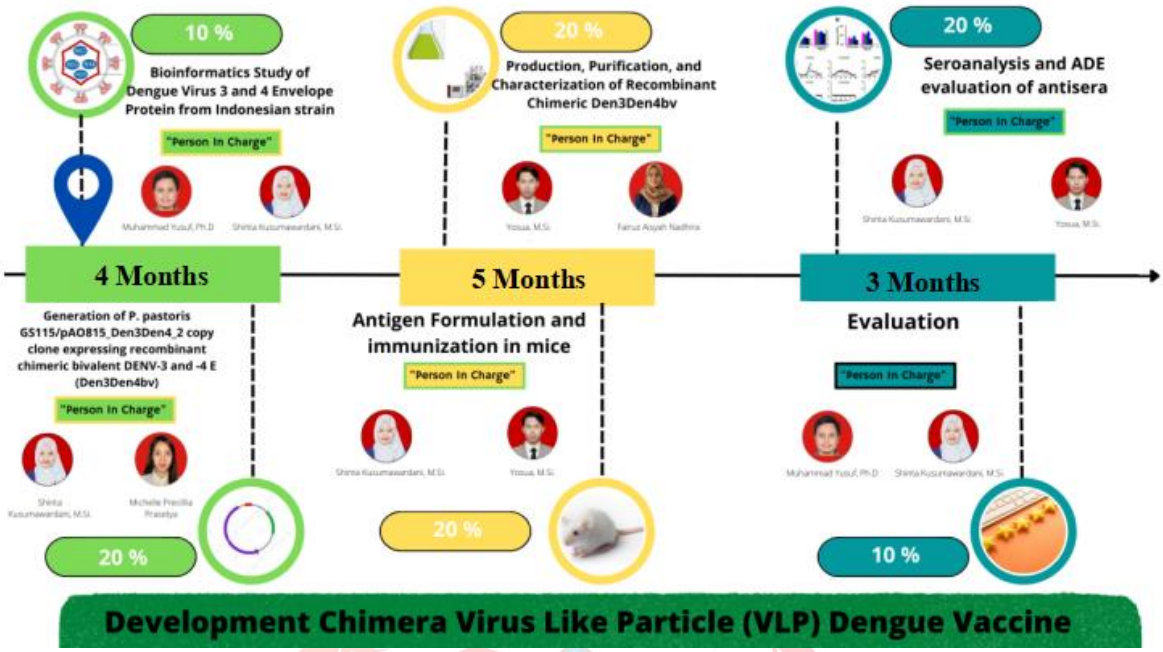

Figure 9.Project Scheduling

8. Target Audience of Project Idea (Users) (5 points)

The target audience of this project are countries that have problems related to dengue disease. Judging from the map of the distribution of dengue disease, countries in Southeast Asia, East Africa, West Africa, and even South America are areas where dengue has been confirmed. More than 3.9 million people are at risk of dengue infection. Expectation of this project is to overcome the spread of dengue infection (Figure 10).

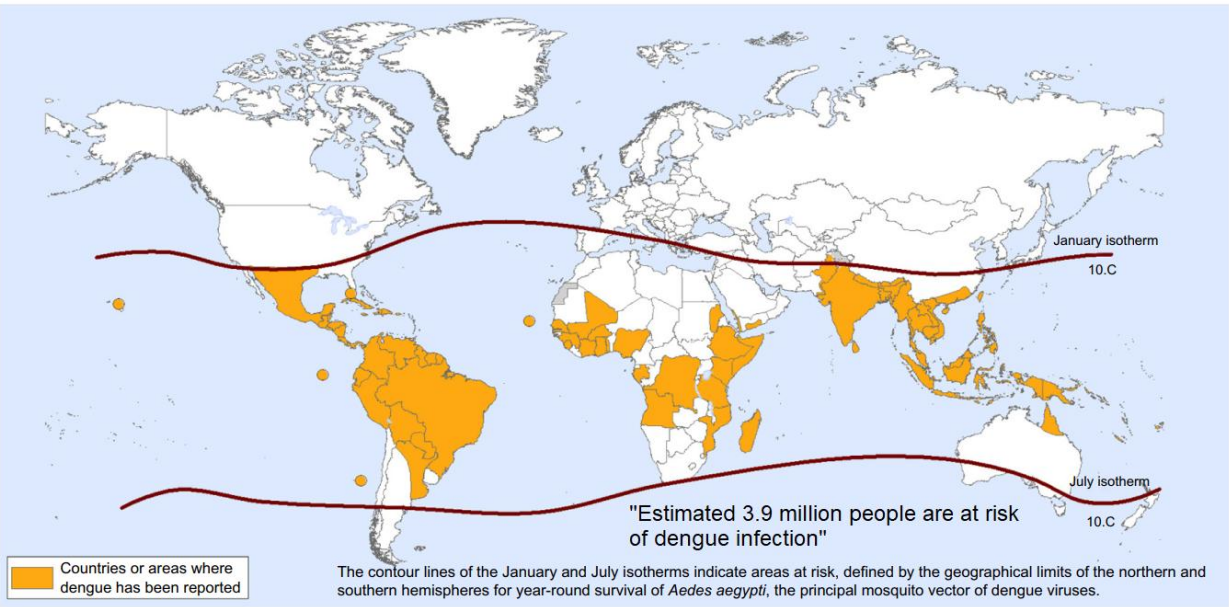

Figure 10. Target Audience of Chimeric Virus-Like Particle Dengue Vaccine (modified from Murray *et.al*., 2013).

9. Risks (10 points)

During this project there is a risk of failure at every stages of development but this project is iterative so any risk of failure can be anticipated by repeating and improving the previous stages. The biggest risk of this project is the time for this project will increase depending on the stages and the level of failure. We have identified four crucial points that can delay the success of this project such as (i) the failure of transformation process at generation of P. pastoris GS115/pAO815_Den3Den4_2 copy clone expressing recombinant chimeric Den3Den4bv step; (ii) low protein yield and (iii) VLP aggregation at purification, and characterization of recombinant chimeric Den3Den4bv; and (iv) low immune response and the presence of ADE potency at seroanalysis and ADE evaluation of antisera step. We have prepared some mitigations to overcome the following crucial points such as increasing the linear plasmid concentration for transformation problem; clone selection and optimization of dialysis process for low yield and VLP aggregation problem; and reformulation for low immune response and presence of ADE potency. Hopefully this risk identification will minimize the risk of failure and bring the project success and finish on schedule (Figure 11).

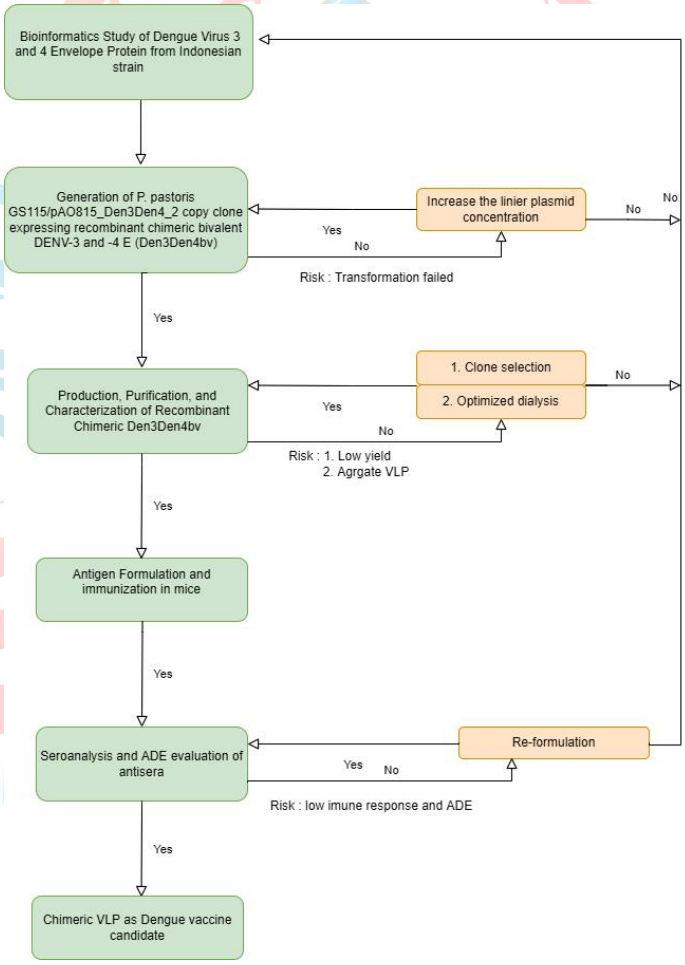

Figure 11. Development of Chimeric Virus-Like Particle Dengue Vaccine risks

10. Resources (References) (5 points)

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