**Abstract**

**Introduction**

Dengue virus in the family Flaviviridae is the causative agent of dengue fever, which is a major growing health problem. Dengue virus has four serotypes. Sometime due to infection or re-infection from different serotypes, dengue fever and dengue haemorrhagic fever enters into severe dengue shock syndrome. Two species of mosquitoes namely Aedes aegypti and Aedes albopictus transmit the virus to the human body. Vector control programs are not valid options in preventing dengue virus and dengue fever. The best option is the production of potential tetravalent vaccine against all four serotypes. Molecular vaccine is a valid source in preventing dengue virus and their four serotypes. In the present review different strategies have been discussed for potent molecular vaccine production. These vaccines include Live Attenuated Vaccine (LAV), Virus-Vectored Vaccine (VVV), Recombinant Protein Vaccine (RPV), Recombinant Chimeric Vaccine (RCV) and DNA Vaccine (DV) which is based on DNA plasmid vector.

**Conclusion**

Plasmid vectors express single open reading frame, which carries domain III of E protein of all dengue serotypes which are separated from each other by proteolytic cleavage sites. These strategies for the formulation of a promising tetravalent vaccine are under various clinical trials, which is the ultimate solution for preventing all dengue serotypes.

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**Introduction**

Dengue virus (DENV) belongs to Flavivirus in the family Flaviviridae and is the causative agent of dengue fever (DF) and dengue haemorrhagic fever (DHF), and sometimes the more severe form as dengue shock syndrome (DSS). Two carrier mosquitoes, Aedes albopictus and Aedes aegypti transmit the virus to humans. The causative virus has four serotypes (DEN-V1, DEN-V2, DEN-V3 and DEN-V4) with 65-70% sequence homology with each other1,2.

Dengue virus is a single stranded RNA virus with a genome size of about 10.6 kb. This single stranded RNA molecule of 11000 bases is encoded for structural and non-structural proteins as shown in figure 1. The structural proteins consists of envelope protein E, capsid protein C and precursor proteins prM (Table 1), while, the non-structural proteins consist of NS2A, NS4A, NS2B, NS4B, NS1, NS3 and NS5 which are important components for replication of the virus1,3. The glycoprotein of structural proteins is responsible for virus attachment and introduction into the host cell, while the non-structural proteins help the virus in replication1.

The structural glycoprotein consists of 3 domains (Domain I, II and III), identified in DEN-V2 and DEN-V3 through X-ray crystallography4. When an infected carrier mosquito bites a normal person, the virus enters into dendritic cells (Langerhans cells) within the incubation period of 4-7 days and finally enters the blood stream after replication. An immune response provokes against the virus by dendritic cells which are antigen-presenting cells5. It has been observed experimentally that the dengue virus first target the dendritic cells. Receptors and co-receptors are involved in the entrance of the virus into the dendritic cells.

These receptors such as C-type lectin and glycosaminoglycans (Figure 2), first bind to the E protein of the virus and then the entrance of the virus into the dendritic cells6,7. After a mosquito bite the dermal macrophages also serves as the first defence as innate immune response against the virus7.

In contrast, C-type lectin in correlation with macrophages activates the pro-inflammatory cytokine release after interaction with the virus8. The viral replication and uptake increases when the virus binds to the Fc-γ receptors after the formation of complexes with sub-neutralizing antibodies and leads to the increased production of virus in the blood stream and finally cause DHF and more severe form of DSS9,10,11.

When the virus enters into the blood stream, the virus targets the white blood cells, reproduces and circulates throughout the body. After triggering the white blood cells, signalling proteins are produced as a defence against the virus such as interferon. Due to the production of interferon, symptoms like fever, flu and severe pain appear. In severe conditions the virus enters into different organs, as a result fluid substances leak out from the blood vessels into the body cavities. In these conditions the blood vessels are unable to circulate the blood into different organs. Blood clotting and bleeding increases, and platelet production decreases due to the dysfunction of the bone marrow cells12, which is the major issue with dengue fever.

Some infected people suffer severely from dengue virus like DHF. This is because the different strains of viruses interacting with people with different immune backgrounds lead to a complex interaction. Among the possible causes is cross-serotypic immune response, through a mechanism known as antibody-dependent enhancement.

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which happens when a person who has been previously infected with dengue gets infected for the second, third or fourth time. The previous antibodies to the old strain of dengue virus now interfere with the immune response to the current strain, leading paradoxically to more virus entry and uptake\textsuperscript{13}.

According to the literature cited, still there are no ideal vaccines that can protect the body from all four serotypes. Even after sixty years of research there are still no licensed vaccines. Repeated infections with all four serotypes may cause catastrophic forms of disease. Protection from serotypes may also cause fatal DHF and dengue shock syndrome\textsuperscript{1,14}.

Protecting the virus may increase the severity of the disease and pathogenesis.

Developing a powerful vaccine against the virus has been a challenge for scientists. An ideal vaccine must level the immunogenicity and pathogenicity. The provoked immunogenicity must produce enough neutralizing antibodies that protect the body from all dengue serotypes.

The dengue vaccine production is a complex phenomenon due to little knowledge of immunopathology and unpredictable mutation in the virus. Therefore to challenge dengue virus, such a potential vaccines should be adopted which contain a common piece from all dengue serotypes that provoke the immune system against all serotypes. The aim of this review was to discuss the possible strategies for molecular vaccine production of dengue virus.

**Discussion**

**Strategies for vaccine production**

**Live Attenuated Vaccines**

Strategies for active live attenuated vaccines have been used to generate such potential vaccines that can be used against all causative dengue serotypes and also balance the immunogenicity and pathogenicity\textsuperscript{15}.

Live attenuated vaccines contain a weakened form of a live virus that produced antibodies for both the structural and nonstructural proteins of the virus\textsuperscript{1}. The yellow fever virus in the family flaviviruses and Japanese encephalitis virus serve as model viruses for live attenuated vaccine production. Live attenuated based vaccines can provide humoral and cellular immunity, and release active antibodies that can protect the body from reinfections\textsuperscript{16,17,18}.

In 1945, Sabin used the first attenuated vaccine by introducing DEN-V1 in the brains of mice, but such vaccines produced unwanted reactions in the form of rashes on volunteer’s bodies. These difficulties can be removed by serial dilutions of dengue virus in primary dog kidney (PDK) cells\textsuperscript{19}. However, such serial dilutions of dengue virus in tissue cultured cells induced unwanted molecular changes. This difficulty can be removed by inducing mutations in a virus gene construct to interfere with viral replication\textsuperscript{20}.

One of the attenuated vaccines has been developed by Oswaldo Cruz Foundation, Brazil (FIOCRUZ) (Figure 3). Yellow fever attenuated vaccine 17DD sub-strain was used as a genetic model. The dengue virus genes were replaced with YFV precursor membrane/E genes to produce chimeric YF17D/Dengue viruses\textsuperscript{21,22}. Monovalent vaccines for all dengue serotypes have been developed and evaluated in non-human primates but...
such vaccines produced only limited viremia as compared to YF 17D vaccines. For chimeric YF 17D/Dengue virus production, the viral strain has been collected from patients in Latin America and the dengue virus strains were received from Asia that provide the basis for tetravalent vaccines as developed by Sanofi Pasteur which is currently under clinical evaluation\textsuperscript{21,23,24,25,26}. Such chimeric vaccines can be used for the development of attenuated tetravalent dengue vaccines.

The Mahidol University of Thailand developed tetravalent live attenuated vaccines. The generated vaccines are safe with immunogenic responses and successfully completed clinical trials in phase-2\textsuperscript{27}. The first clinical trial (US volunteers) of this vaccine revealed that it can only inhibit the DEN-V3, but, however, they have lower potential to interact with the other three dengue serotypes\textsuperscript{27}. Further clinical trials revealed that when the concentration of DEN-V3 was reduced, it enhances the safety of live attenuated vaccines.

During clinical trials, it has been noted that 35% of recipients seroconverted against all four serotypes, while 58% were seroconverted against 3 or more after a single dose of vaccine. In contrast, 71% and 76% seroconversion was recorded after a second dose of the vaccine\textsuperscript{14}.

Another live attenuated vaccine has been formulated by Walter Reed Army Institute of Research (WRAIR) in the US. The WRAIR design vaccine has been tested in different experimental models. In humans, 100% seroconversion was observed against DEN-V1, while, 92% against DEN-V2, 46% against DEN-V3 and 58% against DEN-V4 (DEN-V1>DEN-V2>DEN-V3>DEN-V4).

The WRAIR tetravalent vaccine in pre-clinical trials has been tested in rhesus monkeys and phase 1 and phase 2 has been tested in human volunteers. Tetravalent vaccine tested in rhesus monkeys revealed that all the animals were seroconverted after two doses of the vaccine (DEN-V1+DEN-V2 100% > DEN-V3 90% > DEN-V4 70%) as reported by Mustafa and Agrawal\textsuperscript{14}.

Finally, live attenuated vaccine or weakened viruses are considered to be immunogenic, but the virus may induce mutations, or the reversion of the virus into virulent form may increase the chances of infection and pathogenicity. However such tetravalent vaccines should be designed which can possibly inhibit all four serotypes of dengue virus.

**Figure 3:** Types of vaccines against dengue four serotypes, Live attenuated vaccine (LAV), Virus-vectored vaccine (VVV), Recombinant protein vaccine (RPV), Recombinant chimeric vaccine (RCV) and DNA vaccine (DV), LAV is mono/tetravalent vaccine and in phase I/II/III clinical trials, VVV is mono/tetravalent and in pre-clinical trials, RPV is tetravalent and also in pre-clinical trials, RCV is tetravalent and in phase I/II/III clinical trials, and DV is tetravalent and in phase I trials. LAV is developed by FIOCRUZ (Oswaldo Cruz Foundation, Brazil) and CMU/MU (Chiang Mai University/Mahidol University). VVV is developed by ICGEB (International Centre for Genetic Engineering and Biotechnology, India), GP/NMRC (GenPhar/ Naval Medical Research Center, USA), UNCP (University of North Carolina at Chapel Hill, USA), UTMB (University of Texas Medical Branch, USA) and TB/IP (Themis Bioscience/Institut Pasteur). RPV is developed by Hawaii Biotech Inc. USA, NIH (National Institutes of Health), LID (Laboratory of Infectious Diseases), NIAID (National Institute for Allergy and Infectious Diseases), CDC (Center for Disease Control), SB (Shantha Biotechnics) and Inviragen. DV is developed by Inovio Pharmaceuticals, RU (Kobe University), CDC and NMRC.

**Virus-Vectored Vaccines**

Themis Bioscience and Institute Pasteur developed a virus-vectored vaccine from a live attenuated measles virus vaccine vector on the basis of expression of a single tetravalent dengue virus antigen construct\textsuperscript{21,28}.

Such a virus-vectored vaccine can carry large fragments of an antigen and provoke strong cellular immunity along with production of strong antibodies. The dengue vaccine containing E protein domain III (DEN-V1-V4) and M protein ectodomain (DEN-V1) and can...
Table 1: Structural and non-structural proteins and their target functions.

<table>
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<tr>
<th>Structures</th>
<th>Function</th>
<th>References</th>
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<tr>
<td>E protein</td>
<td>They are found on the surface of virus. E proteins are responsible for attachment of viruses to the host cells. ICAM3-grabbing non-integrin, CD209, GRP 78, Rab 5 and Mannose Receptor in interaction with E protein mediating attachment and viral entry.</td>
<td>[2, 15, 16, 21, 22]</td>
</tr>
<tr>
<td>prM/M protein</td>
<td>The structural precursor membrane protein (prM), which helps the virus in maturation and consists of 7 antiparallel β-strands which is stabilized by three disulphide bonds.</td>
<td>[2, 15, 16, 21, 22]</td>
</tr>
<tr>
<td>NS3 protein</td>
<td>The NS3 non-structural protein of dengue virus is a serine protease, as well as an RNA helicase and RTPase/NTPase.</td>
<td>[2, 15, 16, 21, 22]</td>
</tr>
<tr>
<td>NS5 protein</td>
<td>The NS5 non-structural protein of dengue virus is a 900 residue peptide with a methyltransferase domain at its N-terminal end and RNA-dependent RNA polymerase at its C-terminal end.</td>
<td>[2, 15, 16, 21, 22]</td>
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Balanced production of neutralizing antibodies against all of the causative serotypes has been documented in mice models after two doses of a single component tetravalent vaccine. The University of Texas Medical Branch (UTMB) developed a virus-vectored vaccine from the West Nile virus vaccine vector based on a single cycle and deletion of a capsid gene.

The infected cells with such a vaccine vector cannot produce viral progeny, however after the completion of a single infectious cycle, target cells produced antigens efficiently. Schwitz et al. documented that when the precursor membrane E protein genes of West Nile virus vaccine vector was replaced by a precursor membrane and E protein genes of DEN-V2 can generate a DEN-V2 vaccine candidate. For immunogenicity and pathogenicity the vaccine was tested in experimental mice. Production of neutralizing antibodies and potential effect on viruses has been recorded.

The Naval Medical Research Center (NMRC), USA in collaboration with Gen-Phar developed a virus-vectored vaccine from an adenovirus vector. Such tetravalent vaccines carry precursor membrane and E protein from two serotypes of the dengue virus. The tetravalent vaccine was tested in non-human primates, and it has been recorded that the released neutralizing antibodies were effective against all the four serotypes.

Schmitz et al. reported in a recent review that DEN-V1 and DEN-V3 was blocked between the virus and vaccine while the DEN-V2 and DEN-V4 was reduced significantly.

Recombinant Protein Vaccines
For the development of recombinant protein vaccines, the coat protein (E) of the dengue virus was used as antigen presenting cells. For the correct folding and expression of dengue coat protein, the precursor membrane protein (prM) proteins act as a chaperone.

Additionally, during intracellular expression the precursor membrane and E proteins automatically generates both free prM and E with the help of cellular furin. In tissue cultured cells 80% of recombinant E protein expression at N-terminal has been documented by Deubel et al., Men et al. and Delenda et al.

The recombinant E protein was successfully expressed in fruit fly cells. Balanced immunogenicity and pathogenicity against dengue virus was recorded by Putnak et al., when rhesus macaques were immunized with two doses of the recombinant E subunit vaccine.

The structural glycoprotein of dengue virus contains three domains as mentioned in the introduction. The domain III has the capability to fold into individual structures and also capable for production of serotype specific antibodies. Different approaches have been applied to enhance the immunogenicity of the dengue virus domain III antigen.

The immunogenicity can be enhanced by fusion with A protein of bacterium staphylococcus, fusion with Escherichia maltose binding protein and fusion with Neisseria P64K protein. Furthermore, Valdes et al. reported that cell mediated immunity can be induced by insertion of the dengue virus capsid protein beside domain III. Fusion of domain III from all dengue serotypes produced a chimeric protein which can be finally obtained by over expression in P. pastoris and finally tested in mice with strong antibody production against all serotypes.

Similarly, Leng et al. also documented that domain III protein can produce neutralizing antibodies in mice against all four serotypes based on the
consensus sequences of all four dengue serotypes.

Recombinant Chimeric Vaccines
A recombinant chimeric vaccine can be developed by using molecular genetics technology and by using flaviviruses as a genetic backbone. The precursor membrane and E protein genes can be replaced with similar genes from the dengue virus. On this basis the production of ChimeriVax dengue vaccine, in which the precursor membrane and E protein genes of yellow fever 17D virus (flavivirus) are replaced with similar genes of wild type serotypes of dengue virus.

Moreover, the ChimeriVax dengue vaccine viruses can be developed by electroporation of Vero cells with RNA transcript obtained from cDNA of the virus. Phase I trials of the vaccine has been completed successfully on animals in USA and Australia, while the vaccines were also safe in non-human primates which produced interferon γ responses for both CD4 and CD8 T cells.

Similarly different approaches have been used by the National Institute of Health (NIH), the Laboratory of Infectious Diseases (LID) and National Institute of Allergy and Infectious Diseases (NIAID) by using cDNA clones of the DEN-V4 strain 814669. The 3’ untranslated region of the clone’s cDNA was mutated by reverse genetics and the deletion of 30 nucleotides as Δ30 leads to successful production of antibodies against two serotypes (DEN-V1 and DEN-V4) of dengue virus in two experimental models including humans and monkeys.

These candidate vaccines were also tested in mosquitoes to check their capability of delivering the virus to mosquitoes but unfortunately did not occur. Furthermore the chimeric DEN-V1, DEN-V2 and DEN-V3 were also regenerated from DEN-V4 (Δ30).

However for the production of intertypic vaccine viruses, the precursor membrane and E protein genes were replaced with 3 other types of dengue viruses. The generated chimeric vaccines [DEN-V1/4(Δ30), DEN-V2/4(Δ30) and DEN-V3/4(Δ30)] were tested on monkeys and mosquitoes and it was found that monkeys were more susceptible than mosquitoes.

Another chimeric candidate vaccine term “DENVax” has been developed by the Center for Disease Control (CDC) in USA, Inviragen and Shantha Biotechnics from cDNA clone of DEN-2(PKD-53) strain developed at Mahidol University, Thailand. The intertypic dengue viruses were developed using the DEN-2(PKD-53) as a backbone and by replacing the precursor membrane and E protein of three serotypes of dengue virus (DEN-V1, DEN-V3 and DEN-V4).

Furthermore, the recombinant chimeric vaccine was successfully introduced into Vero cells. Similarly, the Inviragen generated vaccine (DENVax) was successfully tested in mice and balanced immunogenicity and pathogenicity responses were recorded, and now the tetravalent vaccine (DENVax) was underway for human trials.

DNA Vaccines
U.S. Centers for Disease and Prevention developed a tetravalent DNA vaccine. The vaccine is based on the expression of precursor membrane and E proteins. The introduction of plasmid vectors produced precursor membranes and E proteins along with the expression of one serotype from each dengue virus (V1, V2 and V3). The tetravalent candidate vaccine (DENVax) was tested on HPV 16/18 and hepatitis C. The tetravalent candidate vaccine was introduced into mice through intramuscular electroporation which produced excellent antibodies against all dengue serotypes. A similar response was recorded in non-human primates after immunizations.

Similarly, Kobe University developed a tetravalent DNA vaccine by using a mixture of 4 plasmid vectors. These plasmids have the ability to express both precursor membrane and E proteins along with the expression of one serotype from each dengue virus.

The vaccines were further modified and introduced through electroporation and are currently under testing in clinical trials (Phase I) for Human Immune Virus and influenza prevention. For phase II clinical trials these vaccines are tested on HPV 16/18 and hepatitis C. The tetravalent candidate vaccine was introduced into mice through intramuscular electroporation which produced excellent antibodies against all dengue serotypes. A similar response was recorded in non-human primates after immunizations.

The non-human primates are selected for immunogenicity response using a mixture of monovalent DNA vaccines. The vaccines were successful in the induction of balanced immunogenicity and pathogenicity for ten months. Furthermore, the immunogenicity can be enhanced by pre-existing antibodies against dengue virus. The immunogenicity can also be increased by the introduction of antigenic-determinants of E protein.

The Inovio Pharmaceuticals developed DNA vaccine which is based on the DNA plasmid vector. In a recent review, Schmitz et al. reported that plasmid vectors express a single open reading frame which carries domain III of E protein of all dengue serotypes which are separated from each other by proteolytic cleavage sites. The in vivo study revealed that the single tetravalent fusion proteins (Domain III of E protein) by the help of cellular protease are processed into monovalent proteins to induce balance response.

The vaccines were further modified and introduced through electroporation and are currently under testing in clinical trials (Phase I) for Human Immune Virus and influenza prevention. For phase II clinical trials these vaccines are tested on HPV 16/18 and hepatitis C.

Through needle free jet injector, the tetravalent vaccine was introduced into mice and production of neutralizing antibodies were recorded after 30 days. Two approaches are used to increase the Immunogenicity of the tetravalent DNA vaccine in mice. First using co-immunization including DEN-V2 VLPs (Virus like Particles) which are produced in tissue culture cells by co-expression of precursor membrane and E protein. The second approach to increase the attenuation is co-immunization with an inactivated JE (Japanese encephalitis) vaccine.
Conclusion

Two types of efforts are under progress to control and prevent dengue, one effort is vector control and the second is vaccine development. In vector control programs, a number of novel methods have been applied to minimize the carrier mosquito’s larvae in which the Poecilia reticulate are introduced into standing water bodies to eat larvae but almost with little success.

The second and best option to control dengue infection is vaccine development. Struggles for production of potent tetravalent dengue vaccine have faced different challenges including the need to provoke balanced immunogenicity and pathogenicity, unpredictable reversion of the virus into the virulent form, mutation, immune enhancement of disease and lack of appropriate experimental animals. In this regard, absolute efforts have been made in recent years resulting in the preparation of a molecular vaccine entering into phase III clinical trials. However, production of potential tetravalent vaccine against all four serotypes should be safe, effective and must be affordable and cost effective because mostly the developing countries are facing the dengue endemic outbreaks.

From the literature cited it is expected that the potential tetravalent vaccine will be commercially available by 2015.

References


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All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

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