CHAPTER 2

2. REVIEW OF LITERATURE - CHIKUNGUNYA

ACUTE FEBRILE ILLNESS:

Acute febrile illness (AFI) is the sudden onset of fever and it is persistent for 2 to 7 days. It can be caused by bacteria, virus, and parasite etc. Viral AFI are usually caused by arboviruses that belong to the families Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae and Rhabdoviridae that include JE virus, Dengue virus, West Nile virus, Chikungunya virus, Yellow fever virus, Rift Valley fever, Zika virus etc, and is a major problem in the developing countries and it leads to the hospitalization of the patients due to disease severity. Large numbers of patients are infected with arboviruses during rainy season which causes acute undifferentiated febrile illness (AUFI). AUFI usually presents with fever less than two weeks and nonspecific symptoms like malaise, head ache and loss of appetite, but when patients present with severe symptoms hospitalization is mandatory. Earlier arboviruses were found in specific regions of world and world and now these diseases are widespread due to globalization.

2.1. Historical perspectives

2.1.1. Chikungunya

Chikungunya (CHIK) was first reported in 1952 in the Southern Province of Tanzania (Lumsden 1955, Robinson 1955). The

name Chikungunya came from Makonde language of southern Tanzania and Northern Mozambique on the east coast of Africa meaning 'that which bends up'. It was isolated from the serum of patients who had high fever with rash and joint pain especially in small joints of hands and toes.

CHIKV can be detected by suckling mice inoculation and serological assays like Hemagglutination assay (HA), Haemagglutination inhibition assay (HAI), complement fixation test (CFT) and neutralisation test (NT) (Clarke and Casals 1958, Pavri 1964), IgM capture enzyme linked immunosorbent assay (MAC-ELISA) (Gadkari and Shaikh 1984, Bodenmann and Genton 2006). Antigen detection from clinical sample was performed by indirect fluorescence antibody detection technique (Kuberski and Rosen 1977); (Yergolkar, *et al.,* 2006). Virus isolation in cell culture (C636 and Vero etc.,) is the gold standard method but it is time consuming and not feasible (Mohan, *et al.,* 2010). In 1990s Reverse-Transcription polymerase chain reaction (RT-PCR) was developed for the early detection of CHIKV infection (Pastorino, *et al.,* 2005, Srikanth, *et al.,* 2010).

2.1.2. Structure of Chikungunya

Chikungunya Virus (CHIKV) a member of alpha virus genus belonged to *Togaviridae* family. The size of the virus is about 50-70 nm and has an icosahedral like nucleocapsid surrounded by an

envelope. The envelope is made up of lipid bi-layer which is derived from host plasma membrane. Envelope has two major virus encoded glycoproteins E1, E2 and a small peptide 6k1. Nucleocapsid consists of single stranded positive sense RNA approximately 11.8 kb surrounded by multiple copies of capsid proteins with size of about 30 kDa (Strauss and Strauss 1994).

2.1.3 Genome of Chikungunya virus

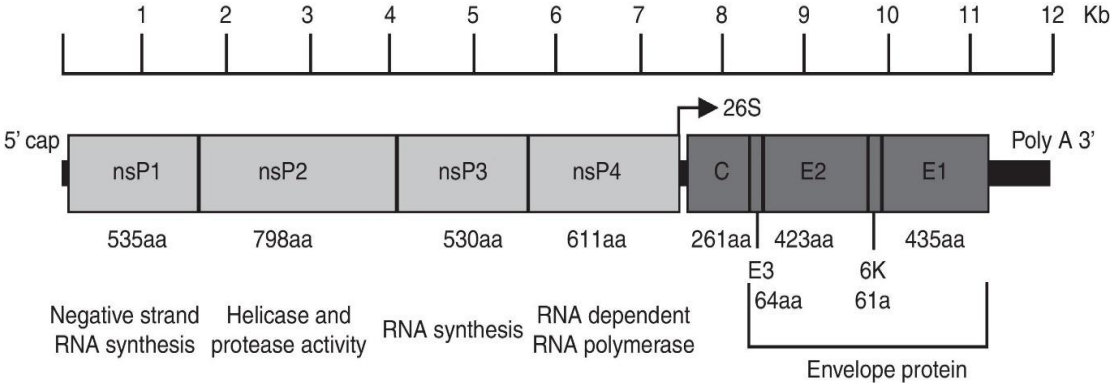

Figure 2.1. Chikungunya genome cassette

(Galan-Huerta *et al.*, 2015)

CHIKV genome is positive-sense single-stranded RNA with 11805 nucleotides long (Fig 2.1). It has genes encoding for non-structural proteins (NSP1, NSP2, NSP3, AND NSP4) and structural proteins (C, E1, E2, E3; 6K). In CHIKV genome capping of 5' end is with a 7-methylguanosine and the 3' end is with poly A tail (Strauss 1986, Faragher, *et al.*, 1988).

2.1.4. Non-structural protein

The non-structural protein (NSP) play a role in transcription and replication of viral RNA. For viral RNA replication, NSP1 is a cytoplasmic capping enzyme. The NSP2 proteins act as helicase and proteinase and cleaves NS to form individual proteins (Strauss, *et al.*,, 1991) (Hardy 1989). The role of NSP3 in replication is not clear. NSP4 codes for RNA polymerase (Strauss and Strauss 1994).

2.1.5. Structural proteins

Capsid, E3, E2, 6K and E1 are structural proteins which are synthesized as single polypeptide and further cleaved to individual proteins and (Strauss 1986, Faragher, *et al.*, 1988) these interact with the host antibodies (Griffin and Johnson 1977).

Junction region promotes transcription of an intracellular sub-genomic 26s RNA. Untranslated region at the 5' end plays a key role in plus-strand RNA synthesis(Ou *et al.*, 1983, Strauss and Strauss 1994) and the region at 3' end helps in proteins translation (Kuhn, *et al.*,, 1991). The 6K is a constitutive membrane protein plays a key role in the processing of glycoprotein, permeabilization of membrane and budding of viral particles.

2.1.6. E2 protein:

E2 protein involves in attachment by binding to the host cell receptor. The p62 precursor is processed by furin at the cell membrane prior to virion budding and it develops a E2-E1

heterodimer. This heterodimer is unstable and dissociate at low pH. Apparently to avoid E1 fusion before its final export to cell surface, processing of p62 occurs at the last step. The C terminal region of E2 protein has ephemeral transmembrane which upon disruption results in change in orientation to the exterior cytoplasm. Critically 2 C-terminus is involved in budding by interacting with capsid proteins. The virus starts secreting the E2 protein and prevents the viral assembly that occurs in the membranes of the endoplasmic reticulum of the infected cell (Sahu et al., 2012).

2.2 Replication:

Virus enters to the cell through receptor-mediated endocytosis and endosomal dependent fusion (DeTulleo and Kirchhausen 1998). Characteristic features of CHIKV supports it replication in both vertebrate host as well as in the invertebrate vector. Mainly, the virus replication is similar in both and little variation was observed in the release of virions in mosquitoes. Viruses were discharged from insect host cells via plasma membrane through exocytosis process (Miller and Brown 1992, Ozden *et al.*, 2007).

The attachment of Alphavirus with different receptors of different types of cells in various host are not well established. Autocatalytic cleavage of the budding structural proteins occurred due to protease activity of capsid proteins. After the self-cleavage, capsid associates with ribosomes instantly and the proteins binds to

viral RNA within few minutes then assembles as icosahedric core particles rapidly.

Transportation of virus into the cell is by endocytosis of clarithrin-coated vesicles. The reduction of pH in the vesicle is essential for the E1 protein activation in the E1-E2 complex. The complex is promoted by viral and endosomal membrane fusion, which results in the release of the nucleocapsid into the cytoplasm. The replication of CHIKV occurs in the cytoplasm (Ozden *et al.*, 2007).

The synthesised nucleocapsid eventually associates with cytoplasmic E2 at the cell membrane, leading to budding and formation of mature virions, which interacts with cellular receptors (Kielian and Rey 2006). During infection, viral fusion process is initiated by E1 trimerization formed by involvement of E1 protein domain I and II. E1-E2 interactions are mediated by domain II during virus maturation and budding (Zhang *et al.*, 2002, Mukhopadhyay *et al.*, 2006).

New virions attach to target cells, and after endocytosis their membrane fuses with the target cell membrane. This leads to the release of the nucleocapsid into the cytoplasm, followed by an uncoating event necessary for the genomic RNA to become accessible. The uncoating might be triggered by the interaction of capsid proteins with ribosomes. Binding of ribosomes would release

the genomic RNA since the same region is genomic RNA-binding and ribosome-binding. E3 protein's function is unknown (McClain *et al.,* 1998, Tsetsarkin *et al.,* 2007).

2.3. Immunopathogenesis

In Italy, 13 cytokines were studied in acute and convalescent sera obtained from CHIKV infected cases (Kelvin *et al.,* 2011)). Level of CXCL9/MIG, CCL2/MCP-1, IL-6 an CXCL-10/IP-10 were significantly elevated in acute phase when compared to the follow up samples. TNF-α, IL-1β, IL-5, IL-10, IL-12 and IFN-γ were low at initial acute phase and significantly elevated at later stage of the disease. Analysis based on the severity of the symptoms such as crippling joint pain mimicking rheumatoid arthritis associated with CXCL-9/MIG, CXCL10/IP-10 and IgG levels.

Several studies have been carried out to better understand CHIKV disease, some studies targeted acute phase of illness, while others used both acute and convalescent sera. Immunopathogenesis among rural population was studied. IFN-γ, IFN-β, IFN-α, CXCL10/IP10 and IL-1β was observed in early acute phase. Other cytokines such as TNF-α, MCP-1, IL-4, IL-6 and IL-10 was extreme in prolonged symptomatic phase and elevated levels were continued in recovered group. IL-4 a maker of Th2 response was highest in the sub-acute illness. Modest positive correlation was observed between myalgia and the pro inflammatory cytokine TNF-

α. This study also reported that IFN-β and IFN-α were significantly raised in seronegative group whereas IL-4 and IL-10 elevated in seropositive group. (Venugopalan *et al.,* 2014).

2.4. Diseases that mimics chikungunya

Dengue is the principal mimic of CHIK fever West Nile and Zika present with similar clinical features of CHIKV. (Bandyopadhyay, 2010)

2.5. Epidemiology-Chikungunya

2.5.1. Global incidence

Year	Country	Genotype
1952	Tanzania	ECSA
1958, 1971, 1985	Uganda	ECSA
1960, 1999-2000	DRC	ECSA
1960, 1962-1964, 1988, 1991-1993, 1995, 2009,2013	Thailand	Asian
1961, 1963	Zimbabwe	ECSA
1963	Cambodia	Asian
1963-1965, 1973, 2006-2016	India	Asian & ECSA
1965, 1967	Vietnam	Asian
1965-1969,1998-1999,2006	Malaysia	Asian
1966, 1982, 1996-1997	Senegal	West African
1967, 2006	Taiwan	Asian & ECSA
1969,1974-1975, 1980	Nigeria	West African
1970	South Africa	ECSA
1970	Kenya	ECSA
1973	Burma	Asian
1973,1980, 1983-1984, 1998-1999, 2000-2005	Indonesia	Asian
1980-1982	Burundi	ECSA
1982	Gabon	ECSA
1983	Pakistan	Asian
1985-1986	Philippines/USA	Asian
1987-1989	Malawi	ECSA
1990, 2006, 2008	Australia	Asian & ECSA
1992	Guinea	West African
2003	Timor	Asian
1999-2000	CAR	ECSA
2004,2005	Kenya	ECSA
2005,2006	Comoros	ECSA
2005,2006	Reunion	ECSA
2005	Seychelles	ECSA
2006	More than 17 countries reported from South East Asia, Europe and USA.	ECSA
2007	Spain, Japan, Gabon	ECSA
2008	China	ECSA
2011	New Caledonia	Asian
2012	Bhutan	ECSA
2013	Caribbean countries and territories, Brazil	ECSA
2014	Caribbean, Mexico	Asian
2014,2015	Brazil	ECSA

Table 2.1. Epidemiological time lines of chikungunya worldwide

In last sixty years CHIKV epidemics were reported in several countries of Africa (Benin, Burundi, Cameroon, Central African Republic, Kenya, Uganda, Senegal, Congo, Nigeria, Sudan, Guinea, South Africa, Tanzania, Zimbabwe, Namibia, Comoros, Mayotte and Ghana, Burkina Faso, Mozambique & Gabon), Indian Ocean region and south east Asia (Mizuno *et al.*, 2011). CHIKV was reported in more than 60 countries (CDC, 2015).

More than 1,00,000 CHIKV infected cases and 200 deaths were reported during 1962 which was massive in Srilanka and India. (http://portalsaude.saude.gov.br/.2015)

2.5.2. Burden in India

Year	State	Genotype
1963	West Bengal	Asian
1964	Tamil Nadu	Asian
1965	Andra Pradesh	Asian
1973	Maharashtra	Asian
1983	Maharashtra	Asian
1996	Haryana	Asian
2000	Maharashtra	ECSA
2001	Madyapradesh	ECSA
2005	Andra Pradesh	ECSA
2006	Andra Pradesh, Karnataka, Tamil Nadu	ECSA
2007	Kerala and Tamil Nadu	ECSA
2008-2009	Kerala	ECSA
2010-2016	Various parts of India (Sporadic cases were reported)	ECSA

Table 2.2. Epidemiological timelines in India

2.5.3. Dengue and Chikungunya co-infection

As dengue and chikungunya are transmitted by same species of *Aedes* mosquitoes, the frequency of coinfection is higher. The regions that are endemic for both the diseases will also increase the rate of co infection. (Myers and Carey 1967, Dayakar *et al.,* 2015). The co-infection of DENV and CHIKV was reported from a patient who returned to Taiwan from Singapore(Chang *et al.,* 2010).

2.6 Transmission:

2.6.1. Vectors:

Mosquitoes involved in the transmission of CHIKV vary based on the geographical regions and conditions of the ecosystem. The mosquito species involved in African countries are *Ae.furcifer, Ae.taylori, Ae. luteocephalus, Ae.neoafricanus* (Jupp, 1988, Diallo *et al.,* 1999). Members of *Ae.furcifer-Ae.taylori* are the major vector in African countries like Senegal, Cote d'Ivoire Central African Republic and South Africa(Jupp and McIntosh 1990). The forest dwelling mosquitoes are the main vectors in Central and West Africa. *Ae. aegypti* an urban vector is most common in Asia. These mosquito species are responsible for the larger regional outbreaks, as these urban mosquitoes maintain close association with humans.

Along with *Ae. aegypti,* other common peridomestic species, like *Ae.albopictus, Aevittatus* and *Anopheles stephensi* are highly reported in CHIKV endemic regions. (Soekiman, *et al.,* 1986,

Mourya and Banerjee 1987, Yadav, *et al.*, 2003). Mother to child transmission of CHIKV has also been reported (Robillard, *et al.*, 2006).

2.7. Mutation

Mutation frequency is seen in CHIKV genome due to its complex replication during host infection (Strauss 1986, Edwards *et al.*, 2007).

2.7.1 E1A226V:

Outbreaks in Kenya, Comoros and Seychelles caused by CHIKV had alanine in 226 position of E1 gene (Tsetsarkin *et al.*, 2007). Studies(Schuffenecker *et al.*, 2006, Tsetsarkin, et al., 2007, Vazeille, et al., 2007) described the amino acid substitution of alanine with valine at 226 position of the E1 protein (E1:A226V) in the Indian Ocean CHIKV isolates from Reunion islands. This specific mutation was reported in 90% of strains isolated after September 2005. A226V mutation enhanced the virus to choose an alternate vector *Aedes albopictus* (Tsetsarkin and Weaver 2011) which had significance in the epidemiology. (Arankalle *et al.*, 2007, Santhosh *et al.*, 2008) and this is responsible for reduction of cholesterol dependence which requires to infect mosquito cells and facilitates in replication and transmission of the virus (Sourisseau *et al.*, 2007). Reduced population of *Ae.aegypti* and death of *Ae.albopitus* species population due to extensive usage of

dichlorophenyl trichloroethane caused ecological pressure which resulted in the amino acid substitution as A226V (WHO 2014). Replacement of primary vector (*Ae.aegypti*) with secondary vector *(Ae.albopictus)* was due to this specific mutation. Increased infectivity of CHIKV with E1:A226V mutation was observed in *Ae. albopictus* than in *Ae.aegypti*. Analysis of numerous factors reveals that *Ae.albopictus* is more lethal and preferred vector for chikungunya (Tsetsarkin *et al.*, 2007).

2.7.2. Other mutations in E1:

E1: K211E, E1; M269V and E1; D284E mutations were reported from Delhi in 2010. Unique amino acid substitutions were also found in certain strain such as E1; V197A, E1; S234P (IND-10-DEL48), E1; R196K (IND-10-DEL88), and E1; R247C (IND-10-DEL108) (Shrinet *et al.*, 2012).

2.7.3. Mutations in E2 gene:

There are reports that mutations in E2 gene play a key epistatic role of E1 gene (Tsetsarkin *et al.*, 2009). In E2 gene, 32 variations at nucleotide level were observed when compared to Tanzania strain isolated in 1952. E2; V264A were found in all samples of Delhi population. E2; V264A was also reported in a patient from France who visited India during 2010. The cases from Tamil Nadu and Andhra Pradesh during 2010 also had this mutation (Grandadam *et al.*, 2011, Sumathy and Ella 2012).

2.8. Clinical Manifestations

2.8.1. Common Chikungunya virus infections:

Several studies documented different symptoms of CHIKV infection (Kennedy, *et al.*, 1980, Borgherini, *et al.*, 2007). In acute stage: CHIKV ranges from mild to severe disease, characterized with high fever (38.5^0C- 40^0C) along with symptoms such as headache, retro-orbital pain, photophobia, lumbar back pain, chills, weakness, malaise, rashes, nausea, vomiting and myalgia. The symptoms and their frequency may vary from one patient to the another. (Munasinghe *et al.*, 1966, Ozden *et al.*, 2007).

2.8.2 Rashes

Rash manifest over the skin, which usually appear on the face, trunk and limbs. The rash along with itch occurs when the temperature declines during acute illness. It has been observed that the rashes in CHIK which were generalized, erythematous, nonpruritic and maculopapular in nature. Presence of aphthous ulcers was also reported. Specifically, symptomatic acute infection is associated with neurological, renal, cardiac, respiratory, hepatic, and haematological complications with a high risk for long term (>30mos) rheumatologic complications among patients older than 30 years(Borgherini *et al.*, 2007).

2.8.3. Neurological complications:

Neurological complications such as meningoencephalitis occurred in a small proportion of patients in first outbreak of India and recent outbreak in reunion (Quatresous 2006). Atypical presentations such as meningoencephalitis and flaccid limb weakness has been observed. (Singh, *et al.,* 2008). However, until recently it has been considered that in chikungunya arthritis radiological findings are normal (Pialoux *et al.,* 2007). A study found that CHIKV was responsible for 14% of suspected CNS infections (Penny *et al.,* 2009).

Persistent viremia after 22 days of illness was observed in an eight months old girl but her acute phase sample was not available. The illness was severe with marked rash, hepatosplenomegaly, and digital gangrene. Alternatively, she may have become infected with CHIKV during her stay in the hospital(Penny *et al.,* 2009). Information on duration of various other symptoms in the acute stage is scanty. Besides, stage signs and symptoms in acute across age groups have also not been understood (Brighton and Simson 1984).

Clinical impact was much more severe in older adults complete loss of self-support, deterioration of health status and sometimes death in debilitated or elderly people (Pialoux, Gauzere *et al.,* 2007). Earlier outbreaks in Reunion and India had few crude deaths reported (Mavalankar *et al.,* 2007, Chua *et al.,* 2010).

Symptoms of acute stage across age groups are not documented. CHIKV infection resulting in rheumatoid arthritis syndrome has been documented (Fourie and Morrison 1979, Bouquillard and Combe 2009).

2.8.4. Chronic arthritis

After the acute phase, polyarthritis may persist for several months to years (Powers and Logue 2007, Sourisseau *et al.*, 2007). In earlier study, chronic arthritis following CHIKV infection has been well documented. In patients with persistent joint pain, high titres of CHIKV antibodies were reported in synovial fluid. (Brighton and Simson 1984). In rheumatoid arthritis, ESR reflect clinical disease activity but usually mimics other symptoms such as morning stiffness or fatigue (Sox and Liang 1986, Wolfe and Michaud 1994). It is postulated that there is a possible role of destructive arthropathy following CHIKV infection which can persist for years. Thus, CHIKV has enormous economic costs in addition to its public health impact(Brighton and Simson 1984). Arthritis following CHIKV infection mimics rheumatoid arthritis (Fourie and Morrison 1979, Bouquillard and Combe 2009) but the underlying mechanism of this arthritis remains elusive (Pialoux *et al.*, 2007). In case of long-lasting symptoms in patients could be explained by discovering CHIKV tropism for muscular satellite cells that are considered as reservoirs for virus or virus-encoded components for longer than expected periods(Ozden *et al.*, 2007).

2.8.5. Arthralgia and Arthritis

Arthralgia is the key feature in clinical diagnosis of chikungunya infection which were experienced by the patients(Staikowsky *et al.,* 2009, Win *et al.,* 2010). Arthralgia is symmetrical with the involvement of more than one joint. The pain can be severe and involve fingers, wrist, elbows, toes, ankles and knees(Tesh, 1982). Joints may be swollen and restriction of movements is common (Suryawanshi *et al.,* 2009).

2.8.6. Severe forms affecting in Reunion

Adults who accompanied CHIKV infected high-school children also contracted the disease. But the adults suffered more severely from the chronic arthritic form of the disease than the children and some cases were with the episodic polyarthritis till 18 months after the onset of the disease(Fourie and Morrison 1979).

2.9. Laboratory diagnosis of chikungunya virus

Isolation of chikungunya virus can be achieved by using Vero and mosquito cell lines which require Biosafety level 3 for processing. Confirmatory diagnosis of CHIKV relies on molecular assays such as RT-PCR and Real time PCR which can be performed for acute phase samples at BSL-2 cabinet (Lanciotti *et al.,* 2007). Serum IgM can be detected from 5 days (sometimes earlier) to several months after the onset of illness. Detection of CHIKV IgG is

possible in between acute-phase and convalescent-phase serum samples.

2.9.1. Virus isolation.

The most definitive gold standard technique which requires Biosafety level 3 for processing CHIKV can be isolated from the blood or serum of patients in viremic phase, infected tissues or blood-feeding arthropods. Virus isolation is less sensitive in post viremic phase (Lanciotti *et al.,* 2007). CHIKV replicates in various cell lines, including insect cells such as C6/36, nonhuman viz, Vero, chick embryo fibroblast-like cells, BHK21, L929 and Hep-2 cells, and human cell lines such as HeLa, MRC5 in which it will often induce a significant cytopathic effect. Confirmatory diagnosis of CHIKV relies on assays such as neutralization test and RT-PCR (Sourisseau *et al.,* 2007).

2.9.2. Serodiagnosis:

Serodiagnosis of CHIKV include Enzyme Linked Immuno Sorbent Assay (ELISA) (Niedrig *et al.,* 2009), Haemagglutination Inhibition Assay (Clarke and Casals 1958), neutralisation, plaque reduction neutralisation assay(Russell and Nisalak 1967) and Immuno Fluorescence Assay (Moi and Takasaki 2016). Detection of IgG antibodies will no longer be useful as they present in the sera of convalescent patients and may persist for several years.

The demonstration of IgM antibodies specific for CHIKV in acute-phase sera from day 5 is used in instances where paired sera cannot be collected. IgM persist for several weeks to 3 months (Kashyap, Morey *et al.,* 2010). The most commonly used test is the IgM capture (MAC)-ELISA(Hundekar *et al.,* 2002). Serological methods are highly sensitive but moderately specific: this is due to the antigenic cross reactivity between CHIKV & other Arboviruses like, Dengue, O'nyong-nyong (Lanciotti *et al.,* 2007). Cross-reaction with other Alphavirus antibodies usually limits the application of MAC-ELISA as a confirmatory test. For a definitive diagnosis, a molecular approach based on reverse transcription (RT)-PCR technologies is useful for early confirmatory diagnosis prior to the development of IgM antibody.

2.9.3. Molecular diagnosis

2.9.3.1. RT-PCR

CHIKV RNA can be detected in plasma within a week of onset of symptoms especially during viremic phase (Jaffar-Bandjee, Das *et al.,* 2009).RT-PCR is a useful diagnostic tool in the early diagnosis of Chikungunya using primer pairs amplifying specific components of three structural gene regions, Capsid (C), Envelope E1 and E2, and part of non-structural protein (NSP) (Sanchez-Seco *et al.,* 2001, Hasebe *et al.,* 2002, Laurent *et al.,* 2007). In 2002, Hasebe *et al* first reported a rapid, sensitive and virus-specific RT-PCR assay based on E1 and NSP1 gene targets for rapid detection and

genotyping of CHIKV, especially in dengue epidemic areas. The sensitivity was 5-50 plaque-forming units (PFUs; corresponding to ~500-5000 copies).

2.9.3.2 Combined RT-PCR & NESTED PCR

A combination of RT-PCR and nested PCR was developed for the specific detection of CHIKV RNA targeting E2 gene to enhance sensitivity and specificity (Pfeffer *et al.*, 1997, Pfeffer *et al.*, 2002) This assay was successful in four CHIKV isolates from Asia and Africa as well as to a vaccine strain developed by The United States Army Medical Research Institute for Infectious Diseases (USAMRIID).

2.9.3.3. Multiplex RT- PCR

Multiplex RT-PCR for CHIKV and DENV was developed for simultaneous detection and the sensitivity of the assay was for DENV 100% and for CHIKV 95.8% and specificity of the assay was 100% for both viruses (Cecilia *et al.*, 2015). Detection limit of the assay ranged from 1 to 50 plaque forming units. Multiplex RT-PCR for Zika, CHIKV and DENV was developed and the assay could successfully differentiate the viruses (Waggoner *et al.*, 2016).

RT-PCR with Alpha virus-specific primers followed by multiplex nested PCR employing species-specific primers was reported for the rapid detection and identification of 14 Brazilian

alphaviruses and was found to be 1000-fold more sensitive as compared with single step RT-PCR(Bronzoni *et al.*, 2004).

2.9.3.4. Real time PCR

Quantitative real time PCR for CHIKV was developed and it is more sensitive, specific and detects wide range of CHIKV concentrations (Carletti *et al.*,, 2007). SYBR Green I based quantitative RT-PCR was also developed and efficiency of the assay was 10-fold more sensitive than conventional RT-PCR. No cross reactivity was detected with other alphaviruses and flaviviruses(Agarwal *et al.*, 2013).

2.9.3.5 Positive and Negative strand quantitative RT-PCR

Development of positive and negative strand quantitative real-time PCR assays for CHIKV NSP3 has helped in diagnosis and studying viral replication. This method had limits of quantification of 1 and 3 log10 RNA copies/reaction. Compared to published E1 diagnostic assay using 30 laboratory confirmed clinical samples, the positive strand nsp3 qRT-PCR higher R^2 and more positives were detected from positive samples (Chiam *et al.*, 2013).

2.9.3.6. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay

LAMP reaction is attributed to continuous amplification under isothermal conditions employing six primers recognizing eight distinct regions of the target. After an amplification LAMP reaction

yields large amounts of by-product (pyrophosphate ion) leading to a white precipitate of magnesium pyrophosphate in the reaction mixture (Notomi *et al.,* 2000). Turbidity measured by real time is directly proportional to the amount of DNA. One-step single tube quantitative RT-LAMP (LAMP for RNA amplification) assay targeting the E1 gene was validated and standardised for rapid and real-time detection of CHIKV(Parida *et al.,* 2008). The detection limit is 20 copies/ml. The specificity of the RT-LAMP primers for the E1 gene of CHIKV was established by ruling out the cross reactivity with members of the *Flavivirus* group.

2.10. Treatment

Other than anti-inflammatory drugs to control symptoms and joint swelling, there are no specific therapeutic agents to treat infected persons. There is no licensed vaccine to prevent chikungunya fever. Passive immunotherapy has been demonstrated in the prevention and cure of CHIKV infection but the same is not proved in humans (Couderc, *et al.,* 2009).

2.11. Prevention

2.11.1. Vaccine Development:

Vaccine strategy for CHIKV infection prevention was initiated in early 1970s. CHIKV was inactivated using two formulations such as formalin fixation and ether extraction. Both inactivated the virus and maintained the ability of hemagglutination-inhibition,

complement fixation and neutralisation (Eckels *et al.*, 1970). CHIKV strain from Thailand outbreak was used for vaccine development (a lyophilised supernatant from human MRC-5 cells). It was studied among 58 study subject, all developed neutralising antibodies but 5 subjects experienced mild to moderate join pain (Eckels, *et al.*, 1970).

Following re-emergence of CHIKV, there was drastic change in the vaccine development. A new formulation using virus like particles has been shown to induce neutralizing antibodies in macaques. These antibodies offered protection following challenge with different strains of CHIKV, and transfer of the macaque antisera into highly susceptible Ifnar$^{-/-}$ mice protected the mice from infection (Akahata *et al.*, 2010).

CHIKV isolated from an acutely infected human patient, developed a defined viral challenge stock in mice that allowed studying the viral pathogenesis and developing a viral neutralization assay. A constructed synthetic DNA vaccine which expresses a component of CHIKV envelope glycoprotein was delivered *in vivo* by electroporation (EP) to evaluate the efficacy. Vaccination induced robust antigen-specific cellular and humoral immune responses, which individually can provide protection against CHIKV. These data suggest the protective role of mAb against

CHIKV disease and support further study of envelope-based CHIKV DNA vaccines(Mallilankaraman, *et al.*, 2011).

2.11.2. Vector control

Large-scale prevention campaigns using DDT has been effective against *A. aegypti* but not against *A. albopictus*. Control of *A. aegypti* has rarely been achieved and never sustained. Recent data show the different degrees of insecticide resistance in *A. albopictus* and *A. aegypti*. However, vector control is an endless, costly, labour-intensive measure and is not always well accepted by local populations whose cooperation is crucial (Savarino, *et al.*, 2003, Lanciotti, *et al.*, 2007).

3. REVIEW OF LITERATURE -DENGUE

3.1. Historical perspective

3.1.1. Dengue

Dengue fever first stated as “water poison” associated with flying insects in a Chinese medical encyclopaedia in 992 from the Jin Dynasty (265-420 AD)(Gubler 1998). The name “dengue” was derived from Ka-dinga pepo meaning “cramp like seizure”. It was also known as "Dandy Fever". The first clinical epidemic dengue occurred simultaneously in Asia, Africa and North America during 1780s and Benjamin Rush reported first clinical case report in Philadelphia and coined the term “break bone fever”. Dengue virus (DENV) was first isolated in Japan during 1943 by inoculating the serum of patients in suckling mice. DHF was documented during 1953-1954 in Manila, Philippines (Sarkar *et al.*, 1964).

3.1.2 Structure of Dengue Virus

Dengue virus (DENV) belongs to the family Flaviviridae and genus flavivirus. It is spherical in shape and size of about 48 to 50nm in diameter surrounded by lipid envelope contains electron dense core of about 30nm. Envelope (E) and membrane (M) proteins are present on the surface of the viral particles. The nucleocapsid contains the capsid (C) protein and genomic RNA (Smith et al., 1970, Lindenbach and Rice 2003).

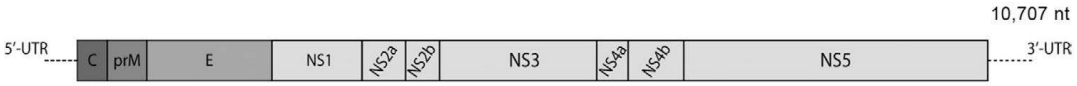

Figure 3.1. Genome of dengue virus (Vasilakis *et al.*, 2011)

Genome is a single stranded positive-sense RNA and 10.7 kb in size (Fig.3.1). It has genes which encodes for ten proteins. Three structural proteins: the capsid (C), envelope (E), and membrane (M) proteins and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

3.2. Proteins of dengue virus

3.2.1 Structural proteins

Capsid (11 KDa) is a basic protein with highly charged residues clustered at the N- and C- termini separated by a short internal hydrophobic domain that mediates membrane association (Trent 1977, Khromykh and Westaway 1996). Membrane Protein (M) is the dimer form of pre membrane (prM) protein and is associated with Envelope Protein (E) to stabilize, (Guirakhoo *et al.*, 1991) and prevent its conformational changes during intracellular transport of immature virions. M protein is found in mature virions (Murray *et al.*, 1993). E protein is a major structural protein that has various biological activities like receptor binding, hemagglutination, inducing major neutralizing antibody in protective immune response, mediation of virus specific membrane fusion of

acidic endosomes and virus assembly (Chambers *et al.*, 1990, Lindenbach and Rice 2003).

3.2.2 Non-structural proteins

The NS1 is a glycoprotein that weighs approximately 46 KDa. It is a hydrophilic monomer, highly conserved and exist in two forms, membrane associated form and secreted form. It plays a significant role in maintaining the viability of the virus. (Falgou & Markoff, 1995). In the early phase of viral replication, the fraction of NS1 remains associated with intracellular organelles. (Mackenzie *et al.*, 1996, Lindenbach & Rice, 2003). *In vivo* studies demonstrated circulating soluble form of NS1 in dengue infected sera (Alcon *et al* 2002). The concentration of soluble NS1 is based on virus serotype, duration of infection, host.

NS2A is a hydrophobic protein of 22 KDa produced by cleavage at the N-terminus by host signal (Falgout and Markoff 1995) and at C-terminus by the viral protease and is thought to be membrane spanning. NS2A is essential for down regulation of type I interferon on response to infection (Liu *et al.*, 2009). NS2A play role in both DENV RNA synthesis and virion assembly/ maturation(Xie *et al.*, 2013). NS2B is a membrane-associated small protein (14 kDa) (Clum *et al.*, 1997) and an essential cofactor for NS3 serine protease which mediate membrane insertion (Falgout *et al.*, 1991, Arias *et al.*, 1993).

NS3 is a multifunctional protein of about 69 kDa (Arias, Preugschat *et al.,* 1993). The trypsin like catalytic triad of flaviviral NS3 protease is distinct from trypsin due to its preference for dibasic residues at P2 and P1 in the substrate (Chambers *et al.,* 1990, Yusof *et al.,* 2000).

NS4A is about 16 KDa, a small hydrophobic protein and C-terminus act as a sequence for translocation of NS4B into the ER lumen. Processing of NS4A/4B junction by signal peptidase requires NS2B-NS3 serine protease cleavage at a site just upstream of the signal peptide to yield 2K peptide (Preugschat and Strauss 1991). NS4B (28 kDa) is a hydrophobic integral membrane protein. Dengue NS4B protein localizes to cytoplasmic foci originating from the endoplasmic reticulum(Miller and Brown 1992). A large NS5 (~103 kDa) is well conserved multifunctional protein involved in RNA replication.

The 5’ and 3’ untranslated regions (UTRs) of the dengue genome are responsible for genome stability, viral replication and translation of the polyprotein and circularization of genome(Filomatori *et al.,* 2006). The 100 nucleotide long 5’ UTR is important for translation of the genome and also acts as a site for initiation of positive strand synthesis during RNA replication. The 3’ UTR is about 450 nucleotides long and lacks a poly (A) tail but ends in a very conserved 3'stem–loop (3'SL). A detailed structure–

function analysis of the 3'SL in many flaviviruses revealed that it is absolutely required for viral replication (Yu and Markoff 2005).

3.2.3. Serotypes of Dengue:

There are four serotypes of dengue virus such as DENV-1, DENV-2, DENV-3 and DENV-4. DENV-1 formed into five clades designated as genotype I (Southeast Asia, China and East Africa), genotype II (Thailand), genotype III (Malaysia), genotype IV (South Pacific) and genotype V (America, Africa) (Mendez *et al.,* 2010). Analyses of E gene sequences have revealed that DENV-1 and DENV-2 can be divided into five and six genotypes, respectively, and DENV-3 and DENV-4 into four genotypes, including the sylvatic lineages found in non-human primates (Ernst *et al.,* 2015). Emergence of DENV-5 was announced in 2013 which was isolated from a 37-year-old patient in Malaysia. Phylogenetic analysis showed it was distinct from DENV-4 and some similarity with DENV-2 and the disease caused by DENV-5 is mild (Mustafa *et al.,* 2015).

3.3. Vectors

Primates are the natural vertebrate hosts and *Aedes* species are their natural vectors and *A. aegypti* is the principal vector. Other Aedes species that could transmit the disease such as *A.albopictus, A.polynesiensis and A.scutellaris (*Franco *et al., 2011).*

3.4. Epidemiology

3.4.1. Dengue in past

During World War II (WWII) the spread of the disease was rapid, when troops began to disperse inland and utilize modern transportation within and between countries, lead to extensive dengue epidemic. Transportation and rapid urbanization led to hyperendemicity of Dengue (multiple serotypes) in most South East Asian countries. Shipping of goods around the world introduced mosquitoes to the coastal destinations (Gubler, 2007).

3.4.2. Current Global Scenario

Dengue is prevalent in 128 countries in that 100 countries are endemic in Asia, the Pacific, the Americas, Africa and Caribbean. Annually 390 million dengue cases were reported, and 96 million cases developed severe manifestations and 2.5% deaths were reported in which majority are children (WHO, 2016).

3.4.3. Dengue in South East Asia (SEA)

Almost 75% of the population is exposed to dengue in Asia-Pacific. Of these 1.3 billion people are at-risk who live in ten dengue endemic countries in SEA (WHO 2011). Eight countries in SEA had reported cases of dengue in 2003 where as by 2009 all SEA member countries excluding the Democratic people of Republic of Korea reported indigenous cases (WHO 2011, WHO 2012). Regular cycles of epidemics in 3-5 years continue to occur in SEA and the number

of reported cases increases along with the severity of disease in many member countries (Shepard *et al.,* 2016).

3.4.4. Dengue in India

Several arboviral diseases have emerged and re-emerged in India during the past three decades. Of these, dengue virus has established its endemicity with regular epidemics every year during the rainy season. The epidemiology of dengue fever in the Indian subcontinent is very complex. In the past six decades is being changed in terms of prevalent strains, affected geographical locations and severity of disease. In 1963-1964, an initial epidemic of dengue fever was reported on the Eastern Coast of India and spread northwards. By 1970s all the four serotypes of dengue were reported across the country. About a lakh dengue cases were reported in 2015 and death rate of 0.22% was observed in Kerala, Karnataka, Tamil Nadu, Gujarat, Orissa, West Bengal and Assam (NVBDCP).

All the Indian DENV-1 isolates belong to the American African (AMAF) genotype and distributed into four lineages, India I, II, III and the Africa lineage. Of these, India III is the oldest and extinct lineage; the Afro-India is a transient lineage while India I is imported from Singapore and India II, evolving *in situ,* are the circulating lineages. The American genotype of DENV-2 which circulated predominantly in India during early-1971 was

subsequently replaced by the Cosmopolitan genotype (Chaturvedi *et al.,* 1970, Myers, *et al.,* 1971). Post-1971 Indian isolates formed a separate subclade within the Cosmopolitan genotype(Dar *et al.,* 1999). The re-emergence of an epidemic strain of DENV type-3 in Delhi in 2003 and its persistence in subsequent years marked a changing trend in DENV circulation in this part of India (Kukreti, *et al.,* 2008). Initially DENV-1 was reported in 1968 epidemic, by 1970s it was replaced by DENV-2. During 1968, all four serotypes co-circulated in South India. In 1997 epidemic of Delhi DENV-1 was isolated along with DENV-2 but the genotype of DENV-2 was different resulting in large numbers of DHFs.

In India, the first clinical case was identified in Chennai in 1780 and the first epidemic was documented during 1963-64 in Calcutta and eastern coast of India (Sarkar *et al.,* 1964). DENV was isolated from Kolkata in 1944 from the infected serum of US soldiers (Sabin and Schlesinger 1945). DENV-2 was also reported from southern India - in Kerala along with DENV-3, DENV-3 has been isolated during the epidemics at Vellore in 1966, Calcutta in 1983 and 1990, Jalore city, Rajasthan in 1985, Gwalior in 2003 and 2004 and Tirupur, Tamil Nadu in 2010. The emergence of DENV-4 has been reported in Andhra Pradesh and Pune, Maharashtra, which was also implicated in increased severity of disease. During 2010-2011 epidemic in Delhi, DENV-1 was reported (Gupta *et al.,* 2012)

and DENV-1, 2 and 3 were reported 2009-2012 epidemic in Uttar Pradesh (Mishra *et al.*, 2011).

3.5. Replication

Dengue virus replication occurs when virion attaches either directly to the host cell receptors (or) through FC portion of dengue virus containing immune complex attaches to FC receptor target cells. DENV infects immature dendritic cells of the skin (Wu *et al.*, 2000), Liver parenchymal cells and macrophages (Jessie *et al.*,2004). Virus enters via receptor mediated endocytosis. The endosomal vesicle triggers conformational change in the virus resulting in the irreversible trimerization of viral envelope and fusion protein of the virus is exposed and fuses with the endosomal membrane resulting in the release of nucleocapsid in the cytoplasm. The viral genome (RNA) is transported to rough endoplasmic reticulum and translated into a single polypeptide which is cleaved by viral and host proteases into ten different proteins (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Viral replication complex is formed, where the translation stops and RNA synthesis, transcription of antisense viral RNA begins. Numerous viral RNA is synthesized and subsequently assembled by capsid protein on the surface of the endoplasmic reticulum. structural proteins and nucleocapsid buds into the lumen of endoplasmic reticulum to produce immature virus particle. They are transported through the Golgi apparatus into trans Golgi network, where the low pH ensures

the conformational change and exposes the furin cleavage sites. Viral maturation occurs by cleaving Pr protein and M protein by host protease and released (Mukhopadhyay *et al.*, 2005, Screaton *et al.*, 2015).

3.6. Pathogenesis of Dengue

Dengue viruses can cause nonspecific febrile illness (pantomorphic) and hemorrhagic fever (visceromorphic) in human host. Clinical spectrum of dengue virus includes dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). When pre-existing dengue antibodies present in the host due to primary (first) DENV infection, antibody dependent enhancement of infection occurs by binding of pre-existing antibody binding to an infecting DENV particle during subsequent infection with different dengue serotype. Partial neutralization of DENV by antibodies during subsequent infection with a different serotype of dengue enhance the attachment of Ab-virus complex to receptors called Fcγ receptors (FcγR) on circulating monocytes. This complex helps the virus to infect monocyte more efficiently and it leads to rapid replication of the virus and development of severe dengue (Whitehead *et al.*, 2007).

3.6.1. Dengue fever

The disease begins abruptly, after a 2- to 14-day incubation period, with high fever, headache, retroocular pain, lumbosacral pain, conjunctival congestion, and/or facial flushing with biphasic fever. Generalized myalgia and arthralgia are reported. Bradycardia associated with fever is seen. Maculopapular rash appears first on the trunk and then spreads centripetally to the face and limbs. A second phase of fever called saddleback fever is seen in some patients. Generalized lymphadenopathy, cutaneous hyperesthesia, and altered (metallic) taste sensation may accompany this stage of the disease. The peripheral white blood cell count is depressed with an absolute granulocytopenia, and the platelet count may fall to less than 100,000 per mm^3. Haemorrhagic phenomena may include petechiae, epistaxis, intestinal bleeding, menorrhagia, and a positive tourniquet test. Unusually myocarditis, various neurologic disorders, including encephalopathy and peripheral mononeuropathy, polyneuritis, and Bell's palsy are associated with Dengue fever.

3.6.2. Dengue haemorrhagic fever and dengue shock syndrome

DENVs being a viscerotropic virus can cause a range of severe disease, including fulminant hepatitis with massive haemorrhage, vascular leak with hypovolemia, organ failure, cardiomyopathy and encephalopathy. The severe form of dengue is a vascular leak syndrome known as DHF. Onset is generally sudden, with fever of 2 to 7 days duration and a variety of nonspecific signs and symptoms

(Nimmannitya 1987). Hemorrhagic manifestations are common, and the tourniquet test may be diagnostically helpful though false positivity is high. Scattered petechiae, gastrointestinal haemorrhage, hematemesis and melena usually occur after prolonged shock.

DHF is classified into four grades of illness based on severity, Grade I is mild, scattered petechiae or a positive tourniquet test. Grade II is more severe with one or more haemorrhagic manifestations. Grade III is characterized by mild shock with signs of circulatory failure; the patient may be lethargic or restless and have cold extremities, clammy skin, a rapid but weak pulse and Grade IV is the most severe form of DSS and characterized by profound shock with undetectable pulse and blood pressure (WHO, 1997). Thrombocytopenia and haemoconcentration indicating plasma leakage are constant findings in DHF and DSS. A platelet count of less than 100,000 per mm^3 is usually found between the third and eighth day of illness. Elevated liver enzymes are common. Pleural effusion or ascites may be detected by physical examination or radiography.

3.7. Dengue Immunopathogenesis

Human cytotoxic factor (hCF) stimulates certain pathways that can shift Th1 response to Th2 response (Gupta *et al.*, 2012). Th1 cells secrete IFN-γ, IL-2, TNF-β which involve in cell mediated immunity, delayed type hypersensitivity, tissue injury. Th2 cells

secrete IL-4, IL-5, IL-6, IL-10 induces antibody production and involve in humoral immunity. Cross regulation of Th1 and Th2 are mediated by IL-10 and IFN-γ. Th1 is linked to the disease recovery and Th2 is linked to severe disease progression. Th1 is raised in dengue fever and Th2 is elevated in severe dengue. Non-Th1, Th2 includes IL-1 and TNF-α, plays a role in the development of acute inflammatory response (Chaturvedi *et al.*, 2000).

DENV infected endothelial cells produce IL-6, IL-8. IL-8 along with ICAM-1 enhances the attachment of polymorphonuclear cells lead to the release of thrombomodulin and vasopermeability (Lei, *et al.*, 2001) IL-8 was raised in patients with dengue infections (Raghupathy *et al.*, 1998). IL-6 levels are elevated in DHF and less in DF. Levels of TNF-α are less in DF and DHF. Levels of IFN-γ, IL-6, IL-8 are elevated in dengue cases. IL-6/IL-8 are elevated in DHF than DF. The association of IFN-γ with ALT levels were significant than with thrombocytopenia. During the analysis of cytokine response, a time trend was noted in IFN-γ and IL-8 response. Decreasing trend was observed in IFN-γ and increasing trend in IL-8 in DHF cases (Priyadarshini *et al.*, 2010).

3.8. Dengue case definition

World Health Organization (WHO) classified dengue as the ‘most important mosquito-borne viral disease in world in 2012

(WHO, 2012) due to the geographic spread of the virus and its vector (Gibbons and Vaughn 2002).

WHO has classified Dengue illness into 3 groups based on certain clinical criteria as probable dengue with live in/travel to endemic area, Fever and 2 of the following criteria such as nausea, vomiting, Rash, aches and pains, Tourniquet test positive, leukopenia and any warning sign and laboratory confirmed dengue, for dengue warning signs abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement >2cm, laboratory: increase in HCT concurrent with rapid decrease in platelet count and the patient with this category requiring strict observation and medical intervention. Criterial for severe dengue includes severe plasma leakage which leads to shock (DSS) and fluid accumulation with respiratory stress and severe bleeding as evaluated by clinician (WHO, 2009).

3.9. Laboratory diagnosis of dengue virus infection:

After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues in 4–5 days. However, viremia persists for more than 5 days in patients with severe disease. Virus isolation, nucleic acid or antigen detection can be performed to diagnose the infection in the early stage. Serology is the method of choice during the end stage of acute infection. (Kuno *et al.,* 1991, Shu *et al.,* 2003). During secondary infection

antibody titres rise rapidly and react broadly against many flaviviruses. Early convalescent stage IgM levels are significantly lower in secondary infections than primary infection and may be undetectable in some cases. To distinguish primary and secondary dengue infections, IgM/IgG antibody ratios are commonly used (Shu *et al.*, 2003).

Conventional methods include virus isolation in animals, embryonated eggs and cell culture. Confirmation of viral isolates is performed by neutralization test using specific antisera. Traditional serological tests like complement fixation test (CFT) and Haemagglutination test (HAI) are helpful in seroepidemiological studies. With the introduction of ELISA test especially immunoglobulin class specific test, the disease was able to detect in about 7 days of onset. Reverse-transcriptase polymerase chain reaction (RT-PCR) used in the detection and quantification of dengue virus. Recently, the development of viral genome (RNA) detection by PCR and its modification like real-time PCR has improved the diagnosis of dengue virus (Ramamurthy *et al.*, 2013).

3.9.1. Virus isolation

DENV was isolated by various techniques such as intracerebral inoculation in suckling mice, inoculation in mammalian cell cultures LLC-MK2 and Vero, intrathoracic inoculation in adult mosquitoes and in mosquito cell lines (Guzman and Kouri 1996,

Vorndam 1997). Four mosquito species have been used such as *A. aegypti*, *A. albopictus*, *Toxorhynchities amboinensis*, and *T. splendens*. This is labour-intensive and requires an insectary to produce large numbers of mosquitoes for inoculation (Rosen, L., and D. J. Gubler. 1974.) C6/36 mosquito cell line was developed which is rapid, sensitive and economical for the isolation of dengue virus (Kuberski and Rosen 1977, Gubler 1988).

3.9.2. Serodiagnosis

Serological methods are relatively inexpensive and easy to perform compared with culture. NS1 antigen and antibody detection will be useful in acute phase diagnosis. NS1 is first marker appear in blood hence many tests have been developed to diagnose DENV infections using NS1 antigen, followed by IgM and IgG (Xu *et al.*, 2006). However, there can be cross reaction with other flaviviruses and these tests cannot differentiate the DENV serotypes. IgM antibodies appear by 3-5 days of the illness and detected in 50% of patients, increasing to 80% by day 5 and 99% by day 10. IgM levels peak in about two weeks and decline generally to undetectable levels over 2–3 months. Anti-dengue IgG is generally detectable at low titres at the end of the first week of illness, increasing slowly thereafter and detectable after several months, probably even for life. Secondary immune response generates high levels of IgG through the stimulation of memory B cells from the previous infection as well as IgM response to the current infection, because

high levels of IgG compete with IgM for antigen binding (Halstead *et al.*, 1970).

A MAC-ELISA for IgM antibody-capture enzyme-linked immunosorbent assay can be used in regions where dengue and Japanese encephalitis virus co-circulate. MAC-ELISA have a sensitivity and specificity of 90% and 98%, when compared with the gold standard hemagglutination techniques. In addition to serum, dengue-specific IgM can be detected in whole blood on filter paper (sensitivity 98.1% and specificity 98.5%) and in saliva (sensitivity 90.3% and specificity 92.0%). False-positive results due to dengue-specific IgG and cross reactivity with other flaviviruses is a limitation of the MAC-ELISA, mainly in regions where multiple flaviviruses co-circulate. IgG ELISA for dengue-specific IgG detection can be used to confirm a dengue infection in paired sera. It is also widely used to classify primary or secondary infections. Similarly, it has been demonstrated that IgG specific for the NS5 protein rather than NS1 protein can potentially discriminate between infections caused by West Nile, dengue and St Louis encephalitis viruses. Neutralization assays like plaque reduction neutralization technique (PRNT) and the micro-neutralization assay are used to define the infecting serotypes following a primary infection. These tests are mainly for research and vaccine studies.

3.9.3. Molecular diagnosis

3.9.3.1. Reverse transcription PCR (RT-PCR)

DENV RNA can be detected from human clinical samples, autopsy tissues or mosquitoes (Guzman and Kouri 1996, Deubel *et al.*, 1997). Several RT-PCR were developed targeting different sites of dengue genome with different approaches (Lanciotti *et al.*, 1992, Deubel *et al.*, 1997). Advantages of performing RT-PCR are confirmation of infection, more sensitive and specific, identifying serotypes and genotypes, results can be obtained in a short duration (24–48 hours). Limitation of RT-PCR are necessitating acute phase sample (0–5 days post onset), well trained personnel, expensive laboratory equipment, RT PCR cannot differentiate primary and secondary infection (Peeling *et al.*, 2010).

3.9.3.2 Nested RT-PCR

A rapid Nested RT-PCR for the detection and typing was developed by targeting 511bp cPrM of DENV. First round of RT-PCR primers amplifies all the DENV and second round primers were serotype specific. The serotypes were identified based on the amplified product size. Sensitivity of the assay 94% and 93% for DENV1 and DENV2 respectively and 100% with DENV 3 and 4 viruses when compared with virus isolation (Lanciotti *et al.*, 1992).

3.9.3.3 Real-time RT-PCR:

Many real-time RT-PCR assays have been developed using TaqMan or SYBR Green chemistries. The TaqMan real-time PCR is

highly specific due to the sequence-specific hybridization of the probe. The real-time RT-PCR assay is a one-step assay that measures the viral load in approximately 1.5 hours. The detection of the amplified virus by fluorescent probes replaces the need for post amplification electrophoresis (Callahan *et al.,* 2001**).** Pei-Yun Shu *et al.,* 2003 developed SYBR GREEN based dengue group and serotype specific real time PCR.

3.9.4.4. Isothermal amplification:

The NASBA (nucleic acid sequence based amplification) assay is an isothermal RNA specific amplification assay that does not require thermal cycling instrumentation. The initial stage is a reverse transcription; single-stranded RNA is converted to double-stranded DNA and amplified. Amplified DNA is detected by electro chemiluminescence or in real-time with fluorescent-labelled molecular beacon probes. NASBA has been adapted to dengue virus detection with sensitivity equal to virus isolation in cell cultures and may be a useful method for studying dengue infections in fields (Balsitis *et al.,* 2009). Loop mediated amplification methods have also been developed (Keller et al., 2006).

3.9.3.5. Luminex-based single DNA fragment amplification assay:

DNA hybridization assay based on the Luminex technology for detecting and serotyping dengue virus (DENV) was developed (Cabral *et al.,* 2016) Results of the RT-PCR/Luminex assay has

86.7% concordance with semi-nested RT-PCR. This is the hybridization assay that can discriminate the four DENV serotypes using probes against a single DENV sequence documented for the first time. This is the rapid test used to detect and serotype DENV.

3.10. Prevention and control

3.10.1. Vector control

Prevention and control of dengue and DHF currently depends on controlling the mosquito vector, *A. aegypti*. Space sprays with insecticides to kill adult mosquitoes are not effective unless they are used indoors. The most effective way to control the mosquitoes is elimination of the larval habitats for *A. aegypti* in the domestic environment (Gubler, 1989., Newton and Rieter 1992).

There are two approaches to effective *A. aegypti* control involving larval source reduction. In the past, the most effective programs have had a vertical, paramilitary organizational structure with a large staff and budget (Soper, *et.al.*, 1965). Use of DDT contributed greatly to get rid of the mosquitoes from the domestic environment.

3.10.2. Vaccine

Several dengue vaccine candidates are under development, Live attenuated vaccines and live chimeric vaccines are in clinical evaluation. Also, inactivated virus vaccines, live recombinant, DNA and subunit vaccines have been evaluated in preclinical animal

models and in clinical trials. For the safety and efficacy of dengue vaccines, the immunopathogenic complications such as antibody-mediated enhancement and autoimmunity of dengue disease need to be studied with caution as the immunopathogenesis of dengue is complex.

The first licensed vaccine for dengue is CYD-TDV in Mexico in December 2015 and is effective in individuals between 9-45 years old living in endemic areas. This vaccine live recombinant tetravalent dengue vaccine developed by Sanofi Pasteur (WHO, 2016). Nilavembu Kudineer, a herbal drug has significant role in reduction of viral fever in all age group whereas found less effect in female group it may be due to different hormonal and metabolic conditions during phases of age group (Christian *et al* 2014).

3.11. Treatment:

There is no antiviral available for dengue virus and treatment is usually symptomatic. Supportive care with analgesics, fluid replacement and bed rest is sufficient for mild cases. Severe cases require administration of plasma expanders, platelet and fresh frozen plasma administrations.