Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

HLA class II allele polymorphism in an outbreak of chikungunya fever in Middle Andaman, India

Itta Krishna Chaaithanya,[1,*] Nagarajan Muruganandam,[1,*] Maile Anwesh,[1] Reesu Rajesh,[1] Sruti R. Ghosal,[1] Chinnaiah Kartick,[1] Kadiyala Nageswara Prasad,[2] Karuppiah Muthumani[3] and Paluru Vijayachari[1]

[1]*Regional Medical Research Centre (ICMR), Port Blair, Andaman & Nicobar Islands, India,* [2]*G.B. Pant Hospital, Port Blair, Andaman & Nicobar Islands, India and* [3]*Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

doi:10.1111/imm.12128
Received 14 October 2012; revised 17 May 2013; accepted 21 May 2013.
*These authors contributed equally to the work.
Correspondence: Dr Paluru Vijayachari, Regional Medical Research Centre (ICMR), Post Bag No.13, Port Blair 744101, Andaman & Nicobar Islands, India.
Email: pblicmr@sancharnet.in
Senior author: Dr Paluru Vijayachari

Summary

A sudden upsurge of fever cases with joint pain was observed in the outpatient department, Community Health Centre, Rangat during July–August 2010 in Rangat Middle Andaman, India. The aetiological agent responsible for the outbreak was identified as chikungunya virus (CHIKV), by using RT-PCR and IgM ELISA. The study investigated the association of polymorphisms in the human leucocyte antigen class II genes with susceptibility or protection against CHIKV. One hundred and one patients with clinical features suggestive of CHIKV infection and 104 healthy subjects were included in the study. DNA was extracted and typed for HLA-DRB1 and DQB1 alleles. Based on the amino acid sequences of HLA-DQB1 retrieved from the IMGT/HLA database, critical amino acid differences in the specific peptide-binding pockets of HLA-DQB1 molecules were investigated. The frequencies of HLA-DRB1 alleles were not significantly different, whereas lower frequency of HLA-DQB1*03:03 was observed in CHIKV patients compared with the control population [$P = 0{\cdot}001$, corrected $P = 0{\cdot}024$; odds ratio (OR) = 0, 95% confidence interval (95% CI) 0·0–0·331; Peto's OR = 0·1317, 95% CI 0·0428–0·405). Significantly lower frequency of glutamic acid at position 86 of peptide-binding pocket 1 coding HLA-DQB1 genotypes was observed in CHIKV patients compared with healthy controls ($P = 0{\cdot}004$, OR = 0·307, 95% CI 0·125–0·707). Computational binding predictions of CD4 epitopes of CHIKV by NetMHCII revealed that HLA-DQ molecules are known to bind more CHIKV peptides than HLA-DRB1 molecules. The results suggest that HLA-DQB1 alleles and critical amino acid differences in the peptide-binding pockets of HLA-DQB1 alleles might have role in influencing infection and pathogenesis of CHIKV.

Keywords: Andaman Islands; chikungunya; chikungunya virus; HLA; immunogenetics.

Introduction

Chikungunya virus (CHIKV) is an important emerging human pathogen that causes a disease syndrome characterized by fever, headache, rash, nausea, vomiting, myalgia and arthralgia and, in its most severe form, the induction of fatal haemorrhagic disease.[1,2] Chikungunya is a mosquito-borne infectious disease, and it is transmitted to humans primarily by the *Aedes aegypti* mosquitoes. CHIKV belongs to the family *Alphaviridae*, and is geographically distributed from Africa to South America and Southeast Asia.[1,3]

CHIKV was first isolated during an outbreak in Tanganyika (now Tanzania) in 1952–53.[4,5] During the past 50 years, numerous CHIKV re-emergences have been documented in both Africa and Asia, with irregular intervals of 2–20 years between outbreaks.[6] The incubation time for CHIKV is relatively short, requiring only 2–6 days with symptoms usually appearing 4–7 days post-infection.[7] The chronic stage of chikungunya fever is

characterized by polyarthralgia that can last from weeks to years beyond the acute stage.[2,8,9] Severe, relapsing and incapacitating arthralgia is the hallmark of CHIKV infection. There are a few precise descriptions of CHIKV-associated joint disorders.[10,11]

India experienced the first confirmed outbreak of chikungunya fever during 1963–64 in Kolkata[12] and in 1965 in Chennai.[12,13] It has recently re-emerged extensively in Andhra Pradesh from October 2005 to July 2009 and has spread to 17 states, including the Andaman archipelago in 2006. The disease burden due to chikungunya is increasing steadily and straining the public health systems.[14]

Currently there is no effective antiviral treatment or vaccine available against CHIKV. Treatment is therefore purely supportive and is based on non-salicylate analgesics and non-steroidal anti-inflammatory drugs.[2] A single mutation from alanine to valine at amino acid position 226 of the Envelope glycoprotein gene (E1) enhances the ability of the salivary gland of *Aedes albopictus* mosquitoes to become infected and so increases the capability of the mosquitoes to transmit the virus to another host.[15,16]

Apart from various virulence factors, host factors play an important role in disease susceptibility. Many studies have reported association between HLA and the outcome of diseases.[17] HLA play a major role in initiating adaptive immune responses. The HLA class I molecules are known to present peptides from pathogens to $CD8^+$ T cells while HLA class II molecules present peptides to $CD4^+$ T cells. HLA molecules are coded in the sixth chromosome and they are the most polymorphic genes known to date. Polymorphisms in the HLA genes are clustered in the peptide-binding regions and affect the repertoire of peptides presented by an HLA molecule to the T cell. Human subjects differ with respect to their HLA genetic makeup and hence might differ in their ability to present the peptides of pathogens to T cells, which would affect their susceptibility to infectious diseases. A recent bioinformatic study analysing sequences of CHIKV strains belonging to different decades has reported that HLA molecules are the driving force behind the selection of more virulent strains, and in newly emerging strains there is a loss of HLA restriction elements.[18] As a result of the robust host adaptive immune responses against the pathogens, which are HLA restricted, the pathogens tend to escape from the adaptive responses by mutating their nucleotide sequences, which might result in occasional changes in the amino acid sequences of their proteins, which might lead to the generation of new peptides or T-cell epitopes that could not be recognized by HLA molecules. Hence when there is loss of HLA restriction elements in the newly emerging strain for the commonly prevalent HLA molecules in the host population, the population is more susceptible to infection with the new strain and development of disease due to the inability to mount an HLA-restricted immune response.[18] Recent studies from Sri Lanka have shown that HLA-A*31, & HLA-DRB1*08 were associated with susceptibility to Dengue Shock Syndrome in the dengue virus-infected Sri Lankan population, during secondary dengue infection.[19]

Studies have shown that HLA class I and II genes were associated with susceptibility or resistance to HIV infection and disease progression.[20] Among the HLA class II molecules, apart from HLA-DR, specific HLA-DQB1 and HLA-DPB1 alleles have been shown to be associated with HIV infection.[21,22] Flavivirus disease and its association with HLA molecules have been studied.[23,24] Recent studies showed that HLA-A*68 and -C*08 might function as 'susceptible' alleles, whereas HLA-B*40 and -C*03 might function as 'protective' alleles in West Nile virus infection.[25]

The pathogenesis and biology behind CHIKV have not been documented. The trend over the past few years indicates that CHIKV has the potential to spread uncontrollably and cause large outbreaks with high attack rates. Even though many outbreaks have occurred worldwide, so far no study exists to explain the host interaction with CHIKV. It is essential to understand the interaction between the aetiological agent and the host to know the chronic sequelae of the disease. Associations of MHC are usually population specific. Many chronic diseases and their host's interaction with the disease have been studied to understand their immunogenetics. HLA-DR3 (HLA-DRB1*0301, -DQB1*0201) and DR4 (HLA-DRB1*04, -DQB1*0302) are markers for type 1 diabetes in Caucasian populations, but not among Japanese patients.[26]

The DRB1*07 allele is associated with protection against Dengue virus infection in the Cuban population;[27] but not in the Sri Lankan population.[19] In view of this gap in understanding the role of host factors associated with this disease, a population cohort was chosen in Middle Andaman Rangat to study the hypothesis. Port Blair is the capital of the Andaman and Nicobar Islands, an archipelago of > 500 islands and islets situated in the Bay of Bengal, 1200 km from peninsular India. Andaman and Nicobar islands witnessed an outbreak of CHIKV fever in 2006, and the attack rate was > 60% of the population.[28,29] After a gap of 4 years a sudden upsurge of fever cases with joint pain was observed in the outpatient department of the Community Health Centre (CHC), Rangat during July–August, 2010. Most of the patients complained with pain/swelling of multiple joints. Recent studies show that the virus causing this severe outbreak belongs to the East Central South African genotype.[30] As MHC is population specific, among these closely related islanders there is likely to be less genetic variance specific to various infections.

It is important to identify the host association markers with chronic diseases like CHIKV. In the present study,

we have investigated the causative agent of the outbreak and HLA class II allelic profiles in patients with CHIKV infection and healthy controls, to understand the possible association, if any, which may influence the disease outcome.

Materials and methods

Study area and patient inclusion criteria

Rangat is situated in Middle Andaman, 210 km from Port Blair and 70 km from southern Mayabunder. Rangat is one of the five local administrative divisions in the Indian district of Andaman and Nicobar Islands, Indian union territory. Rangat's population, according to the 2011 Census of India, was 38 824 (http://en.wikipedia.org/wiki/Rangat). The study was carried out from June to August 2010 in a CHC, Rangat. People from the surrounding area of Rangat (Middle Andaman) and people attending the CHC with fever and joint pain were considered as inclusion criteria.

The patients were counselled and informed written consent was taken at the outpatient department of the CHC. The study was approved by the Ethics Review Committee of the Regional Medical Research Centre (Indian Council of Medical Research) Port Blair. No age criteria were followed while collecting the samples. A clinician interviewed all the patients, and a structured questionnaire was used for eliciting the necessary information. Details regarding personal identification and demographic details, pre-existing joint pain and its nature, other pre-existing diseases, onset, evolution and duration of various symptoms and the joints involved were taken. Five millilitres of venous blood were collected from all the suspected patients and healthy control samples into anti-coagulated tubes and sent to the Regional Medical Research Centre in cold chain condition. There, 500 μl of plasma was separated for serology and for further studies, the rest of the blood were stored at $-80°$ until use.

One hundred and one patients (mean age 33·3 years) with features suggestive of CHIKV infection and positive for either IgM ELISA or RT-PCR were enrolled in the present study. Another 104 samples were obtained from apparently healthy subjects (mean age 31·6 years) who did not have any clinical features of CHIKV infection. Healthy controls who were negative for IgG antibodies against CHIKV and reported no symptoms were included in the study. Both Patients and Controls were living in the same geographical location and belonged to mixed ethnic groups.

Serology

Presence of anti-CHIKV IgM/anti-DENG antibody was tested by an IgM capture ELISA developed by National Institute of Virology, Pune, India.[13] Healthy control samples were screened for the presence of IgG antibodies against CHIKV by NovaLisa™ (chikungunya virus IgG capture ELISA Nova Tec Immundiagnostica GmbH, Germany). The ELISA was performed and results were interpreted as per the manufacturer's instructions. As Rangat is known to be an endemic area for leptospirosis[31] all the samples were tested for anti-leptospiral antibodies by using an in-house developed Latex agglutination test at Regional Medical Research Centre.

RNA extraction and RT-PCR

RNA was extracted from 140-µl ELISA-negative samples using a QIAamp Viral RNA Minikit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. A high-capacity cDNA reverse transcription kit (Applied Bio-systems, Foster City, CA) was used following the manufacturer's instructions. Previously published[32] primer sets were selected to amplify the non-structural protein 1 (NSP1) and envelope glycoprotein 1 (E1) genomic regions of the CHIKV genome. The PCR was carried out following a *Taq* polymerase activation step of 94° for 2 min, 40 cycles of PCR (94° for 60 seconds, 55° for 60 seconds, and 72° for 2 min for each cycle), and a final extension at 72° for 10 min. Amplified PCR products were electrophoresed in 1% agarose gel to confirm the presence of the expected bands of the genes nsP1 and E1 with sizes of 354 and 294 bp, respectively.

DNA extraction and HLA typing

DNA was extracted from anti-coagulated blood samples from the patients and healthy controls using the QIAamp DNA blood Midi Kit (Qiagen, Manchester, UK) following manufacturer's instructions. DNA was eluted in 300 µl elution buffer. Genotyping of HLA class II (DRB1 and DQB1) alleles was performed as previously described using PCR with sequence-specific primers (PCR-SSP) described in refs 33 and 34, respectively. All healthy control samples were processed ($n = 104$) for both DRB1 and DQB1 typing and 101 patient samples were used for DRB1 typing, whereas only 94 patient samples were available for DQ typing.

Each primer mix contained the specific primers and a pair of positive internal control primers, primer concentration was optimized. The PCR was carried out in an Applied Bio-systems AB 2720 Thermal cycler. The amplified PCR products were electrophoresed in 2·0% agarose gels, containing 0·5 μg/ml ethidium bromide, and were viewed under UV trans-illuminator. A prototype was considered to be successful when the control amplifications were positive and at least one allele or group was present in each locus.

Analysis of amino acid variation in peptide-binding pockets of HLA-DQ molecule

As most of the HLA gene polymorphisms are associated with changes in the amino acids at critical positions of the peptide-binding groove of the HLA molecules,[35,36] we investigated whether the critical amino acid differences in the specific peptide-binding pockets of HLA-DQB1 molecules are associated with susceptibility or protection against CHIKV infection. The amino acid sequence of the β-chain of HLA-DQB1 alleles was retrieved from the IMGT/HLA database (www.ebi.ac.uk). Analysis of critical amino acid variation in the peptide-binding pockets of HLA-DQB1 was performed based on amino acids at the 57th position of pocket 9 (aspartic acid/non-aspartic acid) and the 86th position of pocket 1 (glutamic acid/non-glutamic acid).

Statistical analysis

The frequencies of HLA class II alleles in patients with CHIKV fever (n = 101) and the normal healthy controls (n = 104) were compared to determine possible association. Phenotype frequencies were calculated by a direct counting method and the StatCalc program of EpiInfo software version 7 was used for statistical analysis. For each HLA allele, degree of association between HLA alleles and disease state was expressed as the odds ratio (OR), For calculation of odds ratio during comparison of groups with zero value, OR and the 95% confidence interval (95% CI) were calculated using an online calculator (http://www.hutchon.net/ConfidORnulhypo.htm) that uses a null hypothesis to provide an estimate of OR, also called Peto's OR and represented as such wherever necessary. The advantage of this approach over direct calculation is that it will accept zero results without generating infinity. The P-value was further subjected to Bonferroni's correction in which the P-value was multiplied by the number of alleles. A corrected P (P_c) value $< 0{\cdot}05$ was considered significant. NetMHCII 2·2 server (www.cbs.dtu.dk/services/netMHCII/) online software was used to predict binding affinities of CHIKV peptides to HLA class II molecules. The population genetic analysis package, Pypop was used for analysis of linkage disequilibrium and haplotype frequencies.

Results

Confirmation of the outbreak and clinical characteristics of CHIKV infection in the study population

Investigations were carried out with a total of 310 suspected blood samples collected at CHC Rangat in 2010. Of the 310 subjects tested for the presence of anti-CHIKV IgM antibody, 110 tested positive for anti-CHIKV IgM antibody, 35·4% (110/310). Twenty ELISA-negative samples were screened for CHIKV by RT-PCR, and eight were positive (40%) (data not shown). The results of these tests indicate that the outbreak was caused by CHIKV infection. Dengue IgM testing was performed on all serum samples and was detected in only four (0·003%) patients. Leptospiral antibodies were found in three patients. In all, 101 patients with laboratory-confirmed CHIKV infection and 104 healthy control (IgG ELISA) samples found to be negative were used for the present study.

Symptoms among the 310 suspected cases were fever (97%), body ache (92%), headache (88%), chills (65%), nausea (60%), fatigue (56%) and conjunctival redness (52%). Among the confirmed patients, these were fever (96%), body ache (93%), headache (88%), nausea (65%), chills (62%), oedema (62%), conjunctival redness (61%) and fatigue (61%) (Fig. 1). Pain in large joints was reported by 94% of all patients and 96% of confirmed patients whereas pain in small joints was reported by 85% of all patients and 87% of confirmed patients (Fig. 2).

Associations of HLA-DR and -DQ alleles with CHIKV patients

The percentage phenotype frequencies of HLA-DRB1 and -DQB1 alleles were calculated and are represented in Tables 1 and 2, respectively. HLA-DRB1*04, -DRB1*07, -DRB1*12 and -DRB1*15 were the alleles that were commonly present in the study subjects. The allelic frequency of HLA-DRB1*07 and -DRB1*09 was higher in controls compared with CHIKV patients, though statistically not significant (Table 1). The allele frequencies of other DRB1 alleles were not significantly different between patients and controls. HLA-DQB1*02:01, -DQB1*05:01 and -DQB1*06:01 were the alleles that had a frequency of

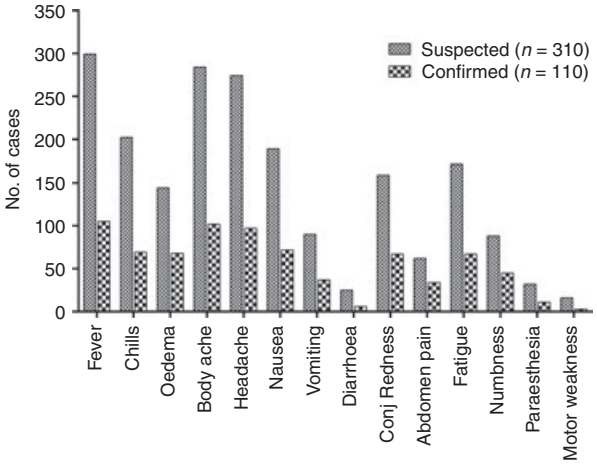

Figure 1. Signs and symptoms among clinically suspected and confirmed patients of CHIKV infection.

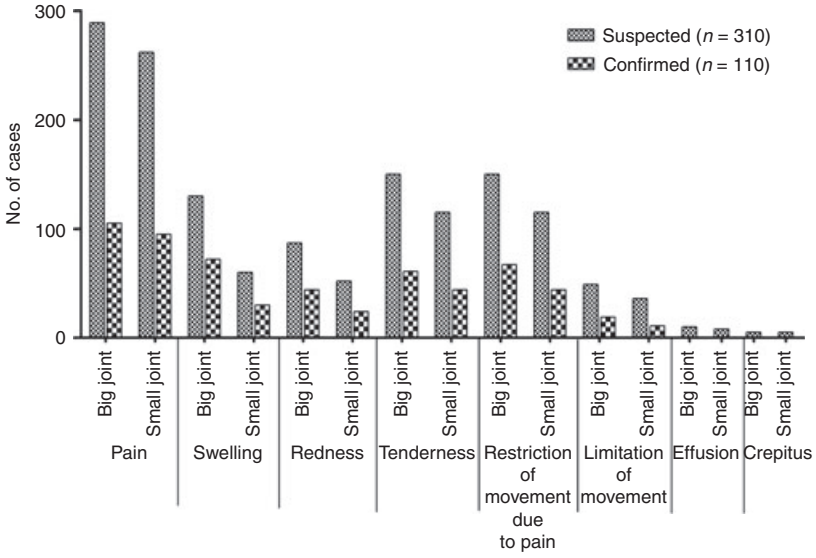

Figure 2. Joint examination of the patients with suspected and confirmed CHIKV infection.

Table 1. Phenotype frequencies of the HLA-DRB1 alleles of the patients with chikungunya virus infection and control population

Allele	Patients (n = 101) (%)	Controls (n = 104) (%)	P value	OR	95% CI
*01	8 (7·92)	6 (5·76)	0·738	1·405	0·409–5·104
*03	8 (7·92)	11 (10·57)	0·678	0·727	0·242–2·091
*04	16 (15·84)	14 (13·46)	0·776	1·210	0·517–2·854
*07	33 (32·67)	46 (44·23)	0·119	0·611	0·338–1·121
*08	4 (3·96)	1 (0·96)	0·347	4·247	0·408–211·1
*09	2 (1·98)	8 (7·69)	0·115	0·242	0·024–1·265
*10	11 (10·89)	13 (12·5)	0·887	0·855	0·328–2·194
*11	6 (5·94)	4 (3·84)	0·710	1·578	0·360–7·834
*12	17 (16·83)	22 (21·15)	0·541	0·754	0·349–1·611
*13	9 (8·91)	12 (11·53)	0·696	0·750	0·265–2·049
*14	16 (15·84)	11 (10·57)	0·363	1·591	0·650–4·013
*15	63 (62·37)	56 (53·84)	0·236	1·459	0·803–2·656
*16	9 (8·91)	4 (3·84)	0·229	2·445	0·652–11·19

> 10% in both patients and controls. Significantly lower frequency of HLA-DQB1*03:03 was observed in patients with CHIKV infection when compared with the control population (P = 0·001, P_c = 0·024; OR = 0, 95% CI 0·0–0·331; Peto's OR = 0·131, 95% CI 0·0428–0·405 (Table 2).Further we compared the percentages of apparently homozygous individuals for the DRB1 (patients 26%, control 28%, P = 0·977) and DQB1 (patients 36%, controls 32% P = 0·715) loci between patients and control groups, no statistical significance was observed suggesting that homozygosity at these loci may not affect the disease outcome. This has to be further confirmed by sequence analysis.

Association of amino acid peptide-binding pockets of HLA-DQB1 with CHIKV infection

We retrieved the amino acid sequences of the β chain of HLA-DQB1 allele-encoded aligned mature protein sequences from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla) and an inquiry of the amino acid sequence variation revealed the presence of polymorphism in positions b57 and b86 in the peptide-binding groove of HLA-DQB1 molecules. A classification was made and presented in Table 3 based on the available data base and the b57 and b86 genotypes were inferred for the subjects based on their respective HLA-DQB1 genotypes.

We examined the critical amino acid variations in the specific peptide binding pockets of HLA-DQB1 molecules based on the HLA-DQB1 genotype of patients and controls. Analysis of critical amino acid variation in the peptide-binding pockets of HLA-DQB1 was performed based on b57 of pocket 9 (aspartic acid/non-aspartic acid) and b86 of pocket 1 (glutamic acid/non-glutamic acid) and the results are given in Table 4.

Analysis based on the amino acid polymorphism in the 57th position of pocket 9 indicated that allele or genotype

Table 2. Phenotype frequencies of the HLA-DQB1 alleles of the patients with chikungunya virus infection and control population

Allele	Patients (n = 94) (%)	Controls (n = 104) (%)	P-value	OR	95% CI
*02 : 01	26 (27·65)	35 (33·65)	0·448	0·753	0·390–1·445
*02 : 03	1 (1·06)	3 (2·88)	0·686	0·362	0·006–4·619
*03 : 02	8 (8·51)	9 (8·65)	0·827	0·981	0·314–3·011
*03 : 03	0 (0·00)	13 (12·5)	*0·001	†0	‡0·000–0·331
*03 : 04	1 (1·06)	3 (2·88)	0·686	0·362	0·006–4·619
*03 : 05	6 (6·38)	4 (3·84)	0·624	1·704	0·388–8·464
*03 : 07	13 (13·82)	17 (16·34)	0·768	0·821	0·343–1·926
*04 : 01	5 (5·31)	6 (5·76)	0·862	0·917	0·213–3·749
*05 : 01	20 (21·27)	23 (22·11)	0·976	0·951	0·455–1·978
*05 : 02	8 (8·51)	5 (4·80)	0·445	1·841	0·507–7·412
*05 : 03	9 (9·57)	8 (7·69)	0·827	1·270	0·413–3·965
*05 : 04	4 (4·25)	2 (1·92)	0·588	2·266	0·315–25·51
*06 : 01	57 (60·63)	55 (52·88)	0·339	1·372	0·750–2·513
*06 : 02	1 (1·06)	0 (0·00)	0·959	–	0·028– −1·000
*06 : 03	8 (8·51)	5 (4·80)	0·445	1·84	0·507–7·412
*06 : 04	0 (0·00)	1 (0·96	0·959	–	0·000–43·14
*06 : 05	7 (7·44)	4 (3·84)	0·427	2·011	0·490–9·659
*06 : 06	3 (3·19)	3 (2·88)	0·772	1·109	0·144–8·492
*06 : 07	3 (3·19)	4 (3·84)	0·891	0·824	0·117–5·018
*06 : 08	1 (1·06)	0 (0·00)	0·959	–	0·028– −1·00
*06 : 10	2 (2·12)	2 (1·92)	0·686	1·108	0·078–15·57
*06 : 11	3 (3·19)	2 (1·92)	0·908	1·681	0·187–20·49
*06 : 12	0 (0·00)	1 (0·96)	0·959	–	0·00–43·14
*06 : 13	2 (2·12)	3 (2·88)	0·908	0·731	0·06–6·547

*P_c 0·024; †Peto's OR 0·1317, ‡95% CI 0·0428–0·405.

frequencies were not different between patients and controls. The allelic frequency of DQB1 alleles coding for non-glutamic acid at position 86 of pocket 1 was higher than in healthy controls (P = 0·008, OR = 1·79, 95% CI 1·16–2·77) The frequency of glutamic acid/glutamic acid at the 86th position of pocket 1 coding HLA-DQB1 genotypes was observed at significantly lower frequencies in CHIKV patients when compared with healthy controls (P = 0·004, OR = 0·307, 95% CI 0·125–0·707). Frequencies of glutamic acid/non-glutamic acid and non-glutamate/non-glutamate coding genotypes did not differ significantly among the different patient groups and healthy controls.

The haplotype frequency between DQB1 and DRB1 was compared between controls and patients with CHIKV infection. The haplotype frequency of HLA-DQB1*03:03–HLA-DRB1*07 was 4% in control samples and was not observed in patients with CHIKV infection (Tables 5 and 6). The linkage disequilibrium between HLA-DQB1 and HLA-DRB1 was moderate in both groups (in patients D' = 0·65942, and in controls D' = 0·65942). Net MHCII analysis using the genomic sequence of CHIKV revealed that HLA-DQ molecules are known to bind comparatively more peptides than HLA-DRB1 molecules (data not shown). Hence, these results suggest that the associations observed for HLA-DQB1 alleles are independent of HLA-DRB1 alleles.

Discussion

In 2006, there was a large outbreak of the *Aedes* mosquito-borne disease, chikungunya fever, in South Andaman.[28] After a gap of 4 years another upsurge of cases was observed in Rangat Middle Andaman. Rangat is a place where leptospirosis is endemic[31] and so far there is no report of chikungunya infection in these islands. The present outbreak was confirmed as a chikungunya infection with 35·4% seropositivity, which was the first record for this area. During the current outbreak, 96% fever and 93% body ache were the main symptoms among the confirmed patients; 96% of the patients showed pain in their large joints during the intervening period. The present study also confirms the findings of various other studies that certain symptoms, such as headache, joint pain/swelling and abdominal pain, are significantly associated with chikungunya infections.[9,37]

We attempted to identify the possible HLA class II alleles that are associated with CHIKV infection. HLA classification with its extensive polymorphism is an excellent marker for population genetic analysis and disease association studies.[38] This is the first study on HLA determinants of susceptibility and/or protection to CHIKV infection in this island population. No study has explained the host factors and their association with chikungunya virus infection. These host factors can influence

Table 3. The classification of observed HLA-DQB1 alleles based on critical inferred amino acid variations in the peptide binding pockets of the β chain at positions 57 and 86 of DQB1 allele

b57 Asp	b57 Non Asp	b86 Glu	b86 Non Glu
	*02 : 01	*02 : 01	
*02 : 03		*02 : 03	
	*03 : 02	*03 : 02	
*03 : 03		*03 : 03	
	*03 : 04	*03 : 04	
	*03 : 05		*03 : 05
	*03 : 07	*03 : 07	
*04 : 01		*04 : 01	
	*05 : 01		*05 : 01
	*05 : 02		*05 : 02
*05 : 03			*05 : 03
	*05 : 04		*05 : 04
*06 : 01			*06 : 01
*06 : 02			*06 : 02
*06 : 03			*06 : 03
	*06 : 04		*06 : 04
	*06 : 05		*06 : 05
	*06 : 06		*06 : 06
*06 : 07		*06 : 07	
	*06 : 08		*06 : 08
	*06 : 10		*06 : 10
*06 : 11			*06 : 11
	*06 : 12		*06 : 12
	*06 : 13		*06 : 13

the outcome of the disease in many viral infections.[17] HLA molecules are coded in the sixth chromosome and they are the most polymorphic genes known to date. Polymorphism in the HLA genes is clustered in the peptide-binding region and affects the repertoire of peptides presented by an HLA molecule to the T cell. The genotypic variants of class II molecules display unique pockets in the peptide-binding groove that interact with specific residues within the antigenic peptides, which influences the nature and specificity of the antigen-specific CD4 T-cell response.[39]

In the present study, a significantly decreased frequency of HLA-DQB1*03:03 was observed in CHIKV patients compared with healthy subjects, suggesting that HLA-DQB1*03:03 might be associated with reduced risk of developing CHIKV symptoms, or severe disease. The frequencies of HLA-DRB1 alleles were not different between patients, although decreased frequency of HLA-DRB1*07 was observed in CHIKV patients. In the present study, moderate linkage disequilibrium was observed between HLA-DRB1 and HLA-DQB1 alleles in both patients and controls. The HLA-DQB1 allele, HLA-DQB1*03:03, which is reported to be associated with reduced risk of CHIKV infection, was not observed among patients. The HLA-DQB1*03:03 was found more frequently in combination with HLA-DRB1*07 allele. Hence the increase in the frequency of HLA-DRB1*07 allele in controls could be a result of its linkage disequilibrium with HLA-DQB1*03:03. However, the HLA-DRB1*07 allele also occurs in combination with HLA-DQB1*02 alleles as a haplotype. As HLA-DRB1*07 was found to occur in multiple haplotypes such as HLA-DRB1*07-HLA-DQB1*03:03 and HLA-DRB1*07-HLA-DQB1*02, this could have compensated for the loss of statistical significance for comparison of frequency of the HLA-DRB1*07 allele between healthy controls and patients. The observation suggests that HLA-DRB1 alleles may not have a role in CHIKV infection. However, the sample size of the present study is limited to delineating minor associations. The HLA-DQB1*03 group of alleles has been shown to be associated with clearance of hepatitis C virus and

Table 4. Comparison of the genotype frequency of the presence or absence of aspartic acid at position 57 and of glutamate at position 86 of HLA-DQ chain between patients and controls

Allele	Patients ($2n$ = 188)	Controls ($2n$ = 208)	*P*-value	OR	95% CI
β57					
Asp	87 (46·2%)	97 (46·6%)			
Non-Asp	101 (53·7%)	111 (53·3%)	NS	–	–
β86					
Glu	57 (30·3%)	91 (43·7%)			
Non-Glu	131 (69·6%)	117 (56·2%)	0·008	1·79	1·16–2·77
Genotype	(n = 94)	(n = 104)			
β57					
Asp/Asp	27 (28·7%)	27 (25·9%)	0·782	1·149	0·585–2·255
Non-Asp/Asp	33 (35·1%)	43 (41·3%)	0·45	0·767	0·413–1·419
Non-Asp/Non-Asp	34 (36·1%)	34 (32·6%)	0·715	1·166	0·621–2·189
β86					
Glu/Glu	10 (10·6%)	29 (27·8%)	0·004	0·307	0·125–0·707
Non-Glu/Glu	37(39·3%)	33 (31·7%)	0·33	1·396	0·746–2·613
Non-Glu/Non-Glu	47(50%)	42 (40·3%)	0·224	1·476	0·809–2·694

Table 5. Haplotype frequency for loci: DQB1-DRB1 in control samples

HLA-DQB1-HLA-DRB1 haplotype	Frequency in %	No. copies
06 : 01–15	16	35·1
02 : 01–07	8	17·7
03 : 03–07	4	9·0
05 : 01–10	4	9·0
06 : 01–12	3	7·0
02 : 01–03	3	7·0
03 : 07–07	2	5·5
02 : 01–15	2	5·1
05 : 01–01	2	5·0
03 : 07–15	2	4·6
03 : 07–12	2	4·3
05 : 01–15	1	4·0
03 : 02–04	1	4·0
03 : 03–09	1	4·0
06 : 01–04	1	3·5
06 : 01–07	1	3·5
04 : 01–04	1	3·0
05 : 01–03	1	3·0
05 : 03–14	1	3·0
06 : 03–13	1	3·0
03 : 04–15	1	2·2
02 : 01–09	1	2·1

Table 6. Haplotype frequency for loci: DQB1-DRB1 in patient samples

HLA-DQB1-HLA-DRB1 haplotype	Frequency in %	No. copies
06 : 01–15	19	35·8
02 : 01–07	9	17·0
05 : 01–01	3	7·0
05 : 01–10	3	7·0
06 : 01–14	3	6·0
02 : 01–03	3	6·0
03 : 07–12	2	4·9
06 : 01–12	2	4·0
06 : 01–16	2	4·0
03 : 02–04	2	4·0
06 : 03–13	2	4·0
03 : 07–04	2	3·9
05 : 03–14	1	3·5
05 : 03–15	1	3·5
03 : 05–15	1	2·9
06 : 05–15	1	2·9
06 : 01–04	1	2·1
04 : 01–15	1	2·1
03 : 05–08	1	2·0
05 : 01–15	1	2·0
03 : 02–07	1	2·0
05 : 04–16	1	2·0
05 : 02–16	1	2·0
06 : 05–13	1	2·0
03 : 02–12	1	2·0
06 : 01–11	1	2·0
06 : 11–15	1	2·0
05 : 02–07	1	2·0

enhanced $CD4^+$ T-cell responses.[40] HLA-DQB1*03:03 has been shown to be associated with psoriasis.[41] It is possible that the HLA-DQB1*03:03 allele might promote diverse antiviral $CD4^+$ T-cell responses and could be associated with reduced risk of CHIKV infection.

Amino acid polymorphism within the peptide-binding site of the b-chain of HLA-DQB1 alleles plays an important role in the selectivity of peptides that bind to it. The widely studied features of the protein product of the HLA-DQB1 locus is the presence of an aspartic acid residue at position 57 pocket 9 in the DQB1 chain, and the role of this mature protein has been identified in diseases like pulmonary tuberculosis.[42] An analysis of HLA-DQB1 alleles based on amino acid variation at the β57 and β86 positions revealed increased frequency of glutamic acid/glutamic acid coding HLA-DQB1 genotypes at the 86th position of pocket 1 in controls when compared with CHIKV patients. This suggests that glutamate at the β86th position might have a role in conferring protection against CHIKV infection. Residue β86 is part of the peptide-binding pocket 1, which is deep and polar in nature because of hydrogen bonding between two positively and negatively charged amino acids. Replacement of glutamate with alanine or glycine will break this network. Presence of glutamate at the β86th and leucine at the 87th position will help to accommodate peptides with large hydrophobic amino acids.[43] This will enhance the ability of glutamate-coding alleles at the 86th position to bind a comparatively large number of amino acids and could have conferred a selective advantage against CHIKV.

Prediction of binding of CHIKV peptides to HLA class II molecules revealed that HLA-DQ molecules are known to bind comparatively more peptides than HLA-DRB1 molecules. Hence, these results suggest that the associations observed for HLA-DQB1 alleles are independent of HLA-DRB1 alleles. However, as computational predictions are not always translated in reality, these results need to be considered cautiously and more functional *in vitro* studies are needed to confirm the higher binding affinity of CHIKV peptides to HLA-DQ molecules.

Populations of both patients and controls were of mixed ethnicity, this may be a limitation of our study. Further follow-up studies with a large number of samples that are ethnically matched are needed to assess the role of HLA alleles in association with post-sequelae of symptoms observed in CHIKV patients. To conclude, the present study identified CHIKV as the agent responsible for the outbreak in Rangat Andaman and suggests that HLA-DQB1 alleles might play a role in influencing CHIKV infection and pathogenesis. However, more research is

required to understand the immunogenetics of CHIKV infection and disease severity.

Acknowledgements

This work was financially supported by funds from the Department of Biotechnology (BT/PR9372/Med/29/24/2007). The authors are grateful to Dr K. Alagarasu, National Institute of Virology India, for his suggestion and kind critical review of the manuscript. We also thank the Department of Health Services (DHS) Port Blair, Clinical and technical staff of Regional Medical Research Centre (ICMR) Port Blair for the collection of samples.

Disclosure

The authors have no conflicts of interests to disclose.

References

1 Sourisseau M, Schilte C, Casartelli N *et al.* Characterization of reemerging chikungunya virus. *PLoS Pathog* 2007; **3**:e89.
2 Thiboutot MM, Kannan S, Kawalekar OU *et al.* Chikungunya: a potentially emerging epidemic? *PLoS Negl Trop Dis* 2010; **4**:e623.
3 Muthumani K, Lankaraman KM, Laddy DJ *et al.* Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. *Vaccine* 2008; **26**:5128–34.
4 Ross RW. The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. *J Hyg (Lond)* 1956; **54**:177–91.
5 Robinson M. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–53 I. Clinical features. *Trans R Soc Trop Med Hyg* 1955; **49**:28–60.
6 Powers A, Logue C. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Gen Virol* 2007; **88**(Pt 9):2363–77.
7 Ziegler SA, Lu L, da Rosa AP, Xiao SY, Tesh RB. An animal model for studying the pathogenesis of chikungunya virus infection. *Am J Trop Med Hyg* 2008; **79**:133–9.
8 Queyriaux B, Simon F, Grandadam M, Michel R, Tolou H, Boutin JP. Clinical burden of chikungunya virus infection. *Lancet Infect Dis* 2008; **8**:2–3.
9 Suryawanshi S, Dube A, Khadse R *et al.* Clinical profile of chikungunya fever in patients in a tertiary care centre in Maharashtra, India. *Indian J Med Res* 2009; **129**:438–41.
10 Brighton SW. Chloroquine phosphate treatment of chronic Chikungunya arthritis. An open pilot study. *S Afr Med J* 1984; **66**:217–8.
11 Kennedy AC, Fleming J, Solomon L. Chikungunya viral arthropathy: a clinical description. *J Rheumatol* 1980; **7**:231–6.
12 Shah KV, Gibbs CJ Jr, Banerjee G. Virological investigation of the epidemic of haemorrhagic fever in Calcutta: isolation of three strains of Chikungunya virus. *Indian J Med Res* 1964; **52**:676–83.
13 Gadkari DA, Shaikh BH. IgM antibody capture ELISA in the diagnosis of Japanese encephalitis, West Nile and dengue virus infections. *Indian J Med Res* 1984; **80**:613–9.
14 Krishnamoorthy K, Harichandrakumar KT, Krishna Kumari A, Das LK. Burden of chikungunya in India: estimates of disability adjusted life years (DALY) lost in 2006 epidemic. *J Vector Borne Dis* 2009; **46**:26–35.
15 Santhosh SR, Dash PK, Parida MM, Khan M, Tiwari M, Lakshmana Rao PV. Comparative full genome analysis revealed E1: A226V shift in 2007 Indian Chikungunya virus isolates. *Virus Res* 2008; **135**:36–41.
16 Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog* 2007; **3**:e201.
17 van Manen D, Kootstra NA, Boeser-Nunnink B, Handulle MA, van't Wout AB, Schuitemaker H. Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection. *AIDS* 2009; **23**:19–28.
18 Tong JC, Simarmata D, Lin RTP, Rénia L, Ng LFP. HLA Class I restriction as a possible driving force for Chikungunya evolution. *PLoS ONE* 2010; **5**:e9291.
19 Malavige GN, Rostron T, Rohanachandra LT *et al.* HLA class I and class II associations in dengue viral infections in a Sri Lankan population. *PLoS ONE* 2011; **6**:e20581.
20 Carrington M, O'Brien S. The influence of HLA genotype on AIDS. *Annu Rev Med* 2003; **54**:535–51.
21 Achord A, Lewis R, Brackin M, Henderson H, Cruse J. HIV-1 disease association with HLA-DQ antigens in African Americans Caucasians. *Pathobiology* 1996; **64**:204–8.
22 Odum N, Georgsen J, Fugger L *et al.* HLA-DP antigens in HIV-infected individuals. *Dis Markers* 1990; **8**:113–6.
23 Nguyen T, Kikuchi M, Vu T *et al.* Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. *PLoS Negl Trop Dis* 2008; **2**:e304.
24 Polizel J, Bueno D, Visentainer J *et al.* Association of human leukocyte antigen DQ1 and dengue fever in a white Southern Brazilian population. *Memórias do Instituto Oswaldo Cruz* 2004; **99**:559–62.
25 Lanteri M, Kaidarova Z, Peterson T *et al.* Association between HLA class I and class II alleles and the outcome of West Nile virus infection: an exploratory study. *PLoS ONE* 2011; **6**:e22948.
26 Alper CA. Major Histocompatibility Complex: Disease Associations. Encyclopedia of Life Sciences (ELS), 2009.
27 Sierra B, Alegre R, Perez AB *et al.* HLA-A, -B, -C, and -DRB1 allele frequencies in Cuban individuals with antecedents of dengue 2 disease: advantages of the Cuban population for HLA studies of dengue virus infection. *Hum Immunol* 2007; **68**:531–40.
28 Manimunda SP, Singh SS, Sugunan AP *et al.* Chikungunya fever, Andaman and Nicobar Islands India. *Emerg Infect Dis* 2007; **13**:1259–60.
29 Singh SS, Manimunda SP, Sugunan AP, Sahina, Vijayachari P. Four cases of acute flaccid paralysis associated with chikungunya virus infection. *Epidemiol Infect* 2008; **136**:1277–80.
30 Muruganandam N, Chaaithanya IK, Senthil GS *et al.* Isolation and molecular characterization of Chikungunya virus from the Andaman and Nicobar archipelago, India: evidence of an East, Central, and South African genotype. *Can J Microbiol* 2011; **57**:1073–7.
31 Sugunan AP, Vijayachari P, Sharma S *et al.* Risk factors associated with leptospirosis during an outbreak in Middle Andaman, India. *Indian J Med Res* 2009; **130**:67–73.
32 Hasebe F, Parquet MC, Pandey BD *et al.* Combined detection and genotyping of Chikungunya virus by a specific reverse transcription-polymerase chain reaction. *J Med Virol* 2002; **67**:370–4.
33 Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; **39**:225–35.
34 Mullighan CG, Bunce M, Welsh KI. High-resolution HLA-DQB1 typing using the polymerase chain reaction and sequence-specific primers. *Tissue Antigens* 1997; **50**:688–92.
35 Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature* 1988; **335**:167–70.
36 Hughes AL, Nei M. Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. *Proc Natl Acad Sci U S A* 1989; **86**:958–62.
37 Druce J, Johnson D, Tran T, Richards M, Birch C. Chikungunya virus infection in traveler to Australia. *Emerg Infect Dis* 2007; **13**:509–10.
38 McNicholl J. Host genes and infectious diseases. *Emerg Infect Dis* 1998; **4**:423–6.
39 Wang JH, Reinherz EL. Structural basis of T cell recognition of peptides bound to MHC molecules. *Mol Immunol* 2002; **38**:1039–49.
40 Harris R, Sugimoto K, Kaplan D, Ikeda F, Kamoun M, Chang K-M. Human leukocyte antigen class associations with hepatitis C virus clearance II virus-specific CD4 T cell response among Caucasians African Americans. *Hepatology (Baltimore, MD)* 2008; **48**:70–9.
41 Schmitt-Egenolf M, Boehncke W, Ständer M, Eiermann T, Sterry W. Oligonucleotide typing reveals association of type I psoriasis with the HLA-DRB1*0701/2, -DQA1*0201, -DQB1*0303 extended haplotype. *J Invest Dermatol* 1993; **100**:749–52.
42 Delgado J, Baena A, Thim S, Goldfeld A. Aspartic acid homozygosity at codon 57 of HLA-DQ β is associated with susceptibility to pulmonary tuberculosis in Cambodia. *J Immunol* 2006; **176**:1090–7.
43 Doytchinova I, Flower D. *In silico* identification of supertypes for class II MHCs. *J Immunol* 2005; **174**:7085–95.