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LETTER TO THE EDITOR

Serological correlates of immune protection conferred by Chikungunya virus infection

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Chikungunya virus (CHIKV), an *Alphavirus* of the family *Togaviridae* is a positive strand RNA virus that is transmitted commonly by the *Aedes* mosquitoes. The characteristic clinical symptom of the virus infection is incapacitating arthralgia that could persist for few weeks to several months in the affected individuals (*1, 2*). High morbidity with severe polyarthralgia, rashes and ocular, hemorrhagic and sensorineural complications are reported in the re-emerging infection (*3-5*). The acquisition of an A226V mutation in the envelope protein E1 has increased the transmissibility of the virus in the widely prevalent *Ae. Albopictus* mosquitoes (*6*). CHIKV infection has become more widespread in the recent years as the mosquito vectors have expanded into new areas. Imported cases of CHIKV have been reported in nearly 40 countries until now *(7*).

Keywords: chikungunya virus; antibody, serum neutralization test; 50% plaque reduction neutralization test; hemagglutination-inhibition test

Several studies on estimation of immune correlates in CHIKV convalescent subjects have identified markers of disease-related morbidity. The cytokines IL-1, IL-6, and IL-10 have been identified as pro-inflammatory markers in the acute phase, and MCP-1, IL-6, IL-8, MIP-1α, MIP-1β, and Th-1 activation with viral persistence in macrophages were identified as markers in the chronic phase of the disease with severe clinical symptoms (*2, 8*, 9). In population studies of CHIKV disease, antibody titers have been estimated by IgG and IgM antibody ELISA (*2*, *10, 11*) and neutralizing antibodies by the hemagglutination-inhibition test (HIT), complement-fixation test and by serum microneutralization (SNT) tests (*12,13*). The 50% plaque reduction neutralization test ($PRNT_{50}$) was used in clinical evaluation of CHIKV vaccine in a Phase II study (*14*). Pre-clinical vaccine studies have relied on the estimation of neutralizing antibodies by either $PRNT_{50}$ (*15*) or by SNT and HIT as a measure of protective efficacy of the vaccine (*13, 16*). Recently, a CHIKV-pseudotyped lentiviral vector using the structural proteins of CHIKV and luciferase as reporter gene has been developed as a safe alternative for the use of virus in neutralization assays (*17*).

The strong hypothesis that CHIKV infection confers sterilizing lifelong immunity in humans and the fact that humoral immunity can protect against CHIKV viremia as observed in animal models is a rationale for using a strong B-cell driven strategy for prophylactic vaccine development. Hence it can be expected that the estimation of the antibody titer, particularly that of neutralizing antibodies, will be central to the measurement of vaccine efficacy in clinical trials. The IgM and IgG antibody ELISA, HIT,

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Abbreviations: CHIKV = chikungunya virus; HI = hemagglutination-inhibition; HIT = hemagglutination-inhibition test; IL-1, IL-6, IL-8, IL-10 = interleukins 1, 6, 8, 10; MCP-1 = monocyte chemoattractant protein-1; MIP-1α, MIP-1β = macrophage inflammatory proteins 1α, 1β; $PRNT_{50}$ = 50% plaque reduction neutralization test; SNT = serum neutralization test; Th-1 = T-helper cell

Table 1. CHIKV antibody titers in sera of convalescents

Assay	No. of subjects	Mean antibody titer (range)[1]
SNT	65	194 (143, 262)
PRNT50	46	1021 (763, 1366)
HIT	59	211 (173, 258)

[1]Geometric mean (95% CI).

$PRNT_{50,}$ and SNT are currently in the battery of tests that are used to evaluate the humoral immune response to CHIKV infection. However, the variability introduced by different methods makes it difficult to assess the threshold of protective efficacy conferred by the virus infection, and to study the efficacy of any CHIKV vaccine in clinical trials in the future. A comparative estimation of serological immune correlates in a serum panel using different methods will be useful to define the cutoff for seroconversion and to understand the nature of protective immunity to the virus. Therefore, we estimated the level of neutralizing antibodies in 85 convalescent subjects of suspected CHIKV infection by SNT, HIT and $PRNT_{50}$ methods in order to obtain comparative correlates of immune protection.

Blood samples were collected under medical supervision during the period December 2009 to January 2010 from Chennai, Villupuram, Theni, Tirunelveli and from two locations in Vellore district of Tamil Nadu in South India, where CHIKV outbreaks were reported. Age, gender, duration and previous history of illness if any, treatment received and the outcome of the treatment were recorded. None of the subjects reported previous history of infection that is clinically symptomatic of CHIKV disease. The sera samples included in the study were at least three weeks after the reported onset of the infection. Six sera samples collected during the viremic phase tested positive for CHIKV by RT-PCR, and were excluded from the assays for neutralizing antibodies. The assay for dengue IgM antibodies was included to study the incidence of co-infection by both arboviruses, as both of the viruses are transmitted by the same mosquito vectors. The CHIKV-IgM and dengue-IgM antibodies were assayed with CHIKV IgM antibody-capture ELISA and dengue IgM antibody-capture ELISA (MAC-ELISA, National Institute of Virology, Pune) respectively, as per the kit protocols. The neutralizing antibodies were estimated in CHIKV IgM antibody positive samples by SNT, HIT, and by $PRNT_{50}$. The serum neutralization titer was the serum dilution that caused complete inhibition of the CPE of 100 $TCID_{50}$ of the virus in 10^5 Vero cells (ATCC CCL-81) when incubated in 5% CO_2 in MEM (Sigma Aldrich, St. Louis, MO) for 5 days at 37°C. The $PRNT_{50}$ titer was the serum dilution that caused 50% reduction in plaques formed by 10^5 PFU/ml of the control virus in Vero cells. The plaques were enumerated on an overlay of 3 ml of 0.85% methyl cellulose in MEM containing 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine after incubation in 5% CO_2 at 37°C for 5 days. Hemagglutination-inhibition (HI) titer was the serum dilution that completely inhibited agglutination of 4 hemagglutinin units of CHIKV antigen in 0.5% goose erythrocytes when incubated at 4°C for 4 hrs. The neutralizing antibody and hemagglutination titers ≥ 20 were considered positive.

Out of the 85 subjects, 46 were females and 39 were males. The age of the subjects varied from 21–65 years with the peak of 30–45 years. About 100% of the subjects reported fever and 96% arthralgia with swelling in joints. Among the 85 subjects included in this study, 70 (82%) tested positive for CHIKV IgM antibodies by MAC-ELISA. Only the 70 CHIKV IgM antibody positive samples were used for the estimation of CHIKV neutralizing antibody titers. About 11/70 subjects tested positive for both CHIKV IgM and dengue IgM antibodies. The post-infection neutralizing antibody titers when expressed as reciprocal of serum dilution ranged from 40 to 5120 by SNT, 320 to 5120 by $PRNT_{50}$ and varied between 40 and 640 by HIT method. The geometric mean of neutralizing antibody titers (with 95% CI) was 194 (143, 262) by SNT, 1021 (763, 1366) by $PRNT_{50,}$ and 211 (173, 258) by HIT methods (see Table). Of these, the number of subjects for whom antibody titers are available by all three methods is 43. However, there was no significant correlation between the neutralization titers estimated by the three methods. Of the sera samples that were assayed for neutralizing antibodies, about 100% tested positive by both SNT and $PRNT_{50}$ and only three samples that tested positive by SNT and $PRNT_{50}$ were negative for HI antibodies.

There is compelling evidence that pre-existing immunity to CHIKV is associated with low infection rates in the community, and is protective both in clinical setting as well as in the animal models of infection (*13, 16, 18*). A reference serum panel of CHIKV convalescent subjects was used to derive comparative titers of neutralizing antibodies by different methods. It should be noted that such data generated at different laboratories will be subject to variables such as differences in the virus strains used in the assays, the extent of host immune response, the duration and severity of clinical illness, and antibody decay over a period of time. Nevertheless, a comparative estimation of the level of neutralizing antibodies in the same serum panel by different methods has provided useful data on the threshold of seroconversion that can be used as a reference to assess the protective efficacy of any CHIKV vaccine in future clinical trials.

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