UNIVERSITI TEKNOLOGI MALAYSIA

SEBB4173-01
CELL & MOLECULAR BIOLOGY FOR BIOINFORMATICS

LET'S CLONE IT!
ENVELOPE PROTEIN (E) - DENGUE VIRUS 2

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Envelope protein (E) – Dengue virus 2

- Introduction and the sequence of the chosen gene [3 Marks] (Ehsan)
- Size of the DNA and protein of interest [5 Marks] (Ehsan)
- What disease related to the gene or protein [5 Marks] (Hanis)
- What is the role of the gene/protein of interest in the pathogenesis [5 Marks] (Hanis)
- Cloning methods employed [30 Marks] (Aiman, Amir & Shahril)
    - PCR amplification of the gene of interest & plasmid vector (Shahril)
    - Purification of the DNA (Amir)
    - Restriction enzyme treatment of the DNA (Aiman)
    - Separation of the DNA (electrophoresis) (Amir)
    - DNA ligation (cloning into plasmid vector) (Shahril)
    - Selection for the correct clones and verification. (Aiman)
- Conclusion [2 marks] (Ehsan)
- References [5 marks] (All)

Proofread + Presentation (Nisha & Dina) (cari jugak info lol)

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https://www.ncbi.nlm.nih.gov/protein/NP_056776.2
https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=1494449
https://opentextbc.ca/biology/chapter/10-1-cloning-and-genetic-engineering/
https://www.nature.com/scitable/topicpage/dengue-viruses-22400925/
https://microbewiki.kenyon.edu/index.php/Dengue_virus_envelope_proteins
https://en.wikipedia.org/wiki/Dengue_fever

https://www.ncbi.nlm.nih.gov/nuccore/NC_001474 (gene sequence)
https://www.ncbi.nlm.nih.gov/nuccore/158976983 (gene sequence)
https://link.springer.com/article/10.1007/BF01538828 (restriction enzyme)
https://www.researchgate.net/publication/287317087_Dengue_virus_RNA_extraction_challenges (extraction of gene)
https://www.sciencedirect.com/science/article/pii/S0042682205003375 (rna replication)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6971860/ (whole genome sequence)
https://www.researchgate.net/publication/15262362_Ligation_of_multiple_DNA_fragments_through_uracil-DNA_glycosylase_generated_ligation_sites (ligation)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5957457/ (NS3 - dengue also? But cloning)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3269289/ (PCR amplification and sheites)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4846332/(separation of dna)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC170421/ (Restriction Enzyme Treatment)
https://europepmc.org/article/cba/441761 (Restriction Enzyme Treatment)
https://www.scielo.br/j/mioc/a/4kbbYYDYtgqBw6r6F9ZYrvD/?lang=en (Selection For Correct Clones And Verifications)

INFO COLLECTION (click the link to see)
Disclaimer: You can add more info from other sources and not necessarily use what has been provided.

Don't forget to cite and do references after writing.

Hope it can help. XD

Sequence of chosen gene
Dengue Virus Genome and Structure

Size of DNA and protein of interest
https://virologyj.biomedcentral.com/articles/10.1186/s12985-018-1043-2#:~:text=DENV%20is%20a,countries%20%5B5

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2581888/#:~:text=In%20supernatants,prM%2FM

https://www.nature.com/scitable/topicpage/dengue-viruses-22400925/#:~:text=The%20structure,human%20cells

Disease related to the gene or protein
https://en.wikipedia.org/wiki/Dengue_fever

https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

Role of the gene/protein in pathogenesis
(Pathogenesis is the process by which a disease or disorder develops)
https://microbewiki.kenyon.edu/index.php/Dengue_virus_envelope_proteins#Role_of_E_Proteins_in_Dengue_Infection

Cloning methods employed
I guess dr will explain this one next class. So nothing for now.

Introduction

What is Virus? Virus is a small organism that infects living organisms and an infective agent that typically consists of a nucleic acid molecule in a protein coat. It is too small to be seen by light microscopy and is able to multiply only within the living cells of a host. Like other viruses, the dengue virus is also a microscopic structure that can only replicate inside a host organism.It also means that , in order to survive, the dengue virus needs a host. Our chosen gene is Envelope protein in Dengue Virus 2. The sequence of DNA that made up the Envelope protein is shown as below.

```
 901 ttcatcttac tgacagctgt cactccttca atgacatgc gttgcatagg aatgtcaaat
 961 agagactttg tggaaggggt ttcaggagga agctgggttg acatagtctt agaacatgga
1021 agctgtgtga cgacgatggc aaaaaacaaa ccaacattgg attttgaact gataaaaaca
1081 gaagccaaac agcctgccac cctaaggaag tactgtatag aggcaaagct aaccaacaca
1141 acaacagaat ctcgctgccc aacacaaggg gaacccagcc taaatgaaga gcaggacaaa
1201 aggttcgtct gcaaacactc catggtagac agaggatggg gaaatggatg tggactattt
1261 ggaaagggag gcattgtgac ctgtgctatg ttcagatgca aaaagaacat ggaaggaaaa
1321 gttgtgcaac cagaaaactt ggaatacacc attgtgataa cacctcactc aggggaagag
1381 catgcagtcg gaaatgacac aggaaaacat ggcaaggaaa tcaaaataac accacagagt
1441 tccatcacag aagcagaatt gacaggttat ggcactgtca caatggagtg ctctccaaga
1501 acgggcctcg acttcaatga gatggtgttg ctgcagatgg aaaataaagc ttggctggtg
1561 cacaggcaat ggttcctaga cctgccgtta ccatggttgc ccggagcgga cacacaaggg
1621 tcaaattgga tacagaaaga gacattggtc actttcaaaa atccccatgc gaagaaacag
1681 gatgttgttg ttttaggatc ccaagaaggg gccatgcaca cagcacttac aggggccaca
1741 gaaatccaaa tgtcatcagg aaacttactc ttcacaggac atctcaagtg caggctgaga
1801 atggacaagc tacagctcaa aggaatgtca tactctatgt gcacaggaaa gtttaaagtt
1861 gtgaaggaaa tagcagaaac acaacatgga acaatagtta tcagagtgca atatgaaggg
1921 gacggctctc catgcaagat ccctttgag ataatggatt tggaaaaaag acatgtctta
1981 ggtcgcctga ttacagtcaa cccaattgtg acagaaaaag atagcccagt caacatagaa
2041 gcagaacctc cattcggaga cagctacatc atcataggag tagagccggg acaactgaag
2101 ctcaactggt ttaagaaagg aagttctatc ggccaaatgt ttgagacaac aatgaggggg
2161 gcgaagagaa tggccatttt aggtgacaca gcctgggatt ttggatcctt gggaggagtg
2221 tttacatcta taggaaaggc tctccaccaa gtctttggag caatctatgg agctgccttc
2281 agtggggttt catggactat gaaaatcctc ataggagtca ttatcacatg gataggaatg
2341 aattcacgca gcacctcact gtctgtgaca ctagtattgg tgggaattgt gacactgtat
2401 ttgggagtca tggtgcaggc cgatagtggt tgcgttgtga gctggaaaaa caaagaactg
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Figure 1: The DNA sequence of DENV-2 for the gene "Envelope protein E"

Size of the DNA and protein of interest

Based on our research, there are few unique sequences in dengue virus 2. The dengue virus genome is a single strand of RNA. The structure of the dengue virus is spherical with the diameter is about 50 nm with an isometric nucleocapsid of 25-30 nm and an ~10.7 kb .The apparent molecular weights are about 20 to 40 kDa. DENV is a single stranded and it has a positive polarity RNA virus with the length of 1484 bases in a genome. The RNA encodes 3 structural proteins (C, prM and E) which form the components of the virion, and 7 non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) which are involved in viral RNA replication. The virus will turn into ‘‘spiky’ or ‘smooth’ forms depending on the pH of the surrounding area.

Disease related to the gene or protein

Dengue is a mosquito-borne viral disease that has rapidly spread in all regions of WHO in recent years. Female mosquitoes, mostly *Aedes aegypti* and to a lesser degree, *Ae. albopictus*, carries dengue virus. Dengue fever is characterised by a high fever and flu-like symptoms. Dengue fever is common in the tropics, with risk levels varying depending on rainfall, temperature, relative humidity, and unplanned rapid urbanisation. Dengue fever is caused by a virus from the Flaviviridae family. There are four different serotypes of the virus that cause dengue fever. They are DENV-1, DENV-2, DENV-3, and DENV-4. Dengue haemorrhagic fever, a severe form of dengue fever, can result in major bleeding, a drop in blood pressure and death. There are a lot of signs and symptoms if you have dengue fever. However, many people have no symptoms or indicators of a dengue infection making it hard to trace if someone is infectious or not. The common symptoms are severe headache, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands and rashes. Some people even experience severe dengue. Severe dengue symptoms are severe abdominal pain, persistent vomiting, rapid breathing, bleeding gums, fatigue, restlessness and blood in vomit.

Any of four types of dengue viruses can cause dengue fever. Dengue fever cannot be contracted by being in the presence of an infected individual. Someone can have long-term immunity to the type of virus that infected them after they recover from dengue fever, however, not to the other three varieties of dengue fever virus. This indicates that you could be infected by one of the other three virus types in the future. If you encounter dengue fever for the second, third or fourth time, your chances of acquiring severe dengue fever is high. If someone has dengue fever or a more severe form of disease, they are more likely to get it if they live in the tropics. Being in tropical and subtropical climates increases the chances of contracting the dengue fever virus. Tropical and subtropical areas are such as Africa, the Caribbean, Central America, Central Pacific, China, India, the Middle East, South America, Southeast Asia and the South Pacific. If someone had dengue fever before, they are more likely to have severe symptoms if they get it again.

There are three types of transmission caused by dengue fever. The first one is transmission from mosquito-to-human. Infected female mosquitoes, typically the Aedes

aegypti mosquito, transmit virus to people through bites. The virus replicates in the mosquito midgut after it feeds on a DENV-infected person before spreading to secondary tissues, such as the salivary glands. Once infectious, the mosquito is capable of transmitting the virus for the rest of its life. The second transmission is human-to mosquito. Mosquitoes can get infected with DENV by biting people who are viraemic. The last transmission made is another mode of transmission. Mosquito vectors are the principal method of transmission of DENV between people while vertical transmission rates appear to be modest, the risk of vertical transmission appears to be connected to when the dengue infection occurs during pregnancy. When a mother has a DENV infection while pregnant, her baby may be born prematurely, have a low birthweight or even experience deadly complications.

The role of the gene/ protein of interest in the pathogenesis

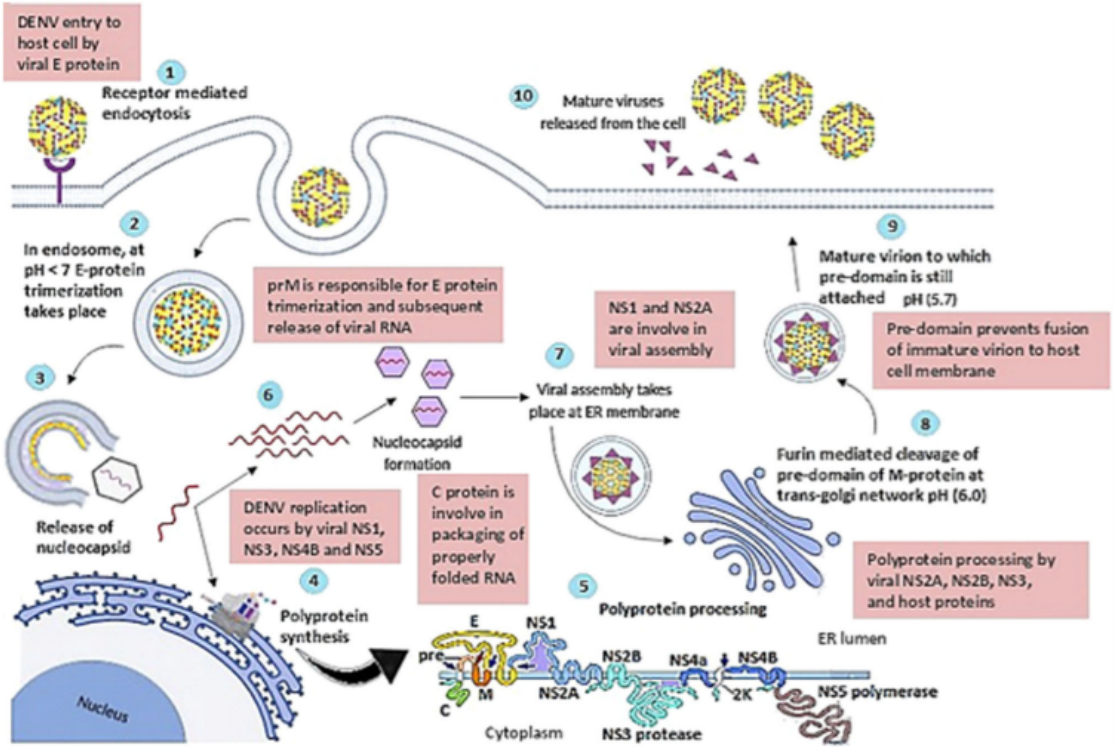

Figure 2: Life cycle of dengue virus inside the cell and role of viral proteins during this process; 1: receptor-mediated endocytosis of dengue virus, 2: structural alteration in viral E-protein, 3: release of viral RNA into cytoplasm, 4: viral poly-protein synthesis using host machinery, 5: release of individual viral protein from translated poly-protein by the action of viral and host proteases, 6: viral genome replication followed by nucleocapsid formation, 7: assembly of virus particle at endoplasmic reticulum surface, 8: processing of viral M protein through furin protease, 9: maturation of virus particle at golgi apparatus, 10: release of infectious virus particle from cell (triangles are representing release of predomain from mature virus)

Envelope protein is a glycoprotein that has been studied for its antigenicity and immunogenicity and especially explored as an epitope type. Envelope protein is also a multifunctional protein that plays a role in cell receptor binding as well as virus entrance by fusion with host cell membranes. It possesses multiple epitopes that respond with neutralising antibodies and is necessary for adhesion to cell membranes during infection. E proteins are found on the surface of dengue viruses and play an important role in cell entry where the shape of the protein influences how the virus interacts with the host cell. The genome and nucleocapsid, which contain the genetic information and surrounding proteins, are surrounded within a lipid-based viral envelope. Multiple proteins, including the E protein, are anchored by the viral envelope. By allowing viruses to enter host cells, E proteins play a critical function in the dengue life cycle. As a result, modifications on E protein may have an impact on the virus' path. During endocytosis, the E proteins undergo structural changes, and targeting the proteins could be an efficient antiviral therapy against dengue. Neutralising antibodies are mostly directed against E protein. Despite the fact that recent research has revealed many new details about the role of E proteins, the precise processes by which the function is still unknown.

The pathogenesis of severe manifestation is unknown, although it is thought that the diverse presentation is owing to intricate virus replication and the host immune response. There has been considerable advancement in DENV vaccine development, however, there is still no effective antiviral on the market. ER stress was found to be higher in viral strains that replicate quickly. This shows that UPR may have a role in DENV pathogenesis and severity. Antibodies to DENV have the potential to be a double-edged sword. As the numbers of cross-reactive antibodies decline over time, these antibodies may become more harmful. The antibody-dependent enhancement (ADE) theory, which states that sub-neutralizing quantities of antibodies might create immune complexes that interact with FcγRs (Fc receptor) in the brain, is the most commonly accepted explanation for this phenomenon. Usually occur in dendritic cells, monocytes, and macrophages leading to increased uptake and infection.

Cloning method employed

- PCR amplification of the gene of interest & plasmid vector

Polymerase Chain Reaction (PCR) is used to amplify a segment of DNA of interest or produce copies continuously from a small sample. The benefit of PCR is that it is allowed to take a little sample of the DNA and amplify it to make it easier to study in detail.

A typical amplification reaction includes a double stranded DNA template, two oligonucleotide primers, a thermostable DNA polymerase being DNA Polymerase (catalyse the new formation of DNA strands, nucleotides (dNTPS), a reaction buffer that contains MgCl2. The reaction is then placed in a thermal cycler, which is an apparatus that exposes the reaction to a series of different temperatures for predetermined periods of time. One cycle of

amplification is defined as a series of temperature and timing modifications. Each PCR cycle doubles the amount of targeted sequence (amplicon) in the reaction, theoretically.

Template denaturation, primer annealing, and primer extension are all steps in the PCR cycle. The target DNA is denatured in the first stage by heating it to 94°C or higher for 15 to 2 minutes. The two entangled strands of DNA split during the denaturation process, resulting in the single-stranded DNA template required for replication by the thermostable DNA polymerase. The temperature drops to around 40–60 degrees Celsius. The oligonucleotide primers can form stable connections (anneal) with the denatured target DNA and serve as DNA polymerase primers at this temperature. This phase takes about 15–60 seconds to complete. Finally, as the reaction temperature is raised to the DNA polymerase's optimum, the synthesis of new DNA begins. This temperature is in the range of 70–74°C for most thermostable DNA polymerases. The extension process takes about 1–2 minutes. The next cycle begins with a denaturation step at 94°C.

For each template and primer pair combination, each step of the cycle should be optimised. If the temperatures in the annealing and extension processes are similar, the two can be integrated into a single step that includes both primer annealing and extension. The amplified result can be evaluated for size, amount, sequence, and other properties after 20–40 cycles, or employed in subsequent experiments (Promega, n.d.)

After successfully amplifying the desired DNA it can be cloned into a TA vector. TA vectors are T vectors which are linearized plasmids that have been treated to add T overhangs to match the A overhangs of the PCR product.

- Purification of the DNA

Purification of the DNA was done by using the QIAamp UltraSens Virus Kit. This kit is commercially used for the purification of the DNA, RNA and protein. According to QIAGEN, this kit allows viral DNA from 1ml samples to be highly concentrated, prior to being purified using QIAamp silica -membrane technology. To make a pellet, buffer AC and carrier DNA was added to the plasma sample and these complexes were centrifuged at low speed. Then, the pellet will be resuspended in Buffer AR and proteinase K, and incubated for 10 minutes at 40°C. In order to bind the DNA to the QIAamp membrane, the binding conditions are adjusted by adding Buffer AB, and the lysate to a QIAamp spin column. After that, the sample was centrifuged. During centrifugation, RNA and DNA selectively bind to the QIAamp membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors are efficiently removed by centrifugation in two wash steps. Lastly, the

pure viral nucleic acids are eluted in low-salt buffer AFE and we got the product of purified DNA.

- Restriction enzyme treatment of the DNA

By definition, restriction enzymes are enzymes that cleaves DNA at specific sites, known as recognition sites. Because of this, the use of restriction enzymes is vital in this research to genetically engineer genes of interest. Restriction enzymes can be categorized as type I and type II restriction enzymes, with type II being the more favoured option. For this research, NotI restriction enzyme was chosen to be used to cleave the DNA in DENV to acquire the envelope protein E. NotI restriction enzyme is a type II restriction enzyme specifically used for 6 to 8 base pairs which produce sticky ends when cleaned. By forming a vast array of direct and water-mediated interactions with individual nucleotide bases, the NotI enzyme approaches DNA from the main groove side and recognises its 8-bp target sequence (Tamulaitiene & Siksnys, 2008). The NotI restriction enzyme will recognize the recognition site 5′-GCGGCCGC-3′ and should be incubated at 37 degrees Celsius in the O buffer to achieve optimum performance.

Figure 3: The base sequence of NotI Recognition Site

- Separation of the DNA (electrophoresis)

To conduct the agarose gel electrophoresis, we need to use the horizontal gel electrophoresis apparatus. The gel is made by dissolving the agarose powder in a boiling buffer solution. Then, the solution is cooled and poured into a casting tray which serves as a mould. A well-former template is placed across the end of the casting tray to form wells when the gel solution solidifies. After the gel solidifies, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode (anode) and a negative electrode (cathode) at each side of the apparatus. The samples are prepared for electrophoresis by mixing them with a loading dyes. Loading dyes in gel electrophoresis are used to provide colour and simplify the loading process. The dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated. Then, these samples are delivered to the sample wells with a clean micropipette.

A power source is connected to the electrophoresis apparatus and electrical current is applied. The charged molecules in the sample enter the gel through the walls of the wells. Molecules that have a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). The buffer serves as a conductor of electricity. Since DNA has a strong negative charge, it migrates through the gel towards the positive electrode during electrophoresis. The bluish-purple dye allows for visual tracking of sample migration during the electrophoresis. The gel is run until the dye has migrated to an appropriate distance.

The agarose gel will have to be post stained after electrophoresis. Ethidium bromide (EtBr) is the most commonly used stain for visualizing DNA. EtBr stains the DNA orange if viewed under UV light. After the orange bands are located, they are cut out of the gel and the DNA is extracted to yield a pure fragment.

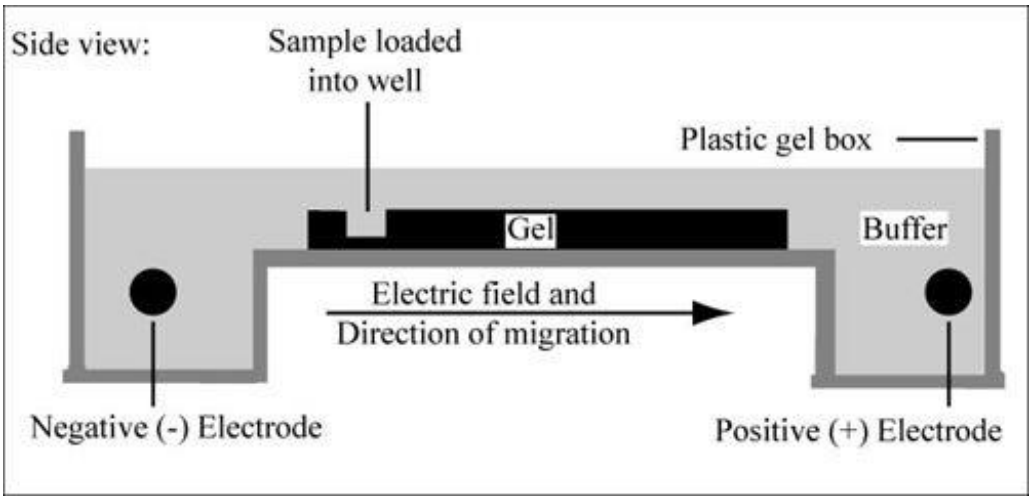

Figure 3: Separation of DNA in the Horizontal Gel Electrophoresis Apparatus

- DNA ligation (cloning into plasmid vector)

PCR cloning is a method where the amplified fragments are ligated directly into a vector. Two simplest PCR cloning methods are represented by TA cloning and blunt-end cloning and it depends on the nature of the vector and the type of PCR enzymes used in cloning. Ligation is the process such as gluing two ends together which can be seen as a vector and PCR-amplified. When ligation is a success, PCR cloning has succeeded.

A thermostable Taq DNA polymerase capable of amplifying short DNA sequences is used in TA cloning. This enzyme has a terminal transferase activity that adds an additional deoxyadenosine to the 3′ end of the amplicons (3′ dA), but no 3′ 5′ proofreading activity. The 3′ dA overhangs in the PCR products can easily be cloned into a linearized TA cloning vector with complementary 3′ deoxythymidine (3′ dT) overhangs.

Meanwhile, the ligation of an insert into a linearized vector with no overhangs is referred to as blunt-end cloning. High-fidelity DNA polymerases with 3′5′ exonuclease or proofreading activity can be used to make blunt-end inserts. Their proofreading activity increases the sequence accuracy of the amplified products; nonetheless, limitations include reduced ligation efficiencies and the inability to clone directionally when inserting into blunt-end cloning vectors. By incubating the amplicons with a Taq DNA polymerase and dATP in a technique known as "3′ dA tailing" (incubate 20–30 minutes at 72°C), then filtering the 3′ dA-tailed products, ligation efficiency can be increased. (Scientific, T., n.d.)

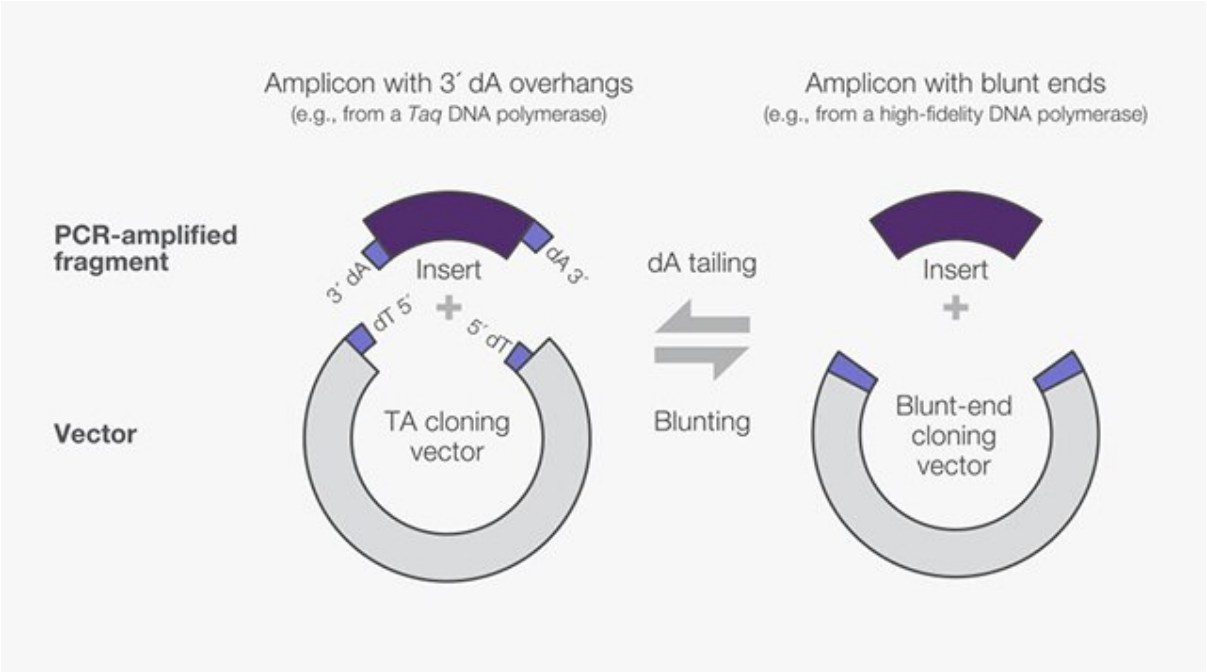

Figure 4: The process of cloning/transformation process

- Selection for the correct clones and verification.

After successfully transforming the plasmid vector containing our cloned gene into the bacterial cell, we have to select the correct clone and verify if the cloned gene is present in the bacterial cell. There are several methods of selection and verification that we can use to determine the correct clones. These include using antibiotics, brute force and ignorance and blue/white screening. For this research, the blue/white screening method was chosen as it is very efficient in determining recombinant bacteria. An article by Julin (2018) states that blue-white screening is a quick and easy approach to tell the difference between bacterial colonies or phage plaques that have a cloning vector with a DNA insert and those that have empty vectors with no insert DNA. The approach is based on the blue pigment that results from the hydrolysis of the synthetic substrate X-gal by beta-galactosidase (Julin, 2018). Grooms (2019) says that the plasmids have a multiple cloning region within the coding sequence of the α-fragment for blue/white colony screening. The reading frame is interrupted when a sequence is placed into this cloning region, resulting in a non-functional α-fragment. α-complementation is not possible with this fragment. You can distinguish between bacterial

colonies created from cells having plasmid with insert and those created from cells having plasmid without insert by growing transformed bacteria on a plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). As a result of the α-galactosidase activity, any colony bearing the plasmid (and hence the functioning α-galactosidase gene) will turn blue. This is referred to as α-complementation. The colour of the colonies carrying plasmids with an insert may be differentiated from those without an insert, with blue being the colony that contains the α-galactosidase gene and white being the colony without the α-galactosidase gene. Since the α-galactosidase gene was disrupted by the insert, the colonies will be white (Grooms, 2019).

Conclusion

In conclusion, dengue viruses are very deadly to human beings. Based on statistics, Malaysia has reported 130,101 cases (over a 60% increase from 2018) & 182 deaths in 2019. (Prudential, 2019). These data are very scary because the number of cases can increase from time to time. We need to take fast and productive action before it takes over us . There should be more future studies on E protein should be conducted so that we can prepare for what is coming next as prevention is better than cure. Government and NGO should work together to eliminate dengue cases from our country. We need to work together as a team to make important changes. For example, there should be more educational talks about dengue to educate the society so that we are aware of our surroundings. There are ways that we can do in general to prevent dengue from infecting our community such as we can reduce the mosquito habitat by getting rid of stagnant water to breed such as tires, plastic covers, flower pots etc. Other than that, we can wear protective clothing such as long sleeves to prevent mosquitoes from biting. We also can use mosquito repellents which can prevent mosquitoes from biting by applying creams on your skin. Hopefully with our effort, we can overcome and reduce dengue cases day by day.

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