Dengue Virus Disease: Recent Updates on Vaccine Development

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Abstract: - This paper reviews the current experimental updates made in the development of a dengue vaccine particularly DNA and plant derived vaccine. Dengue is an endemic viral disease affecting human health particularly children. Till today there is no medication or treatment available for dengue. Vector control measures are not yet successful in controlling dengue transmission. Introduction of *Wolbachia* bacteria might be the new ray of hope for the effective dengue vector control measures. The development of an efficient dengue vaccine is difficult because vaccine must be tetravalent so that it includes all the serotypes. Therefore, a tetravalent formulation plays an important role in developing a dengue vaccine. Recently tetravalent French dengue vaccine, Dengvaxia (CYD-TDV) (Sanofi Pasteur's, France) available (limited to a few countries) on the market since 2015. Sanofi branded, Dengavaxia (CYD-TDV) is the most promising one and has recently successfully completed the phase III clinical efficacy trials in Asia and Latin America. Dengavaxia (CYD-TDV) has been shown to be safe and has different levels of efficacy against the four serotypes. However, DNA vaccination has not yet successful mainly due to the insufficient immunogenicity. Botanical dengue vaccine production is also safe and have many advantages but there are still challenges that limit the rate of successful production of plant expressed vaccines. There are numerous dengue vaccine candidates in pipeline but none of them not yet promoting vaccination.

***Key words: Aedes aegypti*, botanical vaccine, dengue, Dengvaxia, immunization, India, mosquito, vaccine, vector control, *Wolbachia* bacteria**

I. INTRODUCTION

Global attention has been given to dengue epidemics since dengue viral fever is the most common mosquito-borne viral disease in tropical and subtropical regions of the world (Malabadi *et al.*, 2010, 2011; Gubler, 2012; Mahoney *et al.* 2012; Bhatt *et al*. 2013; Ganguly *et al*. 2013a, 2013b, 2013c, 2013d; Ganguly *et al*. 2014, 2015 ; Bhatnagar *et al*. 2012a, 2012b, 2014; Sood *et al*. 2015; Wilder-Smith *et al*. 2010; Murray *et al*. 2013; Gottschamel *et al*. 2016; Halstead, 2007; Khetarpal and Khanna, 2016; Pang and Loh, 2017). Dengue virus and vectors were found to be abundant, harmful, significant burden to worlds population and a major challenge to the modern medical sciences (Malabadi *et al.*, 2011; Ghosh and Dar, 2015; Ganguly *et al*. 2013a, 2013b, 2013c, 2013d ; Murray *et al.* 2013; Ganguly *et al*. 2014, 2015; Gottschamel *et al*. 2016; Pang and Loh, 2017). Dengue viruses are primarily maintained in a human-to-mosquito-to-human cycle. The primary vector is the *Aedes aegypti* mosquito, which is highly domesticated. The main arthropod vector involved in the transmission of dengue virus is *Aedes aegypti* (*A. aegypti)*, and another second dengue virus vector is *Aedes albopictus (A. albopictus)*, which is less active and feeds on multiple species of vertebrates (Halstead, 1974; WHO, 2011, 2013; Murray *et al.* 2013; Gottschamel *et al*. 2016; Malabadi *et al.*, 2011; Bhatnagar *et al*. 2012a, 2012b, 2014). In addition to this, *Aedes aegypti* (*A. aegypti)* also acts as a major common vector for the transmission of other viral diseases such as Zika, Ebola and Chikungunya too (Malabadi *et al*. 2016b; Gottschamel *et al*. 2016; Chaturvedi and Nagar, 2008). According to World health Organization report (WHO, 2013), 3.9 billion people in 128 countries are known to be dengue endemic (Bhatt *et al*. 2013; Murray *et al*. 2013; Gottschamel *et al*. 2016; Beesetti *et al*. 2014; Pang and Loh, 2017). There are many factors such as increased population, globalization and travel, unhygienic conditions, and climatic factors such as temperature and rainfall facilitate mosquito populations and their ability to transmit dengue (Bhatt *et al*. 2013; Murray *et al.* 2013; Gottschamel *et al*. 2016; Pang and Loh, 2017).

Dengue viruses (DENV) have been classified as four closely related but antigenically and genetically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) which causes 100 million cases of dengue fever (DF) and 380, 000 cases of dengue hemorrhagic fever (DHF) each year (Bhatt *et al*. 2013; Halstead SB, 1988; Raviprakash *et al*. 2000; Malabadi *et al.*, 2011; Ganguly *et al*. 2013a, 2013b, 2013c, 2013d; Ganguly *et al*. 2014, 2015). This is mainly due to the development of a secondary dengue infection in individuals naturally develop immunity to only one dengue serotype, and no long lasting immunity towards other dengue serotypes has led to the failure to control the dengue viral infection (Wilder-

Smith *et al*. 2010; Murray *et al*. 2013; Gottschamel *et al*. 2016; Pang and Loh, 2017). Secondary dengue infections are very serious and uncontrolled phenomenon which is associated with antibody dependent enhancement (ADE) of infection (Raviprakash *et al*. 2000; Jaiswal *et al.* 2004; Malabadi *et al.*, 2011, 2012b; Ganguly *et al*. 2013a, 2013b, 2013c, 2013d; Ganguly *et al*. 2014, 2015 ; Wilder-Smith *et al*. 2010; Murray *et al*. 2013; Gottschamel *et al*. 2016). During dengue hemorrhagic fever (DHF), the interaction between dengue virus and secondary infection leads to the formation of immune complexes and these complexes are efficient in human tissue targeting.

The majority of dengue infections are either asymptomatic or mild. The incubation period of dengue is usually 4-7 days but can range from 3-14 days (WHO, 2009). The common dengue virus diseases symptoms are sudden onset of high fever accompanied by abdominal pain, nausea, cold, headache, pain in the neck, eyes, myalgia and arthralgia, flushing of the face, anorexia (WHO, 2009). Rash is frequently seen on the trunk, on the insides of the arms and thighs. Laboratory abnormalities may include leukopenia and thrombocytopenia(WHO, 2009). Warning signs of severe dengue include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or restlessness, liver enlargement of >2 cm, or an increase in haematocrit concurrent with a rapid decrease in platelet count (WHO, 2009). Criteria for severe dengue include any sign of severe plasma leakage leading to shock or fluid accumulation with respiratory distress, severe bleeding, or severe organ impairment (WHO, 2009). There is no specific anti-viral treatment for dengue (WHO, 2009). According to World Health Organization guidelines (WHO, 2009) dengue is diagnosed either by virus isolation, serology MAC-ELISA (IgM antibody capture ELISA), IgG ELISA, (IgG antibody capture ELISA), PRNT (Plaque Reduction and Neutralization Test), NS1 ELISA (The non-structural protein NS1), and micro neutralization PRNT or molecular methods (RT-PCR) (Gubler, 1996; WHO, 2009; Balmaseda *et al.* 2003; Shu and Huang, 2004; Malabadi *et al.* 2012b; Ganguly *et al*. 2014). Diagnosis by serology typically does not allow for serotyping the infecting virus (except by PRNT), and is also susceptible to cross-reactivity, variable sensitivity by timing of specimen collection, and the need for multiple samples (IgG acute and convalescent samples) (Gubler, 1996; WHO, 2009; Balmaseda *et al*. 2003; Shu and Huang, 2004). PCR and detection of NS1 antigen offer more specific and early diagnosis (for PCR, 80-90% sensitivity and 95% specificity if applied in the appropriate time window) (WHO, 2009; Shu and Huang, 2004; Ganguly *et al*, 2014). The NS1 antigen is an important target for developing a quick diagnostic largely due to its long presence in the blood. Very recently Ganguly *et al.* (2014) developed a simple to-use heterosandwich immunoswab-based diagnostic procedure employing monoclonal antibodies and the second generation quadromas (Ganguly *et al.* 2014).The detection limit for NS1 has been established to be in the sub nanogram range (Ganguly *et al.* 2014).The assay is very sensitive, has a visual end point, and also being extremely inexpensive (Ganguly *et al.* 2014).With this assay, screening time for a dengue-infected person would be very rapid (Ganguly *et al.* 2014).

This review paper highlights the recent literature updates on the dengue vaccine development particularly DNA vaccine and **plant derived vaccine**, also discussed about current dengue vaccine clinical trials in pipeline developed by different research groups throughout world.

1) Dengue virus: Vaccine development

Dengue is still a major health problem, and endemic to the tropical and subtropical region of the world (De Roeck *et al.* 2003; Malabadi *et al*, 2010, 2011; Bhatnagar *et al.* 2012a, 2012b, 2014; Sood *et al.* 2015; Ganguly *et al*. 2013a, 2013b, 2013c, 2013d; Ganguly *et al*. 2014, 2015; Wilder-Smith *et al*. 2010; Murray *et al*. 2013; Villar *et al.* 2015; Gottschamel *et al*. 2016; Pang and Loh, 2017). Dengue is one of the leading arthopod-borne viral disease belongs to a member of the genus *Flavivirus* (Malabadi *et al.*, 2010, 2011; Khanam *et al.* 2006a, 2006b, 2007; Rao *et al.* 2005; Martin and Hermida, 2016). Dengue virus contains a positive-sense single-stranded RNA genome encoding three structural (C, prM and E), and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Gubler, 1988; Halstead, 2007; Jaiswal *et al*. 2004; Martin and Hermida, 2016). Dengue viruses are transmitted by the mosquito *Aedes aegypt* and dengue viral agent was first isolated in suckling mice by Sabin and Schlesinger in 1944 nearly 73 years ago (Sabin and Schlesinger, 1944; Halstead, 1974, 1988, 2008; Burke and Monath, 2001;Gubler, 1988; Murray *et al*. 2013; Slon Campos and Jose Luis, 2017). Despite the seriousness of the dengue disease, no effective control measures were developed.

The first efforts to develop a vaccine against dengue started 60 years ago and currently the vaccines were produced using wide range of approaches including: live attenuated virus vaccines, molecularly attenuated live virus vaccines, live chimeric virus vaccines, inactivated virus vaccines, recombinant subunit vaccines and genetic (DNA/RNA) vaccines. The simplest form of non-living vaccine is the purified, inactivated vaccine (PIV). Generally vaccines may need to be formulated with adjuvants to elicit acceptable levels of immunogenicity (Vannice *et al*. 2015, 2016; Slon Campos and Jose Luis, 2017). However, adjuvants may increase reactogenicity, and have occasionally raised safety concerns (Vannice *et al.* 2015, 2016; Slon Campos and Jose Luis, 2017). A variety of increasingly powerful expression systems are now available of bacterial, yeast, insect or mammalian origin, allowing economical production of antigen. An insect cell-derived product has entered clinical evaluation (Jaiswal *et al*. 2004; Collar *et al.* 2011;Vannice *et al.* 2015, 2016; Slon Campos and Jose Luis, 2017).

Till today there is no medication or antiviral drug except only one tetravalent formulated French vaccine

(**Dengvaxia**) (CYD-TDV) (Sanofi Pasteur's, France) available (limited to a few countries such as Mexico, El Salvador, Brazil, Singapore, Philippines) on the market since 2015 after 50 years of intensive efforts against all the four dengue serotypes (Villar *et al.* 2015; Mahoney, 2014; Thomas, 2015; Gottschamel *et al*. 2016; Sirivichayakul *et al*. 2016; Pang and Loh, 2017; Slon Campos and Jose Luis, 2017). Dengvaxia (CYD-TDV) is a live chimeric virus (LCV) vaccine that uses the 17D attenuated strain of Yellow Fever virus as a backbone to express the *prM* and *E* genes of each dengue serotype (Villar *et al.* 2015; Martin and Hermida, 2016; Slon Campos and Jose Luis, 2017). Furthermore, live chimeric virus (LCV) vaccine have several advantages like mimicking natural infection, inducing potent humoral and cellular responses, and conferring long-lasting immune memory (Villar *et al.* 2015; Martin and Hermida, 2016). The ChimeriVax Technology was first developed by St. Louis University to generate a molecular clone of Yellow Fever Virus (YFV) 17D strain with Japanese Encephalitis Virus (JEV) structural proteins (Chambers *et al*. 1999; Guirakhoo *et al*. 1999, 2000, 2001, 2002, 2004; Pang and Loh, 2017). This chimeric virus was then tested in mice and demonstrated an effective protection profile (Guirakhoo *et al*. 1999, 2000, 2001, 2002, 2004; Pang and Loh, 2017). On the basis of this successful prototype, the first YFV 17D/ DENV2 chimera was engineered that harboured heterologous prM and E proteins (Guirakhoo *et al*. 1999, 2000, 2001, 2002, 2004; Pang and Loh, 2017). After a continuous efforts a final tetravalent formulations were finalized and finished product was referred as Chimeric- Yellow fever- dengue (CYD) (Guirakhoo *et al.* 1999, 2000, 2001, 2002, 2004; Pang and Loh, 2017). Among the currently tested vaccine candidates, Sanofi Pasteur's tetravalent dengue vaccine candidate Dengvaxia (CYD-TDV) is the most promising one and has recently successfully completed the phase III clinical efficacy trials in Asia and Latin America (Villar *et al.* 2015; Thomas, 2015; Gottschamel *et al*. 2016; Pang and Loh, 2017). This tetravalent dengue vaccine candidate (Morrison *et al.* 2010; Guy *et al.* 2011; Gottschamel *et al*. 2016; Pang and Loh, 2017) is composed of four recombinant live, attenuated monovalent chimeric yellow fever dengue vaccine strains (Guirakhoo *et al.* 2001; Guy *et al*. 2011; Gottschamel *et al*. 2016; Pang and Loh, 2017) and showed an overall vaccine efficacy of 56.5 % in the trail conducted in Asia (Villar *et al.* 2015; Capeding *et al*. 2014; Gottschamel *et al*. 2016; Pang and Loh, 2017) and 60.8 % in the Latin American trial (Villar *et al*. 2015; Gottschamel *et al*. 2016; Pang and Loh, 2017) with the efficacy against DENV2 still being the lowest (35% and 42.3%, respectively) (Capeding *et al*. 2014; Villar *et al.* 2015; Guy *et al*. 2011; Pang and Loh, 2017).

Furthermore, **Dengvaxia** (CYD-TDV), a Sanofi branded vaccine can only be administered to individuals aged between 9 and 45 years in endemic areas (Mahoney, 2014; Hadinegoro *et al*. 2015; Wilder-Smith and Massad, 2016; Pang and Loh, 2017). The vaccination series consists of three injections at 0, 6 and 12 months. Dengvaxia (CYD-TDV) successfully completed phase III clinical studies in 2014 to evaluate the primary objective of vaccine efficacy (Villar *et al.* 2015). Additional pooled efficacy and integrated safety analyses in volunteers aged 9-16 who participated in the 25-month two Phase III efficacy studies and the ongoing long-term studies, respectively (Villar *et al.* 2015). Phase III clinical study reconfirmed the Dengvaxia® (CYD-TDV) consistent efficacy and longer-term safety profile in populations 9 years of age and older (Villar *et al.* 2015). Dengvaxia®(CYD-TDV) was shown to reduce dengue due to all four serotypes in two-thirds of the participants (Villar *et al*. 2015). Furthermore, this pooled efficacy analysis showed that Dengvaxia®(CYD-TDV) prevented 9 out of 10 cases of severe dengue and 8 out of 10 hospitalizations due to dengue in this age group (Villar *et al.* 2015; Whitehead, 2016).

Dengvaxia (CYD-TDV) is the first dengue vaccine to be licensed. It was first licensed in Mexico in December 2015 for use in individuals 9-45 years of age living in endemic areas (Mexico dengue vaccine first, *Nature biotechnology*, 2016). Therefore, World Health Organization (WHO) recommended Dengvaxia (CYD-TDV) immunization program as a part of comprehensive global dengue control strategy. Dengvaxia (CYD-TDV) can prevent up to 66% dengue cases and 93% severe dengue cases leading to the regulatory approvals in Brazil, El Salvador, Mexico, Singapore, Thailand, Costa Rica, Paraguay, Indonesia, Peru, Guatemala, and the Philippines. Therefore, 10 dengue endemic countries have adopted the vaccine and 5 lakh people globally have been vaccinated using Dengvaxia (CYD-TDV). Philippines already has introduced the Dengvaxia (CYD-TDV) through a national immunization drive. However, some of the critical issues that need to be addressed by the Sanofi Pasteur branded Dengvaxia (CYD-TDV) are 1) serotype interferences, 2) imbalance viral replication of the four monovalent serotypes along with epitopes-linked immunodominance had been observed when the vaccine was administrated as tetravalent formulation (Guy *et al*. 2009; Whitehead, 2016; Martin and Hermida, 2016; Pang and Loh, 2017). Another major concern of live chimeric virus (LCV) vaccine candidates is the poor immunogenicity and attenuation leading to immune imbalance with the potential risk of undesirable immunopathogenic responses (Guy *et al*., 2009; Martin and Hermida, 2016). Therefore, in a long run, more data should be needed for the tetravalency protection for the safe release of the Sanofi Pasteur branded Dengvaxia (CYD-TDV) throughout world. Therefore, next generation dengue vaccines such as viral vectored subunit, VLP's, peptide chimeras, and DNA vaccines would be better option and might play an important role in the production of suitable dengue vaccines. As a good news in 2017 more than 11 countries have approved and licensed the Dengvaxia (CYD-TDV) by National Regulatory Authorities (NRA) of several dengue endemic areas (Vannice *et al*. 2016). The Sanofi Pasteur branded Dengvaxia's (CYD-TDV) regulatory file has already been submitted in more than 20 countries, in Asia including India and Latin America, reflecting the global

burden of dengue. The regulatory review process is still ongoing in most of the dengue endemic countries.

2) In **India**, International Centre for Genetic engineering and biotechnology (**ICGEB**), New Delhi has developed an indigenous dengue vaccine candidate with the scientific name **DSV4** that offers protection from all four dengue serotypes in clinical stages (IndiaToday. in, 2015; FiercePharma, Pharma Asia, 2016a, 2016b; http://www.fiercepharma.com/pharma-asia; The Economic Times, Science, 2016; The Times of India, 2016; The Indian Express, 2016). DSV4 is a synthetic subunit vaccine constructed from dengue virus antigens (FiercePharma, Pharma Asia, 2016a, 2016b; The Economic Times, Science, 2016). A tetravalent dengue chimeric EDIII fusion protein was generated, which consists of the EDIII domains of DENV-1,-2,-3,-4 joined by flexible peptide linkers (Etemad *et al*. 2008; Poddar *et al.* 2016;Tripathi *et al*. 2015; Swaminathan *et al*. 2013; Swaminathan and Khanna, 2009; Mani *et al.* 2013). The tetravalent dengue chimeric EDIII protein was expressed in a yeast *Pichia pastoris* (Etemad *et al.* 2008). Immunization of mice with the tetravalent dengue vaccine candidate adjuvanted with montanide resulted in neutralizing antibody responses against all dengue serotypes (Etemad *et al.* 2008). This single tetravalent approach avoids the complexities related to producing tetravalent mixtures of monovalent vaccine components (Etemad *et al.* 2008). In another effort, the methylotrophic yeast *Pichia pastoris* was used to develop dengue envelope (E) protein based VLPs (Mani *et al.* 2013; Tripathi *et al.* 2015; Poddar *et al*. 2016). **Virus-like particles** (VLPs) which can elicit robust immunity in the absence of infection offer potential promise for the development of non-replicating dengue vaccine alternatives (Mani *et al.* 2013; Arora *et al.* 2013; Tripathi *et al*. 2015; Poddar *et al.* 2016). The formation of immunogenic DENV-2 E VLPs in the absence of pre-membrane protein highlights the potential of a yeast *P. pastoris* in developing non-replicating, safe, efficacious and affordable dengue vaccine (Mani *et al.* 2013; Tripathi *et al.* 2015; Poddar *et al.* 2016). These VLPs elicit very high levels of virus neutralizing antibodies which protected mice significantly against lethal dengue challenge (Mani *et al*. 2013; Tripathi *et al.* 2015; Poddar *et al.* 2016). The use of the high yielding yeast system *P. pastoris* for producing Virus-like particles (VLPs) holds great promise for the development of dengue vaccine that may be not only safe and efficacious but also inexpensive, for use in the resource-poor nations where dengue is endemic (Mani *et al.* 2013; Arora *et al.* 2013; Tripathi *et al.* 2015; Poddar *et al.* 2016; Swaminathan *et al.* 2013; Swaminathan and Khanna, 2009; Khanam *et al*. 2006a, 2006b, 2007; Rao *et al.* 2005; Etemad *et al.* 2008).

3) Apart from dengue vaccine development, a new vector control strategy will be introduced very soon which highlights the growth of a bacteria ***Wolbachia*** to combat the dengue virus vector *Aedes aegypti* mosquito population (Moreia *et al.* 2009a, 2009b; Bian *et al.* 2010; Hertig and Wolbach, 1924; Hertig, 1936; Rasgon, 2008; Hedges *et al*. 2008; Brelsfoard and Dobson, 2011; Pinto *et al*. 2012; Iturbe-Ormaetxe *et al*. 2011; Guruprasad *et al*. 2013, 2014). ***Wolbachia* bacteria** can stop dengue viruses from growing and being transmitted to people (Moreira *et al*. 2009a, 2009b; McMeniman *et al.* 2009; Hoffmann *et al.* 2011; Nguyen *et al.* 2015; Schmidt *et al.* 2017; Turelli and Barton, 2017; Jiggins, 2017). This discovery has the potential to transform the fight against life-threatening viral diseases (Moreira *et al.* 2009a, 2009b; McMeniman *et al.* 2009; Hoffmann *et al.* 2011; Nguyen *et al.* 2015; Schmidt *et al.* 2017; Turelli and Barton, 2017; Jiggins, 2017). Therefore, cultivation of bacteria *Wolbachia* and set up a large facility to breed them before releasing them into the environment so that they compete with the normal mosquito population and check the dengue virus (The Times of India, 2016; The Indian Express, 2016; (Moreia *et al*. 2009a, 2009b; Bian *et al.* 2010; Hertig and Wolbach, 1924; Hertig, 1936; Rasgon , 2008; Hedges *et al*. 2008; Brelsfoard and Dobson, 2011; Pinto *et al.* 2012; Iturbe-Ormaetxe *et al.* 2011; Guruprasad *et al.* 2013, 2014). *Wolbachia* is an intracellular maternally inherited endosymbiotic bacteria found in arthropods (Guruprasad *et al.* 2013, 2014). Such endosymbiotic bacterial strains have been introduced in *Aedes aegypti* mosquito populations to reduce their life span, thereby reducing the extrinsic incubation period (Moreia *et al*. 2009a, 2009b; Bian *et al*. 2010; Hertig and Wolbach, 1924; Hertig, 1936; Rasgon, 2008; Hedges *et al*. 2008; Brelsfoard and Dobson, 2011; Pinto *et al.* 2012; Iturbe-Ormaetxe *et al.* 2011; Guruprasad *et al*. 2013, 2014). The other prospect of exploiting ***Wolbachia* bacteria** is using its ability to interfere with viruses and parasites (Guruprasad *et al.* 2013, 2014; Moreira *et al.* 2009a, 2009b; McMeniman *et al.* 2009; Hoffmann *et al.* 2011; Nguyen *et al*. 2015; Schmidt *et al*. 2017; Turelli and Barton, 2017; Jiggins, 2017). *Wolbachia* is known to interact with a wider range of pathogens in transfected mosquitoes including dengue and chikungunya viruses (Moreira *et al.* 2009a, 2009b; McMeniman *et al*. 2009; Hoffmann *et al*. 2011; Nguyen *et al.* 2015; Schmidt *et al*. 2017; Turelli and Barton, 2017; Jiggins, 2017; Guruprasad *et al.* 2013, 2014). A major advantage of *Wolbachia*-based control approach for mosquitoes is that cytoplasmic incompatability acts as a self-spreading mechanism for *Wolbachia* to rapidly invade populations from the release of relatively small numbers of individuals (Guruprasad *et al.* 2013, 2014).*Wolbachia* bacteria provides a biological method to manipulate mosquito populations and reduce disease transmission and health burden in humans (Guruprasad *et al.* 2013, 2014). Findings have prompted researchers to aid in the control of mosquito-transmitted diseases. It has the benefit of being more environment-friendly than insecticide-based approaches (Moreia *et al.* 2009a, 2009b; Bian *et al.* 2010; Hertig and Wolbach, 1924; Hertig, 1936; Rasgon, 2008; Hedges *et al.* 2008; Brelsfoard and Dobson, 2011; Pinto *et al.* 2012; Iturbe-Ormaetxe *et al.* 2011; Guruprasad *et al.* 2013, 2014). The Wolbachia-based technology will assess a novel strategy for mosquito control by using virulent *Wolbachia* (Guruprasad *et al.* 2013, 2014). It will also deliver new tools for the accurate assessment of the

impact on population age structure in mosquitoes based on ***Wolbachia*** interventions (Guruprasad *et al*. 2013, 2014; Dutra *et al*. 2016; Jiggins, 2017). Dutra *et al* (2016) reported that *Wolbachia*-carrying mosquitoes are highly resistant to Zika virus and display reduced virus prevalence and intensity (Dutra *et al.* 2016). Saliva from *Wolbachia*-carrying mosquitoes did not contain infectious virus, suggesting the possibility to block Zika virus transmission (Dutra *et al.* 2016). As an alternative to traditional control measures, the bacterial symbiont *Wolbachia* has been transferred from *Drosophila* into the mosquito *Aedes aegypti*, where it can block the transmission of dengue and Zika viruses (Jiggins, 2017). A recent paper has reported large-scale releases of *Wolbachia*-infected *Ae.aegypti* in the city of Cairns, Australia (Jiggins, 2017). *Wolbachia*, which is maternally transmitted, invaded and spread through the populations due to a sperm±egg incompatibility called cytoplasmic incompatibility (Jiggins, 2017). Over a period of 2 years, a wave of *Wolbachia* infection slowly spread out from 2 release sites, demonstrating that it will be possible to deploy this strategy in large urban areas (Jiggins, 2017). In line with theoretical predictions, *Wolbachia* infection at a third, smaller release site collapsed due to the immigration of *Wolbachia*-free mosquitoes from surrounding areas (Jiggins, 2017). This remarkable field experiment has both validated theoretical models of *Wolbachia* population dynamics and demonstrated that this is a viable strategy to modify mosquito populations (Jiggins, 2017).

4) A monovalent DENV-1 candidate adjuvanted in alum was evaluated in non-human primate models (NHPs) using a three dose schedule (intradermal injection, biweekly intervals (Maves *et al.* 2011; Raviprakash *et al.* 2013; Vannice *et al.* 2015). Immunized monkeys with a purified psoralen-inactivated DENV-1 vaccine candidate developed by the NMRC showed a DENV-1 neutralizing antibody response (Maves *et al.* 2011; Raviprakash *et al.* 2013;Vannice *et al*. 2015). This dengue vaccine also showed a reduced duration of viraemia upon viral challenge on day 132 (Maves *et al*. 2011; Raviprakash *et al*. 2013; Vannice *et al.* 2015). This clearly indicates that the dengue vaccine candidate provides partial protection in non-human primate models (NHPs) (Maves *et al*. 2011; Raviprakash *et al*. 2013; Vannice *et al.* 2015). It has been recently shown that the binding capacity of a panel of monoclonal antibodies was reduced 30–60% when DENV-2 was inactivated by formaldehyde and iodonaphthyl azide compared to psoralen (Maves *et al*. 2011; Raviprakash *et al.* 2013; Vannice *et al*. 2015). Therefore, research work is still ongoing to establish the superior immunogenicity of psoralen inactivation compared to formalin. (Maves *et al.* 2011; Raviprakash *et al.* 2013; Vannice *et al*. 2015).

5) Very recently a collaboration between Chiang Mai University, **Mahidol University**, **Thailand** and the Thailand National Science and Technology Development Agency (NSTDA) developed a live attenuated dengue vaccine candidate (Keelapang *et al.* 2013; Vannice *et al.* 2015). DEN/DEN chimeric viruses were constructed which contain the prM/E coding region of recent dengue clinical isolates in the genetic background of attenuated DENV-2, including a pr-M cleavage enhancing mutation (Keelapang *et al.* 2013; Vannice *et al*. 2015). Furthermore, this study also confirmed that in non-human primate models (NHP's) that one dose better protects against viraemia when challenged with DENV-1 or DENV-2 compared to the chimeric virus without the pr-M cleavage enhancing mutation (Keelapang *et al.* 2013; Vannice *et al*. 2015). The monovalent DENV-1 prM + E-chimeric dengue vaccine is being planned for GMP manufacturing and toxicology studies in Thailand (Keelapang *et al.* 2013; Vannice *et al*. 2015). The live attenuated virus (LAVs) vaccine have the capacity to replicate in the host, thereby mimicking natural infection, and inducing similar immune responses (Bhamarapravati and Sutee, 2000; Keelapang *et al*. 2013; Vannice *et al*. 2015). Attenuation has been achieved through serial passage of wild-type (wt) virus on nonhuman host cells, in particular using primary dogkidney (PDK) cells (Bhamarapravati and Sutee, 2000; Keelapang *et al*. 2013; Vannice *et al.* 2015). Attenuated strains have classically been tested separately for safety and immunogenicity before being formulated into a tetravalent product. The first such vaccine was developed by Mahidol University, Thailand followed by other developers (Bhamarapravati and Sutee, 2000; Keelapang *et al*. 2013; Vannice *et al.* 2015).

6) Another tetravalent formulated dengue vaccine has been developed by **Takeda/Inviragen** (**Japan**) under a registered name as TDV (formerly known as DENVax) (Osorio *et al*. 2011, 2015; Pang and Loh, 2017). TDV was designed on the basis of live-attenuated DENV2 strain designated as PDK-53, which was then used as the genetic background for the other three chimeric viruses by replacing the prM and E proteins with wild-type DENV1, DENV3 and DENV4 (Osorio *et al*. 2011, 2015; Pang and Loh, 2017). Phase I clinical trials of TDV designated as PDK-53 was successful in inducing neutralizing antibodies against all four serotypes of dengue but tetravalent protection varied between 46% to 80% (Rupp *et al.* 2015; Sirivichayakul *et al*. 2016; Osorio *et al*. 2011, 2015; Pang and Loh, 2017). Furthermore, during Phase 2 clinical trials, TDV induced a neutralising antibody against all serotypes and the vaccine was well tolerated in all age groups irrespective of pre-vaccination dengue serostatus (Rupp *et al.* 2015; Sirivichayakul *et al*. 2016; Osorio *et al.* 2011, 2015; Pang and Loh, 2017). Another Phase 2 clinical trial of TDV is still ongoing among paediatric volunteers living in dengue endemic countries and results are yet to be declared (Rupp *et al*. 2015; Sirivichayakul *et al*. 2016; Takeda Clinical trials reports, 2016A, 2016B). In another separate clinical study programme, Takeda also marked Phase 3 clinical trials of TDV which will enrol 20,000 healthy children as a part of immunization programme against dengue hemorrhagic fever (DHF) (Rupp *et al.* 2015; Sirivichayakul *et al.* 2016; Takeda's Clinical Trials 2016A, 2016B .gov. Safety and immunogenicity of different

schedules of Takeda's tetravalent vaccine) (Pang and Loh, 2017).

Dengue vaccine candidate (TDV) is composed of an attenuated DENV-2 virus strain (TDV-2) and three chimeric viruses containing the pre-membrane and envelope protein genes of DENV-1, DENV-3, and DENV-4 genetically engineered into the attenuated TDV-2 genome backbone (TDV-1, TDV-3, and TDV-4) (Saez-Llorens *et al*. 2017). Furthermore, TDV is safe and immunogenic in individuals aged 2–17 years, irrespective of previous dengue exposure (Saez-Llorens *et al*. 2017). A second TDV dose induced enhanced immunogenicity against DENV-3 and DENV-4 in children who were seronegative before vaccination (Saez-Llorens *et al*. 2017). These data supported the initiation of phase 3 evaluation of the efficacy and safety of TDV given in a two-dose schedule 3 months apart, with analyses that taken into account baseline age and dengue serostatus (Saez-Llorens *et al.* 2017). The levels of immunogenicity induced by TAK-003 (also referred to as TDV) against all four dengue serotypes, even in seronegative participants, are encouraging because seropositivity after vaccination may be an important measure of vaccine performance (Saez-Llorens *et al.* 2017). Very recently Takeda Pharmaceutical Company Limited, Japan has also released data from their six-month interim analysis of the ongoing DEN-204 trial of its live-attenuated tetravalent dengue vaccine candidate, TAK-003 (also referred to as TDV) (Saez-Llorens *et al.* 2017). The trial investigated the safety and immunogenicity of TAK-003 (also referred to as TDV) in 1,794 participants ages two through 17 living in dengue-endemic areas (the Dominican Republic, Panama and the Philippines) (Saez-Llorens *et al*. 2017). At the time of the analysis, participants had either received one dose of TAK-003 (also referred to as TDV), two doses of TAK-003 (also referred to as TDV) administered three months apart, or a placebo (Saez-Llorens *et al.* 2017). On the basis of this study, TAK-003 (also referred to as TDV) elicited a broad antibody response against all four dengue virus serotypes, regardless of previous exposure to the dengue virus (Saez-Llorens *et al.* 2017). The increased presence of antibodies in the blood against the four serotypes ranged between 87-100 percent by month 1 and was sustained at month 6 (85-100 percent), in both the one-dose and two-dose groups (Saez-Llorens *et al.* 2017). This study analysis also showed that, in participants who were not previously exposed to dengue infection before vaccination, seropositivity rates against dengue virus types 3 and 4 were improved after a second dose of vaccine (Saez-Llorens *et al.* 2017). For this reason, a two-dose regimen, administered three months apart, was selected for Takeda's ongoing global pivotal Phase 3 efficacy trial (Saez-Llorens *et al.* 2017).

7) In another parallel effort, a monovalent vaccine **TV003** was jointly developed by National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) and Butantan Institute (Durbin *et al.* 2011, 2013; Pang and Loh, 2017). On the basis of the Phase 1 trial, TV003 was confirmed as the effective tetravalent candidate giving 90% of seropositivity after a single dose with a relatively weaker seroconversion against DENV2 like CYD (Durbin *et al*. 2011, 2013; Pang and Loh, 2017). As a part of the continuous study, optimization was done to generate another tetravalent candidate named as TV005 (with increased DENV2 dose) (Kirkpatrick *et al*, 2015, 2016; Whitehead, 2016; Pang and Loh, 2017). In another clinical challenging study against DENV2, maximum protection efficacy was confirmed by tetravalent TV003. However, TV005 showed higher immunogenicity as compared to TV003 (Pang and Loh, 2017). Therefore, Phase 3 clinical trials of TV005 are under progress and results are yet to be announced for the long term efficacy over TV003 (Kirkpatrick *et al*, 2015, 2016; Whitehead, 2016; Pang and Loh, 2017).

8) The Beijing Institute of Microbiology and Epidemiology, together with the Chengdu Institute of Biological Products and others, has developed a chimeric DENV based on the JE live vaccine strain SA 14-14-2 as a backbone (Chin DENV) (Li *et al.* 2013, 2014; Vannice *et al.* 2015). Mice vaccinated with Chin DENV were protected against lethal JEV challenge (Li *et al.* 2013, 2014; Vannice *et al*. 2015). All animals receiving these doses were protected from viraemia post challenge with DENV-2 virus (Li *et al*. 2013, 2014; Vannice *et al.* 2015).

9) The preclinical development of a dengue vaccine composed of recombinant subunit carboxy-truncated envelope (E) proteins (DEN-80E) for each of the four dengue serotypes has been reported by **Merck Research Laboratories**, USA, Hawaii Biotech Inc., USA, and Immune Design Corporation, USA (Govindarajan *et al.* 2015). During this study, immunogenicity and protective efficacy studies in Rhesus monkeys were conducted to evaluate monovalent and tetravalent DEN-80E vaccines formulated with ISCOMATRIX™ adjuvant (Govindarajan *et al*. 2015). Furthermore, three different doses and two dosing regimens (0, 1, 2 months and 0, 1, 2, and 6 months) were evaluated in these studies (Govindarajan *et al*. 2015). According to this study the two antigens, monomeric (DEN4-80E) and dimeric (DEN4-80EZip) versions of DEN4-80E evaluated at 6, 20 and 100 μg/dose formulated with ISCOMATRIX™ adjuvant, were equally immunogenic (Govindarajan *et al*. 2015). A group immunized with 20 μg DEN4-80E and Alhydrogel™ induced much weaker responses (Govindarajan *et al*. 2015). On the other hand when challenged with wild-type dengue type 4 virus, all animals in the 6 and 20 μg groups and all but one in the DEN4-80EZip 100 μg group were protected from viremia (Govindarajan *et al*. 2015). Two out of three monkeys in the Alhydrogel™ group had breakthrough viremia (Govindarajan *et al*. 2015). A similar study was conducted to evaluate tetravalent formulations at low (3, 3, 3, 6 μg of DEN1-80E, DEN2-80E, DEN3-80E and DEN4-80E respectively), medium (10, 10, 10, 20 μg) and high (50, 50, 50, 100 μg) doses (Govindarajan *et al*. 2015). All doses were comparably immunogenic and induced high titer, balanced neutralizing antibodies against all four DENV (Govindarajan *et al.* 2015). On the other hand upon challenge with the four

wild-type DENV, all animals in the low and medium dose groups were protected against viremia while two animals in the high-dose group exhibited breakthrough viremia (Govindarajan *et al*. 2015). This study also indicated that a 0, 1, 2 and 6 month vaccination schedule is superior to the 0, 1, and 2 month schedule in terms of durability (Govindarajan *et al.* 2015). Therefore, the subunit dengue vaccine which induced strong neutralization titers resulting in protection against viremia following challenge even 8-12 months after the last vaccine dose (Govindarajan *et al.* 2015). The vaccine adjuvant that has yielded the best neutralizing antibody responses (as compared to other evaluated adjuvants), in murine and non-human primates, is the saponin based ISCOMATRIX™ adjuvant (Govindarajan *et al.* 2015).

10) As a continuation of a previous study (Govindarajan *et al*. 2015), in another development by **Merck Research Laboratories** (Infectious Diseases and Vaccines), Merck & Co., Inc., Kenilworth, NJ, USA (Swaminathan *et al.* 2016), a novel dengue recombinant subunit vaccine candidate that contains truncated, recombinant, dengue virus envelope protein from all four dengue virus serotypes (DEN-80E) formulated with ionizable cationic lipid nanoparticles (LNPs) has been developed (Swaminathan *et al*. 2016). According to this study, immunization studies in mice, Guinea pigs, and in *Rhesus macaques*, revealed that ionizable cationic lipid nanoparticles (LNPs) induced high titers of dengue virus neutralizing antibodies, with or without co-administration or encapsulation of a Toll-Like Receptor 9 agonist (Swaminathan *et al.* 2016). Furthermore, ionizable cationic lipid nanoparticles (LNPs) were also able to boost DEN-80E specific CD4+ and CD8+ T cell responses (Swaminathan *et al.* 2016). Cytokine and chemokine profiling revealed that ionizable cationic lipid nanoparticles (LNPs) induced strong chemokine responses without significant induction of inflammatory cytokines (Swaminathan *et al.* 2016). In addition to being highly efficacious, the vaccine formulation proved to be well-tolerated, demonstrating no elevation in any of the safety parameters evaluated (Swaminathan *et al.* 2016). Notably, reduction in cationic lipid content of the nanoparticle dramatically reduced the LNP's ability to boost DEN-80E specific immune responses, highlighting the crucial role for the charge of the LNP (Swaminathan *et al*. 2016). Therefore, this study across multiple species, revealed a promising tetravalent dengue virus sub-unit vaccine candidate (Swaminathan *et al*. 2016). Hence ionizable cationic lipid nanoparticles (LNP) containing dengue sub-unit vaccine formulations are well-tolerated, and elicit strong DEN-80E specific B-cell and T-cell responses in rodents and non-human primates (Swaminathan *et al.* 2016; **Merck Research Laboratories**). In another combined efforts of Merck and NIAID, the vaccine candidate (V180) has recently completed the Phase 1 clinical trials for evaluation of its safety and immunogenicity profiles in healthy adults (ClinicalTrials.gov, 2016A, 2016B; Pang and Loh, 2017).

11) A live attenuated dengue vaccine lacking 2-O-methyltransferase activity has been developed by The Agency for Science, Technology and Research in **Singapore** together with the Novartis Institute for Tropical Diseases (NITD) (Zust *et al.* 2013; Vannice *et al.* 2015). This mutation prohibits the virus from shielding viral RNA from host innate immune factors, thereby triggering an interferon response in the infected cell (Zust *et al.* 2013; Vannice *et al.* 2015). The mutant viruses replicate to high titres in cell culture but they are highly attenuated in mice and non-human primate models (NHPs) (Zust *et al*. 2013; Vannice *et al.* 2015). A single dose of monovalent vaccine protected non-human primate models (NHPs) from viraemia when challenged with DENV-2 (Zust *et al*. 2013; Vannice *et al*. 2015). A 1/2 bivalent vaccine in mice did not diminish antibody responses compared to monovalent vaccines, suggesting a lack of interference, and were mostly protected against challenge with lethal doses of either DENV-1 or DENV-2 virus (Zust *et al*. 2013; Vannice *et al*. 2015). *Aedes aegypti* mosquitoes were fed blood containing wild type virus or vaccine; while even at low doses some mosquitoes were always infected, the mutant virus did not infect any mosquitoes (Zust *et al.* 2013; Vannice *et al.* 2015).

12) **TDEN PIV** is a tetravalent purified inactivated vaccine currently being evaluated jointly by **Glaxo SmithKline** (GSK) and Walter Reed Army Institute of Research (WRAIR) (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, and 2015C). A phase I study of high and low doses in flavivirus native adults has already been conducted in the US (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C). Additionally, Glaxo SmithKline (GSK) is testing TDEN PIV with several adjuvants used in other vaccines (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C). Aluminum hydroxide, AS01E, and AS03B have already been assessed as adjuvants with Glaxo SmithKline GSK's hepatitis B, malaria, and pandemic influenza vaccines, respectively (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C). A recent phase I clinical trial examined the safety and immunogenicity of TDEN PIV with these adjuvants at different doses in the US (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C). Another trial, scheduled to end December 2016, is examining the vaccine in Puerto Rican adults, a dengue primed population (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C). A prime-boost strategy with TDEN PIV and a live attenuated dengue vaccine is also under evaluation in a phase II trial (Schwartz *et al.* 2015; ClinicalTrials.gov, 2015A, 2015B, 2015C).

13) **Arbovax** has developed a live attenuated tetravalent vaccine using host range (HR) mutations to select for viruses that replicate well in insect cells but not in mammalian cells (Briggs *et al.* 2014; Vannice *et al*. 2015). This tetravalent formulation (called HR-Tet, composed of 106 PFU/mL of each vaccine strain) was tested for immunogenicity in non human primates (NHPs) (Briggs *et al.* 2014; Vannice *et al.* 2015). Vaccine viruses for each serotype were developed by truncating transmembrane domain 1 of the E protein (Briggs *et al.* 2014; Vannice *et al.* 2015). All

animals seroconverted to all four strains by 62 days post vaccination. HR-Tet has been approved by the FDA at the pre-IND stage for further development. It is anticipated that clinical studies will start in early 2016 (Briggs *et al*. 2014; Vannice *et al.* 2015).

14) Another new vaccination strategy for dengue virus (DENV) was evaluated in rhesus macaques by priming with tetravalent purified inactivated virus (TPIV) or tetravalent plasmid DNA vaccines expressing the structural prME gene region (TDNA) then boosting 2 months later with a tetravalent live attenuated virus (TLAV) vaccine (Simmons *et al.* 2010; Kanesa-Thasan *et al.* 2003). Both vaccine combinations elicited virus neutralizing (N) antibodies (Simmons *et al*. 2010; Kanesa-Thasan *et al.* 2003). The **TPIV/TLAV** combination afforded complete protection against DENV 3 challenge at month 8 (Simmons *et al*. 2010). In a second experiment, priming with TPIV elicited N antibodies against all four serotypes (GMT 1:28 to 1:43) (Simmons *et al*. 2010; Kanesa-Thasan *et al*. 2003). Boosting with TLAV led to an increase in the GMT for each serotype (1:500 to 1:1200 for DENVs 1, 3, and 4, and greater than 1:6000 for DENV 2), which declined by month 8 (GMT 1:62 for DENV 3, 1:154 for DENV 1, 1:174 for DENV 4, and 1:767 for DENV2) (Simmons *et al.* 2010; Kanesa-Thasan *et al.* 2003). After challenge with each one of the four DENV serotypes, vaccinated animals exhibited no viremia but showed anamnestic antibody responses to challenge viruses (Simmons *et al*. 2010; Kanesa-Thasan *et al.* 2003).

15) In another report, Themis Bioscience and **Institut Pasteur** in collaboration developed a virus-vectored dengue vaccine candidate (Brandler *et al.* 2010; Vannice *et al*. 2015). The virus vectored dengue vaccine is based on expression of a single tetravalent DENV antigen construct from a live attenuated measles virus vaccine vector (strain Schwarz) to produce MV-DEN (Brandler *et al*. 2010; Vannice *et al*. 2015). One of the main feature of this vaccine vector technology is that allows integration of large antigen inserts which has been shown to induce strong neutralizing antibodies and cellular immune responses even in the presence of pre-existing immunity to measles virus (Brandler *et al*. 2007, 2010; Vannice *et al*. 2015; Ramsauer *et al*. 2015). The dengue vaccine candidate expresses a construct containing the EDIII domains of DENV-1–4 as well as the DENV-1M protein ectodomain (ectoM) (Brandler *et al.* 2007, 2010; Vannice *et al.* 2015; Ramsauer *et al* 2015). Inclusion of ectoM in the dengue vaccine construct was found to provide an adjuvant activity (Brandler *et al*. 2007, 2010; Vannice *et al.* 2015; Ramsauer *et al.* 2015). The single component dengue tetravalent vaccine induced neutralizing antibodies against all DENV serotypes in mice (Vannice *et al.* 2015; Ramsauer *et al.* 2015) and non-human primate models (NHPs) (Ramsauer, personal communication). A Phase 1 clinical study for MV-DEN was under preparation and results were not yet declared (Brandler *et al*. 2007, 2010; Vannice *et al*. 2015; Ramsauer *et al.* 2015).

16) **Taiwanese** National Health Research Institutes (NHRI) reported the development of a single tetravalent dengue subunit vaccine (Vannice *et al.* 2015; Leng *et al.* 2009; Chang *et al.* 2003; Chen *et al*. 2013; Chiang *et al*. 2011, 2013, 2014). During this approach, a consensus EDIII amino acid sequence was derived by sequence analysis of different dengue virus-1–4 strains to avoid immune interference between the serotypes, and the consensus EDIII protein was expressed in *E. coli* (Vannice *et al*. 2015; Leng *et al*. 2009; Chang *et al*. 2003; Chen *et al*. 2013; Chiang *et al*. 2011, 2013, 2014). Furthermore, single tetravalent vaccine candidate adjuvanted with alum induced neutralizing antibody responses against all DENV serotypes in mice (Leng *et al*. 2009; Vannice *et al*. 2015). When evaluated in non-human primate models (NHPs) on an immunization schedule of two doses over 8 weeks, 2/3 of the monkeys developed neutralizing antibody titres again DENV-2, but not against the other three serotypes (Chen *et al*. 2013; Vannice *et al*. 2015). Aluminium adjuvant was critical to generate these responses (Chen *et al*. 2013; Vannice *et al*. 2015). Additionally, Taiwanese National Health Research Institutes (NHRI) has evaluated a recombinant lipidated EDIII, both monovalent and the consensus EDIII (Chiang *et al*. 2011, 2013, 2014; Vannice *et al*. 2015) to improve the immunogenicity without the need for adjuvant (Chen *et al*. 2013; Chiang *et al*. 2011, 2013, 2014; Vannice *et al*. 2015). In mice, neutralizing antibody titres were significantly higher with a monovalent dengue 4 lipidated EDIII than for the non-lipidated (Chiang *et al*. 2014; Vannice *et al*. 2015). This has significantly reduced viraemia when challenged with DEN-4 (Chiang *et al*. 2014; Vannice *et al*. 2015).

17) Very recently the Oswaldo Cruz Foundation**, FIOCRUZ** reported the development of a live attenuated dengue vaccine candidate (Robert-Putnak *et al*. 2005; Caufour *et al*. 2001; Galler *et al*. 2005; Mateu *et al*. 2007; Trindade *et al*. 2008; Vannice *et al*. 2015). This dengue vaccine candidate utilizes the live attenuated yellow fever vaccine 17DD sub-strain as a genetic backbone (Caufour *et al*. 2001; Vannice *et al*. 2015). Chimeric YF 17D/DEN viruses were constructed by replacing the Yellow fever virus prM/E genes with those of DENV strains from Latin America (Caufour *et al*. 2001; Vannice *et al.* 2015). Neutralizing antibody responses and protection against viral challenge have been confirmed (Galler *et al*. 2005; Mateu *et al*. 2007; Trindade *et al*. 2008; Vannice *et al*. 2015).

18) A recombinant sub unit dengue vaccine candidate which includes the ectodomain of the envelope (80E) antigen genetically fused to bacterial flagellin, a TLR5 ligand from Salmonella *typhimurium flagellin* (STF2) has been reported by **VaxInnate** (McDonald *et al*. 2007; Vannice *et al*. 2015). Physical linkage of TLR ligand to antigen has been shown to be more specific and efficient than co-administration (McDonald *et al*. 2007; Liu *et al*. 2015; Vannice *et al*. 2015). Furthermore, STF2 fusion proteins can be expressed and purified in baculovirus/insect cells. In a parallel study, four lead monovalent candidates using 80E

have been identified in mouse studies (McDonald *et al*. 2007; Liu *et al*. 2015; Vannice *et al*. 2015). This kind of expression platform has been used for influenza vaccines in clinical development and was well-tolerated and found to be immunogenic (Treanor *et al*. 2010; Taylor *et al*. 2011; Vannice *et al*. 2015).

19) **Global Vaccines approach** is based on an adjuvanted inactivated DENV (iDV) vaccine using adjuvants **GVI3000 and GVI3A**, based on VEE replicon particles (Vannice *et al*. 2015). These adjuvant genomes lack VEE structural protein genes or heterologous antigen genes (Vannice *et al*. 2015). Studies of monovalent and tetravalent dengue vaccines with adjuvant have been completed in mice and non-human primate models (NHPs) and demonstrated enhanced neutralizing antibody titres, detectable T cell responses, and protective efficacy (Vannice *et al*. 2015; White, personal communication). A virus-vectored vaccine candidate developed at the University of North Carolina at Chapel Hill (UNC), USA and Global Vaccines is based on expression of DENV antigens from a single-VEE virus vaccine vector (Khalil *et al*. 2014; White *et al*. 2007, 2013; Vannice *et al*. 2015; Malabadi *et al*. 2016a). The use of virus vectors plays an important role in the production of vaccine (Malabadi *et al*. 2016a). In addition to this, VEE VRPs have been shown to infect human dendritic cells and express high levels of recombinant antigens, inducing both innate and adaptive immune responses in mice and non-human primate models (NHPs) (Khalil *et al*. 2014; White *et al*. 2007, 2013; Vannice *et al*. 2015). Two doses of a tetravalent E85-VRP vaccine (108 IU of each serotype-VRP) given 6 weeks apart induced 100% seroconversion against all 4 dengue serotypes, with no evidence of interference (Khalil *et al*. 2014; White *et al*. 2007, 2013; Vannice *et al*. 2015). Animals were challenged 18 weeks after the second vaccination with each serotype. Viraemia was undetectable after DENV-3 and DENV-4 challenges, and there was a significant reduction in viraemia duration after challenge with DENV-1 and DENV-2 (White *et al*. 2007, 2013; Vannice *et al*. 2015). Therefore, VRP vaccine may be able to overcome interfering maternal antibodies (White *et al*. 2007, 2013; Vannice *et al*. 2015). Tetravalent E85-VRP vaccination induced similar antibody and T cell responses to monovalent vaccine, and could be boosted (Khalil *et al*. 2014; White *et al*. 2007, 2013; Vannice *et al*. 2015).

20) A domain III-capsid (DIII-C), a tetravalent vaccine has been developed based on a combined approach tested only in mice (Valdes *et al*. 2009; Vannice *et al*. 2015, 2016). This tetravalent dengue vaccine has been developed by the Pedro Kourí Tropical Medicine Institute (IPK) and the Centre for Genetic Engineering and Biotechnology (CIGB) in **Cuba** (Valdes *et al*. 2009; Vannice *et al*. 2015). In the first approach, the DENV EDIII protein was fused to the carrier protein p64k of *Neisseria meningitidis* for each of the four serotypes (Valdes *et al*. 2009; Vannice *et al*. 2015, 2016). The EDIII-p64k fusion protein was expressed in *Escherichia coli* (Valdes *et al*. 2009; Vannice *et al*. 2015). Monkeys were immunized subcutaneously with four doses of the monovalent vaccine (50–100 g protein per dose, formulated in Freund's adjuvant) (Valdes *et al*. 2009; Vannice *et al*. 2015). The monovalent vaccine candidates were found to be immunogenic and provided protection against viral challenge (Hermida *et al*. 2006; Bernardo *et al*. 2008; Suzarte *et al*. 2014; Valdes *et al*. 2009; Vannice *et al*. 2015). The second approach used a DENV EDIII-capsid fusion protein (DIIIC-2) expressed in *E. coli* and mixed *in vitro* with oligo deoxynucleotides to obtain particulated aggregates (serotype 2) (Suzarte *et al*. 2014; Valdes *et al*. 2009;Vannice *et al*. 2015). The aggregated DENV-2 EDIII-capsid fusion protein aggregated with a specific oligodeoxynucleotide, selected for high cellular immune response, and adjuvanted in alum induced both humoral and cellular immune responses in non-human primate models (NHPs) and completely protected two of three animals immunized with four doses (Gil *et al*. 2015; Izquierdo *et al*. 2014; Vannice *et al*. 2015). When these two approaches were combined into a tetravalent formulation and evaluated in mice, the antibody response to serotype 4 was low (GMT < 1:10) and survival post challenge ranged from 40% to approximately 70% for DENV-1, DEVN-2, and DENV-4 (DENV-3 virus challenge did not reduce survival for any animal) (Izquierdo *et al*. 2014; Vannice *et al*. 2015). The tetravalent formulation of EDIII-capsid fusion protein is currently under evaluation in non-human primate models (NHPs) (Valdes *et al*. 2009, 2010, 2011; Vannice *et al*. 2015).

2) Dengue virus: DNA vaccine

DNA vaccine technology has been considered as one of the novel and simplest approach for immunization programmes (Zheng *et al*. 2017; Slon Campos Jose Luis, 2017). DNA vaccines have been tested for inducing serum antibodies against various antigens such as bacteria, virus, parasites and tumors (Ulmer, 2002). In addition to this, mucosal antibodies were also induced by using DNA vaccines against certain pathogens for example influenza virus (Ulmer, 2002). Therefore, **DNA vaccine** technology has been introduced as one of the new and reliable mode of vaccination. DNA vaccines are more stable than any other traditional vaccine and several different DNA vaccines can be mixed as one combined priming immunization (Xu *et al.* 2009; Zheng *et al*. 2017; Slon Campos Jose Luis, 2017). There are many cases where DNA vaccines were found effective, and some of them appear to be very promising in inducing neutralizing antibodies (Ulmer, 2002; Zheng *et al.* 2017). Furthermore, **DNA vaccines** are simple, not infectious, contains *E-coli* plasmids, do not replicate during and after immunization programmes, and encode only proteins of interest (Ulmer, 2002). DNA vaccines contain the sequence for target antigens and sequence coding for immune adjutants. Both the sequences are present in the same construct and presented at the same time too. Also there is no protein component in the DNA vaccine, and therefore, the plasmid inserted in a particular vector can induce a broad spectrum of protective immune responses for the corresponding antigen (Ulmer, 2002). On the other hand one of the major drawbacks

of DNA vaccines is that plasmids are effective and immunogenic only in small animals but less effective in large animals, including non-human primates (NHPs) and humans (Dobano *et al.* 2007). This might be due to the fact that inefficient uptake of DNA by cells *in situ*. Generally high doses of DNA were required to induce quality immunogenicity in large animals (Dobano *et al.* 2007). However, high doses of DNA vaccine might create health problems in terms of high toxicity of viral particles in larger animals including human models and hence failed to solve the fundamental issues of controlling the viral infections. Therefore, toxicity and immunogenicity are two different factors which play an important role in the development of DNA vaccine against viral diseases.

Dietrich *et al.* (2001) reported a novel approach which employs attenuated mutant strains of Gram positive and Gram negative intracellular bacteria as carriers for the delivery of DNA vaccines. This method of approach highlights the direct delivery of plasmid DNA to macrophages and antigen presenting cells, dendritic cells (DC), and thus Gram negative bacteria could be exploited for delivering plasmid vectors to human DC (Dietrich *et al*. 2001). Therefore, DNA vaccine has proven to induce strong immune responses, able to mediate prevention or therapy of infectious diseases in small animal models (Dietrich *et al.* 2001). In general, **DNA vaccines** are very effective in mice models than large animals, and this might be due to decreased transfection efficiency leading to a low level expression of plasmid vectors in large animals (Babiuk *et al*. 2003). Babiuk *et al*. (2003) reported the enhanced immune responses using different methods inducing gene gun delivery or suppositories as delivery vehicles to mucosal surfaces, as well as electroporation for systemic immunization (Babiuk *et al.* 2003). Two different antigens—a membrane antigen from bovine herpesvirus glycoprotein (BHV-1) gD and a particulate antigen from hepatitis virus B has been used (Babiuk *et al.* 2003). Gene gun and suppository delivery of BHV-1 gD to the vagina resulted in the induction of mucosal immunity not only in the vagina, but also at other mucosal surfaces (Babiuk *et al*. 2003). Therefore, this study showed the contention of a common mucosal immune system (Babiuk *et al*. 2003). Furthermore, significant enhancement of gene expression following electroporation with surface electrodes (non-invasive electroporation) as well as invasive electroporation using single or six-needle electrodes has been observed (Babiuk *et al.* 2003). On the basis of these studies it was confirmed that various combinations of delivery systems can enhance immunity to DNA-based vaccines and make them practical for administration of these vaccines in large animals (Babiuk *et al.* 2003). Furthermore, Wang *et al.* (2004) evaluated the immune response by a DNA vaccine encoding ESAT6 protein of *Mycobacterium tuberculosis* by DNA prime-protein protocol in a mouse model. This study confirmed that the formation of ESAT6 DNA prime protein boost inoculation could improve antigen specific cellular immune responses, which is an important factor for protection against TB infection (Wang *et al.* 2004). Therefore, DNA vaccines offer a promising alternative to conventional vaccines, and DNA prime-protein boost protocol could be used as a new strategy to improve the efficacy of TB DNA vaccine (Wang *et al*. 2004). Yoshida *et al*. (2006) reported the immunogenicity and protective efficacy of **DNA vaccine** combinations expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) using gene gun bombardment and the hemagglutinating virus of Japan (HVJ)-liposome method. In this study, a mouse IL-12 expression vector (mIL-12 DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed, and a high degree of protection against challenge with virulent *Mycobacterium tuberculosis*; bacterial numbers were 100-fold lower in the lungs compared to BCG-vaccinated mice has been noticed (Yoshida *et al*. 2006). The HVJ-liposome method improved the protective efficacy of the Hsp65 DNA vaccine compared to gene gun vaccination (Yoshida *et al*. 2006). Hsp65 + mIL-12/HVJ induced CD8+ cytotoxic T lymphocyte activity against Hsp65 antigen (Yoshida *et al*. 2006). Most importantly, Hsp65 + mIL- 12/HVJ vaccination resulted in a greater degree of protection than that evoked by BCG (Yoshida *et al*. 2006). This protective efficacy was associated with the emergence of IFN- secreting T cells and activation of proliferative T cells and cytokines (IFN- and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis* (Yoshida *et al*. 2006). These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine (Yoshida *et al*. 2006).

Dobano *et al.* (2007) demonstrated the application of electroporation (EP) to increase immune responses to DNA vaccines encoding the pre-erythrocytic *Plasmodium yoelii* antigens PyCSP and PyHEP17 to mice (Dobano *et al.* 2007). This study concluded that immunization with 5 µg of DNA *via* electroporation (EP) was equivalent to 50 µg of DNA *via* conventional needle, thus reducing by 10-fold the required dose to produce a given effect (Dobano *et al*. 2007). This has also increased the effect of IFN_ responses by 2 fold in malaria vaccine in rodent models (Dobano *et al.* 2007). Therefore, this study demonstrated the potential of EP as an approach to increase the DNA vaccine induced immunity towards clinical application against plasmodium and other infectious agents (Dobano *et al.* 2007). In another report, an application of a short CD11c promoter based DNA vaccines has been considered as one of the safest tool for inducing antitumor immunity in mouse tumor models (Ni *et al.* 2009). This has been used for inducing selective antigen expression in dendritic cells, and successfully induced specific B- and T-cell responses including IFN secretion in mouse tumor models (Ni *et al.* 2009). Therefore, this approach provides a unique strategy for DC-targeted vaccines which induces DC-specific gene expression (Ni *et al.* 2009). In another subsequent study, DNA vaccine expressing a cholera toxin B subunit (CTB) showed both systemic and mucosal anti-CTB antibody responses in mice (Xu *et al*. 2009). This study demonstrated

that DNA vaccination plays an important role in the development of novel vaccination programmes against mucosally transmitted diseases (Xu *et al*. 2009). DNA vaccine is also effective in priming the immune system for antigens delivered at gut mucosal sites (Xu *et al.* 2009).

Several traditional approaches towards the development of vaccine against four dengue serotypes have failed, and **DNA vaccine** as a new approach might play an important role in controlling the dengue disease (Khan, 2013). Therefore, there is an urgent need for DNA vaccine development as the control measure to combat the dengue disease. DNA vaccine could produce long lasting neutralizing antibodies against all four serotypes of dengue. Raviprakash *et al.* (2000) reported that mice immunized by a candidate DNA vaccine produced dengue specific neutralizing antibodies. Furthermore, this study also confirmed that the immunity was long lasting (Raviprakash *et al*. 2000). The plasmid expressing prM and full length E produced virus particles in transfected cells, and showed a positive sign of immunogenicity in mouse models (Raviprakash *et al*. 2000). A DNA vaccine named as pcD2ME incorporated with envelope (E) and pre-membrane (prM) genes of New Guinea C strain of dengue type 2 viruses has been developed and immune responses have been tested in mouse model (Konishi *et al*. 2000). During this study, CHO-K1 cells were transfected with pcD2ME plasmid for the prM and envelope gene expression (Konishi *et al.* 2000). A low level of neutralizing antibody (1:10 at a 90% plaque reduction) development was observed in mouse models inoculated intramuscularly with 100 mg of pcD2ME two or three times at an interval of 2 weeks (Konishi *et al.* 2000). On the other hand immunization twice with 10 mg or 1 mg of pcD2ME or three times with 100 mg of pcDNA3 failed to induce detectable levels of neutralizing antibody (Konishi *et al.* 2000). Mice immunized two or three times with 100 mg of pcD2ME raised neutralizing antibody titers to 1:40 or greater on days 4 and 8 after challenge with 3×10^5 plaque forming units (PFU) of the New Guinea C strain of dengue type 2 virus, showing strong anamnestic responses to the challenge (Konishi *et al.* 2000). In contrast, mice immunized two or three times with 100 mg of pcDNA3 developed no detectable neutralizing antibody on days 4 and 8 after challenge (Konishi *et al.* 2000). These results indicate that immunization with pcD2ME induces neutralizing antibody and dengue type 2 virus-responsive memory B cells in mice (Konishi *et al.* 2000). In another parallel study, Konishi *et al.* (2006) developed a dengue tetravalent DNA vaccine consisting of plasmids expressing premembrane and envelope genes of each of four serotypes of dengue viruses (Konishi *et al.* 2006). BALB/c mice immunized twice with the tetravalent vaccine at a dose of 100 µg (25 µg for each serotype) using a needle-free jet injector developed neutralizing antibodies against all serotypes (Konishi *et al.* 2006). There was no interference among the four components included in this combination vaccine (Konishi *et al.* 2006). Tetravalent vaccine-immunized mice showed anamnestic neutralizing antibody responses following challenge with each dengue serotype: responses to challenges from serotypes different to those used for neutralization tests were also induced (Konishi *et al.* 2006).

A first successful report of DNA dengue vaccine has been tested in 22 healthy adult volunteers in USA (Beckett *et al*. 2010). The US Naval Medical Research Centre (NMRC) have produced the most advanced DNA vaccine candidates and the only published a Phase 1 study using a plasmid expressing the PrM and E proteins of DENV1 (D1ME100) (Beckett *et al*. 2010). During DNA vaccine immunization programme, a monovalent DENV-1 DNA vaccine candidate has been utilized and the vaccine (D1ME100) elicited neutralizing antibody responses in 5 of 12 (41.6%) subjects in the high dose (5.0 mg) dose group (Beckett *et al*. 2010). D1ME100 is a closed circular double-stranded plasmid DNA molecule produced under current Good Manufacturing Practices conditions in the United States by Althea Technologies, Inc. (San Diego, CA) (Beckett *et al*. 2010). The **D1ME100** vaccine construct expresses the prM and E genes of DENV-1 virus under the control of the human cytomegalovirus promoter/enhancer of plasmid vector VR1012 (Beckett *et al*. 2010). The final vaccine product consisted of 1.2 milliliters (mL) of a sterile, phosphate buffered saline (PBS) in glass vials, with a DNA concentration of 5.0mg/mL (Beckett *et al*. 2010). Furthermore, during this study, a concentration of 1.0 mg/mL has been used as the low dose group and, the DNA vaccine was diluted in normal saline per study specific procedures (Beckett *et al*. 2010). Beckett *et al*. (2010) have also confirmed that five subjects (41.6%) in the high dose group and none in the low dose group developed detectable anti-dengue neutralizing antibodies. T-cell IFN gamma responses were detected in 50% (4/8) and 83.3% (10/12) of subjects in the low and high dose groups, respectively. Therefore, safety profile of the DENV-1 DNA vaccine is acceptable at both doses administered in the study (Beckett *et al*. 2010). These results demonstrated a favorable reactogenicity and safety profile of the first in human evaluation of a DENV-1 DNA vaccine (Beckett *et al*. 2010). Due to low immunogenicity, the vaccine was reformulated using a new adjuvant named Vaxfectin® that increased the immune response induced by tetravalent DNA vaccine in non-human primates (NHPs) (Porter *et al*. 2012; Porter and Raviprakash, 2015). Tetravalent DNA vaccine formulations are well-tolerated and safe in humans and low immunogenicity remains a main concern (Danko *et al*. 2011; Coban *et al*. 2011).

Poggianella *et al*. (2015) reported a novel design of a DNA immunisation strategy which induced strong antibody responses with high neutralisation titres in mice against all four viral serotypes (Poggianella *et al*. 2015). The immunogenic molecule is an engineered version of the domain III (DIII) of the virus E protein fused to the dimerising CH3 domain of the IgG immunoglobulin H chain (Poggianella *et al*. 2015). The DIII sequences were also codon-optimised for expression in mammalian cells (Poggianella *et al*. 2015). According to this study, DIII alone

was very poorly secreted; the codon-optimised fusion protein is rightly expressed, folded and secreted at high levels, thus inducing strong antibody responses (Poggianella *et al*. 2015). Mice were immunised using gene-gun technology, and the vaccine was able to induce neutralising titres against all serotypes (Poggianella *et al*. 2015). Additionally, all sera showed reactivity to a recombinant DIII version and the recombinant E protein produced and secreted from mammalian cells in a mono-biotinylated form when tested in a conformational ELISA (Poggianella *et al*. 2015). Sera were also highly reactive to infective viral particles in a virus capture ELISA and specific for each serotype as revealed by the low cross-reactive and cross-neutralising activities (Poggianella *et al*. 2015). The serotype specific sera did not induce antibody dependent enhancement of infection (ADE) in non-homologous virus serotypes (Poggianella *et al*. 2015). Therefore, this study confirmed that a tetravalent immunisation protocol in mice showed induction of neutralising antibodies against all four dengue serotypes (Poggianella *et al*. 2015). Furthermore, Slon Campos Jose Luis, (2017) confirmed that efficiency of DNA vaccine was determined by the antigen secretion (Slon Campos Jose Luis, 2017). Slon Campos Jose Luis, (2017) also developed a novel DNA gene-gun immunisation strategy using an engineered version of DIII fused to the CH3 domain of the IgG H chain, which is efficiently secreted from transfected cells and induced strong antibody responses that neutralise all dengue serotypes (Slon Campos Jose Luis, 2017). The antibody responses were stable over long periods of time and different tetravalent formulations of the vaccine showed induction of neutralising antibodies against all four dengue serotypes (Slon Campos Jose Luis, 2017). The results of the study conducted by Slon Campos Jose Luis, (2017) also indicated that the polyclonal antibody responses against DI/DII are highly cross-reactive, poorly neutralising and promote ADE towards all dengue serotypes, Zika virus, WNV and YFV. Conversely, anti-DIII antibodies are type-specific, with no ADE towards related flaviviruses, and with strong neutralisation activity restricted only to dengue (Slon Campos Jose Luis, 2017).

Very recently the immunogenicity and protection efficiency of **DNA vaccine** candidate pVAX1-D1ME expressing the prME protein of dengue serotype1 was evaluated by vaccination via intramuscular injection or electroporation in BALB/c mice (Zheng *et al*. 2017). These results were compared with traditional intramuscular injection, administration with 50 µg pVAX1-D1ME with three immunizations *via* electroporation induced persistent humoral and cellular immune responses (Zheng *et al*. 2017). Furthermore, DNA vaccination effectively protected mice against lethal dengue serotype 1 (DV1) challenge (Zheng *et al*. 2017). In addition, immunization with a bivalent vaccine consisting of pVAX1-D1ME and pVAX1-D2ME *via* electroporation generated a balanced IgG response and neutralizing antibodies against dengue serotype 1 (DV1) and dengue serotype 2 (DV2). This could protect mice from lethal challenge with DV1 and DV2 (Zheng *et al*. 2017). Therefore, this study showed a positive response for the development of dengue tetravalent DNA vaccine (Zheng *et al*. 2017). In another development, research of phase 3 clinical trials of the attenuated dengue vaccine developed by the NIH in Brazil (NIAID News Releases, 2016) has been highlighted (Zheng *et al*. 2017). Therefore, it is necessary to develop safer, more economical and effective dengue virus vaccines (Zheng *et al*. 2017). However, DNA vaccination has not yet achieved much success in large animals, mainly due to the insufficient immunogenicity, and no licensed DNA vaccine is currently available in humans (Zheng *et al*. 2017). The immune response of DNA vaccines have been modified by improving DNA uptake, antigen expression and immune stimulation (Porter and Raviprakash, 2015). Another major concern about DNA vaccine technology is that the possibility of integration into the host genome leading to the induction of anti-DNA antibodies that could lead to the development of autoimmune diseases. Therefore, practical implementation of DNA vaccines remained difficult process. Therefore, till today there is no safe and effective DNA vaccine available against dengue virus infection. Gene delivery and nanomedicine approaches become more popular strategies in controlling human diseases (Khan *et al*. 2011; Khan *et al*. 2012).

3) Dengue virus: Plant based vaccine

Plants have been utilized as a photosynthetic factory for the production of high value proteins vaccines, enzymes, biopharmaceuticals, and chemicals etc (Malabadi, 2008; Malabadi *et al*. 2010, 2011; Malabadi *et al*. 2012a; Ma and Wang, 2012; Malabadi *et al*. 2016b, 2016c, 2016 d;Kim *et al*. 2016; Laere *et al*. 2016). The phytochemicals used as botanical drugs plays an important role in controlling many human diseases and now a days *in vitro* micropropagation protocols of many medicinal plants have been developed for the pharmaceutical applications (Malabadi and Nataraja, 2001, 2002; Malabadi, 2002a, 2002b, 2005; Malabadi *et al*. 2004, 2005a, 2009, 2011, 2005b; Malabadi and Vijay Kumar, 2005, 2007, 2008; Malabadi *et al*. 2007, 2010; 2012a, 2012b,2012c, 2012d; Chalannavar *et al*. 2011, 2012, 2013a, 2013b, 2015a, 2015b; Narayanaswamy *et al*. 2013, 2014a, 2014b; Laere *et al*. 2016; Malabadi *et al*. 2016b, 2016c, 2016d). Many useful drugs from plants have been discovered by following up ethnomedical uses (Gleiser *et al*. 2007, 2011; Gillij *et al*. 2008; Malabadi *et al*. 2007, 2010; Narayanaswamy *et al*. 2013, 2014a, 2014b; Laere *et al*. 2016; Malabadi *et al*. 2016b, 2016c, 2016d). For example, few medicinal plants such as insulin plant, *Costus speciosus* (Malabadi, 2002a, 2005; Malabadi *et al*. 2004, 2005a; Malabadi *et al*. 2016), *Catharanthus roseus* (Malabadi *et al*. 2009), *Clitoria ternatea* (Malabadi and Nataraja, 2001, 2002; Malabadi, 2002b; Malabadi *et al*. 2005b), and papaya (*Carica papaya* L.) **(**Subenthiran *et al*. 2013; Siddique *et al*. 2014; Malabadi *et al*. 2011) have been tested to control various human disorders. The use of papaya leaf juice significantly increased the platelet count among dengue infected patients (Ahmad *et al*. 2011; Subenthiran *et al*. 2013; Siddique *et al*. 2014).

The attempt to produce **vaccines in plants** was made by Hiatt and coworkers in 1989 (Saxena and Rawat, 2014; Laere *et al*. 2016). The concept of utilizing transgenic plants to produce and deliver subunit vaccines was introduced by Dr. Arntzen and his colleagues and proved that this concept can overwhelm the limitations in traditional vaccine production (Haq *et al*. 1995; Thanavala *et al*. 1995; Mason *et al*. 1996; Tacket *et al*. 2000; Huang *et al*. 2006; Saxena and Rawat, 2014; Malabadi *et al*. 2012a; Laere *et al*. 2016). The first subunit vaccine was produced by them in tobacco plants by expressing surface protein antigen of *Streptococcus mutants* (Saxena and Rawat, 2014; Malabadi *et al*. 2012a; Laere *et al*. 2016). They also initiated the production of hepatitis B and heat-labile toxin B subunit in potato tubers as well as potato plants (Saxena and Rawat, 2014; Malabadi *et al*. 2012a; Laere *et al*. 2016). In 1998, it was proven, for the first time, by National Institute of Allergy and Infectious Diseases (NIAID) that significant immunogenicity can be induced safely by an edible vaccine utilizing the concept of plants as bioreactor (Saxena and Rawat, 2014; Malabadi *et al*. 2012a; Laere *et al*. 2016; Malabadi *et al*. 2016b). Genetic transformation particularly plastid transformation in plants has been used for the production of vaccines (Gottschamel *et al*. 2016). However, high-level accumulation of recombinant proteins in chloroplasts can also have a negative impact on plant growth (Gottschamel *et al*. 2016). In genetic transformation, transient gene expression *via Agrobacterium* or partical bombardment (Malabadi and Nataraja, 2003, 2007a, 2007b, 2007c, 2007d) plays an important role in the botanical vaccines (Gottschamel *et al*. 2016). The use of plants for the production of vaccines could be a promising tool to help reduce the spread of dengue fever. In addition to this plant products are the natural sources of substances which are biodegradable and help to control dengue (Malabadi *et al*. 2016b; Kim *et al*. 2016; Laere *et al*. 2016). **Plant derived vaccines** are very simple, safe for consumption and low production cost which is very beneficial for the developing countries. Botanical vaccines are also very important due to their potential for protecting the protein antigen in gastric acid environment (Malabadi *et al*. 2016b; Kim *et al*. 2016; Laere *et al*. 2016). Plant made vaccines provide many benefits to the vaccine industry but there are still challenges that limit the rate of successful production of these third-generation vaccines (Kim *et al*. 2016; Laere *et al*. 2016; Malabadi *et al*. 2016b). However, such vaccines have been explored for the past 30 years but unfortunately none has yet been licensed. As reviewed by Laere *et al*. (2016), plant based vaccine approach faces many problems like 1) how to increase the antigen concentration in the transgenic plants, 2) immunogenicity of plant based vaccines, 3) consistency of dosage which vary from one plant species to another, 4) major challenge to maintain Good Manufacturing Practice (GMP) standard for the product in plant-based vaccine industry (Laere *et al*. 2016). There are many examples of antigens of human infectious diseases have been introduced into plant cells and tested as potential oral vaccines (Malabadi, 2008; Malabadi *et al*. 2012a; Malabadi *et al*. 2016b; Chan and Daniell, 2015; Kim *et al*. 2016).

Recently Gottschamel *et al*. (2016) reported a dengue recombinant subunit vaccine candidate using tobacco plants as the production platform (Gottschamel *et al*. 2016). Chloroplast genome engineering was applied to express serotype specific recombinant dengue envelop domain EDIII proteins in tobacco chloroplasts using both constitutive and ethanol-inducible expression systems (Gottschamel *et al*. 2016). Expression of a tetravalent antigen fusion construct combining envelop protein domain EDIII polypeptides from all four serotypes was also attempted (Gottschamel *et al*. 2016). Transplastomic envelop protein domain EDIII expressing tobacco lines were obtained and homoplasmy was verified by Southern blot analysis (Gottschamel *et al*. 2016). Northern blot analyses showed expression of dengue envelop domain EDIII antigen-encoding genes (Gottschamel *et al*. 2016). The dengue envelop domain EDIII protein accumulation levels varied for the different recombinant envelop domain EDIII proteins and the different expression systems, and reached between 0.8 and 1.6 % of total cellular protein (Gottschamel *et al*. 2016). This study confirmed the suitability of the chloroplast compartment as a production site for an envelop protein domain EDIII-based vaccine candidate against dengue fever and presents a Gateway plastid transformation vector for inducible transgene expression (Gottschamel *et al*. 2016). The envelop protein domain III (EDIII) induces serotype-specific antibodies (Gottschamel *et al*. 2016). Although it has low intrinsic potential for eliciting cross-reactive antibodies against heterologous serotypes (Gottschamel *et al*. 2016). Therefore, Gottschamel *et al*. (2016) reported the expression of dengue virus envelop protein domain III-based tetravalent fusion protein (EDIII-1-4) and the monovalent forms (EDIII-1, EDIII-3 and EDIII-4) in tobacco chloroplasts (Gottschamel *et al*. 2016). The chloroplast expression system offers transgene confinement, high levels of foreign protein expression and highly precise, site-specific transgene integration by homologous recombination (Gottschamel *et al*. 2016). Therefore, dengue envelop protein domain III (EDIII) has emerged as one of the most promising region for subunit vaccine development (Gottschamel *et al*. 2016). In this work, an ethanol-inducible expression system has been employed which is based on a nuclear-encoded and plastid-targeted T7 RNA polymerase for the expression of EDIII-4 and EDIII-1-4 (Gottschamel *et al*. 2016). So far, only the expression of a dengue virus serotype 3 premembrane and envelope polyprotein has been reported in plastids (Kanagaraj *et al*. 2011; Gottschamel *et al*. 2016). The transformation of the plastid genome represents a promising possibility for the high-level, cost-effective, clean and safe expression of therapeutically relevant proteins in commercial applications (Wani *et al*. 2015; Gottschamel *et al*. 2016). Therefore, this study confirmed that plants can be highly competitive production platforms for vaccines and other biopharmaceuticals (Tuse *et al*. 2014; Gottschamel *et al*. 2016).

Recombinant antigens based on dengue envelop protein domain (EDIII) have been produced using bacteria,

yeast, insect cells and plants (Batra *et al*. 2007, 2010a, 2010b; Cardoso *et al*. 2013; Clements *et al*. 2010; Etemad *et al*. 2008; Martinez *et al*. 2010; Saejung *et al*. 2007; Gottschamel *et al*. 2016). Importantly, a recombinant fusion protein linking the EDIII domain of the four dengue viruses as a tetravalent antigen (EDIII-1-4) was able to elicit neutralizing antibodies against all four serotypes (Batra *et al*. 2007; Etemad *et al*. 2008; Gottschamel *et al*. 2016). A gene fragment encoding domain III of the dengue 2 envelope protein (D2EIII) was successfully expressed in a model plant system *Nicotiana benthamiana* using a *Tobacco mosaic virus* (TMV)-based transient expression system (Kim *et al*. 2009; Martinez *et al*. 2010; Saejung *et al*. 2007; Malabadi *et al*. 2010, 2011; Gottschamel *et al*. 2016). The intramuscular immunization of mice with D2EIII induced the production of the antibodies against dengue (Kim *et al*. 2009; Martinez *et al*. 2010; Saejung *et al*. 2007; Malabadi *et al*. 2010, 2011; Gottschamel *et al*. 2016). The induced antibodies demonstrated neutralizing activity against DEN-2 (Kim *et al*. 2009; Martinez *et al*. 2010; Saejung *et al*. 2007; Malabadi *et al*. 2010, 2011; Gottschamel *et al*. 2016). Therefore, results of this study indicate that the plant system produces dengue virus antigen, which possesses appropriate antigenicity and immunogenicity (Kim *et al*. 2009; Martinez *et al*. 2010; Saejung *et al*. 2007; Malabadi *et al*. 2010, 2011; Gottschamel *et al*. 2016). Therefore, transgenic plants demonstrate the feasibility of using botanical vaccines to prevent infection by the dengue virus (Malabadi, 2008; Kim *et al*. 2009; Martinez *et al*. 2010; Saejung *et al*. 2007; Malabadi *et al*. 2010, 2011; Malabadi *et al*. 2012a, 2012b; Gottschamel *et al*. 2016).

In one of the study reported by Gjorvad, (2012), dengue monovalent antigen EDIII4 and tetravalent antigen "Tetra" by combining all the four monovalent antigens together were introduced into nuclear and chloroplast genomes of tobacco (Gjørvad, 2012). An ethanol inducible promoter T7 RNAP was utilized to control the expression of EDIII4 and Tetra in tobacco nuclear genome, whereas *Prrn promoter* which drives transcription of the plastid ribosomal RNA (rrn) operon was used to control the expression of EDIII4 and Tetra in tobacco chloroplast genome (Gjørvad, 2012). For nuclear transformation, *Agrobacterium* mediated transformation method was used; while biolistic particle gun bombardment was utilized in chloroplast transformation of tobacco (Gjørvad, 2012). Regenerated putative transformants from both nuclear and chloroplast transformation experiments were produced and molecular methods including DNA and protein analyses as well as morphological characterization were carried out on the nuclear transformants (Gjørvad, 2012).

In another **plant made** dengue vaccine developmental study, Kim *et al*. (2009) demonstrated that oral immunogenicity against a fusion protein comprised of proven adjuvant CTB and porphyromonas gingivalis fimbrial protein antigen expressed in *E. coli* (Kim *et al*. 2009a, 2009b, 2010a, 2010b, 2013, 2016), and evaluated its oligomerization and biological activity when expressed in plants (Kim *et al*. 2009a, 2009b, 2010a, 2010b, 2013, 2016). This study also confirmed that the orally administrated CTB fusion proteins could be internalized by M cells and enterocytes in Peyer's patches (Kim *et al*. 2009a, 2009b, 2010a, 2010b, 2013, 2016). Recently in another development, Kim *et al*. (2016) generated an edible dengue vaccine by expressing the dengue fusion protein in tomatoes, which is a desirable expression system owing to the inherent adjuvanticity of alpha tomatine and immunogenicity of the tomato lectin/microbial antigen complex (Kim *et al*. 2009a, 2009b, 2010a, 2010b, 2013, 2016). Tomato is particularly amenable for production of vaccines because it contains two important substances: alpha tomatine (an endogenous plant adjuvant) and tomato lectin (an immunogen by forming tomato lectin/microbial antigen complex) (Kim *et al*. 2016). Alpha tomatine, a saponin contained in green tomatoes at high concentrations, can induce a strong immune response at low doses (Kim *et al*. 2016). Therefore, successful oral vaccination was achieved with hemagglutinin H5 antigen protein purified from transgenic plants plus 10g of adjuvant saponin (Kim *et al*. 2016). The vaccine induced protection against a highly pathogenic avian influenza virus in immunized mice (Lee *et al*. 2015; Kim *et al*. 2016). Therefore, transgenic tomato plants could similarly induce a strong immune response and protect against dengue infection by oral administration of green tomatoes containing CTB–EDIII fusion protein antigen (Kim *et al*. 2016). The B subunit of Vibrio cholera toxin (CTB) was genetically fused to dengue envelope antigen for improved delivery to antigen-presenting cells and enhanced immunogenicity, while avoiding immunological tolerance (Kim *et al*. 2009a, 2009b 2010a, 2010b, 2013, 2016). Kim *et al*. (2016) utilized domain III of the dengue envelope protein (EDIII), as it has been shown to induce serotype-specific neutralizing antibodies (Kim *et al*. 2016). The CTB–EDIII (Kim *et al*. 2016) fusion gene construct containing an endoplasmic reticulum target sequence was introduced into tomato plants by *Agrobacterium tumefaciens*-mediated gene transformation, and the expression of CTB–EDIII (Kim *et al*. 2016) in transgenic plants was confirmed by DNA, RNA and protein analyses (Kim *et al*. 2016). Accumulated fusion protein accounted for up to 0.015 % of total soluble protein, and it assembled into fully functional pentamers as demonstrated by binding to GM1 ganglioside (Kim *et al*. 2009b, 2016). Therefore, during this study CTB was genetically fused to dengue antigen and directed to the ER for expression (Kim *et al*. 2009b, 2016). The fusion protein was expressed in tomato plants, and consuming the tomato would enhance the oral immune response by increasing the chance of interaction with GM1 ganglioside on epithelial cells of the gut (Kim *et al*. 2009b, 2016). The assembled CTB–EDIII fusion protein was verified by immunoblotting and GM1 ELISA assays (Kim *et al*. 2016). The 130-kDa expression product corresponding to the pentameric form was detected under NR conditions (Kim *et al*. 2016). The CTB–EDIII protein in the ER was largely intact, and evidence of slight degradation was observed in reduced samples by anti-CT antiserum but not by anti-dengue antibodies (Kim *et al*. 2016). These results

suggested that the CTB–EDIII fusion protein (Kim *et al*. 2016) could be stably expressed and assembled efficiently into pentamers in the ER (Kim *et al*. 2016). The CTB–EDIII protein showed a strong affinity for GM1-ganglioside, which is an important characteristic for a potential oral immunogen (Kim *et al*. 2016). Although the expression level of biologically active CTB–EDIII (Kim *et al*. 2016) was relatively low, at 0.015 % of TSP in fresh plants, which anticipated that much higher yields could be achieved in freeze-dried green tomato fruits (Kim *et al*. 2016). Therefore, transgenic tomato-derived CTB–EDIII could induce a strong immune response against dengue antigen by oral vaccination (Kim *et al*. 2016). This study showed that CTB–EDIII could be expressed and assembled into a biologically active form in transgenic tomato plants (Kim *et al*. 2016). These transgenic tomatoes will be evaluated for their immunogenic potential in mice as an oral vaccine candidate against dengue infection (Kim *et al*. 2016). Furthermore testing of transgenic tomatoes for immunogenicity in mice following oral delivery is yet to be confirmed (Kim *et al*. 2016).

II. CONCLUSION

Eradication of dengue viral disese and development of an effective dengue vaccine is one of the biggest challenge to the medical and pharmaceutical biotechnology and remains an unresolved task. There are many dengue vaccine candidates in pipeline under the clinical trials and in near future these vaccines might be approved for the commercilization. In spite of many problems and hurdles in the development of a dengue vaccine, there is a licensed dengue vaccine is available. As of today, only Sanofi-Pasteur branded Dengvaxia (CYD-TDV) (Sanofi Pasteur's, France) has made it through phase III clinical trials. Dengvaxia (CYD-TDV) is the first dengue vaccine has been approved and licensed in few countries. However, Dengvaxia (CYD-TDV) confirmed unbalanced protection against the different dengue serotypes and increased risk for haemorrhagic disease particularly among children. In spite of all these difficulties, Dengvaxia (CYD-TDV) has been approved immidiately to reduce the dengue viral disease as a global burden. Dengvaxia (CYD-TDV) has also been shown to work better in people with some prior dengue immunity, and to be less efficacious in those with no prior dengue immunity. Therefore, Sanofi-Pasteur branded Dengvaxia (CYD-TDV) is now available in many dengue endemic countries except in India. Furthermore, next-generation strategies have emerged as a new alternatives to overcome the traditional vaccine problems with new candidates based on tetravalent DNA vaccines, viral-vectored genetic vaccines, attenuated live viruses, recombinant VLPs, molecularly attenuated live viruses, and all moving closer to Phase III studies. New vector control measures of using a bacteria ***Wolbachia*** to combat the *Aedes aegypti* mosquito population would be the best scenario but still it is too early to conifrm experimental potentiality of the method. Another major problem is the cost of the production of **bacteria *Wolbachia*** and much work needs to be done in controlling dengue vector and other viral diseases. Curently plant based vaccine approach is also gained much attention since many of these **photosynthetic** vaccines also reached phase I, II and phase III clinical trials. Till today there is no **plant based vaccine** is available for the medical treatment and not yet reached commercilization. On the othe hand if a **botanical vaccine** is successful, then it would be simple, cost effective for developing countries, more potenital than classical vaccine approach will definately revolutionise the vaccine industry. Plant derived vaccines have higher therapeutic value in controlling human health diseases. Therefore, plant based oral delivary method plays an important role in mass immunization programmes of poor conuntries in controlling the dengue viral disease.

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