Discussion
5.0 Discussion:

Any attempt to prepare a vaccine is need-driven and the commercial availability is usually market/ economics-driven. This is true for Chikungunya infection. With the initial outrage of the disease during 1950-60s, attempts were made to prepare the vaccine by using the technologies available at that time. These included both the attenuated and killed vaccine formats (Levitt et al 1986, Harrison et al 1967). One of the vaccines did go to clinical trials but the trial had to be withdrawn on account of the reactogenicity. No further attempts were made probably because the virus was almost dormant for a few decades and neither researchers nor industry were keen in pursuing this goal.

With the forceful come back of Chikungunya during the first few years of the 21st century, it was natural for the scientists to revisit the issue of vaccine development. India, being one of the worse-affected countries was not an exception. The National Institute of virology at Pune, India was responsible for the identification of Chikungunya virus as the aetiology of outbreaks of dengue-like outbreaks in the rural Karnataka during October 2005. As the ELISA reagents for the diagnosis of Chikungunya (IgM-anti-CHIK antibodies) could be quickly prepared and made available to government agencies, diagnosis of large-scale outbreaks in newer areas could be quickly identified. It was also shown that the current episode is caused by the virus of ECSA genotype (causing outbreaks in Africa and Indian Ocean islands) and not the Asian genotype responsible for the earlier epidemic (Yergolkar et al 2006, Arankalle et al 2007).

With the rapid spread of the virus, continued morbidity in a significant proportion of the patients, serious complications involving various organs and high mortality, a definite need for (1) specific anti-viral/ immunomodulatory therapy leading to uneventful recover (2) potent vaccine for the individuals residing in the unaffected areas and (3) protection from the infected mosquitoes (almost complete eradication of the vector, most desirable) was strongly felt. We decided to concentrate on the development of a vaccine.
For any virus that does not replicate efficiently in cell cultures (most preferable) or small animal models (not really desirable), use of recombinant technology remains the only alternative. Fortunately, CHIKV grows very rapidly in cell-lines certified for the preparation of vaccines for human use and therefore, the conventional approach is possible. However, with the earlier experience of failure of an attenuated vaccine candidate in phase II clinical trial and availability of newer platforms and required expertise, various technologies were applied to the development of much needed chikungunya vaccine.

In our department, we have successfully developed recombinant protein-based vaccines for hepatitis E (Deshmukh et al 2007) and Chandipura (Venkateswarlu et al 2009) and combination vaccines for hepatitis B and E (Shrivastava et al 2009) and Chandipura and DPT (Venkateswarlu et al 2010). We therefore, opted for the development of a recombinant protein-based vaccine. As the virus replicates efficiently in cell culture, it was also thought equally important to develop conventional, whole virus-based killed vaccine.

Considering the fact that E1 and E2 proteins are the major envelope proteins forming spikes on the surface of the virion, we initially planned to use both the proteins as immunogens. However, due to inability to express the entire E1 and E2 genes, the strategy was modified to include only E2 protein. In this respect, it may be noted that most of the neutralizing epitopes are present on the E2 protein of CHIKV and other related viruses such as VEEV (Hunt et al 2010). Subsequently, this decision proved to be correct, leading to a candidate vaccine producing sterilizing immunity in adult mice. Once we decided to opt for the E2 protein, obtaining the protein in a soluble form was an important for consideration. For this, we removed the trans-membrane region (rich in hydrophobic amino acids) of the protein and cloned the ectodomain region. For the expression in the bacterial system, BL-21(DE3) RIL Codon plus competent cells containing extra plasmids coding tRNAs for rare codons (for Arginine, Leucine and Isoleucine) was used. Use of these cells led to satisfactory expression of rE2p when compared to only BL21 (DE3) cell. This protein reacted with serum samples from CHIK patients.
confirming the immunoreactivity. Importantly, after purification, a single band was obtained that was reactive with patient’s sera in immunoblot. Thus, we could get the immunoreactive rE2p of reasonable purity (Figure 15). The yield of purified rE2p was ~ 1.5 mg of purified protein from 50 ml of induced bacterial culture.

The next step was the evaluation of immunogenecity of the recombinant protein. Requirement of adjuvants for the generation of immune response against recombinant proteins is well known and accepted. We evaluated 3 adjuvants in enhancing immune response of the rE2p in mice, CadB (liposome-based), MW (from mycobacteria) and alum (universally accepted adjuvant). Earlier, encapsulation of either protein and corresponding DNA or protein alone in liposomes / CadB was shown by us to be the best approach for the development of recombinant hepatitis E protein vaccine (Shrivastava et al 2009, unpublished observations). CadB, therefore, was the adjuvant of choice. As MW predominantly induces Th1 (Andersen et al 2009) type response, its evaluation was thought important in case the protective immunity is mainly of Th1 type. Alum was included as the standard adjuvant for the evaluation of the performance of other adjuvants.

Contrary to most recombinant proteins, it was indeed satisfying and interesting and to note that rE2p itself was a good immunogen, 50% of mice developing neutralizing antibodies after 1st dose, increasing to 100% after the 2nd dose and, with high antibody titres (Table 10, Figure 25). As the antibody titres were immunogen dose-dependent, 50ug dose inducing highest titres was used for all subsequent comparisons. All the adjuvanted preparations significantly enhanced the antibody titres. However, the adjuvants did not differ in enhancing immunogenecity of rE2p. CadB had an edge over the other adjuvants as 83% mice seroconverted after the 1st dose and also exhibited higher antibody titres at 2 weeks post-2nd dose.

Before dealing with further experiments with rE2p, let us consider similar data with the inactivated vaccine preparations.

The primary requirement of rapid in-vitro replication of the virus was fulfilled by CHIKV and therefore it was possible to undertake this approach. The virus was grown in Vero cells approved for the production of vaccines by
the regulatory authorities. As we needed to harvest the virus 48-72hrs after infection, it was possible to use serum free media, making virus purification easier and convenient (Figure 18 A, B).

Two inactivating agents, formalin and BPL, were tried. After understanding kinetics of inactivation by these agents, inactivation time was finalized for both the preparations and followed for all the experiments. For inactivation, virulence in infant mice and cell lines was assessed by serial 2-3 passages. At this time, both the inactivated formulations were ready for further evaluation.

Formalin is commonly used as a preservative for biological samples and a potent disinfectant as well. It is also a commonly used inactivated agent for vaccines. Incidentally, the first killed vaccine developed for CHIKV was formalin-inactivated (Harrison et al 1967). Formalin causes minimal alterations in the protein structure as it lacks hydrophobic groups thereby maintaining antigenic properties of the treated viruses (Fraenkel-Conrat H, 1981). However, the concentration of formalin and time for inactivation should be optimum, to avoid reduction / loss of immunogenicity. For many medically important viruses viz. FMDV, EEEV, JEV, RRV etc., formalin is used for the preparation of vaccines that are under use (Barteling and Woortmeyer 1984, Maire et al 1970, Appaiahgari et al 2004, Kistner et al 2007).

Alum and MW adjuvants were evaluated with formalin-inactivated vaccine candidate. We decided against the CadB adjuvant as encapsulation of the whole virus was not considered necessary for enhancing immune response. CHIKV seems a very good immunogen as 50% mice after the first dose and 100% mice after the 2nd dose exhibited neutralizing antibodies of reasonably good titres. Thus, both the virus as well as rE2p based vaccine candidates were immunogenic when used even without any adjuvants. After the administration of the 1st dose of formalin inactivated virus to mice, the alum-adjuvanted and MW-adjuvanted preparations induced 100% seroconversion to neutralizing antibodies. The antibody titres were significantly higher for alum than Mw (p=0.0013).

The third candidate that we need to look into is the BPL-inactivated preparation. Though BPL has carcinogenic properties, it has a major
advantage as well. During inactivation at room temperature, within few minutes, it gets completely hydrolysed to β-hydroxypropionic acid, a non-toxic substance naturally present in human body as a by-product of fat metabolic pathway. As a result, no additional chemicals are introduced in the body. Inactivation by BPL is thought to be due to its affinity for purine bases of nucleic acids. Several DNA and RNA viruses have been inactivated by this method (SARS virus by Roberts et al, 2010, CHPV by Jadi et al, 2011, IBRV by Kamraj et al 2008).

Based on the results obtained with formalin inactivated CHIKV, we did not evaluate MW adjuvant and restricted ourselves only to alum. Overall, BPL-inactivated CHIKV was the best preparation, the virus alone inducing 100% seroconversion to neutralizing antibodies 2 weeks after the first dose. Alum did not significantly enhance the antibody titres generated by the virus alone (p = 0.273). The highest reciprocal neutralizing (2560) and ELISA (102400) titres were recorded for this formulation, others being 2-fold less (51200 for formalin and rE2p each) in terms of ELISA titres and 2 to 8 fold less in terms of nAb titres (highest reciprocal titre was 1280 and 320 for formalin and rE2p preparation respectively).

For performing ELISA, we had used the same immunogen for immunization and coating ELISA wells. However, sera from mice receiving rE2p as the immunogen were equally reactive when CHIKV-coated wells were used for ELISA confirming that the antibodies generated against the truncated recombinant protein expressed in bacteria could bind to the native virus. Vice-versa was naturally true.

Next, the type of immune response generated (Th1/Th2) was classified on the basis of IgG subtype analysis and secretion of cytokines by the splenocytes of immunized mice stimulated by the respective antigens. Overall, alum adjuvanted formulations led to balanced Th1/Th2 response indicative of activation of both arms of immune response. The discrepancies between the type of immune response indicated by isotyping and CBA analysis could be that undefined cell populations of splenocytes are involved in cytokine generation and analysis through CBA, while serum is used for
isotyping. The adjuvant-driven shift in the immune response for the same antigen needs further in-depth analysis.

Thus, we could generate three variants of vaccine candidates worth exploring further: a recombinant protein-based and two inactivated preparations. Based on the neutralizing antibody titres, we selected following preparations for challenge studies in mice:
1. 50 µg rE2p/ dose with Alum,
2. 50 µg rE2p/ dose with CadB,
3. 50 µg formalin inactivated CHIKV/ dose with Alum,
4. 50 µg formalin inactivated CHIKV/ dose with MW,
5. 50 µg BPL inactivated CHIKV/ dose with Alum and
6. 50 µg BPL inactivated CHIKV/ dose without adjuvant.

While the vaccine development work was going on, a study on the development of animal model for Chikungunya was undertaken by another researcher. He was looking for reproducibility of symptoms observed in humans. Though adult mice of different species were first to be evaluated, in the absence of symptoms, were not pursued further. Instead, 8days old mice were symptomatic (Patil et al 2011) that could not be used for vaccine evaluation. As our need was adult mice, we revisited this age-group (Balb/c mice used for immunization). Three routes of infection were tried:
1. Intravenous (highest possibility of reaching the target organ for initial viral replication)
2. Intramuscular (the target tissue for CHIKV) and

Though we looked for symptoms such as fever and loss of weight, our major thrust was evidence for viral replication. For this, following parameters were considered:
1. Detection / quantitation of viral RNA in blood and the target organ (muscles) as well as brain and spleen (Real time RT-PCR).
2. Sero-conversion to anti-CHIK antibodies.
3. Tissue pathology.
The results (Tables 8, 9; Figures 23, 24) clearly showed that mice could be infected by all the three routes although none of them were febrile (Figure 22) or exhibited significant weight loss (Figure 21). Therefore, we could not assess vaccine efficacy in terms of reduction / elimination of clinical symptoms. Viral loads in infected muscles and spleen as well as viremia could be detected till 4th day post-infection, highest being mice infected by the intranasal route. Why only brains of mice infected by intranasal route were viral RNA positive for long time remains unclear. Consistent with the absence of clinical symptoms, histopathological changes were not very severe (Figure 24). The criteria for protection from intranasal challenge of CHIKV was decided to be the absence/ reduction of viral RNA in blood and muscles, though other tissues were examined as well and behaved similarly. We did not do histopathology on the mice tissues from immunized and challenged mice.

We would like to point out here that other investigators also faced similar problems for efficacy testing and have used various options for the evaluation of vaccine candidates generated by them, the best being rhesus monkeys (Akahata et al 2010, Mallilankaraman et al 2011). However, it is not easy to get permission for use of monkeys when alternative small animal model approaches have not been explored. As far as mice are concerned, the first reported study during the current episode (Tiwari et al 2009), tried 3-4 week old swiss albino mice. Though very high PRNT titres (1:6400) were obtained, in-vivo protective titres were only 1:30 by the method adopted. Wang et al (Vaccine 2008) used three week or older C57BL/6 mice strain for vaccination purpose and mouse adapted CHIKV strain (Ross strain, passaged 175 times in newborn mice) for challenge studies by intranasal route. 8 week or older female BALB/c mice was used for studies by Mallilankaraman et al (PLoS NTDS 2011). However, CHIKV strain used to challenge vaccinated mice by intranasal route was a highly virulent one (isolated from a patient). Akahata et al (Nat Med 2010) studied their vaccine protective efficacy by passive transfer of immunized mice sera into immune-compromised mice (IFN α/βR⁻-deficient) and challenge with virulent CHIKV strain (LR2006 OPY-1 isolated from recent epidemic in ReUnion Island).
As the vaccine requirement could be during epidemics / at the time of re-emergence (an emergency situation) or after detecting a case in the family, a two-dose schedule, 3 weeks apart was followed for all the immunization experiments. Challenge experiments were carried out at two time points on different mice group. The first challenge was to assess if the vaccine candidates could induce neutralizing antibodies to offer protection against the challenge virus dose. This was carried out two weeks post-2nd dose. This would be the best response one can achieve. The second challenge experiment was done at 20 weeks post-2nd dose. This was primarily done to understand the extent of long-lasting protective response.

Let us first consider the initial set of experiments. It was indeed heartening to note that most of the vaccine formulations did induce protective immune response, not permitting detectable viral replication. Viral RNA was consistently absent in the blood as well as tissues indicative of sterilizing immunity. Alum emerged to be the best adjuvant as it induced sterilizing immunity with all the three immunogens, i.e., rE2p, formalin inactivated or BPL inactivated virus. MW-adjuvanted formalin inactivated candidate vaccine also induced complete protection (Table 17B). Importantly, BPL-inactivated, non-adjuvanted formulation also yielded similar results. Mice immunized with CadBadjuvanted rE2p exhibited delayed (on day 4 post-challenge) and reduced viral load (4logs reduction) in blood and brain. Virus could not be detected in muscles and spleen. Our data is the first report of vaccine efficacy based on the inhibition of replication of virus in the infected mice.

Our next aim was to assess if the vaccine (prepared from ECSA genotype) induced antibodies could neutralize diverge CHIKV strains. For this, we had the Asian genotype virus circulating in Asia during the sixties. An Indian strain used by us could be neutralized, though the titres were less. When we compared, the titres were ~ 4fold lower with the Asian genotype. When we compared the amino acid sequence of the protein used for vaccine and that of the Asian strain, a total of 10 amino acid substitutions were recorded (Figure 37). One of these (G205D) was in the immunodominant region shown for the Semiliki virus. The role of amino acid substitutions on the immunorectivity needs to be studied further by molecular modelling.
Neutralizing antibody kinetics clearly demonstrated that the neutralizing antibodies continue to circulate at high titres over an observation period of 5 months (Figure 34A, B and C). These results encouraged us to conduct the additional challenge experiments at this time point. The results were identical to the earlier challenge experiment, only CadB-adjuvanted rE2p group exhibiting viremia on day 2 post-infection. These results confirm that our vaccine candidates lead to long-lasting sterilizing immunity.

Overall, we could successfully prepare three vaccine candidates. The fact that bacterially expressed rE2p could induce protective immunity clearly shows that glycosylation is not critical for the generation of protective immune response and E2 protein alone (not needing E1 or E3) contains an important neutralizing epitope capable of virus neutralization in-vitro and in-vivo. Though several vaccine candidates have been developed, so far, no reports of clinical trials have been published. The reason for this remains unclear. Whether the peculiar epidemiology of long silence is prohibiting vaccine manufacturers to undertake this vaccine production is an important concern. We do hope that the results presented in thesis do not remain just as a publication but get translated in a product protecting our communities from this troublesome disease. Those who have suffered from this disease during the current episode strongly feel the need for such a vaccine as they know the pain and the duration.