CHAPTER 6

DISCUSSION

Arboviral infections are major problem in the developing countries and it leads to the hospitalization of the patients due to disease severity. Large number of patients are infected with arboviruses especially which presents as Acute Undifferentiated febrile illness (AUFI) during rainy season. AUFI presents with fever less than two weeks duration and nonspecific symptoms like malaise, head ache and loss of appetite. However, some patients present with severe & chronic symptoms which may require hospitalization.

AUFI in Western countries is commonly due to arboviral disease caused by West Nile virus, St. Louis encephalitis virus, Eastern equine encephalitis virus (EEEV) and Lacroose virus. Surveillance organization like ArboNET by CDC, Atlanta USA regularly monitor arboviral incidence in the Western countries, whereas in the developing countries like India where viral, bacterial and parasitic disease occurs, inadequate public health measures are taken, like vector control and improper sanitation and lack of awareness among public are the major factors which hinder the control of the disease despite of existing disease control programmes.

Patients presented with etithecal symptoms of CHIKV such as acute fever with or without joint pain and head ache were observed in majority of the patients and haemorrhagic manifestations and maculopapular rash were presented in small percentage of patients. A study reporting of CHIKV clinical manifestation suggested that the severity of CHIKV disease is increased in that epidemic. Atypical manifestations of CHIKV also were reported from Kerala (Conference Proceedings of the National workshop on Emerging fevers with focus on Chikungunya, 2007). CHIKV patient with oral candidiasis suggested that it may be due to the immune-suppression (Kumar *et al.*, 2008). CHIKV infection in CNS showed its involvement in nervous system (Lewthwaite *et al.*, 2009) and other study also documented about neurovirulence of the disease (Vanlandingham *et al.*, 2005).

All patients enrolled in the study had acute onset of fever and 85% of them had joint pain which was crippling especially among patients sampled in 2009. The joint pain of CHIKV is characteristic, which allows for differentiation from dengue as previously described. Arthralgia affecting joints such as ankle, knee joint, wrist and phalanges has been reported (Mackenzie and Smith, 1996). Majority of our study subjects complained of joint pain of the small joints. Acute onset of fever with joint pain does appear to be pathognomonic of CHIK.

Many patients affected belonged to the age group 20-50 years ($p<0.001$) (Srikanth *et al.*, 2010) and 18% of cases belonged to the age group of less than 20 years. However, an analysis of patients affected in 2009 revealed that more cases were affected between 31-45 years (44.6%) of age as with arthralgia increased from 18.2 % to 87.3%. The limitation of our study is that we were able to collect clinical details only at one point of time. At the time of collection of samples, no cases of meningitis were reported. Prospective follow up studies need to be undertaken to determine the clinical impact of CHIKV and disease progression.

Among 354 patients analysed in a study from Kerala in 2007, 194 were women & 160 were men. Females appear to be more frequently affected than males. In the La Reunion Island outbreak, women were predominantly affected and the male/female ratio was 0.68. (Kannan *et al.*, 2009 & Paquet *et al.*, 2006**)**. In our study, females (55%) were more commonly affected in 2008 whereas in 2009 males (60.4%) were more frequently affected.

In all, 51 cases of *Chikungunya* were found to be RT PCR positive. Correlation of fever with RT-PCR positivity showed that 8 (21%) of 38 patients samples collected in 2009 were positive within day 2 of fever with as many as (66%) samples testing positive on day one. RT-PCR positivity has been documented from day 0 to

approximately 10 (Kam *et al.*, 2009). While the outbreak in 2008 appeared to show preponderance towards certain age groups (20-50) in 2009, the number of cases that were RT-PCR positive was found across all ages.

CHIKV acute phase infection usually continues from few days to couple of weeks. Though myalgia/arthralgia may persist for weeks, months or even years. Some cases may develop into chronic arthritic syndrome (Win *et al*., 2013 Borghani *et al*., 2007).

Serological methods such as ELISA based IgM detect CHIKV after 5 days of infection in acute phase; IgG antibodies in convalescent phase can persist for several years in serum or plasma. Diagnosis by this method is moderately specific due to cross reaction with other Alpha viruses such as, Dengue, O'nyong-nyon viruses (Kam 2015).

Confirmatory diagnosis of CHIKV can be achieved by using rapid, sensitive method such as RT-PCR (Laurent *et al* 2007) within first week (day 2 of illness to 10 days) of disease onset of illness using E1, E2 and NSP regions. Other methods such as nested (Pfeffer *et al*., 1997, Pfeffer *et al*., 2002), multiplex PCR (Bronzoni *et al*., 2004)), Real time quantitative RT –PCR (Pastorino *et al*., *2005*, Santhosh *et al*., 2007) are also useful for diagnosis but their sensitivity vary widely. The tests are either qualitative or

quantitative and the cost effectiveness of the test varies depending on the assay used.

A new rapid test RT-LAMP (Reverse Transcription Loop-Mediated Isothermal Amplification Lamp Assay) targeting E1 gene allows for real time detection of CHIKV (Parida *et al.*, 2007). The RT-LAMP was found to be 10-fold more sensitive than RT-PCR, with a detection limit of 20 copy numbers, against 200 copy numbers for RT-PCR. LAMP does not require a thermal cycler and thus can be performed simply with a heating block and/or water bath.

Therefore, the use of confirmatory tests such as RT-PCR in the early onset of the disease is important to detect CHIKV before disease progression occurs and also to prevent transmission.

The distribution of CHIKV cases in the three districts of Kerala that were sampled clearly indicated that Kozhikode had the maximum number of infected patients. State Disease Control & Monitoring in Kerala state had documented CHIKV positivity rate (by IgM Antibody assay) in 2007 & 2008, such as Kozhikode 3.5%, Kannur 3.0% and Kasargode 1.4% of all the total cases. In the study conducted by Kumar NP *et al* in 2007, Kottayam and Pathanamthitta contributed 44.33% and 14.37% respectively, of the total cases in Kerala. These areas were situated near by the Alapuzha district which was severely affected in 2006 and contributed to 7.70% of the

total cases in 2007. All the samples were collected from the areas in the border of the coast, however, Iritti was a hilly region, where 9 positive cases were isolated. Thus, the vector appears to cause the spread of the disease to interior regions of Kerala. Public health measures need to address such locations while planning for control measures.

Dengue is the most prevalent disease in India among the vector viral borne diseases. Incidence of dengue is predominant in south east Asian countries. Number of epidemic cycle is increasing every 3 to 5 years. Most of the cities in India have become endemic (Cecilia, 2014). Nearly one lakh dengue cases and 0.22% death rate are documented in India during 2015 (NVBDCP). It was the highest incidence since last 5 years. During 2016, 15,099 cases were reported until July 2016 and the death rate was 0.17%. The numbers are expected to increase during rainy season (NVBDCP).

In India, dengue has become hyperendemic in most of the cities, Delhi (15867), Punjab (14128) reported highest number of cases and Tamil Nadu with 4535 cases in 2015. CHIKV reported high number of cases in 2015(27,553) and 2013 (18,840). Karnataka reported 20,763 cases being the highest and Tamil Nadu with 329 cases the least. However, there are reports that reveal under reporting of dengue cases in developing countries (Amarasinghe *et al.*, 2011).

Dengue disease is acute and develops to severe life threatening complication, whereas consequence of CHIKV disease may develop to chronic disease (Kalawat *et al.*, 2011). Until few years ago, CHIKV was a neglected vector borne disease. It emerged to an epidemic level with quite number of cases reported with CHIKV all around the globe. Re-emergence of CHIKV in 2005 in India, spread to several states of India and millions of cases were reported during this epidemic.

Confirmation of the infection is very essential to decide the course of patient management. It creates demands for the differential diagnosis to prevent disease progression. Due to overlapping of clinical features with dengue (endemic), actual number of the cases for CHIKV is under reported. From its re-emergence, occurrence of CHIKV is increasing every year with 45%-63% incident rates in numerous epidemic areas (Mavalankar *et al.*, 2007).

Due to cocirculation of the causative agent in tropical countries, clinical diagnosis of AUFI is complicated. Both dengue and chikungunya are transmitted by similar type of vectors such as *Ae. aegypti* and *Ae. albopictus* and they spread widely in the same geographic region. CHIKV and DENV samples have been isolated concurrently from blood samples which were collected during the acute phase of dengue like illness (Myers and Carey, 1967). However, since

viruses like CHIKV and DENV are self-limiting, it is possible only during the viremic stage (1-7 days).

Though virus isolation is the gold standard and most sensitive method for DENV and CHIKV, it is time consuming as it takes 2 to 3 weeks to confirm the presence of virus. It is laborious and also requires well trained personnel. As it is not producing unique CPE, confirmatory assays like HAI, IFA and RT-PCR need to be performed to detect the specific virus. Due to extensive cross reactions among the flavivirus based serological test, false positives are observed some times and this is common issue among the flaviviruses.

Primer selection for multiplex RT-PCR for CHIKV and DENV performed in our study were selected carefully checking the primer sequence quality with BLAST online tool of NCBI. The related primer sequences showed 100% identity with the reference sequences.

Among traditional serological techniques, plaque reduction and neutralization assay with specific antibodies were used for serotyping of dengue, but these techniques have some limitation due to their cost and time consumption (10-14 days) (Kao *et al.*, 2005). After the development of molecular detection technique for rapid diagnosis and typing of dengue, slowly replaced the virus isolation as a new gold standard technique for diagnosis of dengue from acute phase samples. (Guzman et al., 2004). Worldwide, most commonly used method for

dengue detection by RT-PCR was developed by Lanciotti *et al*., 1992, later this method was developed into single tube nested PCR (Parida *et al*. 2013)

Molecular diagnosis such as RT-PCR is the best choice for detection of virus from the clinical specimen during acute phase as well as from cell culture isolate. RT-PCR results can be obtained within 2-3 hours and it is more sensitive as it can detect low copies of viral RNA and more specific than other methods. Performance of RT-PCR also helps in the identification of serotypes and genotypes by sequencing the amplicons. In CHIKV, though RT-PCR is an effective tool for early diagnosis of virus, sometimes it may be negative due to low copy number and inhibitors present in the specimen.

CHIKV IgM antibody detection by indirect ELISA method and genus specific RT-PCR was developed for detection of alpha viruses (Pfeffer *et al*., 1997). Real-time PCR is an important milestone in the viral diagnostic method. Since specific probes are used in the taqman assay, Real-time RT-PCR is more specific.

As viral load plays significant role in severity of the disease, Real-time PCR is also helpful in quantification of the viral load in the patient samples. Viral load, also helps in patient management and follow up. Though Real-time RT-PCR is more efficient technique, it

requires extensive care in planning and analysing the Real-time multiplex RT-PCR results (Bustin, 2004).

Major advantage of Real-time RT-PCR is quantification of target RNA. In quantitative PCR, every step is important, starting from receiving the specimen, template preparation, Reverse transcription and till the last amplification step. Accuracy in each stage is important to obtain accurate quantitative data. Moreover, choice of chemistries, primers, probes and instrument should be appropriate when performing quantitative assay (Bustin *et al*., 2004).

RT-PCR resolving power is dependent on the efficiency of RNA to cDNA conversion. It varies based on the RT enzyme used. RNA to cDNA conversion is significantly less when template RNA is less (greater than 3-fold) and is affected by presence of background nonspecific RNA in the RT reaction (Curry *et al*., 2002).

Diagnosis of dengue is based on clinical case definitions and is often a challenge to clinicians due to the overlapping symptoms (Andrews *et al.,* 2014). Also, due to the changing epidemiology, there is always a need for a reliable laboratory diagnosis to complement the clinical diagnosis.

6.1. Multiplex RT-PCR

As multiplex RT-PCR is capable of detecting more than one target in the simultaneous assay, it is more suitable for differential diagnosis of CHIKV and DENV. Advantage of performing multiplex RT-PCR is 3-fold reduction in the cost since the reagent usage is getting minimized, as assay can be completed within a shorter duration and it is very useful in differential diagnosis especially during epidemics.

Limitation in our multiplex RT-PCR was that the serotyping of DENV was not included, which we are planning to work in the future as serotyping of DENV will be helpful in patient management as severity dengue is associated with certain serotypes of dengue. Even though the number of samples were less for evaluation, convenient sampling size was done. We have optimized conventional multiplex RT-PCR. Though Real-time PCR is more sensitive than traditional RT-PCR, cost of the Real-time RT-PCR is high when compared to traditional RT-PCR.

6.2. Molecular Characterization of CHIKV

Phylogenetic tree constructed using geographic sequences of different genotypes has shown that all the study sequences belonged to ECSA genotype. ECSA genotype clade is rooted with strains isolated from Congo during 1960. Three clades were observed in the

ECSA lineage. Majority (n=32) of the study sequences were found in clade III along with sequences isolated from South East Asian countries and Italy. Rest of the study sequences (n=19) were found in clade I along with the sequences of Indian Ocean strains, Indian, African strains and the sequences related to Indian Ocean lineage (IOL). None of the study sequences found in clade II which had sequences isolated from South East Asian countries and strains isolated in 2013 from India. The strains isolated from India after 2012 were found in Clade II. It may be due to the amino acids divergence. No specific genetic diversity was noted in the consensus sequence of 2007-2010 isolated from different periods since they were formed in the same cluster. Nucleotide divergence was found to be 4.1%, 5.28% and 6.41% in clade I, clade II and clade III respectively (Figure 16). Branch length of 2011-2016 consensus sequence was more when compared to all other sequences which may be due to nucleotide and amino acid divergence. Over all 20 nucleotide substitutions were observed in our study sequence, of which only 7 substitutions were noticed at the amino acid level.

Nucleotide divergence of IOL when compared to S27 (ECSA) is 2.7%, Asian clade of Nagpur strain showed 5.1% and with west African clade from Senegal showed 15% divergence (I Schuffenecker *et al.*, 2006). Indian Ocean strain outbreak started from Kenya in 2004 and it emerged from ECSA group. Phylogenetic analysis

showed two different lineages for Indian ocean and Indian subcontinent as described earlier (Volk *et al*, 2010). The most recent common ancestor (MRCA) in our analysis was La reunion 2005 strain.

In Africa, Aedes mosquito species such as *Ae. furcifer, Ae. taylori, Ae. Africanus, Ae. luteocephalus and Ae. neofricanus* are involved in transmission of CHIKV between non-human primates and canopy-dwelling mammals (Yergolkar *et al.*, 2006, Pavri *et al.*, 1986, Burke *et al.*, 1985, Powers and, Logue. 2007, Weaver and, Reisen WK. 2010). *Ae. aegypti* was the principal vector in transmitting Asian genotypes; however, the ECSA genotype of CHIKV switched to *Ae. albopictus* mosquitoes as vector and resulting in wide spread epidemic in Asia (Kumar *et al.*, 2010, Eapen, *et al.*, 2010). The earlier isolates from India (1963-1973) were the Asian genotype. Among isolates of the Asian genotype, all older isolates *i.e.* India 1963-1973 and Thailand 1962-1978 clustered together, while Philippines (1985), Indonesia (1985), Thailand (1788, 1995, 1996) and Malaysia (1998) form a separate cluster (Arankalle VA *et al.*, 2007).

An analysis of the isolates of CHIKV causing the epidemic since 2005 indicate the prevalence of ECSA type replacing the Asian type. The Asian type was last reported from Malaysia (AbuBakar *et*

al., 2007) and parts of India in Kolkata (Shah KV *et al.*, 1964), Vellore (Jadhav *et al.*, 1965) & Chennai.

The Yawat isolate (2000) from Maharashtra was the first ECSA strain identified in India (Yadav *et al.*, 2003). The Asian genotype had affected the urban areas while the African genotype had mainly affected rural areas. Also, the Reunion Island outbreak and the epidemic in India were caused by same strain (Joseph AY *et al.*, 2008).

The ECSA and the West African strains appear to produce adequate antibody titre and based on Plaque reduction neutralization tests (PRNT) have shown distinct antigenic subtypes which have more than 4-fold difference. (Powers *et al.*, 2000, Calilsher *et al.*, 1980).

Previously E1-A226V mutation was documented (Kumar NP *et al.*, in 2007). Recently newly identified L210Q mutation has been documented among Kerala strains (Niyas *et al.*, 2010). The E1-A226V mutation has allowed the views to adapt to new vector *Ae. albopictus*, apart from *Ae. aegypti* thus allowing for greater epidemic potential. The significance of L210Q mutation is not clearly known. It is postulated that it may have increased virulence; Further studies need to be conducted to determine significance of L210Q.

Tsetsarkin *et al., (2009*) reported E2 – G60D and E2-I211T mutations that were found in the viruses derived from *Ae. albopictus* and *Ae. aegypti* mosquitoes. The E2-G60D mutation was an important determinant of CHIKV infectivity for both *Ae. albopictus* and *Ae. aegypti*. CHIKV infectivity for *Ae. aegypti* was not influenced by the E2-1211T mutation. The occurrence of the E2-60G and E2-211I residues among CHIKV isolates was analysed, revealing a high prevalence of E2-211I among strains belonging to the Eastern/Central/South African (ECSA) clade. This suggests that E2-211I position might be important for adaptation of CHIKV to certain conditions prevalent in areas occupied by ECSA strains.

In a study conducted by NIV (Yergolkar *et al*., 2006) during the CHIKV epidemic of 2005 in the states such as Andhra Pradesh, Karnataka and Maharashtra in India stated that, the predominant mosquito species in the affected regions were *Ae. aegypti*, and *Ae. albopictus* was either absent or present in negligible numbers.

Yergolkar *et al*, (2006) found that the 2005 outbreak of the areas studied were predominantly rural. Our study also confirms the spread of CHIKV to rural areas; recently there has been a re-emergence of CHIKV in India following an outbreak in the La Reunion Islands. *Ae. albopictus* was found to be the vector for the La Reunion outbreak. In India, the main vector is *Ae. aegypti;*

however, recent studies also document transmission through *Ae. albopictus.*

Data Monkey software was used in the analysis and positive selection pressure was observed among our study sequences. E2 variability is also increasingly being reported (Sahu et.al, 2013). We have also encountered variability in E2. E2 protein exhibited indicative adaptive evolution. Adaptive evolution may enhance binding with the host cell receptors in different tissues or multiple organs resulting in virulence of the disease.

As previously described, E2 glycoprotein is vector determinant which indicated by sequential of adaption of alpha virus to a new mosquito (Ravi *et al*., 2006, Sourisseau *et al*., 2007**)**. Different mechanisms of host cell interactions and spread can occur due to specific genotype residues in the transmembrane domain. Transmembrane helix hold E2 protein or pE2 protein in virus induced proliferated intracellular membranes for stronger interaction with the host receptors (Sourisseau *et al*., 2010).

Overall, 7 amino acid substitutions such as S299N, T312M, A344T, S375T, V386G, W339R and S375P were observed. Novel substitutions W339R and S375P were noted in two of our sequences. V386A substitution has previously been reported in sequences isolated during 2006-2010, we have also observed V386G in our

study sequences from strains isolated during the same period. S375T was reported in E2 gene of Asian and African genotypes which is concordant with our report. Also, S375K in E2 gene and other two substitutions E2-M384N and E2-M384I were reported (Sahu *et al*., 2013).

Specific conformational changes were not observed in the structure of the domain C of E2, which indicates that amino acid substitutions observed in particular region does not affect the structural stability and topology of the protein. However, amino acid changes in E2 may affect interaction with other host proteins and tissue tropism (Niyas and Abraham *et al*. 2010), which means if there is any mutation in the amino acids which are involving in the interaction directly or indirectly will affect the interaction which leads to conformational changes. Multiple amino acid substitutions in the envelope glycoprotein may help CHIKV to adapt to a new mosquito vector and may also contribute to disease severity (Tsetsarkin *et al*., 2009). More studies are required to understand the effects of novel substitutions in the E2 region. Our study results along with published reports (Niyas *et al*., 2010, Chevillon *et al*., 2010, Sahu *et al*., 2013) indicate that there is a persistent change in the genome of CHIKV strains.

The amino acid positions 356-379 and 365-385 of E2 codes for transmembrane protein and can be used as drug target for CHIKV(Sahu and Das. 2013). Mutations in domain C impact in variation of binding strength of CHIKV to the host cell. Continuous monitoring of the virus evolution using a molecular approach may help to understand the changes in genome of CHIKV and aid towards drug discovery.

CHIKV continues to be reported till date from different regions of India suggesting the need for continued surveillance that can be done by serological and molecular tests such as RT-PCR in early diagnosis and containment. Strategy for the containment of CHIKV should include RT-PCR to detect CHIKV viremia since it can detect the virus during the acute illness. All these reported mutations in the transmembrane domain of E2 suggest the further studies in understanding the disease severity and designing anti-viral drugs are necessary.

The virus has undergone mutation in A226V in E1, and this mutation has been extensively described (Kumar NP *et al.*, 2007). It is also postulated that this mutation may contribute to the changing manifestation of CHIKV. What is well known is that the A226V mutation allowed for a mosquito vector *Aedes albopictus* to emerge. Another mutation E2-L210Q gene indicates the increase in

percentage of patients suffering joint pain may be attributed to it. (Niyas *et al.,* 2010) However further studies need to be conducted to conclusively prove such an association. Experimental studies in animal models indicate that the virus infected macrophages may reside in intra articular space and accumulate following virus replication (Karine *et al.,* 2010). Release of pro-inflammatory cytokines may contribute to the debility.

CHIKV E1 gene was extensively studied and limited data was available on E2 region which is highly conserved and responsible for host cell interaction (Tsetsarkin, *et,al,* 2007). Characterization of E2 will assist in understanding disease severity and pathogenesis. Recent epidemic resulted in severe clinical manifestation therefore we investigated E2 region. Adaptation of CHIKV to a new vector, *A. albopictus* supported the wide spread of CHIKV in India (Vazeille, et,al, 2007). Alpha virus E2 protein has three distinct subdomains such as N terminal domain A located in the center of the 3D structure of E2, domain B; at distal tip of the ecto-domain that interact with a cellular receptor and the terminal domain C is close to the viral membrane. Domain C which was known to be conserved was sequenced in this study for amino acid or nucleotide substitution (Kielian, 2006).

Detection of IgM and IgG by ELISA is significant in acute phase (after 5 days infection and convalescent phase (persist for several years) from plasma or sera. Serological assays are moderately specific due to cross reaction with Alphaviruses (Kam *et al.*, 2015). RT-PCR detects CHIKV within a week (day 1 of illness to 10 days) by targeting E1, E2 and NSP region.

Co-existence of CHIKV and DENV-2 were reported earlier in India (Chahar *et al.*, 2009). DENV and CHIKV multiplex RT-PCR was developed and optimized to differentiate both viruses from a clinical specimen in single tube one step multiplex RT-PCR and the assay can be completed within shorter duration (5-6 hr.). Multiplex RT-PCR was 100% sensitive when compare uniplex RT-PCR for CHIKV and DENV, as performing separate RT-PCR for each virus is time consuming. Multiplex PCR is time efficient, convenient, reduce the expenses to three-fold; and prevents risk of contamination (De Paula *et al.*, 2004).

Three genotypes (Asian, ECSA and West African) have been reported so far. Recently ECSA from Indian Ocean lineage (IOL) caused epidemics in India Ocean islands, mainland India and Europe during 2004 (Volk SM *et al.*, 2010). Asian genotype was reported in India during 1963-1973CHIKV epidemic whereas the re-emergence of recent epidemic was reported with ECSA genotype (Arankalle, *et al*

2007). The first ECSA strain in India is Yawat isolate (2000) from Maharashtra (Yadav *et al.*, 2003). All the isolates of CHIKV in our study was characterized as ECSA genotype and these were belonged to Indian Ocean lineage (IOL). Several studies reported that Asian genotype was replaced by ECSA genotype during recent epidemic period (Patrick. *et al.*, 2008). Nucleotide divergence in clade I, clade II and clade III were 4.1%, 5.28% and 6.41% respectively.

Studies reported S375T substitution in E2 gene which is concordant with our report. A study also reported S375K mutation apart from S375T in E2 gene and two other substitutions such as E2-M384N and E2-M384I (Sahu *et al.*, 2013). Multiple amino acid substitutions in the envelope glycoprotein may lead the virus to adapt a new mosquito vector and contribute to disease severity (Tsetsarkin *et al.*, 2013). In our study isolates, novel V386G mutation was found in all the sequences and W339R and S375P were noted in two of our sequences. Since the mutation at the amino acid position 386 was noted in all the sequence, the significance of this needs to be revealed. Further genetic characterization like *in silico* study may be carried out to experiment the changes in the protein structure.

One of the predicted effects of such amino acid changes is the exposure of buried protein surfaces. Possibly, this may alter the interaction of E2 with other proteins, particularly with cellular

receptors, and may impact on tissue tropism (Niyas *et al.*, 2010). However, further studies are required to understand the effects of novel substitutions in the E2 region. The results from this study, along with the previous observations (Sreekumar *et al.*, 2010) indicate that there is a constant genomic evolution among circulating CHIKV strains. The amino acid positions 356-379 and 365-385 codes for transmembrane helices can be used as choice of drug target. Mutations in domain B can modulate binding of CHIKV to host cell. Continuous monitoring of the virus evolution with a molecular approach may help to understand the changes in genome of CHIKV and towards vaccine development and drug discovery.

6.3. Immunopathogenesis of Dengue

Th1 type of response is characterised by IFN-γ, IL-2 and TNF-β. Th2 response is characterised by IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines. These Th1/Th2 responses have been studied to develop a better understanding of disease progression in DEN. Certain non Th1/Th2 cytokines such as TNF-α, IL-8 and IL-1 are also implicated in promoting Th2 response indirectly. Th1 and Th2 responses have also been studied for better understanding of the severity of arthralgia/arthritis.

6.3.1. Viremic group and non viremic group:

In this study, we have analysed the cytokine IFN-γ (Th1) IL-6 and IL-10 (Th2) and TNF-α and IL-8 (non th1/th2) in both CHIKF and DEN. There is a direct correlation between viremia and severity of the disease (HY Cheng *et al.*, 2014). We analysed the representative cytokines among those who were viremic as evidenced by RT-PCR positivity versus non viremic that is RT-PCR negative. Among those who had viremia 71% (25/35) showed detectable levels of IL-8 (Range=11.8-1564.4pg/ml, mean=200.28pg/ml, median=52.62pg/ml) followed by elevation of TNF-α (48.57%, 17/35) and IL-10 (45.71%, 16/35). Among the inflammatory mediators, cytokine IL-6 is known to inhibit the replication of the virus (Kuo *et al.*, 2009) and this was detected in 42.85% (15/35). In the study group, 6.66% (3/45) of patients did not show presence of IL-6, (one patient was non viremic and two were in the viremic group). Due to cross regulation of Th1 and Th2 response, secretion of IFN-γ by Th1 response may inhibit IL-6 production which is secreted by Th2 cells. IL-6 suppresses virus, therefore patient is more viremic IL-6, released to suppress replication of virus in turn these cytokines promotes Th2 response. Non Th1/Th2 response also promotes Th2 then IFN- γ promotes Th1 response. Elevation of IFN-γ in the viremic group may prevent disease progression as majority of the patients were in probable dengue group (85.71%, 12/14).

Response of non Th1/Th2 cytokines TNF-α (64%, n=16) and IL-8 (96%, n=24)) found more in viremic phase of dengue and it was statistically significance. Among Th2 cytokines, IL-10 response in non viremic patients was more than IL-6 response it indicates that IL-10 may have significant role in clearance of virus. At the same time IFN-γ (Th1 cytokine) was not expressed much in non viremic group, as IL-10 is known to be an inhibitor of IFN-γ. IL-10 is also called as immunoregulatory cytokine which involves in the balance of Th1/Th2 response. IL-10 and IFN- γ are involved in the cross regulation of Th1 and Th2 respectively (Chaturvedi *et al.*, 2000) Higher response of IL-10 in non viremic group also indicates it has significant role in inhibition of viral replication and viral infection stimulated NK cell activity. IL-10 may play a significant role in clearance of virus as it mediates B-cell activity and function which is responsible for antibody production (Wilson *et al.*, 2011).

Th2 response (IL-6 and IL-10) in dengue viremic group was found to be 32.06% (n=15) and in non viremic IL-10 was found to be 19.56% (n=9), IL-6 was 15.21% (n=7) patients. Existing data on dengue immunopathogenesis suggests that TNF-α is secreted by macrophages which are induced by CD4+ T cells by secreting human cytotoxic factor (hCF), TNF-α causes increased vascular permeability which leads to DHF. In our study TNF-α level was found to be raised in both viremic and non viremic group. TNF-α

was not associated with severe dengue, elevation was observed in dengue fever and dengue warning sign group and this was concordant to a study (Bozza *et al.*, 2008). However, some studies reported that TNF-α associated with severe dengue mainly during DHF in a Brazilian population (Hober *et al.*, 1993, Braga *et al.*, 2001).

6.3.2. Acute and convalescent:

Cytokines were analysed from samples both during acute phase and convalescent phase of illness. Cytokines IL-10, IL-8, IL-6, IFN-γ were elevated during the acute phase of illness and declined during the convalescent phase which was found to be statistically significant. IL-6 is a pyrogen and was elevated in 48.88% (22/45) of patients who had evidence of fever clinically. Elevated IL-8 also correlated with thrombocytopenia (46.66%, 21/45), and liver damage (AST-40%, 18/45 ALT-35.55%, 16/45). In addition, TNF-α and IL-10 correlated with ALT/AST and thrombocytopenia. IL-8 causes tissue damage as evidenced by increased AST/ALT due to liver damage and cause platelet destruction evidenced by thrombocytopenia (Chaturvedi *et al.*, 2000, Lei *et al.*, 2001). IL-8 increases the process of increased vascular permeability leading to DHF. Patients with increased IL-8 had high viral load (Ct value – 21.16) of dengue serotype-1 and presented with myalgia and severe head ache. Increased IL-10 and IL-8 correlated with increased in

liver enzymes indicating hepatic involvement. Difference in the TNF-α response may be due to different serotypes of dengue or in host immune response such as different TNF-α genetic polymorphism (Fernandez-Mestre MT *et al*., 2004).

Among acute group of dengue, a non Th1/Th2 cytokine (IL-8) elevation was found in all patients. Only one patient showed elevated level of IL-8 at convalescent phase remaining all showed decreased level at convalescent phase. Though elevated of IL-8 was found in one patient, he did not develop to in severe dengue and he recovered from the illness. Th2 response was found to be high in acute group than Th1 response. Among Th2 cytokines IL-10 was elevated in majority of acute phase of dengue. IL-6 a Th2 cytokine, was elevated in convalescent phase of two patients, none of other cytokines were elevated in convalescent phase except IL-8 in one patient.

6.3.3.Cytokine profile in Probable dengue, DWS and Severe dengue:

A Thirty-two-year-old male presented with severe head ache and myalgia with fever of 5 days which progressed to multi organ failure on 8th day and patient succumbed to death. This patient had shown High IL-10 and IL8 with absence of IFN-γ was low. Patient had also elevated liver enzymes (ALT (4043) and ALT (11196). As

this patient had elevation of IL-8 and IL-10, it indicates that production of IL-8 mediates Th2 response (IL-10). Absence of IL-6 reveals that when TNF-α get suppressed and IL-6 is also suppressed.

TNF-α elevated level (n=10) also correlates with thrombocytopenia. However, these patients recovered. Suppression of TNF-α and IL-6 promotes secretion of IL-8 and IL-10 which results in severity of the disease. In IFN-γ elevation average probable DEN was observed in 85.71% (12/14) and 40% (2/5) in severe DEN and none of the patients in dengue warning signs group. Decreased levels of IFN-γ (mean=21.32pg/ml) suggest that it has minimum role in severe DEN. IL-8 was elevated in majority of the patients in the severe DEN group (80%, 4/5) and the range was 0.62-1121.35, mean=106.09.

In our study, Th1 response of dengue fever and severe dengue was found in 33.33% (n=12) and 40% (n=2) respectively None of the cases of DWS group showed Th1 response. As non Th1/Th2 cytokines (TNF-α and IL-8) were elevated in the DWS group it may block the pathway of Th1 response. As association of Th2 cytokines were found to be high in Dengue fever and severe dengue than dengue warning signs. Th1 cytokine response was found in less number of the patients of dengue fever, DWS and severe dengue than Th2 and non Th1/Th2 cytokines. Among Th2 cytokines IL-10

(58.53% (n=21)) and was found to high in DF than IL-6 (52.77% (n=19)). IL-6 as pyrogen (Dinerello *et al*., 2004) may have been responsible for acute fever in dengue fever group and it acts as myokine which results in muscle contraction due to elevated IL-6 concentration, though more number of patients with myalgia are associated with IL-8 elevation than IL-6 elevation.

Patients who had shown elevated level of >100pg/ml of IL-8 were more than 50% with increased level of SGPT (61.11% (n=11)) and SGOT (55.55% (n=10) IL-8 causes tissue damage and involved in the acute and chronic liver disease. For the diagnosis of various hepatitis virus infection, IL-8 has been used as a marker (Masumoto T *et al*., 1998), which shows the significant role of IL-8 in liver damage. Association of elevation of IL-8, TNF-α and IL-10 correlated with the elevation of AST and ALT enzymes. DENV is known as hepatotropic and has been detected in hepatocytes in the liver biopsy of patient with DHF (Rosen L *et al*., 1989).

In our study, IL-8 (46.66%, n=21) highly correlated with thrombocytopenia of DEN patients. IL-6 and IL-10 (28.88%, n=13) also associated with patients with thrombocytopenia. The characteristic features of DHF/DSS includes capillary leakage, thrombocytopenia and coagulopathy. As IL-8 is involving in tissue and cell damage it may also be involved in the platelet destruction

and IL-6 and IL-10 involving in the Th2 response which mediates antibody production, those antibodies behave as cross reactive antiplatelet autoantibodies. As per earlier report, over production of IL-6 might have major role in the enhanced production of antiplatelet autoantibodies. In contrast in our study IL-8 was highly associated with thrombocytopenia cases. It indicates that IL-8 promotes Th2 response as it is antibody mediated. Antibodies produced by Th2 response will also lyse the platelets as it behaves as autoantibodies to platelets.

In cytokine level analysis in the acute and convalescent patients, two patients showed levels of all the 5 cytokines in convalescent phase and one patient showed elevated TNF-α at convalescent phase, both the patients reported with grade II and grade III thrombocytopenia. Association of IL-6, IL-8 was found in some patient both acute and convalescent phase which developed in to severe dengue. As IFN-γ is involved in the Th1 response, it causes DF.

However, sometimes mixed response of Th1/Th2 may occur due to difference in dengue serotypes and host immune response and other factors etc., Earlier identification of disease will help management of the severe cases and it can protect the patients from death due to dengue. More reports suggested that deregulation of

cytokines and inflammatory response play a key role in the development of severe clinical manifestations (Clyde *et al.*, 2006). Cytokines can be used as a better biomarker and elevated cytokine levels will indicate disease condition. By using immunomodulatory agents against these cytokines severity of the illness can be reduced.

6.4. Immunopathogenesis of CHIK

Our study suggests that majority of the CHIKV patients had non Th1/Th2 cytokine (IL-8) elevated among those who were viremic (80%, 16/20, mean = 73.51). Th1 (IFN- γ) response was detected in 36.66% (n=11) patients in acute phase when compared to healthy controls and this was statistically significant. IL-6 was marginally elevated in both viremic and non viremic groups as compared to controls and this was statistically significant ($p<0.05$). Since IL-6 is a pyrogen, it may be responsible for acute fever that characterises CHIKV. IL-6 may also have significant role in myalgia of CHIKV patients.

TNF-α was not elevated in the non viremic group in any of the patients but was elevated in the viremic group (35%, n=7/20, mean=2.97$\pm$9.94). A Non Th1/Th2 cytokine, TNF-α was not elevated among majority of cases of CHIKV, but the other non Th1/Th2 cytokine (IL-8) and Th1 cytokine (IFN-γ) were found to be significantly high. This specifies that expression of IL-8

(Mean=106.67± 93.33pg/ml) alone could not block Th1 response as is evidenced by increased IFN-γ (Mean=108.57± 61.13pg/ml) level in the absence of TNF-α (Mean=0) in the non viremic group. TNF-α is found repeatedly in patients with rheumatoid arthritis. TNF- α was slightly elevated only in the viremic group which is in agreement with the previous report (Ng *et al.*, 2010). TNF-α may be produced locally and detection of TNF- α in synovial fluid reveals the role of TNF-α in CHIKV arthralgia. High levels of TNF-α, have been observed in convalescent group within four weeks of illness (Anuradha *et.al.*, 2014). A limitation of our study is that convalescent samples could not be collected.

Majority of the patients with severe joint pain had elevated level of IL-8 and some of them were associated with elevated IL-6 and IFN-γ. As IL-8 and IFN-γ were found to be elevated in majority of the patients in the viremic group and also in the non viremic which may have significant role in CHIKV pathogenesis. All the patients had strong clinical features of CHIKF (the samples were collected from CHIKV epidemic area). Severe joint pain cases were scored by severity pain in the joints, restricted movement of the patients due to joint pain and joint inflammation (http://www.chikungunya.in/chikungunya-joint-pain.shtml).

Several studies have reported elevated levels of IL-8 in serum and synovial fluid of Rheumatoid arthritis (RA) patients. However, this was not attempted in our study as this is an invasive procedure. Ross river virus (RRV) infected macrophages and fibroblasts are involved in the upregulation of IL-8 and MCP-1, showing that IL-8 plays a similar role in epidemic polyarthritis (EPA) due to alphavirus RRV also (Mateo *et al*., 2000). Since IL-8 was elevated in majority of patients with severe joint pain, IL-8 might have a crucial role in CHIKV arthralgia.

In our study, majority of the patients presented predominantly with Th1 cytokine (IFN- γ) response. Though IL-8 elevation was observed in many of the patients, Th1 response was not blocked and Th2 response was less among CHIK group.

Cytokine level was high in DEN patients than CHIK patients. Among the DEN viremic group it was observed that cytokine levels were high, as well as DEN acute phase of illness, in the convalescent phase cytokine levels dropped. TNF-α was not detected in the non viremic group of CHIKV, however it was detected in the non viremic group of DEN (one patient had 503pg/ml of TNF-α). In both CHIKV and DEN, IL-8 was associated with disease severity. Majority of patients with DEN showed elevated level of IL-8 and IL-10, IL-8 is a non Th1/Th2 cytokine that mediates Th2 response (IL-10) whereas in

CHIKV group a Th2 response mediated by IL-10 was not observed. Probably, Th2 response in CHIKV was mediated by IL-6 as this was elevated in most of the patients than IL-10. Usage of immunomodulatory agents was aimed at suppressing the immune response which causes over production of cytokines as a response against the virus. Anticytokines antibodies can be used as immunomodulatory agents in controlling the elevated cytokine levels.