CONTRIBUTION OF HOST FACTORS TOWARDS ARBOVIRAL INFECTIONS IN EASTERN INDIA AND EFFECT OF CURCUMIN AGAINST ARBOVIRAL INDUCED ARTHRITIS

Thesis submitted for the Degree of

Doctor of Philosophy (Ph.D) (Science)

By

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Index no.88/19/Life Sc./26

Jadavpur University

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2023

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**CONTRIBUTION OF HOST FACTORS TOWARDS ARBOVIRAL INFECTIONS IN EASTERN INDIA AND EFFECT OF CURCUMIN AGAINST ARBOVIRAL INDUCED ARTHRITIS**" Submitted by **Sri Siddhartha Sengupta** who got his name registered on **13.09.2019** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Anusri Tripathi** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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DECLARATION

I declare that the work in this thesis entitled "**CONTRIBUTION OF HOST FACTORS TOWARDS ARBOVIRAL INFECTIONS IN EASTERN INDIA AND EFFECT OF CURCUMIN AGAINST ARBOVIRAL INDUCED ARTHRITIS**" has been carried out by me in the Department of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, under the supervision of **Dr. Anusri Tripathi**.

Siddhartha Sengupta
25/4/2023
Siddhartha Sengupta

I dedicate this thesis

To

Maa and Baba

Wherever you are, thanks for everything. I love you.

ACKNOWLEDGEMENTS

My work, the entirety of this thesis would not have been possible without the guidance and support of my guide and supervisor, **Dr. Anusri Tripathi,** Assistant Professor (Molecular Biology), Dept. of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine. I am and will be forever grateful and indebted to her for providing me the opportunity, and in turn guiding, motivating and mentoring me through all possible nooks and crannies of research work and manuscript or thesis writing. I offer my deepest gratitude to her and wish this guidance continues.

I would like to thank **Dr. Mandira Mukherjee**, Head of the Department, Dept. of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, for providing me with the opportunity to work with the departmental research instruments.

I am grateful to the past and present **Directors** of Calcutta School of Tropical Medicine, for granting me permission to work at this institute of great repute and extending all facilities related to research work.

I am thankful to **Prof. Nemai Bhattacharya**, ex Unit-in-charge, Virology, Department of Microbiology, Calcutta School of Tropical Medicine, for providing me with the huge sample pool, without which this thesis would not have materialized. His wise words and valuable guidance enriched my clinical research knowledge. I would also like to thank **Surja Kumar Halder,** technologist from this department for helping me out whenever needed.

I would like to thank **Dr. Chinmay Kumar Panda**, NASI Senior Scientist Platinum Jubilee Fellow, Officer-in-charge (Research) – Former, Senior Assistant Director Grade (Retired), Department of Oncogene Regulation, Chittaranjan National Cancer Institute,

Kolkata; for his advice and thought-provoking insights regarding my research, along with providing his laboratory research facilities whenever needed.

I express my deep regard and gratitude to **Dr. Rajib Bandopadhyay,** Professor, Department of Botany, The University of Burdwan, for invoking my interest in research during my post-graduate days. Without him, I would've never trodden this path of exploring the intricacies of research.

Also, I will be forever thankful to the **Indian Council of Medical Research**, for awarding me the ICMR-Senior Research Fellowship and helping me financially throughout.

I express my heartiest love and gratitude to my lab seniors **Sudip Kumar Dutta**, **Lena Dhara** and **Arijit Pal**; to my fellow compatriot **Saikat Mukherjee**; and juniors **Sumoyee Mukherjee**, **Priyanka Ghosh**, **Srijan Manna** and **Akash Shaw**. These people are not my lab-colleagues, but friends, without whom, this thesis work would've been incomplete.

There are certain people who made my dwelling easier through the ebbs and flows of research life. I thank **Swe Yein Aung Mog**, **Advaita Chakraborty**, **Sayan Gupta**, **Suvaditya Roy**, **Abhishek Pramanik**, **Abhishek Dasgupta**, **Prajesh Kumar Saha** and **Anabadya Chatterjee**. I cannot imagine my life without these people.

I would like to thank my Pishi, Pishemoshai, Chordi, Bordi, Didun and Chotomama. I thank my late uncle, Borobhai, who was my friend and is responsible for shaping my interest in Science, Literature, Music and Movies. Finally, I thank **Maa (Late Nibedita Sengupta)** and **Baba (Late Sushanta Sengupta)** for everything. I miss you and I wish you could see this day.

Siddhartha Sengupta

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LIST OF ABBREVIATIONS

ACR/EULAR	American College of Rheumatology/European League Against Rheumatism
AA	Acute arthralgia
AA+PCA	Acute and post chikungunya arthralgia
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
Anti-CCP	Anti-cyclic-citrullinated-peptide
Arbovirus	Arthropod-borne virus
ARRIVE	Animal Research: Reporting of In Vivo Experiments
AST	Aspartate aminotransferase
BEB	Bengali Bangladeshis
CCHF	Cytomegalo and Crimean–Congo Hemorrhagic fever virus
CDC	Centers for disease control and prevention
CHIKV	Chikungunya virus
C.I.	Confidence interval
COL2A1	Anti-mouse type II collagen
COMP	Cartilage oligomeric matrix protein
CPCSEA	Committee for the Purpose of Control and Supervision on Experiments on Animals
CRP	C-reactive protein
CSE	Centre Square Entries
Ct	Cycle threshold

CT	Co-treatment
DAB	3,3-diaminobenzidine
DENV	Dengue virus
DHF	Dengue hemorrhagic fever
dNTP	Deoxyribonucleotide triphosphate
DSS	Dengue shock syndrome
ECM	Extracellular matrix
ECSA	East Central South Africa
ESR	Erythrocyte sedimentation rate
GWAS	Genome Wide Association Studies
HBV	Hepatitis-B virus
HCV	Hepatitis-C virus
HD	High-dose
HPV	Human papillomavirus
HSV	Herpes simplex virus
HVL	High-viral-load
IFN	Interferon
Ig	Immunoglobulin
IL-1β	Interleukin-1-beta
IL-2R	Interleukin-2 receptor
IL-6	Interleukin-6
IRF3	Interferon regulatory factor 3
JEV	Japanese encephalitis virus
L	Leucine
LD	Low-dose

LDpair	Linkage disequilibrium pair
LOD	Limit of detection
LVL	Low-viral-load
M	Methionine
MAF	Minor allele frequencies
MD	Mid-dose
MDA5	Melanoma differentiation-associated protein 5
MFC	Medial femoral condyle
MMP	Matrix metalloproteinases
MTP	Medial tibial plateau
NA	Patients without any arthralgia
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nano gram
NIH	National Institute of Health
NIV	National Institute of Virology
NS1	Non-structural protein 1
NSAID	Non-steroidal anti-inflammatory agent
NVBDCP	National vector borne disease control programme
OARSI	Osteoarthritis Research Society International
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PCA	Post chikungunya chronic arthritis
PCA	Post-chikungunya arthritis

PCR	Polymerase chain reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
pg	Pico gram
Post-T	Post treatment
PRR	Pattern recognition receptor
PT	Pre-treatment
Q	Glycine
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I like receptor
ROC	Receiver operating characteristic curve
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription-Polymerase chain reaction
SAS	South Asian population
SD	Severe dengue
SMASH	Standardized Microscopic Arthritis Scoring of Histological sections
SNP	Single nucleotide polymorphism
SPase	Signal peptidase
SPC18	Signal peptidase complex catalytic subunit
ssRNA	Single stranded ribonucleic acid
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha

US FDA	United States Food and Drug Administration
UTR	Untranslated region
V	Valine
WA	West African
WHO	World health organisation
WOMAC	Western ontario and mcMaster universities arthritis index
ZIKV	Zika virus
µg	Micro gram
µl	Micro liter

ABSTRACT

Arboviral infections caused by various arthropod-borne viruses viz. dengue (DENV) and chikungunya (CHIKV) have emerged as one of the most serious emerging threats to global health, leading to high mortality and morbidity. Both DENV and CHIKV are transmitted by same vector *Aedes aegypti* in India. Dengue endemic areas of India overlap with regions having chikungunya infection, leading to DENV-CHIKV co-infection. CHIKV mono-infection and DENV-CHIKV co-infection among symptomatic Eastern Indian patients was determined by RT-PCR, anti-CHIKV/DENV IgM and dengue-NS1 ELISA. 26.05% (167/641) CHIKV mono-infection and 19.96% (128/641) DENV-CHIKV co-infection was detected among suspected febrile patients presenting WHO-recommended symptoms. 40-60% of CHIKV-infected patients develop chronic inflammatory rheumatism and musculo-skeletal disease known as post-chikungunya chronic arthritis (PCA). This study revealed serum-levels of arthritic biomarkers viz. C-reactive protein (CRP), cartilage oligomeric matrix protein (COMP), anti-cyclic citrullinated peptide (anti-CCP) antibody, interleukin-2 receptor (IL-2R) significantly increased among PCA patients. Also, patients with IL-2R-rs743777-GA, G-allele and COMP-rs144778694-GA genotypes were susceptible to chikungunya infection. COMP-rs144778694-GA genotype was susceptible towards development of arthralgia. Upon DENV-CHIKV co-infection, Toll-like receptors (TLR) and CRP triggers innate immunity activation resulting in elevated cytokines leading to a plethora of defensive mechanisms. Thus, this study identified patients with certain single nucleotide polymorphisms (SNPs) of TLR3,7,8 and CRP were susceptible to co-infection. Moreover, probable role of specific genotypes of TLR7,8 and CRP SNPs towards imparting high dengue/chikungunya viral load during competitive viral replication among co-infected patients were identified. Till date, no FDA-approved analgesic drug is available for treatment of CHIKV-induced arthritis. Hence, in this study analgesic and prophylactic effect of curcumin, among CHIKV-induced arthralgic mice was explored. Curcumin treatment significantly alleviated CHIKV-induced arthritic pain by improving pain-threshold, locomotory behavior and reduced feet swelling of CHIKV-induced arthralgic mice. Also, decreased proteoglycan loss and cartilage erosion with lower arthritic scores were observed after curcumin treatment. Compared to infected mice, 1-2-fold increased intensity of type II collagen in knee articular regions was observed among curcumin-treated mice by immunohistochemical staining. Thus, the entire study imparted valuable inputs regarding contribution of host factors towards arboviral infections in eastern India and analgesic and prophylactic effect of curcumin against arboviral induced arthritis.

INTRODUCTION

1. INTRODUCTION

Emerging infectious diseases caused by various arthropod-borne viruses (arbovirus) leads to devastating illnesses in humans, viz. Chikungunya, Dengue etc. - making them one of the major challenging aspects in global health-care. Arboviral infection is an important public health problem in India, causing high mortality or morbidity of infected patients [1]. Dengue (DENV) and chikungunya (CHIKV) viral infections with potentially fatal complications are reported from various parts of India every year [1]. India is a dengue-endemic country with seroprevalence rate of 70% or higher, whereas chikungunya outbreak in India was reported from 1963–1973, after which it disappeared for three decades only to re-emerge during 2005 [1-3]. Both viruses are transmitted by same vector, i.e., *Aedes aegypti* - thereby resulting in overlap of dengue endemic areas of India with chikungunya infection [4, 5]. Chikungunya infection is characterised by fever, rash, vomiting, myalgia, joint-swelling and arthralgia, whereas, dengue infected patients mostly manifest fever, rash, vomiting, myalgia, arthralgia, abdominal pain and haemorrhages [1, 6]. Since, several symptoms of these two infections are overlapping, it is a challenge to distinguish CHIKV infection from DENV clinically, which co-circulate in same geographical area [7]. Persons infected by these arboviruses present a parallel clinical manifestation, such as rash, myalgia, exanthema, arthralgia, joint pain, headache, and fever [7]. DENV and CHIKV co-infections were first reported in Calcutta, India, in 1967. DENV and CHIKV co-infections have been reported in 13 of 98 countries/ territories where both viruses were transmitted locally [8]. Transmission period of both viruses in India mostly occurs during post-monsoon period (September–November) favouring mosquito breeding [9]. Thus, there might be misdiagnosis of chikungunya as dengue and vice versa among infected patients [10].

CHIKV-infection causes both acute and chronic polyarthralgia along with stooped appearance of patients with severe joint pain [11]. Acute arthritis might lead to development of devastating arthritis involving 4 or more joints of both upper and lower limbs [12]. Approximately, 88-100% of infected patients experienced post-chikungunya sub-acute arthritis (6 weeks) and 40-60% demonstrated chronic arthritis even after 1.5 to 5 years of infection [13, 14]. After inoculation through an infected mosquito bite, CHIKV enters subcutaneous capillaries of hosts and invades as well as infects susceptible cells in skin, such as macrophages, fibroblasts or endothelial cells [15]. They travel to organs such as liver, spleen, muscles and joints, where further viral replication transpires– resulting in rapid innate immune responses, bone erosion, degradation of extracellular matrix and release of pro-inflammatory cytokines [15, 16]. An important family of pattern recognition receptors (PRRs), Toll-like receptors (TLRs) are responsible for detecting pathogen-associated molecular patterns (PAMPs) of various pathogens such as viral single stranded ribonucleic acid (ssRNA) of dengue and chikungunya, thus, activating innate immunity against them within an infected patient body [17]. TLR3, TLR7, TLR8 and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are the most important class of PRRs which recognize viral RNA and act as pathogen sensors [18, 19]. These receptors activate a signalling cascade that leads to activation of type I interferons (IFNs) and transcription of cytokines and chemokines [20]. Inflammatory biomarker C-reactive protein (CRP) is a pentameric protein synthesized by liver, whose level rises in response to inflammation [21]. It is a part of innate immune system where it acts as a pattern recognition molecule to activate the adaptive immune response [22]. Interleukin-6 (IL-6), interleukin-1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are also stimulated by CRP. It acts as a scavenger protein by binding to damaged tissues, nuclear antigens, and pathogens, thus, removing

toxic molecules and neutralizing invasive harmful microorganisms [23]. Arthralgic manifestation during acute CHIKV-infection resembles rheumatoid arthritis (RA), both are characterized by increased rheumatoid factor (RF) and anti-cyclic-citrullinated-peptide (anti-CCP) antibody related articular damage [24]. Detection of anti-CCP antibodies in serum is one of the most specific biomarker for diagnosis of RA [25]. Also, anti-CCP antibody cross-reacts with type II collagen, resulting in proteoglycan depletion and severe arthritis [26]. Elevated level of several serum biomarkers viz. Cartilage oligomeric matrix protein (COMP), interleukin-2 receptor (sIL-2R) has been used as diagnostic and /or prognostic markers of RA [27, 28]. COMP, a non-collagen, extracellular matrix protein biomarker for cartilage degradation found in articular cartilage, ligament, meniscus, synovial membrane, and tendon, has been associated with cartilage turnover and joint destruction related to osteoarthritis and rheumatoid arthritis [29]. Soluble sIL-2R, a pro-inflammatory cytokine related to CHIKV-infection, has been implicated in development of synovitis and exacerbation of disease severity of RA [28]. Moreover, RF, anti-CCP antibody and CRP has been recognized as serological and acute phase response markers for rheumatoid arthritis, according to 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria [30]. But, their prognostic value for post-chikungunya chronic arthritis has not been tested.

Arthritis is a major feature of both dengue and chikungunya arboviral infections and agents that can alleviate pain among arboviral arthritic patients' needs to be identified [31]. Plant derived chemical compounds that have minimal toxic side effects and are readily absorbed in bloodstream to reach concentrations that have therapeutic potential might emerge as source of novel therapeutics [32]. Curcumin, a natural polyphenol isolated from turmeric (*Curcuma longa*) has been approved by the US Food and Drug

Administration (FDA) for usage [33]. It has been found to be safe and tolerable in human clinical trials and systematic reviews without any toxic or adverse effects [34]. Curcumin supplementation has been reported to improve walking time, morning stiffness, and joint swelling of rheumatoid arthritic patients [35]. But, Curcumin has not yet been tested for treatment of arbovirus induced arthritis, in this case, CHIKV-induced arthritis.

1.1 Arboviral infections:

Arboviral disease is a general term used to describe infections caused by a group of viruses spread to people by bite of infected arthropods (insects) such as mosquitoes [36]. These infections usually occur during warm weather months, when mosquitoes are active. Endemic infectious diseases viz. chikungunya and dengue caused by arthropod-borne arboviruses are a reason for high morbidity and mortality among those infected [1]. CHIKV belongs to Alphavirus genus of *Togaviridae* family [37]. Alphaviruses are arboviruses and are therefore transmitted chiefly by infected mosquitoes. *Aedes aegypti* and *Aedes albopictus* are known as main vectors for CHIKV transmission in Americas, Africa, Europe, Asia and Oceania [Figure 1.1] [38].

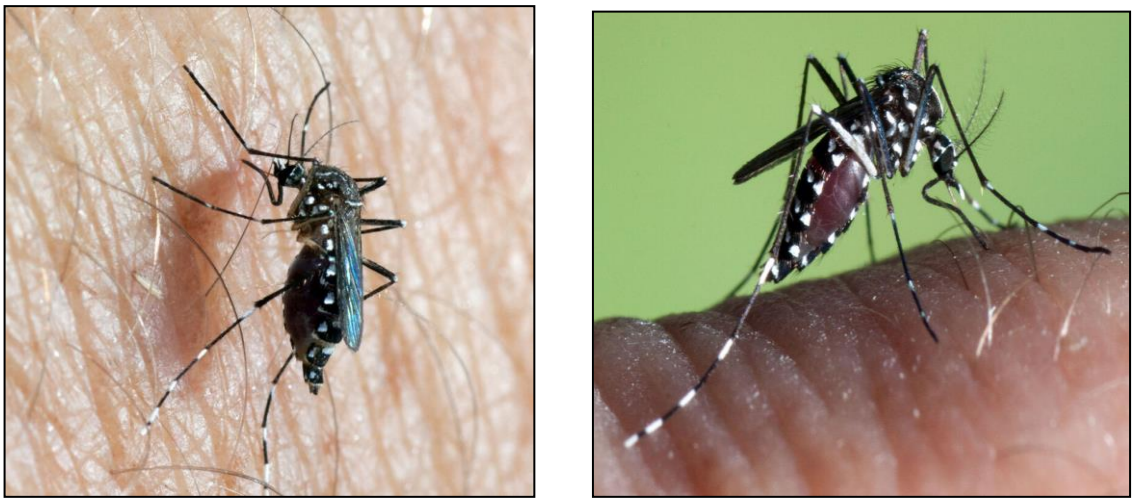

Fig 1.1: Representative photograph of *Ae. aegypti and Ae. albopictus*

1.2. Chikungunya prevalence around the world

Incidence of chikungunya has rapidly increased in number of patients over last decades. First recorded outbreak of Chikungunya infection was reported from Tanzania in 1952 [39]. In 2004, one of the largest chikungunya outbreaks was noted in Kenya during which virus spread to other regions of world [39]. Regions of Indian Ocean, Southeast Asia and India were highly affected by the virus. The disease re-emerged in several countries in Central and West Africa, and small outbreaks have been reported in Europe since 2007. Pacific region has experienced several CHIKV outbreaks since 2011 and from 2013 it rapidly spread from Caribbean to neighbouring islands and Central, South, and North America [Fig 1.2, 1.3].

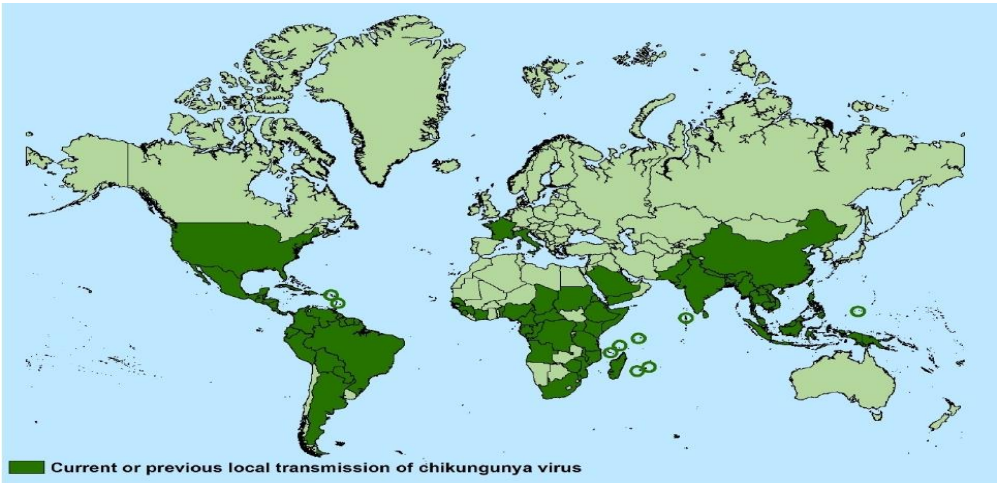

Fig 1.2: Countries and territories where chikungunya cases have been reported

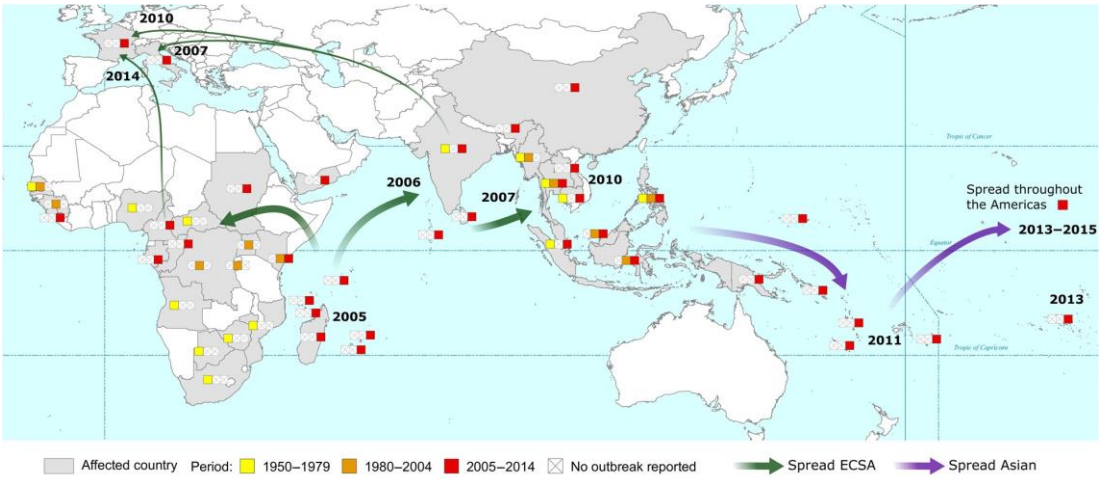

Fig 1.3: Chikungunya geographic spread [Courtesy ref. 39].

1.3. Chikungunya prevalence in India

Earliest record of chikungunya infection in India was reported from Calcutta in 1963 [40]. It was followed by several epidemics in Chennai, Pondicherry, Vellore, Visakhapatnam, Rajmundry, Kakinada, Nagpur and Barsi between 1964 and 1973. Since then, there have been several reports of Chikungunya outbreaks in different parts of India. A high number of cases of Chikungunya were recorded in India from 2006 to 2010 [2]. According to data from National vector borne disease control programme (NVBDCP), from 2017 to October 2022 a total of around 58,000 confirmed cases of chikungunya have been noted in various states of the country [41]. States which generally have the highest reporting of Chikungunya include West Bengal, Karnataka, Tamil Nadu, Telangana, Delhi, Rajasthan and Gujarat [2]. Currently, CHIKV-infection is endemic in 24 Indian states and 6 union territories [Fig 1.4].

West Bengal was the first state in India to record first instance of chikungunya epidemic in 1963 [40]. After 32 years of disappearance, chikungunya infection was again noted in West Bengal in 2006, with occasional spurts in the number of cases there onwards [42]. After that number of chikungunya cases reduced until a massive outbreak was again noted in 2016 [43].

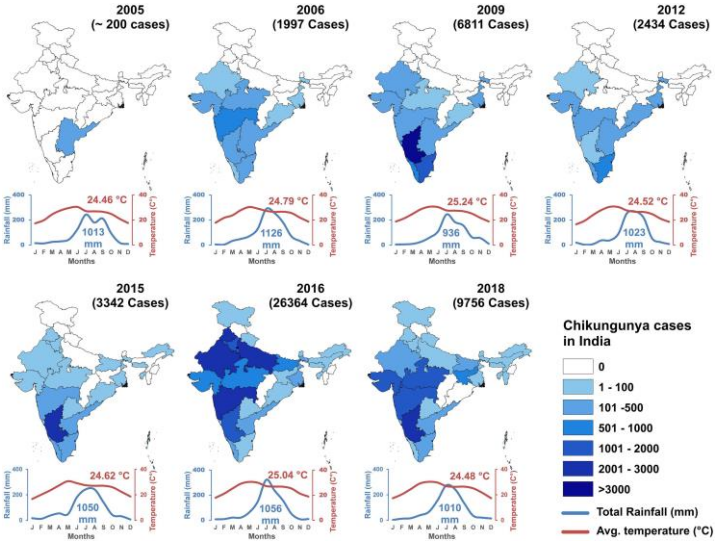

Fig 1.4: Chikungunya distribution in India [Courtesy ref. 2].

1.4. Dengue prevalence around the world

Dengue infection is the most common vector borne disease in world [44]. During last 50 years, incidence of dengue has increased 30-fold (Centers for disease control and prevention-CDC 2014) [45] [Fig 1.5]. DENV epidemics occur annually in the Americas, Asia, Africa, and Australia, and also affect travellers from endemic regions [46]. According to World health organisation (WHO), dengue is endemic in more than 100 countries across the globe [47]. In 2020, dengue affected several countries, with an increased number of cases in Bangladesh, Cook Islands, Brazil, India, Ecuador, Maldives, Indonesia, and other countries [48].

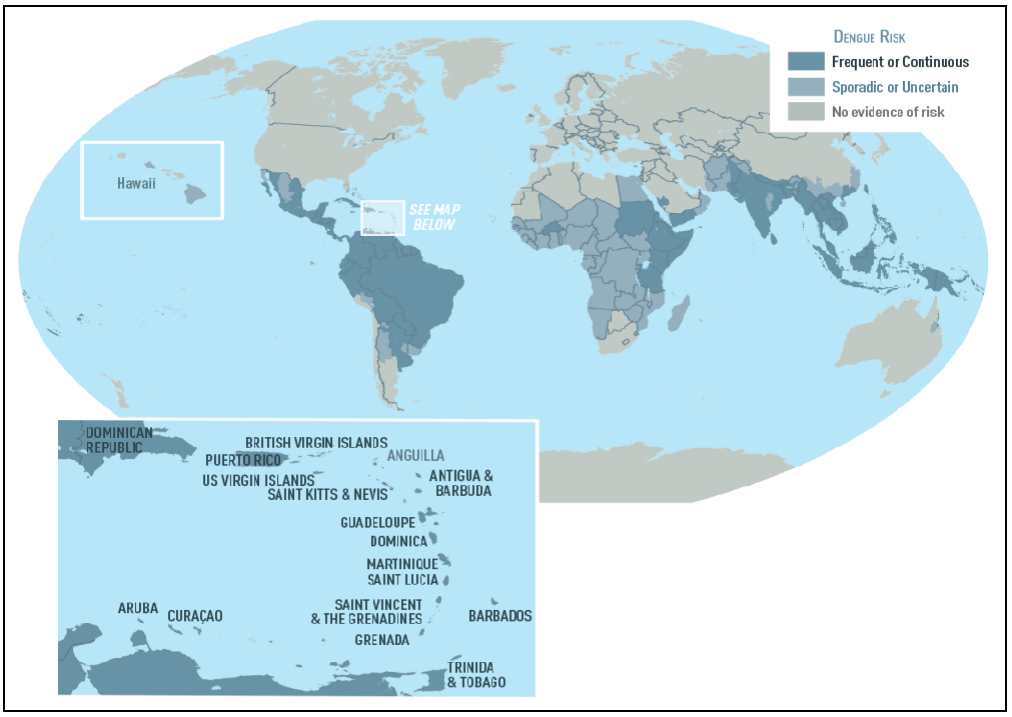

Fig 1.5: Countries and territories where dengue cases have been reported [Courtesy ref. CDC].

First dengue outbreak was reported in 1779 in Jakarta, Indonesia and Cairo, Egypt [46]. But, a confirmed outbreak in North America, by DENV, occurred in Philadelphia in 1780. In 2010, there were 49,000 severe dengue cases all over North and South America.

Largest outbreak of dengue was seen in 2016 in US, with more than 2.38 million cases reported. Dengue cases have drastically increased in US in 2019, with more than 3 million cases [46].

Dengue epidemics were reported in East, West, and South Africa from beginning of 19th century [46]. From 1980 to 2000, several dengue outbreaks in both East and West African countries were caused by dengue virus. In Africa estimates from 2010 suggest that there were 15.7 million apparent DENV infections during the year [49]. Dengue also exists in sylvatic cycles in African forests between mosquitoes and non-human primates. Dengue is endemic in at least 34 African countries and from 2011-2021 outbreaks have occurred in East Africa, followed by West Africa, North Africa, and then Central Africa.

In Asia, first two outbreaks of dengue hemorrhagic fever were reported in the Philippines in 1953 and 1956, respectively [50]. After that, dengue epidemics occurred cyclically every year in Southeast Asian countries, including Philippines, Bangkok, Thailand, Bhutan, Brunei, Cambodia, East Timor, Indonesia, Laos, Malaysia, Myanmar, Singapore, and Vietnam [46]. Between 2004 and 2010, Indonesia had the second highest number of dengue cases after Brazil.

1.5. Dengue prevalence in India

In India, dengue is endemic in almost all states and is the leading cause of hospitalization. Dengue fever had a predominant urban distribution a few decades earlier, but is now also reported from peri-urban as well as rural areas [51]. Dengue is now considered to be endemic in almost all regions of India. In southern region of India transmission occurs all through year while in northern regions of the country dengue outbreaks are commonly noted from April to November [52].

Dengue hemorrhagic fever (DHF) first reported in Kolkata during year 1963-64 [53]. Dengue outbreaks in West Bengal have been increasing every year. Data from studies show that within 2010-2019 a total of 151 outbreaks were reported from different districts of West Bengal [54]. According to data from NVBDCP, from 2017 to October 2022 a total of around 7,95,000 confirmed cases and 1151 deaths due to dengue-infection have been noted in various states of the country [55] [Fig 1.6].

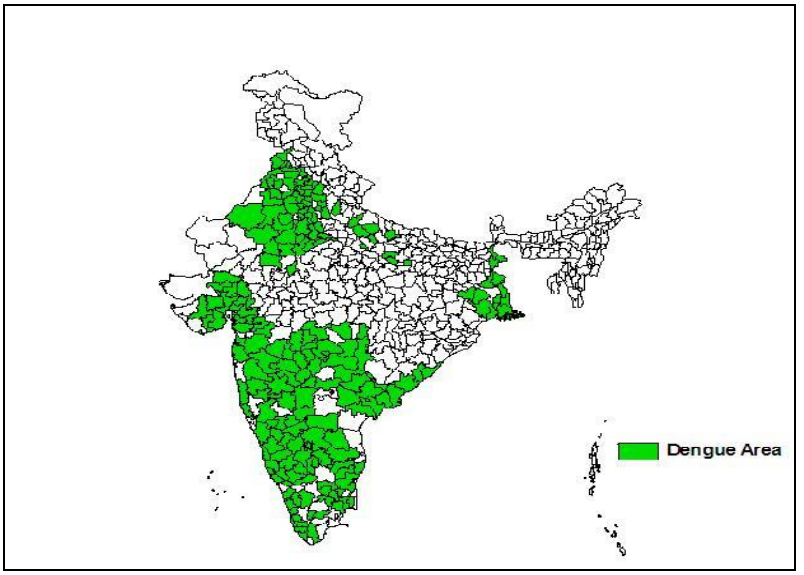

Fig 1.6: Regions prone to dengue infection in India [Courtesy ref. 55].

1.6. Dengue-chikungunya co-infection around the world and Eastern India

Dengue and chikungunya are vector borne viral diseases mostly prevalent in tropical and subtropical regions of the world. Both dengue and chikungunya have seen a rise in number of reported cases during last decade. Both these diseases are spread by *Aedes sp.* Mosquitoes [4]. Dengue chikungunya co-infection occurs when both viruses infect one individual at the same time. The earliest recorded case of DENV-CHIKV co-infection dates back to 1969 when 9 cases of co-infection were reported in Thailand [10]. Recent cases diagnosed were in South America, India and Nigeria in 2013-2014. Most co-infections are found in South Asia and Africa. DENV-CHIKV co-infections have been reported from 26 countries [56] [Fig. 1.7].

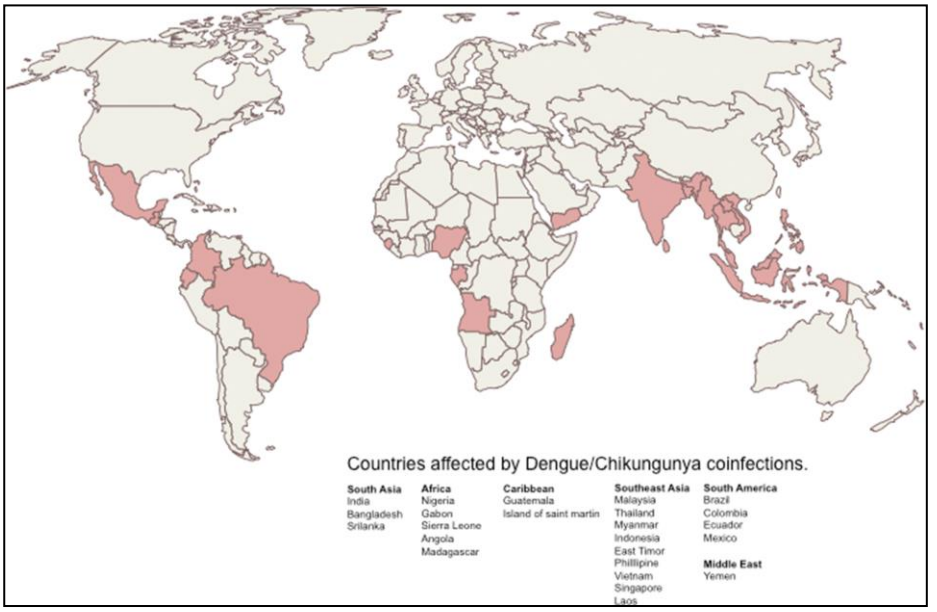

Fig 1.7: Global distribution of Dengue-Chikungunya co-infections [Courtesy ref. 56].

During spike in chikungunya outbreaks in 2006 and after 2016 many cases of co-infection have been reported in India [57]. DENV and CHIKV pathogens are also known to be co-circulating in West Bengal. A 2010 report identified 12.4% co-infected patients among 550 febrile cases [9].

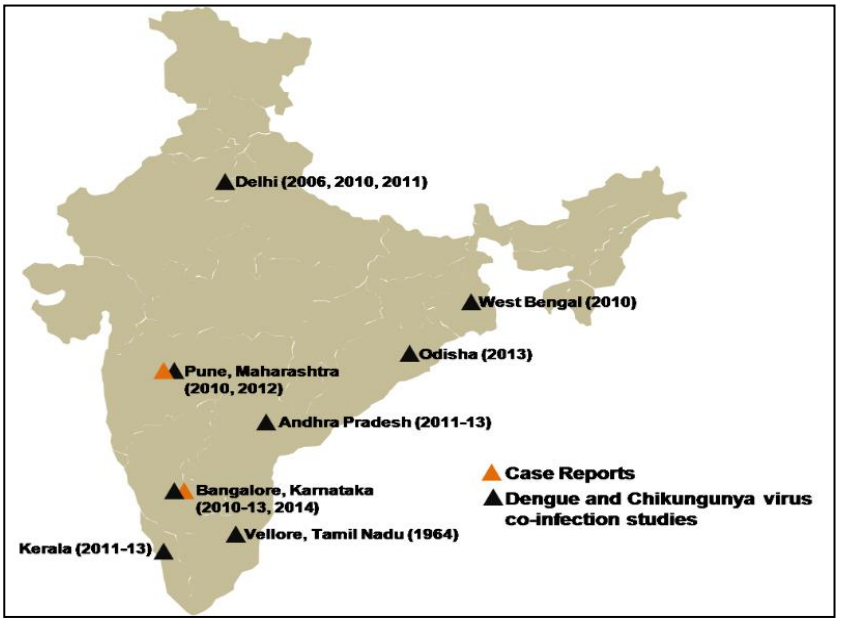

Fig 1.8: Map of India showing DENV-CHIKV co-infection prevalence [Courtesy ref. 57]

1.7. Detection and laboratory diagnosis:

Laboratory confirmation and early detection of dengue and chikungunya infection is crucial as dengue hemorrhagic fever often leads to mortality and chikungunya causes extreme morbidity and may lead to chronic post-chikungunya arthritis. Chikungunya infection is confirmed by detecting anti-CHIKV IgM antibody by ELISA or CHIKV genome by quantitative real time PCR in patient's sera [11]. Detection of dengue specific IgM or IgG antibodies is standard for serologically confirming dengue infection [47]. NS1- based ELISA is an important diagnostic tool for detecting NS1 antigen among acute samples in which IgM is not detectable. Real-time RT-PCR assay is a one-step assay that allows virus titre to be quantified within approximately 1.5 hours [58]. One advantage of this assay is its ability to determine viral titre early in dengue and chikungunya illness, which is believed to be an important predictor of disease severity [11, 47].

1.8. Chikungunya symptomatic classifications

In Makonde, chikungunya means "that which bends up" and refers to bent posture of CHIKV-infected patients with severe arthralgia [59] [Fig 1.9]. Clinical symptoms associated with CHIKV infection are classically divided into acute and chronic phases.

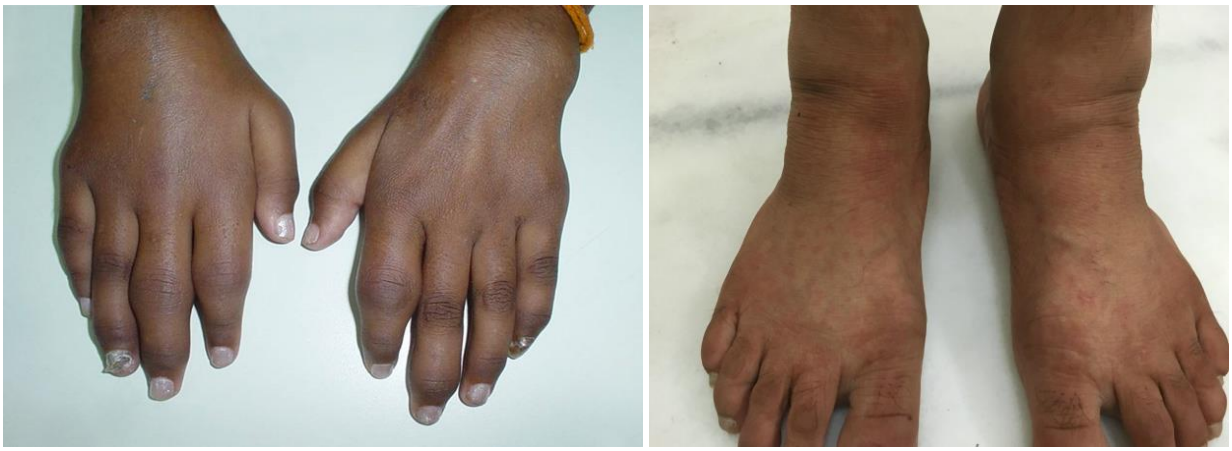

Fig 1.9: Chikungunya induced clinical manifestations viz. joint swelling and rash.

1.8.1 Acute phase

Acute stage is febrile phase and can extend until 5-10 days since onset of fever [7]. In this phase, approximately 50–97% of individuals infected with CHIKV develop clinical disease with fever and arthralgia [7]. CHIKV infection has been associated with sudden onset of febrile illness, arthralgia, back pain, headache and fatigue [60]. Most common symptom in CHIKV-infection is polyarthralgia, affecting mainly peripheral joints (ankles, wrists and phalanges) and some large joints (knees and elbows). Different studies have demonstrated that CHIKV-infection can reach high viral loads, ranging from 10^5 to 10^9 copies of viral RNA/mL, which seem to be correlated with presence and severity of clinical signs and symptoms [61]. Acute phase may be further subdivided into viraemic (5–10 days) and sub-acute post-viraemic (6–21 days) phases [62].

1.8.2 Chronic phase

Approximately, 88-100% of infected patients experience post-chikungunya sub-acute arthritis (6 weeks) and 40-60% demonstrated chronic arthritis even after 1.5 to 5 years of infection [13, 14]. Polyarthralgia has been described to persist for varying periods of time, lasting from weeks to several months and, in some cases, up to five years [63]. Persistent musculoskeletal symptoms were reported in 54–79% of CHIKV-infected patients 15–36 months post-infection [15]. Fingers, wrists, knees, ankles, and toes are the most frequently affected areas during chronic phase [64]. Majority of patients report intermittent pain, however, 35% of them had permanent effect. It may be accompanied by other symptoms such as local swelling, morning stiffness, fatigue and asthenia. Persistence and intensity of symptoms often impacts quality of everyday life, which leads to reduction of daily activities, job invalidity and depression, etc..

1.9. Dengue symptomatic classification

Characteristic symptoms of dengue infection are sudden-onset fever, headache, muscle and joint pains, and rash [3]. An alternative name for dengue, "breakbone fever", comes from associated muscle and joint pains [65]. According to clinical presentation, patients are categorized into three groups: dengue patients without warning signs, with warning signs, and severe dengue (SD), according to WHO classification. Warning signs include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed and lethargy or restlessness. Severe dengue includes severe plasma leakage leading to shock (dengue shock syndrome: DSS), fluid accumulation with respiratory distress, severe bleeding and organ involvement. Severe dengue has a higher risk of death when not managed appropriately [66].

1.10. Pathogenesis of CHIKV-induced chronic arthralgia

Clinical presentation of chikungunya infection mimics rheumatoid arthritis [67]. After infected mosquito bite, CHIKV enters subcutaneous capillaries, infecting susceptible cells in skin, such as macrophages, fibroblasts or endothelial cells and invades liver, spleen, muscles and joints, where further viral replication occurs [15]. CHIKV-infection triggers rapid innate immune responses, primarily by strong activation of type 1 IFN and by production of proinflammatory cytokines. Underlying mechanism by which CHIKV induces chronic arthritis has three possible reasons: 1) persistence of infectious virus; 2) persistence of viral nucleic acids which induce persistent immunopathology; 3) triggering of persistent immune activation in certain individuals after virus has been cleared [15]. Persistent viral replication in synovial tissue may lead to sustained inflammatory response in several probable mechanisms, e.g. by release of proinflammatory cytokines viz. IL-6, TNF-α, IL-1β, IL-2R, or by inducing apoptosis of infected cells [68]. Replication of CHIKV in joint tissue can induce expression of

osteoclastogenic cytokines, such as IL-6 and receptor activator of nuclear factor kappa-B ligand (RANKL) [15]. CHIKV replication in joint tissues induced expression of pro-osteoclastogenic cytokines, IL-6, NF-κB, TNF-α, IL-1β and RANKL [16].

According to 2010 ACR/EULAR classification criteria, any patient or healthy individual having active clinical synovitis (i.e., swelling) in at least 1 joint and those patients in whom observed synovitis is not caused to another diagnosis viz. systemic lupus erythematosus, psoriatic arthritis, and gout, are categorized on a score of 0–10, with a score of ≥6 being indicative of presence of definite RA, which can be CHIKV induced arthritis also [30]. To classify a patient, a history of symptom duration, a thorough joint evaluation, and at least 1 serologic test (of RF or anti-CCP) and 1 acute-phase response measure (of erythrocyte sedimentation rate [ESR] or CRP) is obtained and subsequently, the patient is categorized.

	Score
Target population (Who should be tested?): Patients who	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA)‡	
A. Joint involvement§	
1 large joint¶	0
2–10 large joints	1
1–3 small joints (with or without involvement of large joints)#	2
4–10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)**	5
B. Serology (at least 1 test result is needed for classification)††	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)‡‡	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms§§	
<6 weeks	0
≥6 weeks	1

Fig 1.10: 2010 ACR/EULAR rheumatoid arthritis classification criteria: domains, categories and point scores [Courtesy ref. 30].

1.11. Host innate immune response against arboviral infections

TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors, melanoma differentiation-associated protein 5 (MDA5) and RIG-I are a class of proteins that acts as first lines of defence in innate immune recognition of single stranded RNA viral genome and act as pathogen sensors resulting in secretion of interferons, cytokines and chemokines along with altered gene expression [69]. TLR family detect viruses that enter endosome through endocytosis. This pathway induces production of interferons through several signalling proteins that ultimately lead to activation of transcription factors NF-kB, Interferon regulatory factor 3 (IRF3) and IRF7 [70]. TLR3 initiates production of IFN-β and immunoregulatory cytokines while TLR7 and 8 initiate IFN-α production, IL-6 and TNF-α [71, 72, 73]. TLR7 stimulation produces proinflammatory cytokines through phosphorylation of IRF7 and liberation of nuclear factor-kB, respectively [74]. These are potent antiviral cytokines and modulators of the adaptive immune system.

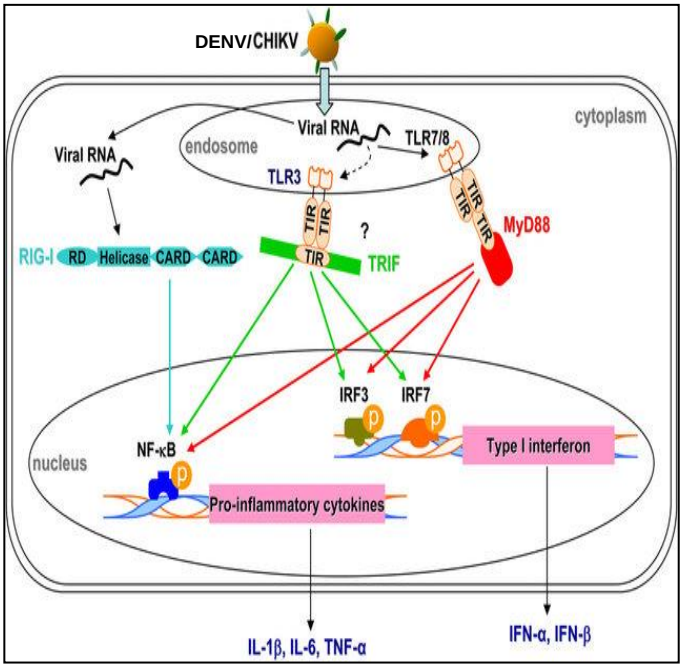

Fig 1.11: A schematic diagram of TLR signaling activated against DENV/CHIKV [Courtesy ref. 73]

1.12. Effect of bioactive compound Curcumin on arbovirus induced arthritis.

Since, arthritic manifestation is a major feature of arboviral infections, it is of utmost importance to identify agents that can alleviate pain among arboviral CHIKV-induced arthritic patients. Existing pharmaceuticals used for treating arthritis are analgesics, steroids and non-steroidal anti-inflammatory drugs, whose long-term use cannot be sustained due to inadequate pain relief, immune disturbances, and serious gastrointestinal & cardiovascular adverse events [75]. Plant derived compounds have minimal toxic side effects and are readily absorbed in bloodstream to reach concentrations that have therapeutic potential, which make them advantageous over chemically synthesized compounds [32].

Fig 1.12: A schematic representation of curcumin.

Curcumin, derived from turmeric, is one such compound that is known to be effective in treating joint inflammation in rheumatoid arthritis and has shown anti-inflammatory potential for treatment of osteoarthritis; patients with osteoarthritis showed improvement in pain, physical function, and quality of life after taking curcumin [76]. Since, it has been reported that curcumin can effectively reduce pain related to rheumatoid arthritis and osteoarthritis, it might also positively do so in chikungunya induced arthritis.

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OBJECTIVES

2. THESIS OBJECTIVES

- ❖ Determination of chikungunya mono-infection and dengue-chikungunya co-infection among symptomatic eastern Indian patients by detecting anti-dengue/chikungunya IgM/NS1 antibody and dengue /chikungunya viral (DENV/CHIKV) genome in patient-serum.

- ❖ Analysis of arthritic viz. C-reactive protein (CRP), rheumatoid factor (RF), cartilage oligomeric matrix protein (COMP), anti-cyclic citrullinated peptide (anti-CCP antibody), interleukin-2 receptor (IL-2R) and hepatic viz. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin and bilirubin biomarker levels in serum for early identification of chikungunya patients with post-chikungunya chronic arthralgia.

- ❖ Identification of specific genotypes of CRP, IL-2R & COMP polymorphisms, associated with chikungunya disease susceptibility and arthritic manifestation among infected patients.

- ❖ Identification of specific genotypes CRP, TLR3, TLR7 & TLR8 polymorphisms associated with chikungunya and dengue co-infection.

- ❖ Determination of analgesic effect of curcumin on arbovirus induced arthritis.

CHAPTER 1

Detection of chikungunya mono-infection and dengue-chikungunya co-infection among symptomatic eastern Indian patients

CHAPTER 1

Objective

- Determination of chikungunya mono-infection and dengue-chikungunya co-infection among symptomatic eastern Indian patients by detecting anti-dengue/chikungunya IgM/NS1 antibody and dengue /chikungunya viral (DENV/CHIKV) genome in patient-serum.

Publications:

❖ **Sengupta S,** Mukherjee S, Haldar SK, Bhattacharya N, Tripathi A. Re-emergence of Chikungunya virus infection in Eastern India. **Brazilian Journal of Microbiology**. 2020 Mar;51(1):177-182. doi: 10.1007/s42770-019-00212-0. Epub 2020 Jan 2. PMID: 31898249; PMCID: PMC7058808.

3.1 INTRODUCTION

One of the most serious emerging threats to global health is caused by various arthropod-borne viruses viz. dengue and chikungunya. Recurrent infection with dengue and chikungunya viruses leads to high morbidity and mortality, making them a major notorious public health problem in India [1]. Chikungunya, a Makonde word means, "that which bends up", is an arthropod-borne neglected tropical disease caused by chikungunya virus, a single stranded positive sense RNA virus, belonging to Alphavirus genus of *Togaviridae* family [2,3]. While, *Aedes albopictus* serves as major vector in Indian Ocean islands, *Aedes aegypti* is the principal vector for CHIKV in mainland India [4,5]. In Africa, CHIKV follows a sylvatic cycle involving non-human primates and a number of forest-dwelling mosquitoes (*Aedes furcifertaylori, Aedes luteocephalus* and *Aedes africanus*) [6]. CHIKV virus is considered to be endemic in certain parts of West and Central Africa [7]. Since 2005, chikungunya infection has spread mainly in tropical and sub-tropical regions [8]. Chikungunya is a re-emerging arboviral disease which achieved high public health significance in South-East Asia Region viz, India, Indonesia, Maldives, Myanmar, Sri Lanka, and Thailand [9]. Alarmingly, the infection spread from Asia and Africa since its first detection in Saint Martin Island late 2013 and subsequently has infected a million people in Caribbean, Latin America, Mexico, and parts of South America viz. Brazil, Bolivia, Colombia, Ecuador, Paraguay, and Venezuela having both imported and autochthonous cases [10]. The worst chikungunya outbreak occurred in Reunion Island in 2005-2006 which affected around 35% of the population [11]. Three genotypes of the CHIKV, namely, West African (WA), Asian and East Central South Africa (ECSA) are known till date with Asian genotype being prevalent in India [12]. In India, CHIKV was first isolated in 1963 at Kolkata (then Calcutta) and several epidemics

broke out at different regions of India, viz. Chennai, Pondicherry, Vellore, Nagpur, and Mumbai between 1964 and 1975 [13, 14]. Subsequently, CHIKV disappeared from India, but, eventually made a sudden re-emergence in 2005 after a gap of 32 years causing an explosive outbreak starting with Andhra Pradesh [15,16]. Kerala was the worst affected state in 2007 with approx. 3.6 million fever cases [17]. Then, recurring spurts of mass infection and spread of disease was reported from 13 different states of India, resulting in 1.4–6.5 million estimated cases across country and till date, it has spread to 30 states [12, 18]. In 2006, West Bengal witnessed a chikungunya outbreak, and since then, chikungunya fever has been recurring time and again [19-22]. Chikungunya infection is classified into three stages: acute stage (3–7days), a post-acute stage (after first 3 weeks until 3rd month after onset), and a chronic stage, which starts from 3 months after disease onset [23]. Clinical manifestations include abrupt onset of high fever, joint pain, myalgia, headache and rash [24, 25]. Joint pain, persisting for months, or even years, is a typical clinical symptom of CHIKV infection and referred to as chronic post-chikungunya arthritis [26]. In some patient's maculopapular rashes are found mainly on trunks or exterior surfaces of limbs, and rashes are usually accompanied by secondary rise in temperature [27].

India is endemic to DENV-infection —causing a full spectrum of the disease which makes it a serious health problem. DENV-infected patients can be categorized into three groups: dengue patients without warning signs, with warning signs, and severe dengue, according to WHO classification [28]. Warning signs are abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed and lethargy or restlessness [28]. Severe dengue includes extreme plasma leakage leading to shock (dengue shock syndrome), fluid accumulation with respiratory distress, severe bleeding

and organ involvement and may lead to death in some cases [28]. Annually, approximately 400 million dengue cases and 40,000 deaths occur worldwide [29]. Multiple DENV outbreaks have been reported from various parts of India, viz. Delhi, Kolkata, Lucknow, Kerala and has spread to sub-urban and rural areas [30-32]. Since, re-emergence of CHIKV in India during late 2005 after massive Indian Ocean islands outbreak, there has been an overlapping of DENV-endemic areas with CHIKV-affected areas in India [33]. In India, although both DENV and CHIKV belong to different families of *togaviridae* and *flaviviridae* respectively, they are transmitted by same vector, i.e., *Aedes aegypti* [31]. Also, every year transmission period of both viruses in India mostly appears to be during post-monsoon period (September–November) when climatic conditions become favourable for mosquito breeding [32]. Thus, frequency of concurrent infection by both viruses has remarkably increased in India since 2005. During early acute febrile phase of illness, both DENV and CHIKV infections have similar disease manifestations of high fever, myalgia, arthralgia, headache, nausea, rash, and vomiting. However, abdominal pain, retro-orbital pain, hemorrhagic indicators and myalgia are frequently reported among dengue patients, whereas prolonged polyarthralgia, joint-swelling is more commonly found among chikungunya patients [15, 32, 34, 35]. However, dynamics of CHIKV and DENV infection during such period of multiple illnesses and its implication during clinical manifestation among patients remain poorly studied.

This chapter focuses on detection and current status surveillance of chikungunya mono-infection and dengue-chikungunya co-infection among symptomatic patients from Eastern India in West Bengal during 2014-2016. The study was also aimed to correlate appearance of characteristic clinical symptoms with these infections.

3.2. MATERIALS AND METHODS

3.2.1. Ethics statement:

Collection of blood from febrile patients and healthy participants as well as experiments were performed in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consents were obtained from patients and healthy individuals before their participation in the study.

3.2.2 Patients and healthy controls:

5ml of blood from all age groups and sexes were collected from each symptomatic febrile patients (acute phase: 1-3 days, critical phase: 4-6 days late phase: ≥7 days), visiting Calcutta School of Tropical Medicine, West Bengal, India from September 2014 to October 2016. Febrile patients exhibiting any two of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, nausea, vomiting, swollen glands, rash, haemorrhagic manifestation, fatigue were selected as per WHO criteria.

3.2.3. Viral RNA isolation:

Viral RNA was extracted from 140µL of patient serum by using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France). Briefly, 560µl of AVL lysis buffer containing carrier RNA was mixed with 140µl serum and pulse vortexed for 15secs, and incubated at room temperature for 10mins. Following brief centrifugation, 96-100% ethanol was added to the mixture and subsequently pulse vortexed for 15secs. Carefully, 630µl solution from above mixture containing AVL buffer, ethanol and serum was transferred to QIAamp mini-Column along with collecting

tubes and centrifuged at 8000rpm for 1 minute. After centrifugation, collecting tube containing filtrate was discarded and QIAamp columns were then placed into fresh collecting tube. 500µl buffer AW1 was added to QIAamp columns and were centrifuged at 8000rpm for 1 minute. 500µl of AW2 buffer was added to each column, and centrifuged at 14000rpm for 3 minutes. The column was then placed in new 2ml centrifuge tube, and 60µl of AVE buffer was added to each tube. The column was incubated at room temperature for 1 minute, followed by centrifugation at 8000rpm for 1 minute. Finally, the elute containing viral RNA was stored at -80ºC.

3.2.4. Determination of chikungunya viral load:

Chikungunya viral load was determined by real-time qRT-PCR in total volume of 20 µl, using 5 µl of patient-extracted viral RNA, One Step qRT-PCR MasterMix, and other components of CHIKV Genesig kit (Primer Design Ltd., UK). Briefly, 5µlRNA extracted from patient sera was mixed with 10µl Oasig OneStep 2x qRT-PCR Mastermix and 1µl CHIKV primer/probe mix. Final volume was adjusted by adding4µl RNAse/DNAse free water. Real-time PCR was performed in 7500 Fast Real time PCR system. The amplification took place in four major steps: Reverse transcription at 55°C for 10 minutes; Enzyme activation at 95°C for 2 minutes; Denaturation at 95°C for 10 second and Data collection at 60°C for 1 minute (both the denaturation and data collection steps were repeated for 50 times). A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided in the kit) [Fig 3.1]. Copy number was calculated from corresponding Ct values [Table 3.1, Fig 3.2]. Each sample was analysed in triplicate, according to manufacturer's protocol.

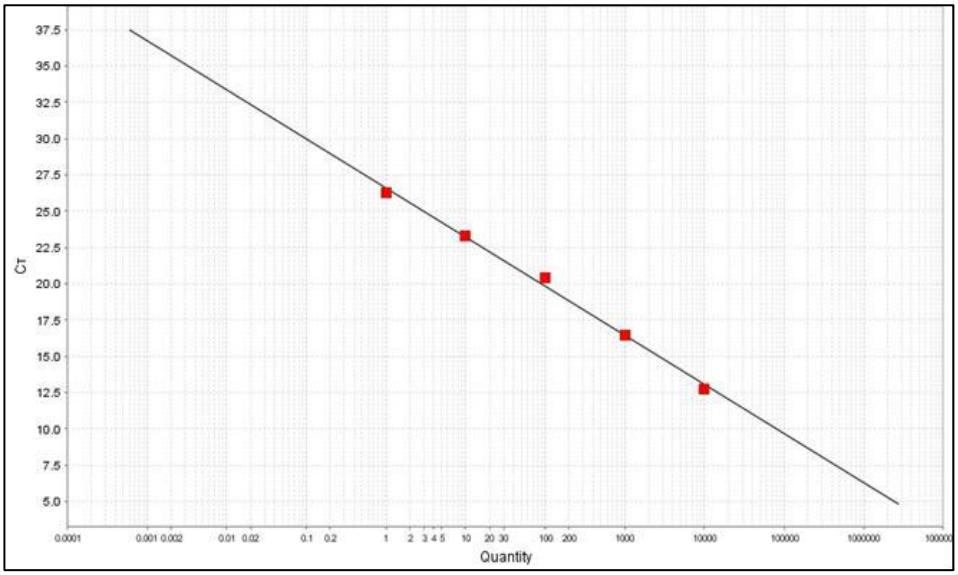

Fig 3.1: Representative Standard curve for CHIKV copy number determination.

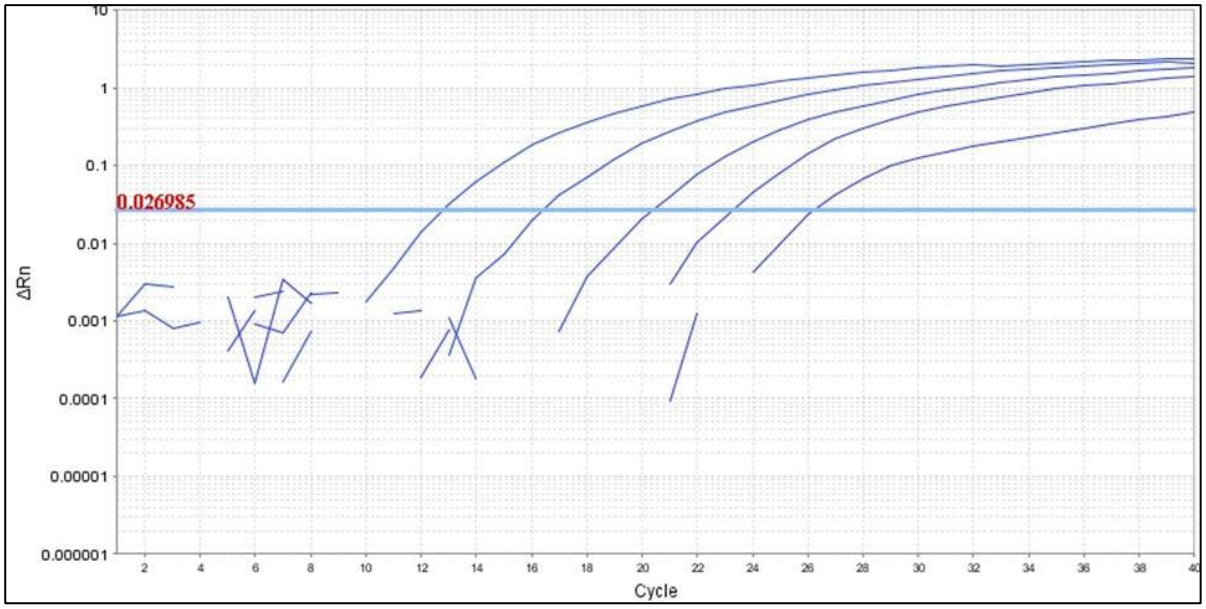

Fig 3.2: Representative amplification plot of CHIKV Real-time PCR.

Table 3.1: C_T values of 10-fold serially diluted cloned CHIKV nsp2 gene

STANDARD NUMBER	QUANTITY (Copies / 5µl)	C_T VALUE
Standard 1	$2X10^4$	12.78
Standard 2	$2X10^3$	16.45
Standard 3	$2X10^2$	20.40
Standard 4	20	23.3
Standard 5	2	26.25

3.2.5. Detection of CHIKV specific antibody by IgM ELISA:

Serum samples of patients were subjected to serological diagnosis for presence of anti-Chikungunya-IgM antibodies using NIV CHIKV MAC ELISA Kits (National Institute of Virology, Pune, India), following manufacturer's instructions [Fig 3.3]. Briefly, 100µL of 100 times diluted serum samples, negative control, positive control and calibrator were added to respective wells and incubated for 1 hour at 37^0C. The wells were washed five times and were incubated with 100µL of 10 times diluted enzyme conjugate for 1 hour at 37^0C. Following incubation, wells were washed five times and further incubated at room temperature with 100µL of TMB. After 30 minutes incubation, reaction was terminated by addition of 50µL of stop solution and absorbance was measured at 450nm wavelength with reference filter of 600-650nm within 30 minutes. Results were interpreted as negative or positive according to the assay manual.

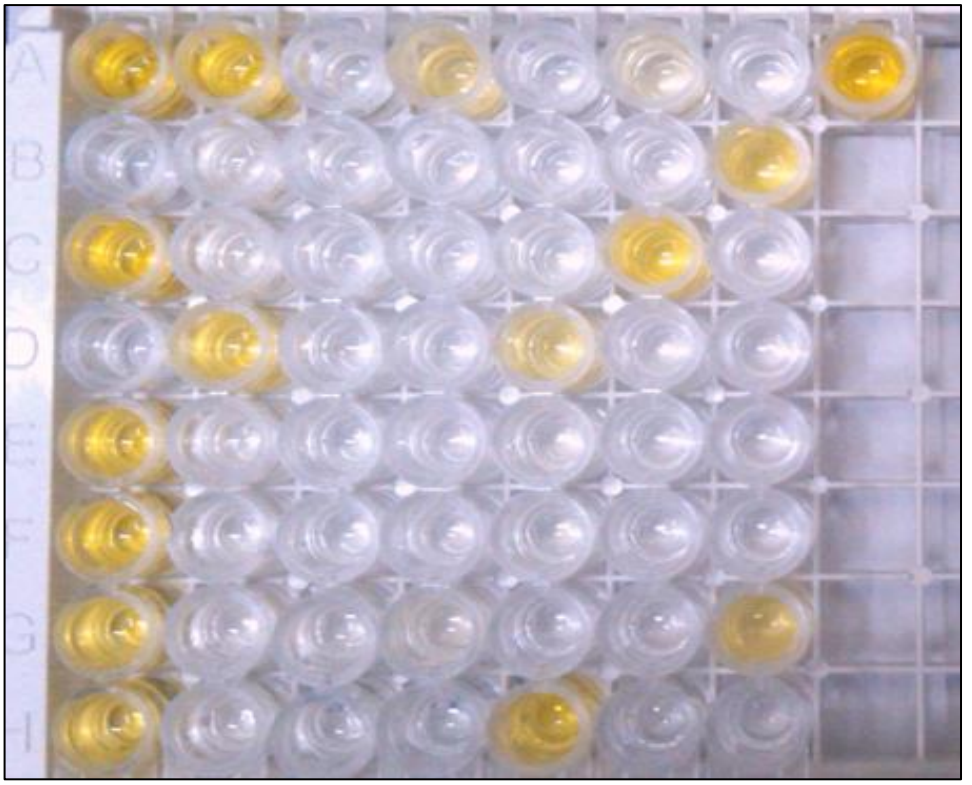

Fig 3.3: Representative picture depicting anti-chikungunya IgM antibody ELISA

3.2.6. Determination of dengue viral copy number:

Viral-RNA was extracted from 140µL of patients' sera, using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France). Presence of DENV genome and viral-load among infected patients' sera was determined by qRT-PCR (Taqman assay) in 20µl, using 8µl viral-RNA, OneStep qRT-PCR MasterMix and other components of DENV Genesig kit according to manufacturer's protocol (Primer Design Ltd., UK). Real time PCR was performed on ABI Prism 7500 fast instrument. A standard-curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided with kit). DENV RNA copy number in patients' sera was calculated from corresponding Ct values. Viral-RNA titre ≥10,000 copies/ml and <10,000 copies/ml was considered as high-viral-load (HVL) and low-viral-load (LVL), respectively.

3.2.7. Detection of anti-dengue-IgM antibody and dengue-NS1 Antigen:

Suspected patients' sera were subjected to serological diagnosis for presence of anti-dengue-IgM, dengue-NS1 antigen using dengue IgM MAC ELISA kit and dengue-NS1 ELISA kit, respectively (National Institute of Virology, India), according to manufacturer's instructions – as described previously.

3.2.8. Statistical analyses

2 × 2 contingency Chi-square and odds-ratio analysis were performed to find any association between frequency of DENV, CHIKV, and DEN–CHIK double-virus infection with different clinical symptoms. Statistical analysis among DENV, CHIKV and DEN–CHIK double virus- infected patients was calculated using healthy individuals as control group. $P<0.05$ was considered as statistically significant. Odds ratio values >1 indicated that a condition/event was more likely to occur in a particular disease group.

3.3. RESULTS

3.3.1. Determination and demographics of chikungunya mono-infection

In this study, conducted during 2014-2016, incidence of chikungunya infection was investigated among 641 symptomatic patients from various parts of West Bengal, located in eastern India. Among them, 26.05% [167/641] were found to be chikungunya infected by real time qRT-PCR and anti-chikungunya-IgM ELISA [Table 3.2, 3.3].

Table 3.2: Demographic scenario of CHIKV mono-infected and DENV-CHIKV co-infected symptomatic patients.

		Suspected (n= 641)	
		Chikungunya positive (n= 167)	**Dengue-Chikungunya co-positive (n = 128)**
Diagnostic Tools	Anti-Chikungunya-IgM ELISA	10.92%	_
	Real time RT-PCR	20.28%	_
	Both anti-Chikungunya-IgM ELISA and Real time RT-PCR	6.55%	_
Age(years)		33.06 ± 12.7 (range: 7-68)	24.28 ± 14.7 (range: 2–60 years)
Sex	Male	91	75
	Female	67	53
Symptomatic prevalence	Fever	100%	100%
	Myalgia	53.89%	55.11%
	Arthralgia	67.06%	48.81%
	Headache	22.15%	33.07%
	Rash	19.76%	14.96%
	Nausea	2.39%	18.11%
	Persistent Vomiting	25.14%	26.77%
	Abdominal Pain	8.98%	11.02%
	Joint Swelling	21.55%	17.32%
	Bleeding	0%	5.51%
	Retro-Orbital Pain	0%	4.72%
Viral Load	High Viral Load (≥ 10,000 copies/ml)	80.77	Dengue 56.32% (49) Chikungunya 47.12% (41)
	Low Viral Load (< 10,000 copies/ml)	19.23%	Dengue 43.67% (38) Chikungunya 52.87% (46)

Table 3.3: Detailed symptomatic history of CHIKV mono-infected patients

SR. NO.	AGE (Years)	SEX	FEVER (Days)	NAUSEA	RASH	BODY ACHES & PAIN	JOINT PAIN & SWELLING	VOMITING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
1	40	M	4	-	-	-	-	-	-	-	+
2	10	F	7	+	-	+	-	-	-	+	-
3	30	M	30	-	+	+	-	+	-	+	+
4	62	F	7	-	-	-	+	-	-	-	+
5	4	M	2	-	-	-	+	-	-	-	+
6	29	F	4	-	-	+	-	-	-	-	+
7	48	M	6	+	-	-	+	-	-	-	+
8	19	M	7	-	+	+	-	-	-	-	+
9	11	M	6	-	-	+	+	-	-	-	+
10	48	M	5	-	-	+	+	-	-	-	+
11	52	M	5	-	-	+	+	-	-	+	-
12	47	F	3	-	-	-	+	-	-	-	+
13	72	M	3	-	+	+	+	-	-	+	+
14	6	M	21	-	-	+	-	+	+	+	+
15	45	M	5	-	-	-	+	-	-	+	+
16	33	M	4	-	-	+	+	-	-	+	-
17	38	F	1	-	-	+	+	-	-	-	+
18	25	F	3	-	-	-	+	-	-	-	+
19	33	M	3	-	-	+	+	-	-	-	+
20	48	M	30	-	-	+	+	-	-	-	+
21	19	M	1	-	-	+	+	-	-	+	-
22	11	M	10	-	-	+	+	-	-	-	+
23	38	F	11	-	-	+	+	-	-	-	+
24	25	M	25	-	-	+	+	-	-	+	-
25	33	M	6	-	-	+	+	-	-	+	+
26	36	F	15	-	-	-	+	-	-	+	+
27	33	F	5	-	-	-	+	-	-	+	+
28	38	M	15	-	-	+	+	-	-	+	-
29	29	M	8	-	-	+	+	-	-	-	+
30	48	F	6	-	-	+	+	-	-	-	+
31	19	F	15	-	-	+	+	-	-	+	-
32	11	M	4	-	-	+	+	-	-	+	+
33	33	F	3	-	-	-	+	-	-	+	+
34	38	M	6	-	-	+	+	-	-	+	+
35	25	M	4	-	-	+	+	-	-	+	-
36	33	M	5	-	-	-	+	-	-	-	+
37	47	M	2	-	-	+	+	-	-	-	+
38	40	F	1	-	-	+	+	-	-	+	-
39	42	M	10	-	-	+	+	-	-	+	-
40	28	M	11	-	-	+	+	-	-	-	+
41	19	M	28	-	-	-	+	-	-	+	-

SR. NO.	AGE (Years)	SEX	FEVER (Days)	NAUSEA	RASH	BODY ACHES & PAIN	JOINT PAIN & SWELLING	VOMITING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
42	37	M	5	-	-	+	+	-	-	-	+
43	33	F	5	-	-	+	+	-	-	+	-
44	42	F	7	-	-	+	-	-	-	-	+
45	14	F	7	-	-	-	+	-	-	+	-
46	33	M	10	-	-	-	+	-	-	+	-
47	38	M	12	-	-	+	+	-	-	+	-
48	40	F	7	-	-	-	+	-	-	+	+
49	45	M	6	-	-	-	+	-	-	+	+
50	16	M	6	-	-	+	+	-	-	+	+
51	33	M	7	-	-	-	+	-	-	-	+
52	47	M	14	-	-	-	+	-	-	-	+
53	40	M	3	-	-	+	+	-	-	+	-
54	42	F	10	-	-	+	+	-	-	+	-
55	28	M	30	-	-	+	-	-	-	-	+
56	19	M	8	-	-	+	+	-	-	+	-
57	33	M	1	-	-	-	+	-	-	+	-
58	32	M	3	-	-	+	-	-	-	+	-
59	50	F	2	-	-	-	-	-	-	+	-
60	49	M	4	-	-	+	+	-	-	-	+
61	11	M	3	-	-	-	+	-	-	+	+
62	30	F	4	-	-	+	+	-	-	+	+
63	20	M	4	-	-	-	+	-	-	+	+
64	40	F	6	-	-	-	+	-	-	-	+
65	23	M	5	-	-	+	+	-	-	+	-
66	12	F	7	-	-	-	+	-	-	-	+
67	33	F	6	-	-	-	+	-	-	+	-
68	26	M	4	-	-	-	+	-	-	+	-
69	37	F	7	-	-	+	-	-	-	-	+
70	43	M	6	-	-	-	+	-	-	+	-
71	32	F	8	-	-	-	+	-	-	-	+
72	36	M	20	-	-	-	+	-	-	-	+
73	23	M	7	-	-	-	-	-	-	+	-
74	21	F	6	+	-	+	+	-	-	+	-
75	69	M	7	-	-	+	-	-	-	+	-
76	23	F	10	-	+	-	+	-	-	+	-
77	40	F	4	-	-	+	-	-	-	+	+
78	21	F	7	-	-	+	+	-	-	+	+
79	42	F	9	-	-	-	-	-	-	+	+
80	10	M	8	-	-	-	-	-	-	+	-
81	8	F	10	-	-	-	+	-	-	-	+
82	21	M	4	-	-	-	-	-	-	-	+
83	52	M	7	-	-	+	+	-	-	-	+
84	13	F	5	+	-	+	+	-	-	-	+

SR. NO.	AGE (Years)	SEX	FEVER (Days)	NAUSEA	RASH	BODY ACHES & PAIN	JOINT PAIN & SWELLING	VOMITING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
85	55	F	14	-	-	+	-	-	-	+	-
86	48	F	11	-	-	+	+	-	-	+	-
87	33	F	12	-	-	-	-	-	-	-	+
88	61	M	5	-	-	+	-	-	-	-	+
89	33	F	13	-	-	-	+	-	-	+	-
90	35	F	7	-	-	+	+	-	-	-	+
91	30	F	14	-	-	-	+	-	-	-	+
92	64	F	7	-	-	+	+	-	-	+	-
93	45	F	9	-	-	-	+	-	-	+	-
94	60	M	10	-	-	+	+	-	-	+	-
95	50	M	7	-	-	+	+	-	-	+	+
96	33	F	6	-	-	-	+	-	-	+	+
97	18	M	14	-	-	+	+	-	-	+	+
98	33	M	30	+	-	-	-	-	-	+	+
99	10	M	12	-	-	-	-	-	-	+	+
100	26	F	21	-	-	-	-	-	-	+	+
101	13	F	7	-	-	-	-	-	-	+	-
102	30	F	5	-	-	+	+	-	-	+	-
103	33	M	2	-	-	-	-	-	-	-	+
104	60	F	12	-	-	+	-	-	-	-	+
105	33	M	3	-	-	-	-	-	-	-	+
106	34	M	7	-	-	-	+	-	-	+	-
107	13	M	4	-	-	-	+	-	-	-	+
108	33	M	3	-	-	-	-	-	-	+	-
109	43	F	5	-	-	+	-	-	-	-	+
110	54	F	12	-	-	-	+	-	-	-	+
111	22	M	6	-	-	+	-	-	-	-	+
112	33	M	5	-	-	-	+	-	-	+	-
113	34	M	7	-	-	-	+	-	-	+	-
114	54	M	4	-	-	+	+	-	-	-	+
115	28	F	8	-	-	+	+	-	-	+	+
116	44	M	7	-	-	+	-	-	-	+	+
117	32	M	9	-	-	+	-	-	-	+	+
118	42	F	3	-	-	+	+	-	-	+	-
119	28	M	4	-	-	+	+	-	-	-	+
120	30	M	9	-	-	-	-	+	-	+	-
121	48	M	10	-	-	+	-	-	-	-	+
122	26	M	22	-	-	+	+	-	-	+	-
123	40	F	3	-	-	+	-	-	-	-	+
124	65	M	15	-	-	+	+	-	-	+	-
125	41	M	11	-	-	+	+	-	-	-	+
126	28	M	7	-	-	+	+	-	-	+	-
127	28	F	6	-	-	-	+	-	-	-	+

SR. NO.	AGE (Years)	SEX	FEVER (Days)	NAUSEA	RASH	BODY ACHES & PAIN	JOINT PAIN & SWELLING	VOMITING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
128	33	M	5	-	-	+	+	-	-	+	-
129	45	F	12	-	-	+	+	-	-	-	+
130	50	F	6	-	-	+	-	-	-	-	+
131	31	F	6	-	-	+	-	-	-	+	-
132	37	F	8	-	+	+	+	-	-	+	+
133	52	M	6	-	+	+	+	-	-	+	+
134	24	M	7	-	-	-	+	-	-	+	+
135	12	F	13	-	-	-	+	-	-	+	-
136	72	M	29	-	-	+	+	-	-	+	-
137	36	F	14	-	-	+	+	-	-	+	-
138	33	M	5	-	-	-	+	-	-	-	+
139	14	M	2	-	-	-	+	-	-	-	+
140	50	F	7	-	-	+	+	-	-	+	-
141	28	M	10	-	-	-	+	-	-	-	+
142	38	M	6	-	-	+	+	-	-	+	-
143	25	M	3	-	-	-	+	-	-	+	+
144	33	M	7	-	-	-	+	-	-	-	+
145	34	M	4	-	-	-	+	-	-	+	-
146	10	M	8	-	-	-	+	-	-	-	+
147	17	M	6	-	-	+	+	-	-	-	+
148	46	F	3	-	-	+	+	-	-	+	-
149	39	M	11	-	-	-	+	-	-	+	+
150	52	F	5	-	-	+	+	-	-	+	+
151	23	F	4	-	-	+	+	-	-	+	+
152	38	F	30	-	-	-	+	-	-	+	-
153	25	F	5	-	-	-	+	-	-	-	+
154	33	M	5	-	-	+	+	-	-	+	-
155	47	M	6	-	-	+	+	-	-	+	+
156	40	F	7	-	-	-	+	-	-	+	+
157	42	M	2	-	-	-	+	-	-	+	+
158	28	F	7	-	-	-	+	-	-	+	+
159	19	F	3	-	-	+	+	-	-	+	-
160	36	F	8	-	-	-	+	-	-	-	+
161	28	F	5	-	-	-	+	-	-	-	+
162	29	F	9	-	-	-	+	-	-	+	-
163	33	F	12	-	-	-	+	-	-	-	+
164	26	M	10	-	-	-	+	-	-	-	+
165	35	F	7	-	-	-	+	-	-	+	+
166	38	M	11	-	-	-	+	-	-	+	+
167	34	M	4	-	-	-	+	-	-	+	+

Among these CHIKV infected patients, 90 were within acute stage (0-5 days of symptomatic onset). Male to female ratio of chikungunya patients was 1.35:1 and mean

age group was 33.95 ± 13.67 years (range 7-72 years). Frequency of CHIKV infection was highest among patients of 21–30-year age group, and gradually decreased with increasing age [Fig 3.4].

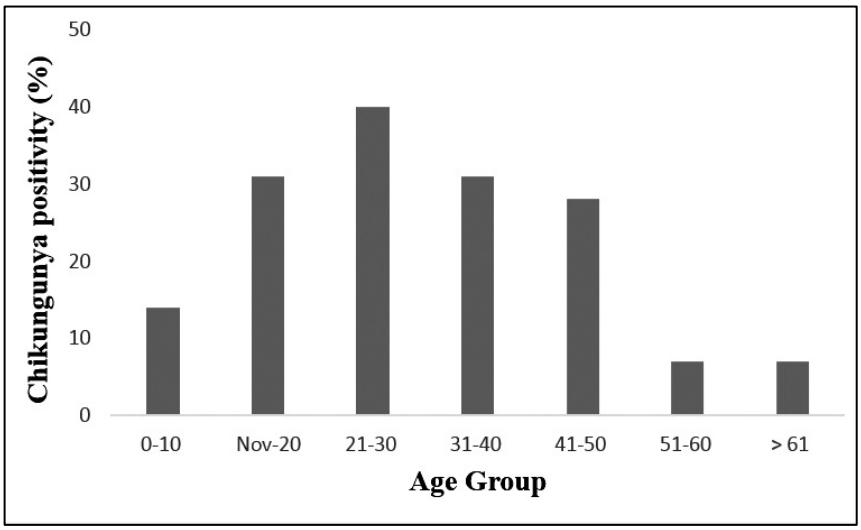

Fig 3.4: Age-wise distribution of CHIKV-infected patients.

Similar to other mosquito-borne infections, CHIKV-infection peaked up after July, which corresponds to the onset of monsoon in India. Maximum percentage of CHIKV-infection was reported during September [(51/158) = 32.27%] [Fig 3.5].

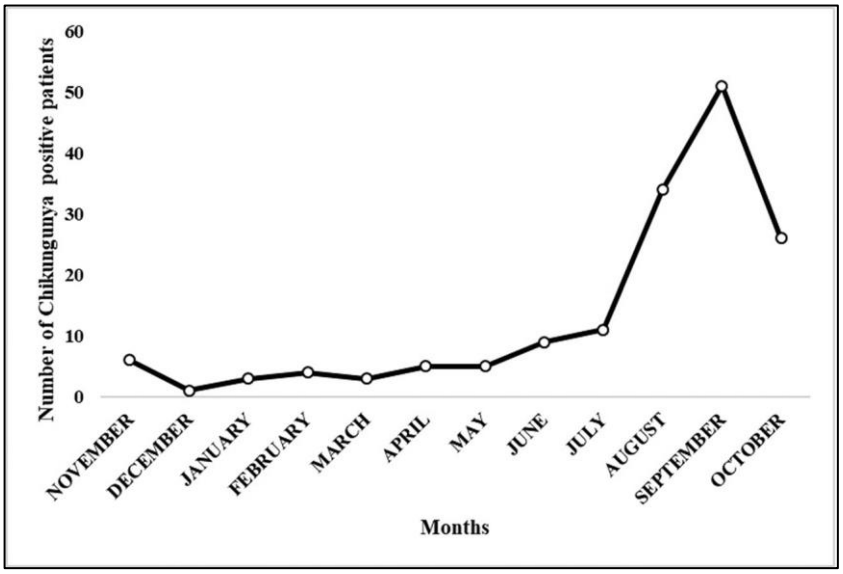

Fig 3.5: Cumulative distribution of CHIKV positivity across monsoon and post-monsoon seasons of 2014–2016.

Depending upon day of sample collection after symptomatic onset, real time qRT-PCR assay could detect highest percentage of CHIKV genome on first day of infection, which gradually decreased with increasing number of days after symptomatic onset [Fig.3.6]. On the contrary; percentage prevalence of anti-chikungunya-IgM followed the reverse trend. Anti-chikungunya-IgM ELISA could detect highest percentage of anti-chikungunya-IgM antibody in patients' sera on sixth day of symptomatic onset.

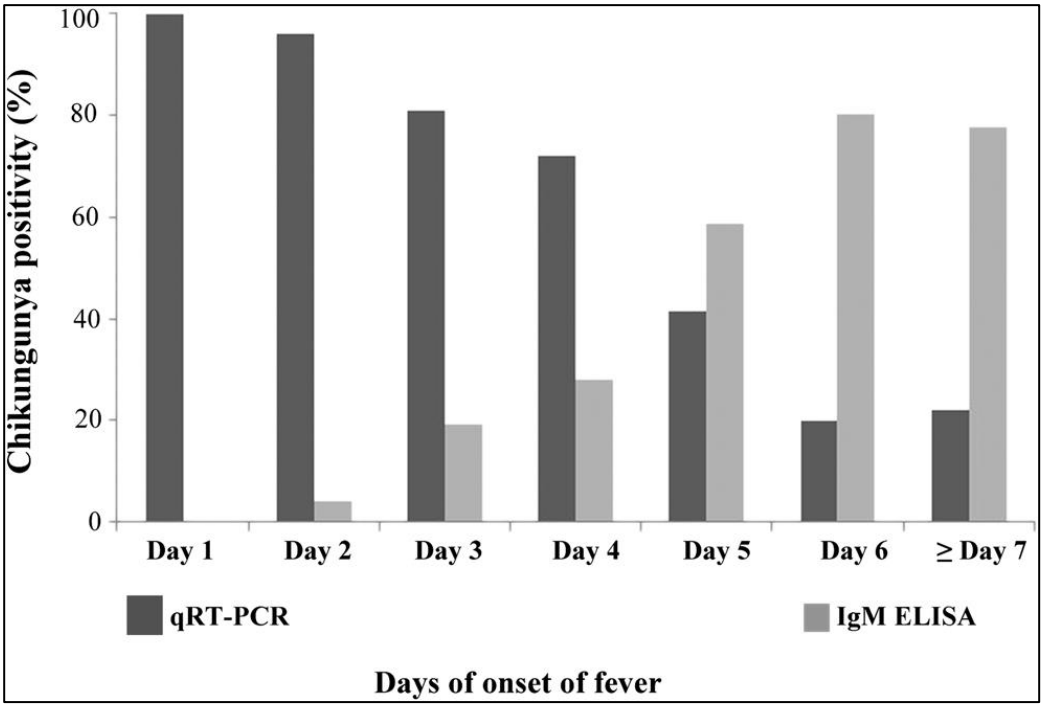

Fig 3.6: Percentage of CHIKV positivity by real-time qRT-PCR and IgM ELISA according to the days of collection from symptomatic onset

Clinically, arthralgia [OR = 2.14, 95% CI 1.46 - 3.14, p= 0.0001] and joint swelling [OR = 1.67, 95% CI 1.03-2.70, p = 0.03] were more frequent among chikungunya positive patients than that of chikungunya negative symptomatic patients [Fig 3.7a]. Approximately 23.8% PCR positive chikungunya patients exhibited HVL of CHIKV, whereas, remaining 76.2% PCR positive chikungunya patients showed LVL. Interestingly, compared to chikungunya LVL group, arthralgia, joint swelling and

myalgia were significantly higher among chikungunya HVL patients [Fig 3.7b]. According to symptomatic history accounted by patients, pain started on the first or second day of fever. It then involved both small and big joints of both the extremities with no upper/lower limb predilection. Vomiting and abdominal pain were the other associated minor symptoms. Majority of the symptoms subsided within 3-5 days, whereas, joint pain persisted for more than 30 days. All the patients were treated symptomatically and no death was observed. Burden of chikungunya infection was markedly noticed within Kolkata metropolitan, West Bengal, India and its adjoining districts, viz. South 24 Parganas, Howrah, Hooghly and North 24 Parganas.

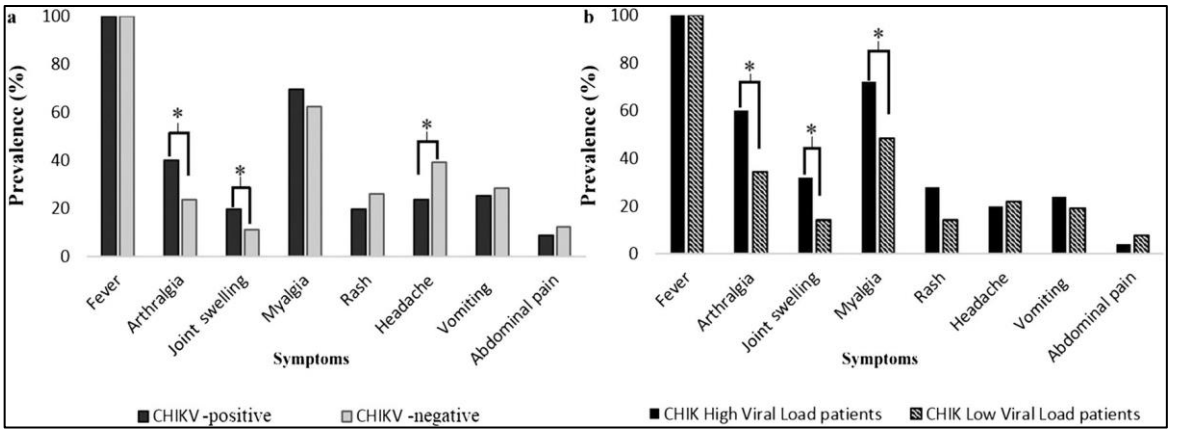

Fig 3.7: Comparison of clinical symptoms between a) CHIKV-infected and uninfected symptomatic patients, b) HVL and LVL group of CHIKV infected patients.

3.3.2. Determination and demographics of dengue-chikungunya co-infection

Among suspected cases, 19.96% (128/641) were co-infected with both DENV and CHIKV as detected by anti-dengue-IgM ELISA/dengue-NS1ELISA/real-time qRT-PCR and anti-CHIKV IgM ELISA/real-time qRT-PCR, respectively [Table 3.2, 3.4].

Table 3.4: Detailed symptomatic history of DENV-CHIKV co-infected patients.

SR. NO.	AGE	SEX	FEVER	NAUSEA	RASH	MYALGIA	HEADACHE	JOINT PAIN	VOMITTING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
1	22	M	4	-	-	+	+	-	-	-	-	+
2	25	F	3	-	-	-	-	-	-	-	+	-
3	14	F	2	-	-	-	+	-	-	-	+	+
4	20	M	4	-	-	-	-	-	-	-	-	+
5	24	M	3	-	-	-	-	-	-	-	-	+
6	35	M	5	-	-	+	+	-	-	-	-	+
7	11	M	2	-	-	+	-	+	+	-	-	+
8	38	M	2	-	-	-	-	-	-	-	-	+
9	36	M	7	-	-	+	-	-	+	-	-	+
10	56	F	2	-	-	+	-	-	-	-	-	+
11	14	M	4	-	-	-	-	-	-	-	+	-
12	28	F	5	-	-	-	-	-	-	-	-	+
13	18	F	3	-	-	-	+	-	-	-	+	+
14	17	M	6	-	-	-	-	-	-	-	+	+
15	7	M	4	-	-	-	-	-	-	-	+	+
16	17	M	7	-	-	-	-	-	-	-	+	-
17	56	M	7	-	-	+	+	+	+	-	-	+
18	15	M	6	-	-	-	-	-	-	-	-	+
19	16	F	7	-	-	-	-	-	-	-	-	+
20	34	M	4	-	-	-	+	+	-	-	-	+
21	25	M	2	-	-	-	-	-	-	-	+	-
22	20	M	1	-	-	+	+	-	-	-	-	+
23	14	F	2	-	-	+	-	-	+	-	-	+
24	25	M	3	-	-	-	-	-	-	-	+	-
25	50	M	15	-	-	+	+	+	-	-	+	+
26	30	M	4	-	-	+	+	-	-	-	+	+
27	28	F	2	-	-	-	-	-	-	-	+	+
28	15	F	3	-	-	-	-	+	-	-	+	-
29	50	M	5	-	-	+	-	-	-	-	-	+
30	23	F	1	-	-	-	-	-	-	-	-	+
31	24	F	4	+	-	+	-	-	-	-	+	-
32	10	F	2	-	-	-	-	-	-	-	+	+
33	20	M	4	-	-	+	-	-	-	-	+	+
34	39	M	4	-	-	+	+	-	-	-	+	+
35	35	M	7	-	-	-	-	-	-	-	+	-
36	28	F	6	-	-	-	+	-	-	-	-	+
37	16	F	2	-	-	+	+	-	-	+	-	+
38	54	F	3	-	-	+	+	-	-	-	+	-
39	14	M	3	-	-	+	+	-	-	-	+	-

SR. NO.	AGE	SEX	FEVER	NAUSEA	RASH	MYALGIA	HEADACHE	JOINT PAIN	VOMITTING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
40	18	M	3	-	-	-	-	-	-	-	-	+
41	2	F	5	-	-	+	+	-	-	-	+	-
42	21	M	5	-	-	+	+	-	-	-	-	+
43	14	M	2	+	-	+	+	-	+	+	+	-
44	45	M	8	-	-	-	-	-	-	-	-	+
45	18	F	7	-	-	-	-	-	-	-	+	-
46	16	M	20	-	-	+	+	-	+	+	+	-
47	30	M	7	-	-	+	+	-	-	-	+	-
48	16	M	3	-	-	+	+	+	+	-	+	+
49	35	M	4	-	-	+	+	-	-	-	+	+
50	50	M	6	-	-	-	-	-	-	-	+	+
51	6	M	9	-	-	-	+	-	+	-	-	+
52	24	M	5	-	-	-	+	+	-	-	-	+
53	15	M	5	-	-	+	-	-	-	-	+	-
54	17	M	7	-	-	+	-	-	-	-	+	-
55	28	M	3	-	-	+	+	+	-	-	-	+
56	30	M	7	-	-	-	+	-	+	-	+	-
57	25	F	5	-	-	-	-	-	-	-	+	-
58	16	M	7	-	-	-	-	-	-	-	+	-
59	10	M	3	-	-	-	+	-	+	-	+	-
60	29	M	7	-	-	+	-	-	+	-	-	+
61	22	M	14	-	-	-	+	-	-	-	+	+
62	30	M	7	-	-	-	+	-	-	-	+	+
63	42	F	6	-	-	-	-	-	-	-	+	+
64	14	M	2	-	-	+	-	-	-	-	-	+
65	25	F	3	-	-	-	-	-	-	-	+	-
66	34	M	4	-	-	+	+	-	-	-	-	+
67	3	F	12	-	-	+	-	-	+	-	+	-
68	23	M	3	-	+	+	+	-	-	-	+	-
69	42	M	3	+	-	+	+	-	-	-	-	+
70	16	F	5	-	-	-	-	-	-	-	+	-
71	15	F	7	-	-	+	+	-	-	-	-	+
72	24	F	5	-	-	+	+	-	-	-	-	+
73	41	M	3	-	-	+	+	+	-	-	+	-
74	20	M	5	-	-	+	+	+	-	-	+	-
75	23	M	6	-	-	-	-	-	-	-	+	-
76	50	F	3	-	-	+	+	-	+	-	+	-
77	28	M	3	-	-	+	+	-	+	-	+	+
78	35	F	4	-	-	-	-	-	+	-	+	+
79	28	F	5	-	-	+	+	-	-	-	+	+
80	17	M	4	-	-	+	+	-	-	-	+	-

SR. NO.	AGE	SEX	FEVER	NAUSEA	RASH	MYALGIA	HEADACHE	JOINT PAIN	VOMITTING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
81	16	M	6	-	-	-	-	-	-	-	-	+
82	35	M	7	-	-	-	-	-	-	-	-	+
83	21	M	2	-	-	+	+	-	-	-	-	+
84	38	F	3	-	-	+	+	+	+	-	-	+
85	11	F	8	-	-	-	-	-	-	-	+	-
86	13	F	3	+	-	+	+	+	+	-	+	-
87	55	F	5	-	-	+	+	+	+	-	-	+
88	29	F	3	-	+	+	-	-	-	-	-	+
89	28	F	5	+	+	+	-	-	+	+	+	-
90	28	F	6	+	-	+	-	-	-	-	-	+
91	41	M	8	+	+	+	-	-	-	-	-	+
92	16	M	5	-	-	+	-	+	+	-	+	-
93	21	M	7	+	+	+	-	+	+	-	+	-
94	25	M	5	+	-	-	-	+	-	-	+	-
95	21	F	4	+	-	+	-	+	-	-	+	+
96	17	F	7	-	+	+	-	-	-	-	+	+
97	10	F	2	-	-	+	-	-	-	-	+	+
98	11	M	7	-	-	+	-	-	-	-	+	+
99	19	F	2	-	+	+	-	-	+	-	+	+
100	29	M	6	+	-	+	-	-	+	-	+	+
101	32	F	8	-	+	+	-	-	+	-	+	-
102	7	M	6	-	-	+	-	-	-	-	+	-
103	9	F	5	-	+	+	-	-	+	-	-	+
104	6	M	3	-	-	+	-	-	-	-	-	+
105	28	M	2	+	+	+	-	+	+	+	-	+
106	18	M	9	+	+	+	-	+	+	+	+	-
107	21	M	6	+	+	+	-	+	+	+	-	+
108	15	M	7	+	-	+	-	+	+	+	+	-
109	37	M	3	+	+	+	-	-	-	+	-	+
110	14	F	5	+	+	+	-	-	+	-	-	+
111	8	M	3	+	+	+	-	-	+	-	-	+
112	25	M	6	+	+	-	-	+	+	-	+	-
113	5	M	2	+	-	-	-	-	+	-	+	-
114	18	M	8	+	+	+	-	+	-	+	-	+
115	28	F	4	-	-	-	-	-	-	-	+	+
116	42	F	3	-	-	-	-	-	-	-	+	+
117	60	F	7	-	-	-	-	-	-	-	+	+
118	28	F	3	-	-	-	-	-	-	-	+	-
119	29	F	2	-	-	-	-	-	-	-	-	+
120	31	F	1	-	-	-	-	-	-	-	+	-
121	26	F	7	-	-	-	-	-	-	-	-	+

SR. NO.	AGE	SEX	FEVER	NAUSEA	RASH	MYALGIA	HEADACHE	JOINT PAIN	VOMITTING	ABDOMINAL PAIN	REAL-TIME PCR	IgM-ELISA
122	45	M	8	+	+	+	-	-	-	-	+	-
123	25	F	6	-	-	-	-	-	-	-	-	+
124	21	F	4	-	-	+	+	-	-	-	+	-
125	17	M	3	-	-	-	-	-	-	-	-	+
126	10	F	2	-	-	-	+	-	-	-	+	-
127	11	F	4	-	-	-	-	-	-	-	-	+
128	28	F	3	-	-	-	-	-	-	-	+	-

Among these DENV-CHIKV co-infected patients, 30.46% (39/128) were within acute stage, 46.87% (60/128) were within critical phase and 22.65% (29/128) were late phase patients. Male to female ratio of co-infected patients was 1.44:1 and mean age group was 24.24 ± 14.7 years (range: 2–60 years).

Frequency of co-infection was highest (around 45%) among patients of 20–40-year age group, and gradually decreased with increasing age. Clinically, arthralgia [48.81%] and myalgia [55.11%] were more frequent among these patients [Fig 3.8]. Around 5.51% patients developed bleeding of some form. Approximately 56.32% PCR positive co-infected patients exhibited HVL of DENV, whereas, 47.12% PCR positive patients showed HVL of CHIKV.

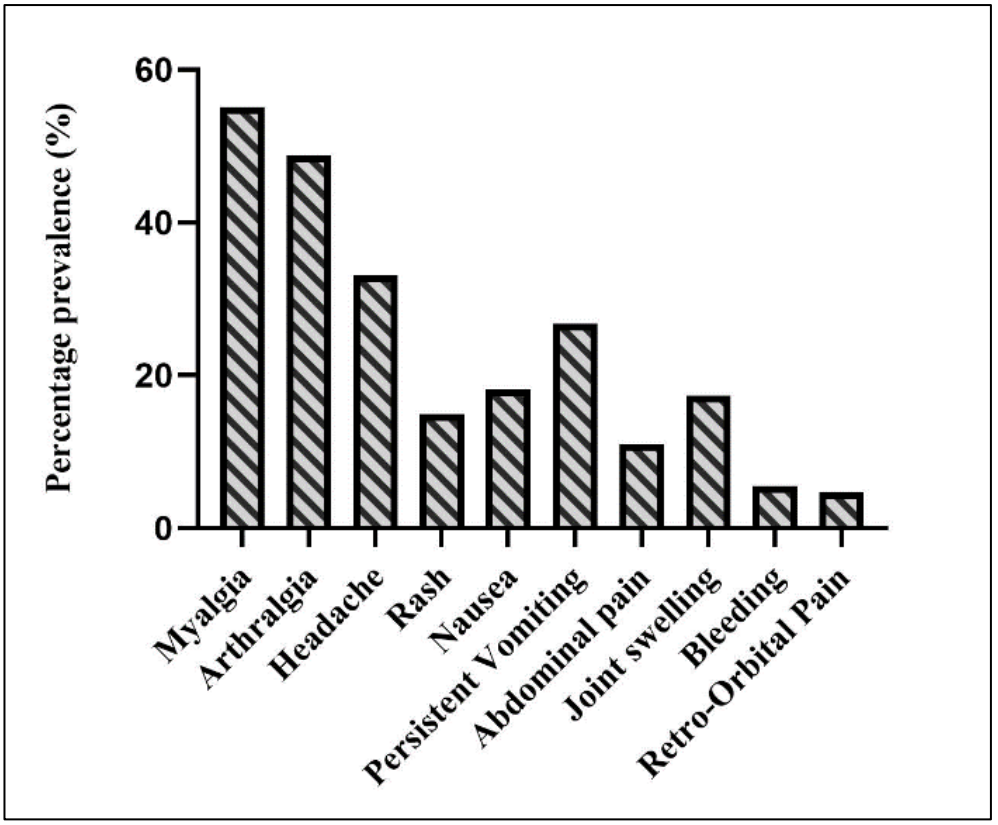

Fig 3.8: Percent prevalence of DENV-CHIKV co-infected patients clinical manifestations.

3.4. DISCUSSION:

In this cross-sectional study, multi-diagnostic approach viz. real time qRT-PCR and IgM ELISA were carried out for laboratory confirmation of chikungunya mono-infection and dengue-chikungunya co-infection. Similar to an earlier study by Dutta *et al*. 2014, present study also confirmed that real time qRT-PCR is the most sensitive diagnostic tool for detecting chikungunya mono-infection and DENV-CHIKV-co-infection during early stage of infection as it confirmed presence of CHIKV, DENV genome among acute stage patients' blood - indicating greater efficacy of molecular diagnosis compared to serology-based techniques for early detection [21]. Maximum number of CHIKV mono-infection was reported during September, the first post-monsoon month in India. Furthermore, a higher rate of CHIKV mono-infection was found among middle age

groups, in accordance to previous reports [22, 36, 37]. In this study, arthralgic manifestation and joint swelling was significantly more prevalent among CHIKV mono-infected patients compared to that of symptomatic uninfected patients which have been mentioned in several earlier studies [22, 36]. Chikungunya patients with HVL were found to demonstrate arthralgia, joint swelling and myalgia at significantly higher frequency compared to patients with LVL. High-viral load has been previously associated with higher production of pro-inflammatory cytokines, which might be associated with development of joint inflammation in CHIKV-infected patients [38]. Also, prevalence of joint swelling among chikungunya patients with HVL has been previously reported by Dutta *et al*. 2014 [21].

Co-infection with both dengue and chikungunya viruses indicated the complexity of arboviral outbreaks in India. Co-infections in general, might result in illness depicting overlapping signs and symptoms, thus, making diagnosis and treatment difficult for physicians, often requiring multiple laboratory tests. Overlapping symptomatic manifestation of arthralgia and myalgia was prevalent among co-infected patients, which was similar to previous reports of co-infected patients depicting common signs and symptoms [39, 40]. Also, DENV–CHIKV co-infected patients had more severe clinical manifestations. DENV-CHIKV co-infection peaked up after July, which corresponded to onset of monsoon season in eastern India. In correspondence to earlier reports, frequency of co-infection was highest among occupationally active patients of 20–40-year age group because of their higher exposure to vector carrying any one/both viruses [40].

In this study, chikungunya mono-infection was detected among 26.05% of symptomatic patients; and co-infection among almost 20% of symptomatic patients. However, it is important to diagnose the type of virus with which patient is infected for proper patient-

treatment and management. Continuous monitoring and rigorous testing for both mono and co-infections is essential for better clinical management of patients.

3.5. CONCLUSION

This study confirmed re-emergence and spread of CHIKV mono-infection and DENV-CHIKV co-infection in West Bengal, eastern India. Though in this study no mortality was reported among patients, a significant presence of joint swelling among chikungunya mono-infected patients implied the severity of arthralgic manifestation, which required prioritized attention for proper management of these patients. Also, arthralgia, myalgia and bleeding were significant clinical manifestations among co-infected patients which may turn severe if untreated. Thus, the health authorities and community should, therefore, keep a strict vigilance for early diagnosis of the disease.

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CHAPTER 2

Significance of arthritic and hepatic biomarkers in prognosis of post-chikungunya chronic arthritis among infected patients

CHAPTER 2

Objective

- Analysis of arthritic biomarkers viz. C-reactive protein (CRP), rheumatoid factor (RF), cartilage oligomeric matrix protein (COMP), anti-cyclic citrullinated peptide (anti-CCP antibody), interleukin-2 receptor (IL-2R) and hepatic biomarkers viz. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin and bilirubin levels in serum for early identification of chikungunya patients with post-chikungunya chronic arthralgia.
- Identification of **s**pecific genotypes of CRP, IL-2R & COMP polymorphisms, associated with chikungunya disease susceptibility and arthritic manifestation among infected patients.

Publication:

- **Sengupta S**, Bhattacharya N, Tripathi A. Increased CRP, anti-CCP antibody, IL-2R, COMP levels in prognosis of post-chikungunya chronic arthritis and protective role of their specific genotypes against arthritic manifestation [published online ahead of print, 2022 Nov 7]. **Virus Research**. 2022;323:198998. doi:10.1016/j.virusres.2022.198998

4.1. INTRODUCTION

Chikungunya viral (CHIKV) infection is characterized by abrupt onset of fever, which is frequently accompanied with joint pain, acute or chronic arthritis, causing stooped appearance of patients [1]. Acute arthritis might lead to development of excruciating oligo- or polyarthritis generally involving 4 or more joints of arms and legs [2]. Approximately, 88-100% of infected patients might also experience post-chikungunya sub-acute arthritis (6 weeks) and 40-60% might demonstrate chronic arthritis even after 1.5-5 years of infection, indicating role of host genetics in determining chronic arthritis among infected patients [3, 4]. Post-chikungunya arthritis is characterized by development of chronic inflammatory rheumatism and musculo-skeletal disease which could be relapsing or unrelenting [4]. Clinical presentation of long term/post-chikungunya infection might mimic autoimmune rheumatological conditions viz. rheumatoid arthritis with features including symmetrical polyarthritis often affecting hands and feet similar to rheumatoid arthritis [5, 6]. Hence, early identification of infected patients who might develop chronic arthralgia in long term could help clinicians in their pain management. Both DNA and serum level biomarkers might help in early differentiation between acute (AA) and post-chikungunya arthritic (PCA) patients [7, 8].

CHIKV primarily infects skin cells, thus, replicating in skin, liver, muscle and bone joints simultaneously invading monocytes and blood vessels – resulting in erosion of bones, extracellular matrix degradation and release of pro-inflammatory cytokines [9]. C-reactive protein (CRP) is a pentameric protein synthesized by liver, whose level increases in response to inflammation [10]. It is a part of the innate immune system where it acts as a pattern recognition molecule to activate the adaptive immune response

[10]. CRP was first identified by its reactivity with C-polysaccharide of pneumococcus and subsequently named as C-reactive protein [10]. It acts as scavenger protein by binding to pathogens, damaged tissues, nuclear antigens, thus, removing toxic molecules and neutralizing invasive microorganisms [11]. Increased level of C-reactive protein (CRP), an acute-phase hepatic protein has been reported among viraemic and arthritic infected patients [6,12]. CHIKV-infected patients may develop hepatotoxic effects and studies of outbreaks in La Reunion Island and Gabon reported elevations of liver enzymes in approximately 20-46% of cases [13, 14]. Elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) levels has been reported among both viraemic and post-viraemic chikungunya patients – indicating involvement of hepatic anomalies during CHIKV infection [13,14]. Arthritic manifestation during CHIKV-infection mimic rheumatoid arthritis (RA), both characterized by increased rheumatoid factor (RF) and anti-cyclic-citrullinated-peptide (anti-CCP) antibody related articular damage [15]. RFs are not only a biomarker of rheumatoid arthritis but prevalent in others viz. autoimmune and non-autoimmune diseases [16]. RFs are antibodies which acts against Fc portion of IgG [17]. RF-positive rheumatoid arthritic patients may experience more aggressive and erosive joint damage with extra-articular manifestations such as rheumatoid nodules and vasculitis [18] Protein citrullination occurs widely in cell differentiation, inflammatory responses, cell apoptosis, gene regulation, and aging process [19]. Several proteins, including vimentin, fibrin, and α-enolase, have been found to be citrullinated, and they can then be recognized by anti-CCP antibodies. [19]. Detection of anti-CCP antibodies in serum is one of the most specific biomarker for diagnosis of RA [20]. Also, anti-CCP antibody cross-reacts with type II collagen, resulting in proteoglycan depletion and severe arthritis

[21]. Elevated level of several serum biomarkers viz. cartilage oligomeric matrix protein (COMP), interleukin-2 receptor (sIL-2R) has been used as diagnostic and /or prognostic markers of RA [22,23]. COMP, a non-collagen, extracellular matrix proteinbiomarker for cartilage degradation found in articular cartilage, ligament, meniscus, synovial membrane and tendon, has been associated with cartilage turnover and joint destruction related to osteoarthritis and rheumatoid arthritis [22, 24]. It has a role in endochondral ossification, interacting with collagen fibrils via each C-terminal globule, for extracellular matrix stabilization [25]. It influences fibril formation for type I and II collagen, accelerating fibrillogenesis and binding to aggrecan, thereby organizing the cartilage matrix for its load bearing function [25]. Soluble Interleukin-2 receptor (sIL-2R), a pro-inflammatory cytokine related to chikungunya infection, has also been implicated in development of synovitis and exacerbation of disease severity of rheumatoid arthritis [23]. Binding of IL-2 to its cell-surface receptor (IL-2R) leads to clonal expansion of antigen-triggered T-cell subsets and is an essential event in generating most immune responses [26]. The induction of IL-2 and its receptor are therefore central to immune activation [23]. Moreover, RF, anti-CCP antibody and CRP has been recognized as serological and acute phase response markers for rheumatoid arthritis, according to 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria [27]. Table 4.1 elucidates the type of arthritic biomarkers (CRP, IL-2R, Anti-CCP antibody, COMP and RF) impacting major arthritic diseases.

Table 4.1: Type of arthritic biomarkers impacting major arthritic diseases.

Name of the Protein/Gene	Type of biomarker	Overexpression in disease
C-Reactive Protein (CRP)	Pro-inflammatory	Acute Chikungunya Osteoarthritis Rheumatoid arthritis
Interleukin-2 receptor (IL-2R)	Pro-inflammatory	Rheumatoid Arthritis
Cartilage Oligomeric Matrix Protein (COMP)	Cartilage/synovial	Rheumatoid arthritis Osteoarthritis
Anti-Cyclic Citrullinated Peptide (Anti-CCP)	Auto-antibodies	Acute Chikungunya Rheumatoid arthritis
Rheumatoid Factor (RF)	Auto-antibodies	Acute Chikungunya Rheumatoid arthritis

Besides protein levels, polymorphic genotypes of these genes might contribute to development of chikungunya induced arthritis among infected patients. Certain genotypes of cis-acting polymorphisms of CRP, viz. rs3091244, rs3093059, have been reported to contribute to variance in protein level among dengue, chikungunya, ankylosing spondylitic and osteomyelitic patients [28-32]. Previously, mutations within COMP gene have been strongly associated with pseudoachondroplasia, multiple epiphyseal dysplasia and osteoarthritis [33, 34]. Similarly, specific genotypes of IL-2R-rs743777 polymorphism, which is localized 6kb upstream of transcription start site (5′near gene region), were associated with rheumatoid arthritis and peripheral arthritis of ankylosing spondylitic patients [35-37]. But, contribution of these polymorphic variants (if any) towards development of chikungunya induced arthritis has not been studied. Moreover, prognostic value of these polymorphic genotypes and protein levels has not been evaluated in case of CHIKV induced arthritis.

Thus, this study explores prognostic values of these serum proteins and their genetic polymorphisms in determining acute and chronic arthritis among CHIKV infected patients.

4.2. MATERIALS AND METHODS

4.2.1 Ethics statement:

Collection of blood from each of the febrile patients and healthy participants was performed in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine, Kolkata, India (CREC-STM/53 dated 26.09.2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consents were obtained from patients and healthy individuals.

4.2.2 Inclusion criteria:

Patients with acute febrile illness and history of headache, body ache, myalgia, arthralgia, rash, with or without haemorrhagic manifestation of all age-groups and both sexes were reviewed by physician according to WHO criteria of chikungunya infection.

4.2.3 Exclusion criteria:

Haematological malignancies, bleeding disorders, chronic liver disease, diabetes mellitus, and renal diseases.

4.2.4 Patients and healthy controls:

Around 2ml of blood of all age groups and sexes were collected from each of 641 symptomatic patients during their first visit at Calcutta School of Tropical Medicine (CSTM), West Bengal, India from September 2014 to October 2016, within acute phase of infection. Febrile patients exhibiting any two of the following symptoms: headache, myalgia, arthralgia, nausea, vomiting, rash, fatigue was selected as per WHO criteria.

Amongst them, 167 were CHIKV-infected, as detected by anti-CHIKV IgM ELISA (NIV, Pune, India)/real-time qRT-PCR. To carry out age-matched case control study, blood from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with DENV, CHIKV, Japanese encephalitis, HIV and Hepatitis A/B/C infection - as tested by IgM and IgG ELISA/RT-PCR, were collected from same community. Symptomatic patients and healthy controls consisted of Bengali population of eastern India having similar ethnic genetic make up to that of Bengali Bangladeshis (BEB) group of SAS included within 1000 genome project of GWAS. Control population size was calculated using EpiInfo™ version 7.2 software of CDC, with 95% confidence interval.

All the following biochemical and genotypic experiments were performed on patient-blood collected during their first visit at CSTM. Detailed follow up study of arthralgic manifestations of CHIKV infected patients was performed both during their first visit and after 2 years from date of collection of blood. Patients were grouped into acute and chronic chikungunya cases according to criteria of World Health Organization (WHO) [38]. Patients were categorised into four groups: NA: Patients without any arthralgia; AA: patients with only acute arthralgia; AA+PCA: patients with both acute and post chikungunya arthralgia and PCA: patients developing only post-chikungunya arthralgia. Depending upon rheumatoid arthritic outcome, CHIKV-infected patients were also categorised according to 2010 ACR/EULAR classification criteria [27].

4.2.5 Extraction of viral RNA and determination of CHIKV

Viral RNA was extracted from 140µL of patients' sera, using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France). Genome presence of CHIKV was determined by real-time qRT-PCR using Chikungunya virus

Genesig kit (Primer Design Ltd., UK), according to manufacturers' protocol. Limit of detection (LOD) of Chikungunya virus Genesig kit was <100 copies of corresponding target genomes. Real-time PCR was performed on ABI Prism 7500 fast instrument. Each sample was loaded in triplicate.

4.2.6 Quantification of serum CRP

Sera collected from infected patients were subjected to CRP quantification. CRP level was determined by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics, India) according to manufacturer's protocols. Briefly, 3µl of patient serum/calibrator was mixed with 500µl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and absorbance was measured at 550nm wavelength after 10 seconds (A1) and 120 seconds (A2). CRP concentration (mg/l) in serum was measured using following formula:

Serum concentration of CRP (mg/l) = [(A2-A1) Serum sample/(A2-A1) Calibrator] x Concentration of calibrator

4.2.7 Quantification of serum COMP, Anti-CCP, IL-2R

Serum levels of COMP (Invitrogen, MA, USA), Anti-CCP (KINESISDx, CA, USA) and IL-2R (Invitrogen, MA, USA) were determined by sandwich ELISA kits, according to manufacturers' instructions. Briefly, 100µL standard and sample was added to each well, and plate was covered and incubated for 1 hour at 37°C. After removal of liquid in each well, 100µL of Detection Ab Working Solution was added. The plate was covered and incubated for 1hour at 37°C. Then, each well was washed with Wash Buffer thrice. 100µL of detection reagent B working solution was added to each well, and plate was covered and incubated for 30 minutes at 37°C. Each well was washed with Wash Buffer five times, 90µL of Substrate Solution was added to each well, and plate was covered

and incubated for 30 minutes at 37°C. After incubation, 50µL of Stop Solution was added to each well. Absorbance was measured at 450nm wavelength. Standard-curve was drawn using GraphPad Prism 9 and levels of each protein were analysed.

4.2.8 Detection of serum RF

RF detection was done using RF-latex agglutination slide test kit (AUTOSPAN, UK), where patient serum was placed within circled area on special slide (provided with kit). One drop of Reagent-1 was added to it and mixed well. Agglutination was observed macroscopically under direct light source. Agglutination indicated serum samples as positive.

4.2.9 Quantification of biochemical parameters

Levels of AST, ALT, ALP, bilirubin and albumin were measured by using a standard automated clinical chemistry analyzer (ERBA Model no: EM360) according to manufacturer's instructions.

4.2.10 Identification of CRP, IL-2R and COMP genotypes susceptible to chikungunya related arthritis

To understand role of CRP, IL-2R and COMP polymorphisms (if any) towards CHIKV infectivity and arthralgic manifestation compared to healthy controls, genotyping of CRP (rs3093059 and rs3091244), IL-2R (rs743777) and COMP (rs144778694) polymorphisms was performed among 167 CHIKV infected and 102 healthy controls, based on their minor allele frequencies (MAF).

Single nucleotide polymorphism (SNP) genotyping was carried out by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using

QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Based on sequences available in GenBank database, four primer pairs were designed using Primer3 software, to amplify polymorphic regions of CRP, IL-2R and COMP genes [Table 4.2]. PCR reaction was performed in 20µl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5mM $MgCl_2$ and 20p.moles of previously mentioned primers [Table S1]. Respective PCR products were digested with Tas I, BfaI, StuI, PspFI (Fermentas, USA) and Taq I (Himedia, India) accordingly. Different RFLP patterns were validated by sequencing of respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

Table 4.2: List of primers and restriction enzymes used in PCR RFLP.

Sr. No.	Gene	SNP Id	5'-Primer-3'	PCR product Size	Restriction Enzymes	Restriction digestion temperature	Restriction digestion pattern
1.	CRP	Rs3093059	F-TATCCTGACTCCTGCCTG R-CCCATCTATGAGTGAGAACACG	224bp	TasI	65º C	C: 224bp T: 164bp + 60 bps
2.	CRP	Rs3091244	F-AATGTGTCCATGGCTCTG R-AATGGGAAATGGTAACATATTAATC	214bp	Bfa I	37 º C	C/T: 214 bp A: 187 bp + 27 bp
3.	CRP	rs3091244	F- AATGTGTCCATGGCTCTG R- AATGGGAAATGGTAACATATTAATC	214bp	TaqI	65 º C	A/T: 214 bp C: 188 bp + 26 bp
4.	IL-2R	rs743777	F- ACTCCCCATCATACACACTC R-GGATGTGGAAGACGTAAGAG	445 bp	StuI	37 º C	G: 445 bp A: 252 bp + 193 bp
5.	COMP	rs144778694	F- TGTGCCTGTAGTACCAGCTA R- TAGTAGAGACGGGGTTTCAC	287 bp	PspFI	37 º C	A: 287 bp G: 201 bp + 86 bp

4.2.11 Statistical analysis

Associations of protein levels and their SNP genotypes with CHIKV infectivity and arthralgic manifestations were analyzed and represented using GraphPad Prism 9. Allelic and genotypic frequencies were compared between different study groups using Pearson's Chi-square test, unpaired Welch's t-test and multi-variant analysis, using one way ANOVA. For genotypic associations; p-values, odds ratio (OR) and risk ratio were calculated. A p-value of <0.05 was considered statistically significant. Among healthy controls, Hardy–Weinberg equilibrium was analyzed for all polymorphisms using Haploview program. Cut-off values, sensitivity and specificity of serum-markers associated with post-chikungunya chronic arthritis (PCA) were analyzed by (receiver operating characteristic curve) ROC curve. A principal component analysis was conducted to identify the markers in different samples' groups.

4.3. RESULTS

In this study, male to female ratio of CHIKV infected patients:1.49:1 and mean age:33.06±14.7years, whereas, that of control group was 1:1.01 and 36.2±11.8 years, respectively [Table 4.3]. Major WHO-defined symptoms of febrile chikungunya patients were arthralgia, myalgia, persistent vomiting and joint swelling. According to arthralgic manifestation, as mentioned in materials and methods section, approximately, 14.10% of infected patients were NA (mean age:42.5±15.5years), 53.84% were AA (mean age:37.57±10.2years), 24.35% were AA+PCA (mean age:40.61±14.2years) and 7.69% were PCA (mean age:33.5±19.1years) groups. Younger and older patients were equally affected eliminating age biasness or any underlying medical condition. In addition, there was no significant difference in male-female distribution among above mentioned groups. PCA patients complained about persistent joint pain even after 2 years of first

visit blood collection. Whereas, AA+PCA patients reported arthralgia both during first visit and after 2 years of blood collection. 2010 ACR/EULAR scoring classification indicated majority of PCA and AA+PCA patients qualified for rheumatoid arthritis (score ≥6/10), with average score of 6 and 6.21, respectively. Principal component analysis of 2010 ACR/EULAR scoring parameters of CHIKV-infected patients indicated similar clustering of PCA and AA+PCA groups [Fig 4.1].

Table 4.3: Comparative demographics, symptomatic diversity and 2010 ACR/EULAR classification of CHIKV infected patients.

		Chikungunya infected patients (N=167)	**Healthy Controls (N=102)**
Mean age (in years)		33.06 ± 14.7 (range: 4-74 years)	36.2 ± 11.8(range: 18–63 years)
Sex	Male	59.88%	50.98%
	Female	40.11%	49.02%
	Male: Female ratio	1.49:1	1:1.01
Diagnostic Tools	Anti-CHIKV-IgM ELISA	44.31%	
	Real time RT-PCR	82.03%	
	Both anti-CHIKV-IgM ELISA and Real time RT-PCR	26.34%	
Symptomatic prevalence	Fever	100%	
	Myalgia	53.89%	
	Arthralgia	67.06%	
	Headache	22.15%	
	Rash	19.76%	
	Nausea	2.39%	
	Persistent Vomiting	25.14%	
	Abdominal Pain	8.98%	
	Joint Swelling	21.55%	
	Bleeding	0%	
	Retro-Orbital Pain	0%	
Patient's arthralgic manifestation	No arthralgia (NA) Mean age (in years)	14.10% 42.5±15.5years (range 7-62years)	
	Acute arthralgia (AA) Mean age (in years)	53.84% 37.57±10.2years (range 12-72years)	
	Acute arthralgia + Post-chikungunya arthritis (AA+PCA) Mean age (in years)	24.35% 40.61±14.2years (range 10-72years)	
	Post-chikungunya arthritis (PCA) Mean age (in years)	7.69% 33.5±19.1years (range 11-46years)	
% prevalence of patients with post-chikungunya rheumatoid arthritis according to 2010 ACR/EULAR scoring (score ≥6/10)*	NA	0%	
	AA	2.43%	
	PCA	83.33%	
	AA+PCA	95.23%	
Mean ACR/EULAR classification score	NA	3.09	
	AA	4.66	
	PCA	6	
	AA+PCA	6.21	

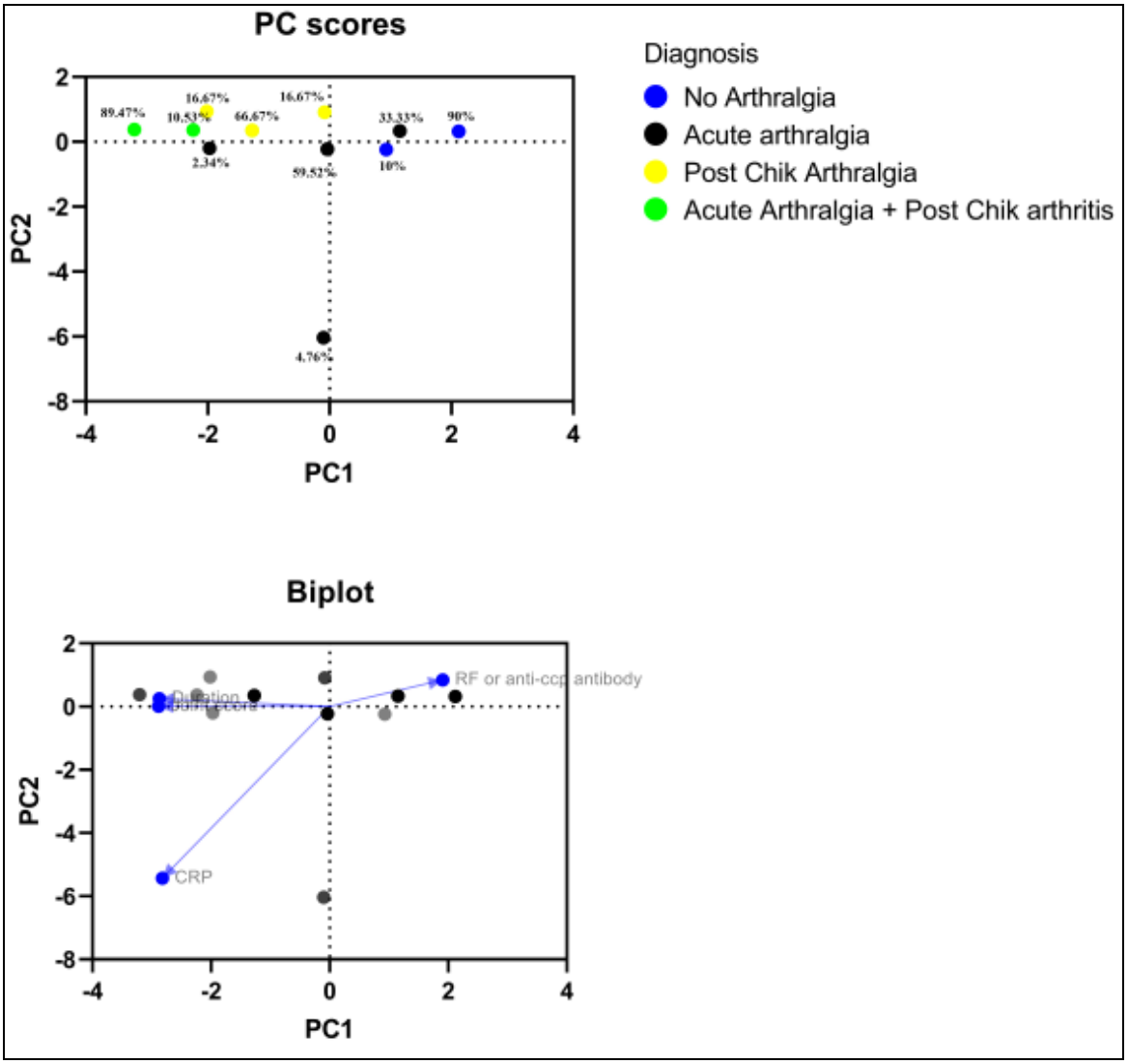


Fig 4.1: Principal component analysis of ACR/EULAR scores representing linkage and variance among AA+PCA, PCA, AA, NA of CHIKV-infected patients

4.3.1 CRP, anti-CCP antibody, COMP and IL-2R levels among chikungunya patients with acute and chronic arthritis

Mean serum CRP concentrations significantly increased among AA (46.03mg/ml, p-value= 0.0291), PCA (60.71mg/ml, p-value=0.0212) and AA+PCA (77.05mg/ml, p-value<0.0001) groups when compared to NA which had a mean level of 19.60mg/ml [Fig 4.2, Table 4.4]. Moreover, CRP level of AA+PCA group was significantly higher than AA patients (p-value=0.0004). Anti-CCP antibody level was significantly high only among AA+PCA group when compared to both NA and AA patients (NA: 133pg/ml, AA: 190.1pg/ml, AA+PCA :419.7pg/ml, p-value=0.0003 and p-value<0.0001, respectively). On a similar trend, COMP level was significantly higher among AA+PCA and PCA groups when compared to both NA and AA groups (AA+PCA: 40.35ng/ml, PCA: 49.35ng/ml, AA: 21.88ng/ml, NA: 21.18ng/ml; p-value<0.0001). AA+PCA and PCA groups also showed higher IL-2R level compared to NA (AA+PCA: 2421.22pg/ml and PCA: 4882.45pg/ml vs NA: 969.78pg/ml; p-value=0.0202 and p-value<0.0001, respectively). Also, PCA group showed highest mean IL-2R level compared to AA+PCA and AA groups (AA: 1128.96pg/ml; p-value<0.0001 and p-value=0.0005). RF was detected among 13.17% (22/167) CHIKV patients which was insignificant when compared to healthy individuals.

Table 4.4: Group-wise detailed serum arthritic and hepatic biomarker history of infected patients and healthy controls.

ID No.	Diagnosis	CRP	Anti-CCP antibody	COMP	IL-2R	Albumin	AST	ALT	AST/ALT ratio	Bilirubin	ALP
1.	AA+PCA	81.71	212.3	34.01	2078.98	2.12	268.19	198.76	1.36	1.65	96.56
2.	AA+PCA	122.5	289.9	47.2	2025.66	2.15	275.26	256.98	1.07	1.23	34.47
3.	AA+PCA	46.87	544.83	56.98	1076.44	4.1	447.65	91.6	4.88	0.92	76.65
4.	AA+PCA	109.37	899	40.12	1037.22	3.7	126.82	147.82	0.85	1.11	104.76
5.	AA+PCA	93.75	582.56	30.98	1049.07	3.85	196.48	117.04	1.67	1.6	129.09
6.	AA+PCA	56.87	278.65	24.55	1067.44	3.11	131.81	75	1.75	1.34	70.98
7.	AA+PCA	65.4	213.71	29.09	2025.66	2.84	176.98	165.9	1.066	1.41	87.95
8.	AA+PCA	129.07	567	20.1	2074.88	2.82	185.36	120.49	1.53	1.43	73.81
9.	AA+PCA	81.71	130.89	50.98	1043.55	2.52	227.07	127.46	1.78	0.89	96.86
10.	AA+PCA	40.48	123.45	30.12	2031.98	4.36	318.82	93.44	3.41	1.23	135.67
11.	AA+PCA	31.26	202.45	54.09	6068.64	4.18	54.68	47.37	1.15	0.23	59.452
12.	AA+PCA	75.11	900	47.89	2013.87	4.09	443.56	232.37	1.9	1.34	46.09
13.	AA+PCA	78.63	190.92	30.97	3024.1	3.27	436.54	264.13	1.65	0.97	140.98
14.	AA+PCA	80.31	166.76	60.19	1078.98	3.91	255.8	92.15	2.77	0.76	54.44
15.	AA+PCA	71.02	650	30.98	1076.12	2.17	444.87	145.28	3.05	1.57	33.734
16.	AA+PCA	29.8	137.39	34.56	2021.9	2.76	269.7	124.4	2.16	1.19	89.011
17.	AA+PCA	69.91	191.85	40.12	10160.8	2.89	106.875	72.13	1.48	0.87	316.46
18.	AA+PCA	66.44	792.3	40.67	3012.87	3.48	453.87	232.56	1.95	1.21	139.26
19.	AA+PCA	133.71	900	62.98	2034.98	3.42	301.21	118.32	2.54	1.34	70.8
20.	PCA	80.31	186.65	40.91	7089.1	2.76	101.24	99.18	1.02	0.338	87.8
21.	PCA	16.071	168.99	46.98	6056.09	3.38	73.21	42.37	1.73	1.89	120.74
22.	PCA	91.87	370.66	53.56	3045.89	3.51	56.74	78.9	0.71	0.98	7.68
23.	PCA	80.31	148.54	39.89	2056.19	3.06	91.36	69.21	1.26	0.264	112.31
24.	PCA	28.57	189.44	52.47	8001.78	2.09	96.96	67.7	1.32	0.305	109.82
25.	PCA	67.12	139.25	62.3	3045.67	3.11	74.66	71.56	1.55	0.364	129.78
26.	AA	20.8	176.05	18.92	1009.12	3.28	63.02	43.72	1.44	0.8	112.39
27.	AA	50.26	164.34	45.67	2001.32	3.33	304	165.16	1.84	0.32	15.865
28.	AA	69.91	192	35.87	1001.98	3.49	110.29	275	0.4	1.35	43.587

ID No.	Diagnosis	CRP	Anti-CCP antibody	COMP	IL-2R	Albumin	AST	ALT	AST/ALT ratio	Bilirubin	ALP
29.	AA	18.48	122.52	20.19	1021.32	2.76	134.39	56.48	2.37	0.51	87.007
30.	AA	93.75	332.78	45.9	1078.19	2.84	52.82	190.16	0.27	0.56	17.58
31.	AA	20.45	129.03	25.05	1034.67	3.08	125.12	65.57	1.9	0.21	32.73
32.	AA	17.85	185.35	32.54	3009.81	3.13	9.26	58.6	0.15	0.46	19.29
33.	AA	10.714	194.64	19.98	1044.56	3.3	82.48	27.04	3.05	0.65	31.3
34.	AA	46.857	99.29	37.9	1023.98	2.17	50.95	43.44	1.17	0.78	30.98
35.	AA	10.714	164.34	30.12	1078.98	2.47	23.17	58.19	0.39	0.46	49.26
36.	AA	26.78	106.16	16.61	1098.7	3.26	98.68	48.9	2.01	0.45	23.33
37.	AA	20.22	138.32	12.2	1002.98	3.84	48.45	79.09	0.61	0.99	48
38.	AA	145.85	670	42.9	1010.89	3.58	152.36	89.9	1.69	0.34	198.73
39.	AA	20.22	119.73	13.9	1004.98	3.27	32.44	56.96	0.56	1.32	53.607
40.	AA	13.4	97.43	15.23	1056.34	3.91	102.95	50.91	2.02	0.56	30.06
41.	AA	31.2	97.43	19.18	1034.78	3.87	81.56	52.86	1.54	0.67	192.85
42.	AA	21.23	81.63	10.9	1089.1	3.14	91.36	69.21	1.26	0.45	34.16
43.	AA	75.11	136.46	18.6	1067.89	3.27	97.31	51.63	1.88	0.33	46.927
44.	AA	43.64	183.86	20.5	1011.34	2.87	50.97	122.54	0.41	0.32	75.317
45.	AA	20.42	137.39	15.37	1012.87	3.73	145.65	193.85	0.75	0.11	193.88
46.	AA	80.4	194.08	25.44	1020.9	3.01	168.86	129.89	1.3	1.13	67.98
47.	AA	105.15	860	26.3	1006.78	2.44	167.75	129.65	1.29	1.61	147.62
48.	AA	32.34	197.8	16.81	1045.87	2.62	137.17	145.62	0.94	0.01	87.07
49.	AA	62.5	60.26	15.56	1032.9	1.43	152.45	122.34	1.24	0.96	161.67
50.	AA	14.44	54.68	26.56	1022.67	1.99	282.68	166.39	1.69	1.51	123.47
51.	AA	65.86	159.7	19.38	1098.76	3.48	173.56	126.56	1.38	1.45	98.01
52.	AA	53.25	167.13	26.76	1020.19	2.98	72.34	61.29	1.18	0.354	66.78
53.	AA	31.2	181.07	18.67	1039.09	3.6	96.96	67.7	1.32	0.241	38.57
54.	AA	30.26	182.93	28.67	1015.9	3.63	78.98	89.8	0.87	0.67	156.98
55.	AA	21.23	208.02	27.88	1027.98	3.67	55.26	49.89	1.07	0.535	88.91
56.	AA	78.63	125.31	11.94	1098.76	2.61	165.99	129.09	1.28	0.56	86.33
57.	AA	61.21	82.56	22.15	1009.2	2.77	132.87	89.09	1.49	0.347	70.06
58.	AA	45.06	73.27	16.49	1003.46	2.53	121.98	131.24	0.92	2.07	24.048

ID No.	Diagnosis	CRP	Anti-CCP antibody	COMP	IL-2R	Albumin	AST	ALT	AST/ALT ratio	Bilirubin	ALP
59.	AA	38.71	122.52	13.79	1043.98	3.14	78.53	49.11	1.6	0.391	66.61
60.	AA	68.1	165.27	20.7	1009.9	3.48	82.38	58.06	0.86	0.361	58.41
61.	AA	68.71	160.62	17.6	2087.9	3.43	56.72	50.73	1.26	0.338	54.59
62.	AA	56.04	291.67	17.08	1045.67	3.63	42.43	48.78	1.1	0.677	102.56
63.	AA	51.26	336.27	13.32	1023.87	3.94	176.07	156.09	1.12	0.21	65.78
64.	AA	28.57	121.59	11.15	1032.67	2.19	45.31	38.31	1.18	0.571	62.35
65.	AA	62.2	116.95	12.67	1009.89	2.87	51.11	52.18	0.98	0.404	67.89
66.	AA	39	316.27	18.6	1023.98	2.17	66.87	52.17	1.28	0.292	66.21
67.	AA	61.21	278.95	13.99	1002.34	2.25	32.48	18.85	1.72	0.34	104.87
68.	NA	15.625	167.13	27.88	1021.98	3.13	67.65	44.62	1.51	0.45	34.56
69.	NA	10.714	132.09	11.94	1002.32	2.42	46	33.7	1.36	0.64	52.77
70.	NA	7.142	97.45	16.81	1001.98	3.8	25.95	74.32	0.34	1.89	40.915
71.	NA	26.78	188.51	15.56	1032.9	2.35	6.48	43.03	0.15	1.32	46.927
72.	NA	12.04	177.35	26.56	1065.09	3.38	39.85	26.63	1.49	0.36	24.16
73.	NA	30.04	171.78	19.38	1009.01	2.31	15.75	13.93	1.13	1.99	16.867
74.	NA	19.21	142.97	26.76	809.9	3.32	26.87	34.3	0.78	1.45	23.881
75.	NA	21.23	123.45	18.67	709.8	2.89	69.51	44.13	1.57	0.25	36.5
76.	NA	26.2	98.45	28.67	1001.9	2.36	22.24	40.57	0.54	0.35	43.87
77.	NA	29.71	82.56	27.88	1021.9	3.06	85.26	40.98	2.08	0.44	25.88
78.	NA	16.93	81.63	12.87	990.87	2.17	50.95	43.44	1.17	0.78	28.96
79.	Healthy	1.67	15	6.79	507.09	4.5	32.26	36.24	0.89	0.468	48.5
80.	Healthy	1.59	19.88	4.5	807.09	4.184	27.78	30.23	0.91	0.472	50.65
81.	Healthy	1.801	21	9.7	405.89	3.996	30.06	34.55	0.87	0.729	48.61
82.	Healthy	2.46	21	6.34	709.08	5.26	22.4	28.01	0.79	0.431	54.49
83.	Healthy	1.97	16	3.09	305.98	4.851	30.22	35.3	0.85	0.291	40.15
84.	Healthy	1.99	13.87	2.1	300.9	3.641	34.56	37.56	0.92	0.207	59.89
85.	Healthy	2.34	12.89	4.5	708.9	4.688	36.76	45.15	0.81	0.624	49.71
86.	Healthy	2.61	22	7.8	405.09	5.096	33.3	37.64	0.92	0.321	43.86
87.	Healthy	2.38	19.89	4.3	609.8	4.69	28.96	22.28	0.79	0.378	56.67
88.	Healthy	1.37	10.09	7.6	708.1	4.213	26.4	32.64	0.8	0.46	52.68

Level of all four arthritic biomarkers was higher among chikungunya patients with chronic arthritis (PCA, AA+PCA) compared to those with only acute or no arthritic manifestation among all age groups with CRP, anti-CCP, COMP and IL-2R significantly high among PCA patients within 19-59 age-group [Fig 4.3]. Similar trend was followed among male and female chikungunya patients [Fig 4.4].

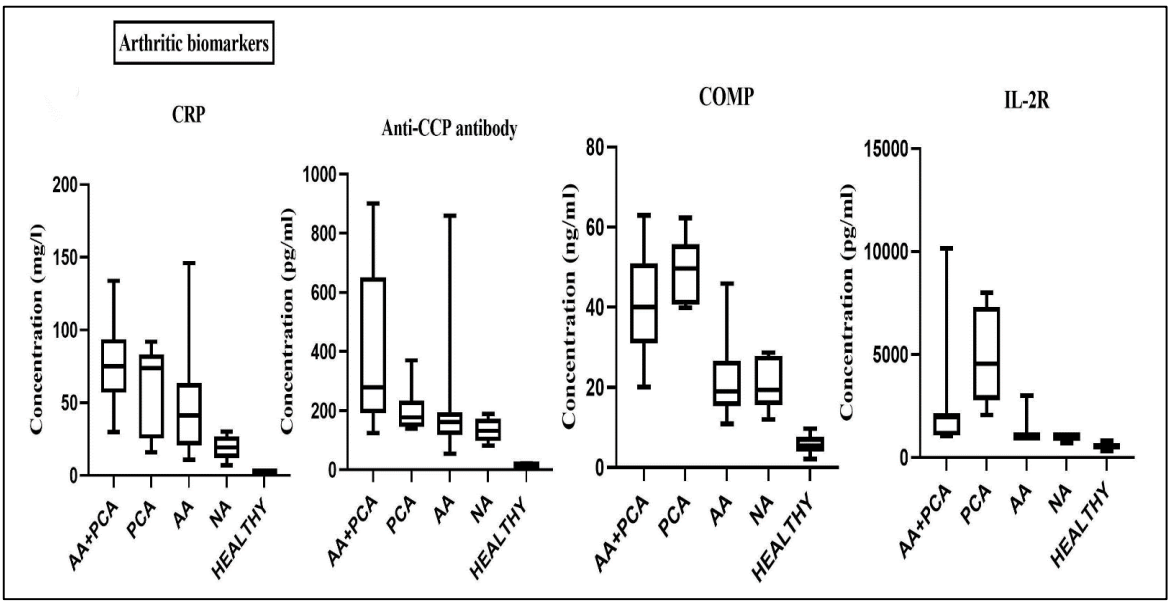


Fig 4.2: Comparative analysis of serum concentration of arthritic biomarkers among AA+PCA, PCA, AA and NA groups of CHIKV-infected patients

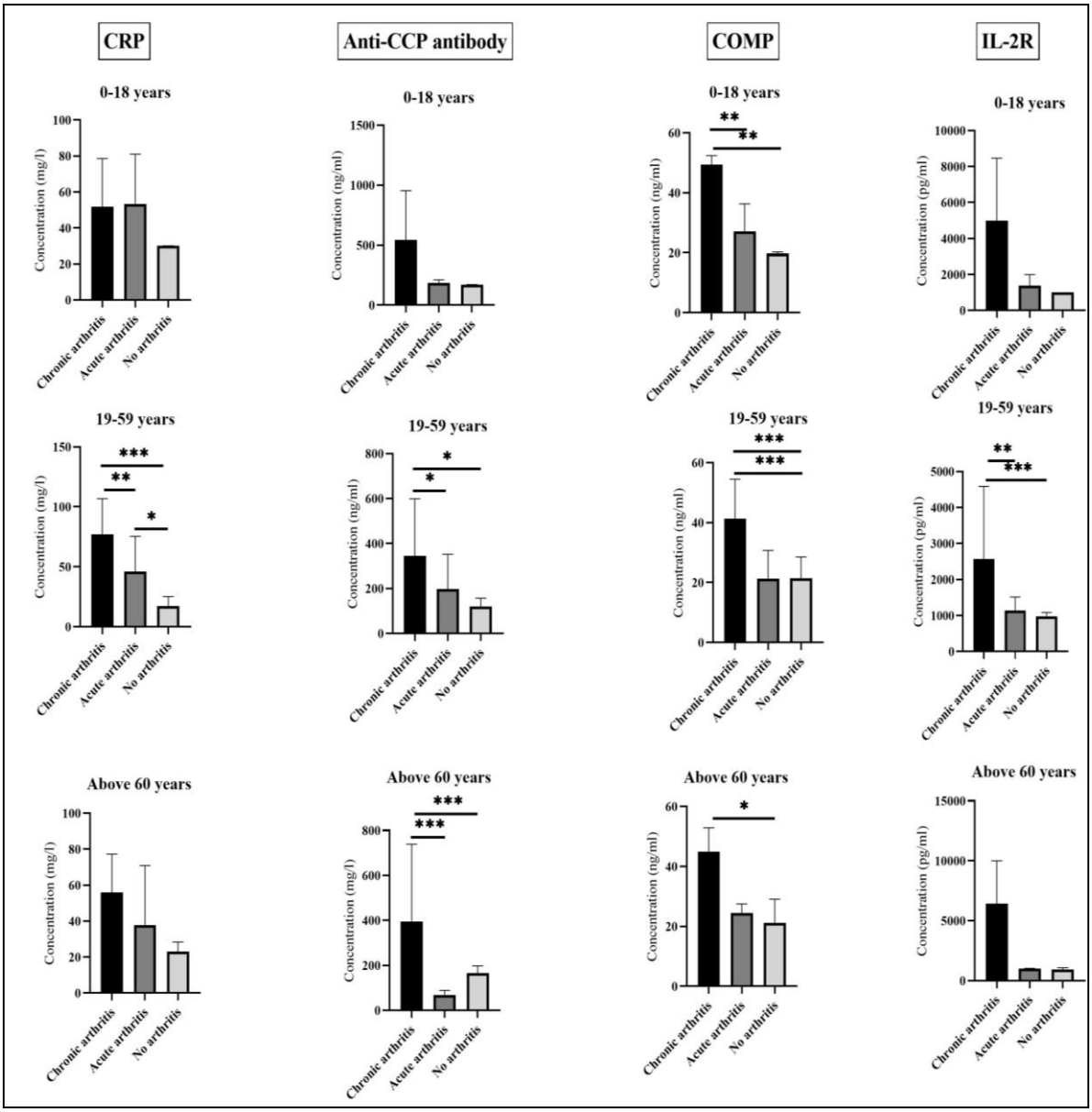


Fig 4.3: Age-wise comparative analysis of serum concentration of arthritic biomarkers among chronic arthritic, acute arthritic and non-arthritic groups of CHIKV-infected patients.

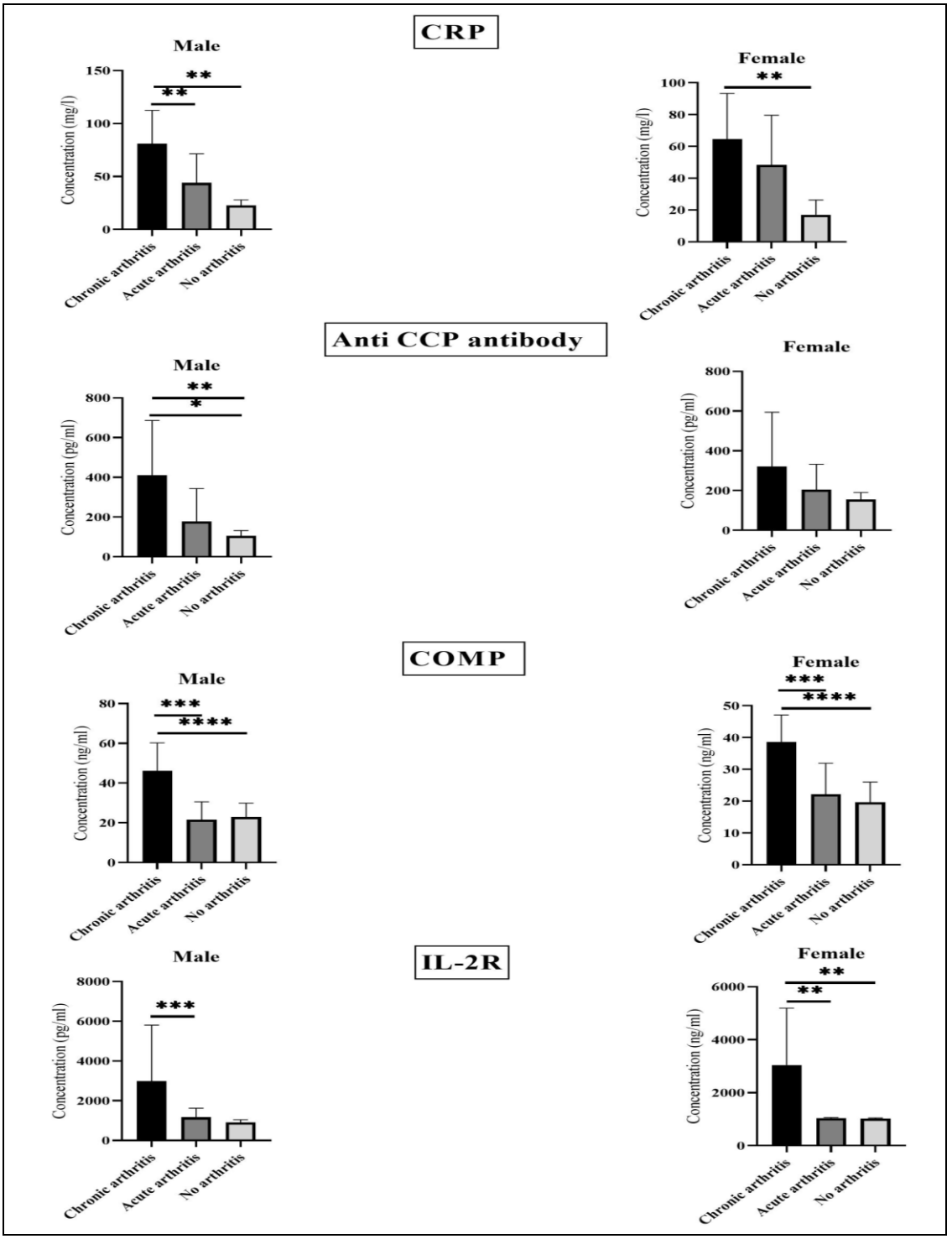


Fig 4.4: Gender-wise comparative analysis of serum concentration of arthritic biomarkers among chronic arthritic, acute arthritic and non-arthritic groups of CHIKV-infected patients

4.3.2 AST, ALT, ALP, bilirubin and albumin levels among chikungunya patients with acute and chronic arthritis

Amongst hepatic markers, gradual increase of AST, ALT, albumin level and AST/ALT ratio was found in arthralgic groups according to following order: AA+PCA>AA>PCA>NA [Fig 4.5]. AST levels were significantly elevated among AA+PCA (269.6IU/L) compared to NA (NA: 41.50IU/L; p-value<0.0001), AA (AA: 102.3IU/L; p-value<0.0001) and PCA (PCA: 82.36IU/L; p-value<0.0001) groups. Similar trend was followed for ALT among AA+PCA (AA+PCA: 143.3IU/L) compared to all the other groups (NA: 39.97IU/L, p-value<0.0001; AA: 89.57IU/L, p-value=0.0016; PCA: 71.49, p-value=0.0217). AST/ALT ratio was significantly high among AA+PCA (2.001) with respect to NA (1.102; p-value=0.0043) and AA (1.257; p-value=0.0008) groups. Also, AA+PCA had significantly higher bilirubin levels compared to AA (AA+PCA: 1.173IU/L vs AA :0.6350IU/L; p-value=0.0004) and ALP compared to NA (AA+PCA: 97.74IU/L, NA:34.12IU/L; p-value=0.0051). Single sample for each of AA and AA+PCA groups were the only exceptions with “maximum outlier” value for CRP, Anti-CCP antibody, COMP, IL-2R, AST, ALT, AST/ALT ratio, bilirubin and ALP. Among AA+PCA group, a single 72years old male patient with fever for 2 months exhibited these outlier values.

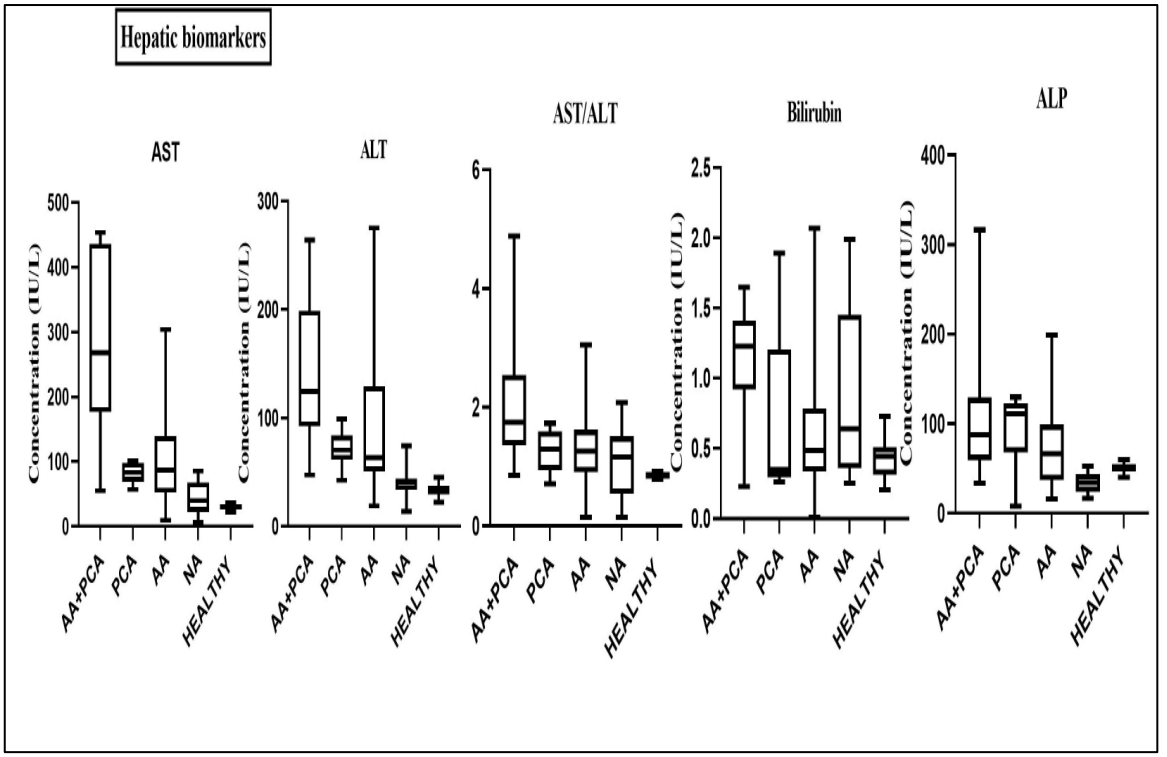
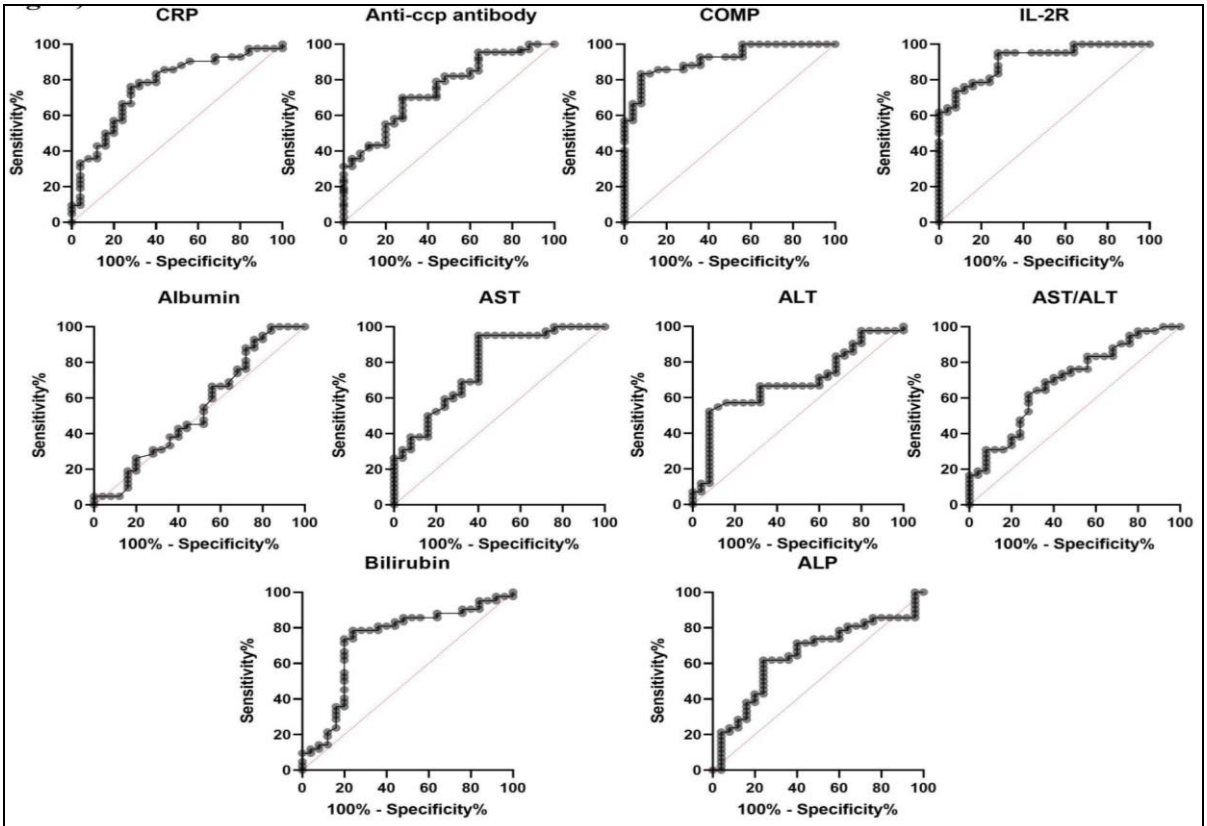


Fig 4.5: Comparative analysis of serum concentration of hepatic biomarkers among AA+PCA, PCA, AA and NA groups of CHIKV-infected patients.

4.3.3. ROC curve analysis of biomarkers among chikungunya patients with and without chronic arthritis

A cut-off value of both arthritic and hepatic biomarkers that are significantly altered among chikungunya induced chronic arthritic patients was analyzed by ROC curve to differentiate between patients with (PCA, AA+PCA) and without (AA, NA) chronic arthritis [Fig 4.6]. Cut off values of arthritic and hepatic biomarkers that significantly increased in case of chronic arthralgia were: CRP: >63.95mg/ml, Anti-CCP antibody: >186.0pg/ml, COMP: >28.88ng/ml, IL-2R: >1066pg/ml, AST: >133.6U/L, ALT: >90.75U/L, AST/ALT: >1.46U/L, Bilirubin: >0.83U/L, ALP: >70.43U/L (p-value: 0.0001-0.0051). High sensitivity and specificity values of COMP and IL-2R indicated their diagnostic potential for chronic arthritis among infected patients. On the contrary, hepatic markers demonstrated lower sensitivity level compared to arthritic ones.

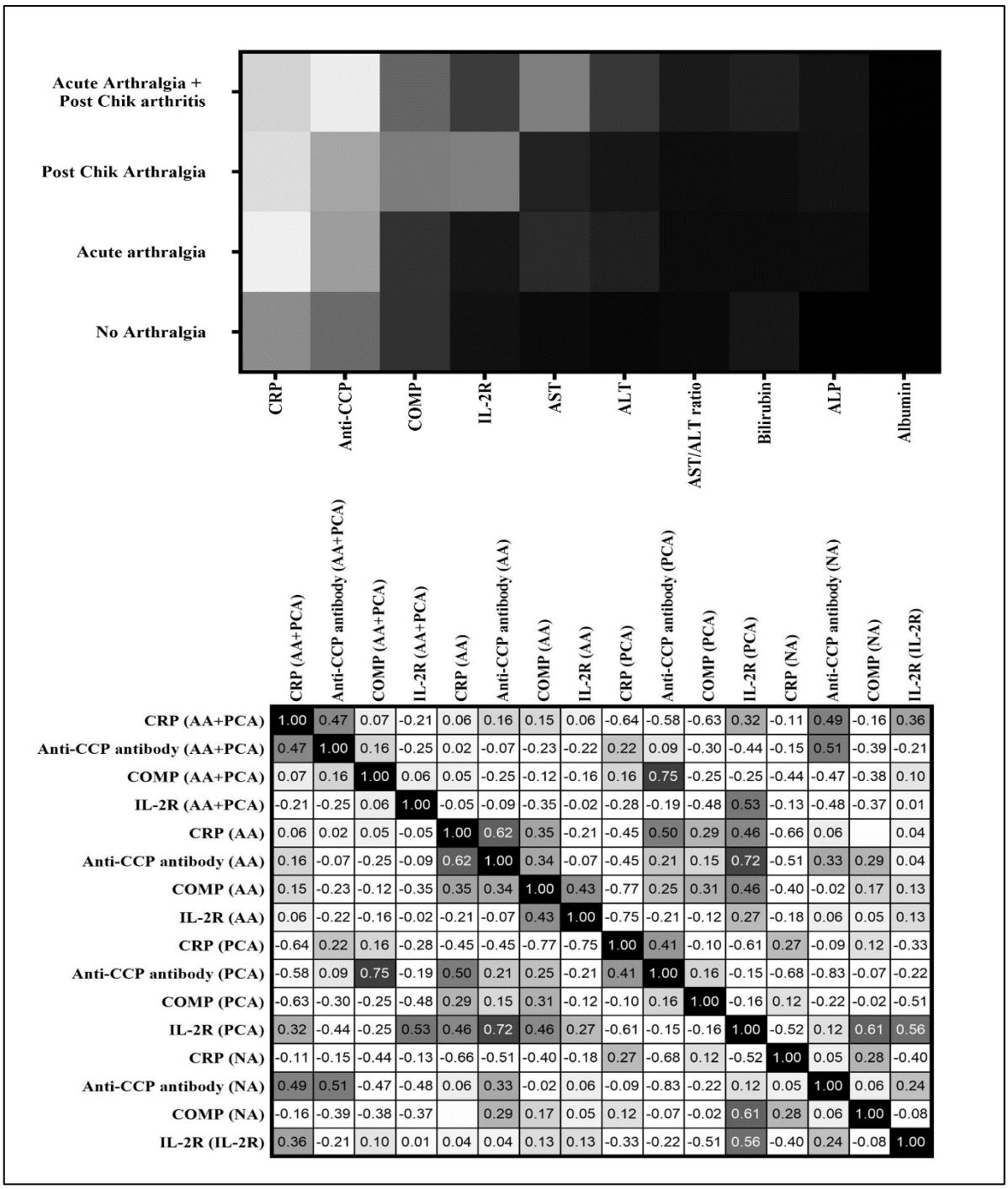


	Area	Sensitivity %	Specificity %	Cut-off value	p-value
CRP	0.8057	81.13	72	> 63.95	<0.0001*
Anti-CCP antibody	0.7826	75.47	72.00	> 186.0	<0.0001*
COMP	0.9257	86.79	92	>28.88	<0.0001*
IL-2R	0.9257	88.00	81.13	> 1066	<0.0001*
Albumin	0.5585	49.06	56	>3.095	NS
AST	0.8219	69.81	72	>133.6	0.0001*
ALT	0.7442	73.58	68.00	> 90.75	0.0005*
AST/ALT	0.7023	67.92	64.00	> 1.46	0.0041*
Bilirubin	0.6974	75.47	76	> 0.83	0.0051*
ALP	0.7026	69.81	76	>70.43	0.004*

Fig 4.6: Receiver operating characteristic curve (ROC curve) analysis of arthritic and hepatic biomarkers with their sensitivity, specificity and cut-off value of chikungunya induced chronic arthritis.

4.3.4. Heatmap, correlation and principal component analysis of biomarkers among chikungunya patients with and without chronic arthritis

Heatmap analysis of arthritic and hepatic biomarkers revealed increase of CRP, anti-CCP antibody as well as COMP levels among all four groups of infected patients compared to healthy controls [Fig 4.7]. Serum albumin level increase was negligible. Correlation studies of these biomarkers among these groups indicated a strong positive significant correlation between Anti-CCP antibody vs. CRP for AA+PCA (p-value=0.044), Anti-CCP antibody vs. CRP (p-value<0.001), COMP vs. CRP (p-value=0.023), COMP vs. Anti-CCP antibody (p-value=0.029) and COMP vs. IL-2R (p-value=0.004) for AA groups [Fig 4.7]. Negative correlation was obtained between AA vs NA and PCA vs NA groups for CRP and anti-CCP antibody levels, respectively. Principal component analysis of all analyzed biomarkers indicated gradual segregation of AA+PCA and PCA groups from AA and NA, which in turn overlapped with each other; NA was in proximity to healthy controls [Fig 4.8]. AA+PCA group was linked with increased anti-CCP antibody, CRP, AST, ALT, AST/ALT ratio and bilirubin levels in patient-serum, whereas, PCA group was associated with increased ALP and IL-2R levels. Highest proportion of variance of PC1 was 37.99%, PC2 was 51.72% and PC3 was 63.75%.



	CRP (AA+PCA)	Anti-CCP antibody (AA+PCA)	COMP (AA+PCA)	IL-2R (AA+PCA)	CRP (AA)	Anti-CCP antibody (AA)	COMP (AA)	IL-2R (AA)	CRP (PCA)	Anti-CCP antibody (PCA)	COMP (PCA)	IL-2R (PCA)	CRP (NA)	Anti-CCP antibody (NA)	COMP (NA)	IL-2R (IL-2R)
CRP (AA+PCA)	1.00	0.47	0.07	-0.21	0.06	0.16	0.15	0.06	-0.64	-0.58	-0.63	0.32	-0.11	0.49	-0.16	0.36
Anti-CCP antibody (AA+PCA)	0.47	1.00	0.16	-0.25	0.02	-0.07	-0.23	-0.22	0.22	0.09	-0.30	-0.44	-0.15	0.51	-0.39	-0.21
COMP (AA+PCA)	0.07	0.16	1.00	0.06	0.05	-0.25	-0.12	-0.16	0.16	0.75	-0.25	-0.25	-0.44	-0.47	-0.38	0.10
IL-2R (AA+PCA)	-0.21	-0.25	0.06	1.00	-0.05	-0.09	-0.35	-0.02	-0.28	-0.19	-0.48	0.53	-0.13	-0.48	-0.37	0.01
CRP (AA)	0.06	0.02	0.05	-0.05	1.00	0.62	0.35	-0.21	-0.45	0.50	0.29	0.46	-0.66	0.06		0.04
Anti-CCP antibody (AA)	0.16	-0.07	-0.25	-0.09	0.62	1.00	0.34	-0.07	-0.45	0.21	0.15	0.72	-0.51	0.33	0.29	0.04
COMP (AA)	0.15	-0.23	-0.12	-0.35	0.35	0.34	1.00	0.43	-0.77	0.25	0.31	0.46	-0.40	-0.02	0.17	0.13
IL-2R (AA)	0.06	-0.22	-0.16	-0.02	-0.21	-0.07	0.43	1.00	-0.75	-0.21	-0.12	0.27	-0.18	0.06	0.05	0.13
CRP (PCA)	-0.64	0.22	0.16	-0.28	-0.45	-0.45	-0.77	-0.75	1.00	0.41	-0.10	-0.61	0.27	-0.09	0.12	-0.33
Anti-CCP antibody (PCA)	-0.58	0.09	0.75	-0.19	0.50	0.21	0.25	-0.21	0.41	1.00	0.16	-0.15	-0.68	-0.83	-0.07	-0.22
COMP (PCA)	-0.63	-0.30	-0.25	-0.48	0.29	0.15	0.31	-0.12	-0.10	0.16	1.00	-0.16	0.12	-0.22	-0.02	-0.51
IL-2R (PCA)	0.32	-0.44	-0.25	0.53	0.46	0.72	0.46	0.27	-0.61	-0.15	-0.16	1.00	-0.52	0.12	0.61	0.56
CRP (NA)	-0.11	-0.15	-0.44	-0.13	-0.66	-0.51	-0.40	-0.18	0.27	-0.68	0.12	-0.52	1.00	0.05	0.28	-0.40
Anti-CCP antibody (NA)	0.49	0.51	-0.47	-0.48	0.06	0.33	-0.02	0.06	-0.09	-0.83	-0.22	0.12	0.05	1.00	0.06	0.24
COMP (NA)	-0.16	-0.39	-0.38	-0.37		0.29	0.17	0.05	0.12	-0.07	-0.02	0.61	0.28	0.06	1.00	-0.08
IL-2R (IL-2R)	0.36	-0.21	0.10	0.01	0.04	0.04	0.13	0.13	-0.33	-0.22	-0.51	0.56	-0.40	0.24	-0.08	1.00

Fig 4.7: Differential heatmap and correlation analysis of arthritic and hepatic biomarkers with AA+PCA, PCA, AA and NA groups of CHIKV-infected patients.

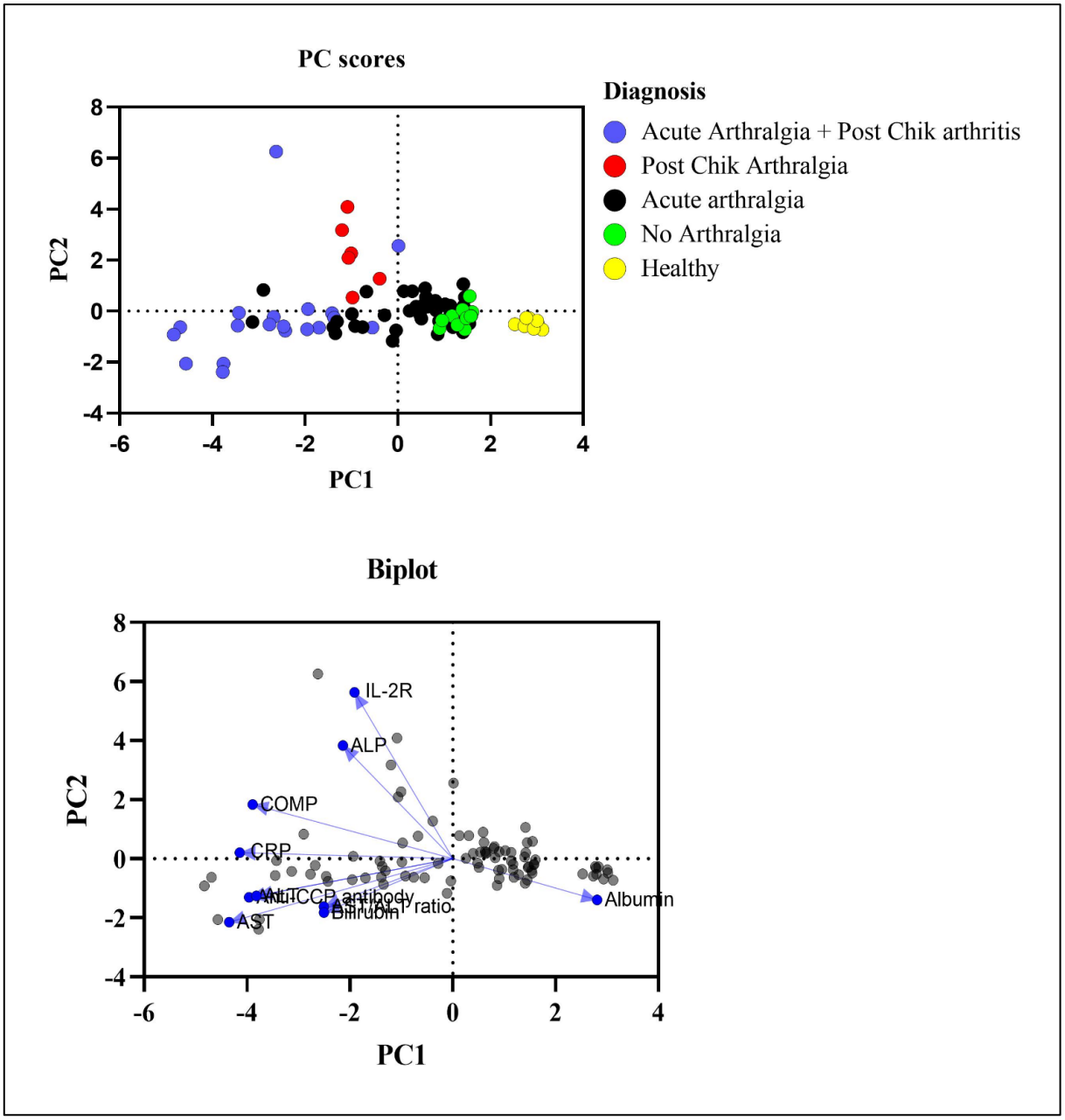


Fig 4.8: Principal component analysis representing linkage and variance among AA+PCA, PCA, AA, NA of CHIKV-infected patients and healthy groups, with arthritic and hepatic biomarkers.

4.3.5. Genotypic association of CRP, IL-2R, COMP polymorphisms with chikungunya susceptibility

Genotypic and allelic distribution of CRP, IL-2R and COMP polymorphisms was analyzed among 167 CHIKV infected patients and 102 healthy controls [Fig 4.9, Table 4.5]. Statistical analysis revealed individuals with CRP-rs3091244-TC genotype were less susceptible to CHIKV infection (p-value=0.0126). Additionally, according to additive model, rs3091244 was significantly associated with infection susceptibility (p-value=0.0286). Furthermore, patients with IL-2R-rs743777-GA genotype and G-allele were significantly susceptible to CHIKV infection (p-value=0.0198 and p-value=0.0079, respectively). Additive model suggested this IL-2R polymorphism to be significantly associated to CHIKV infection susceptibility (p-value=0.0179). Subsequently, COMP-rs144778694-GA genotype and A-allele were positively associated with CHIKV infection when compared to healthy control population (p-values= 0.0032, 0.0020, respectively). Similar trend was also followed by its additive model [p=0.0051].

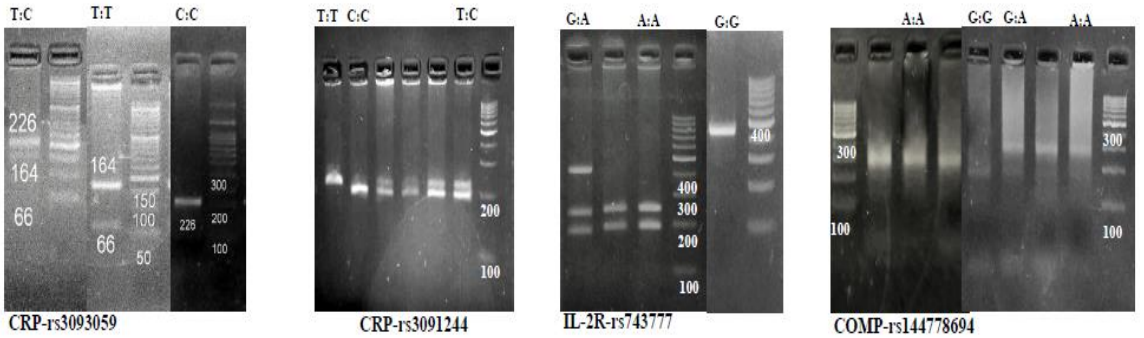


Fig 4.9: Representative PCR-RFLP images of specific genotypes of CRP, IL-2R and COMP polymorphisms

Table 4.5: Genotypic and allelic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV-infected patients and healthy controls.

SNP Ref. No.	Genotype and allele distribution	Healthy Controls (%)	Chikungunya infected patients (%)	OR (95% C.I)	Relative risk (95% C.I)	p-value
CRP rs3093059		**n=102**	**n=167**			
	CC	2	3	1.093 [0.1913 to 5.429]	1.056 [0.3082 to 2.098]	>0.9999
	CT	21	43	0.7476 [0.4144 to 1.374]	0.8304 [0.5526 to 1.196]	0.3776
	TT	79	121	ref		
	C allele	25	49	0.8123 [0.4783 to 1.377]	0.8757 [0.6125 to 1.199	0.5191
	T allele	179	285	ref		
	Additive	0.6280				
CRP rs3091244		**n=102**	**n=167**			
	TT	3	11	2.327 [0.6932 to 7.942]	1.812[0.7990 to 5.176]	0.1915
	TC	39	40	1.965 [1.166 to 3.323]	1.489 [1.092 to 1.996]	**0.0126***
	CC	60	116	Ref		
	T allele	45	62	1.242 [0.8138 to 1.907]	1.140 [0.8736 to 1.452]	0.3243
	C allele	159	272	Ref		
	Additive	**0.0286***				
IL-2R rs743777		**n=102**	**n=167**			
	GG	0	3	2.512 [0.4077 to 31.01]	1.929 [0.6057 to 10.69]	0.6523
	GA	22	59	1.987 [1.139 to 3.422]	1.567 [1.079 to 2.353]	**0.0198***
	AA	80	105	Ref		
	G allele	22	65	1.999 [1.195 to 3.323]	1.596 [1.119 to 2.364]	**0.0079***
	A allele	182	269	Ref		
	Additive	**0.0179***				
COMP rs144778694		**n=102**	**n=167**			
	AA	0	3	2.452 [0.3981 to 30.27]	1.900 [0.5966 to 10.54]	0.4103
	GA	11	42	2.848 [1.409 to 5.897]	2.058 [1.241 to 3.630]	**0.0032***
	GG	91	122	Ref		
	Aallele	11	46	2.822 [1.444 to 5.524]	2.088 [1.268 to 3.655]	**0.0020***
	G allele	193	286	Ref		
	Additive	**0.0051***				

4.3.6. Genotypic association of CRP, IL-2R, COMP polymorphisms with chikungunya induced arthritis

Role of these CRP, IL-2R and COMP polymorphisms was analyzed between patients with (n=112) and without arthralgia (n=55) [Table 4.6]. Analysis revealed CRP-rs3093059-CT, CRP-rs3091244-TT, IL-2R-rs743777-GA and COMP-rs144778694-AA genotypes were significantly associated with patients without any arthralgic manifestations (p-value<0.0001, p-value=0.0004, p-value<0.0001 and p-value=0.0255, respectively). In contrast, COMP rs144778694-GA genotype was significantly linked to patients with arthralgia (p-value=0.0471). Also, CRP-rs3093059-C, CRP-rs3091244-T and IL-2R-rs743777-G alleles were associated with patients without arthralgia (p-value<0.0001, p-value=0.0004 and p-value=0.0017, respectively). Interestingly, CRP-rs3093059-CT genotype was significantly more prevalent among patients who never developed PCA (p-value=0.0317) [Table 4.7].

Table 4.6: Genotypic and allelic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV infected patients with or without arthralgia.

SNP Ref. No.	Genotype and allele distribution	With Arthralgia (%)	Without Arthralgia (%)	OR (95% C.I)	Relative risk (95% C.I)	p-value
		n=112	**n=55**			
	CC	3	0	2.036[0.3239 to 25.32]	1.207[0.5633 to 1.531]	0.6419
	CT	8	35	0.04396[0.01826 to 0.1106]	0.2218[0.1157 to 0.3914]	**<0.0001***
	TT	101	20	Ref		
CRP rs3093059	C allele	14	35	0.1429[0.07313 to 0.2746]	0.3878[0.2411 to 0.5797]	**<0.0001***
	T allele	210	75	ref		
		n=112	**n=55**			
	TT	2	9	0.09293 [0.01981 to 0.3832]	0.2579 [0.07262 to 0.6821]	**0.0004***
CRP rs3091244	TC	22	18	0.5025 [0.2510 to 1.029]	0.7761 [0.5532 to 1.012]	0.0626
	CC	88	28	Ref		
	T allele	26	36	0.2699 [0.1519 to 0.4839]	0.5761 [0.4156 to 0.7555]	**<0.0001***
	C allele	198	74	Ref		
		n=112	**n=55**			
	GG	1	2	0.2387 [0.01635 to 2.108]	0.4925[0.09066 to 1.187]	0.2097
IL-2R Rs743777	GA	24	35	0.1558[0.07779 to 0.3111]	0.4992 [0.3534 to 0.6680]	**<0.0001***
	AA	87	18	Ref		
	G allele	26	39	0.2391 [0.1356 to 0.4223]	0.5434[0.3908 to 0.7166]	**0.0017***
	A allele	198	71	Ref		
		n=112	**n=55**			
	AA	0	3	0.1184 [0.009611 to 0.7509]	0.2947 [0.05328 to 0.9292]	**0.0255***
COMP rs144778694	GA	32	10	2.343 [1.024 to 5.225]	1.256 [1.003 to 1.516]	**0.0471***
	GG	80	42	Ref		
	Aallele	32	16	0.9792 [0.5266 to 1.925]	0.9931 [0.7738 to 1.196]	0.9493
	G allele	192	94	Ref		

Table 4.7: Genotypic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV infected patients with (PCA, AA+PCA) or without (AA, NA) post-chikugnunya arthrtitis.

SNP Ref. No	Genotype and allele distribution	AA + NA (%)	PCA, AA+PCA(%)	p-value
CRP rs3093059	CC	1.88	8	0.2392
	CT	37.73	12	**0.0317***
	TT	60.37	80	0.1229
CRP rs3091244				
	TT	1.88	0	0.3063
	TC	30.18	40	0.4453
	CC	67.92	60	0.6111
IL-2R Rs743777				
	GG	1.88	16	0.5411
	GA	24.52	24	0.99
	AA	73.58	72	0.97
COMP rs144778694				
	AA	1.88	8	0.2392
	GA	39.62	28	0.4536
	GG	58.49	64	0.8077

4.3.7. Association of CRP, IL-2R, COMP polymorphic genotypes with serum concentration among patients with and without chronic arthritis

Association between polymorphic genotypes and serum level of CRP, IL-2R and COMP was analyzed among AA, PCA and AA+PCA groups [Fig 4.10]. Serum level of CRP-rs3093059-TT genotype was significantly higher among patients of AA+PCA and AA groups when compared to CC genotype (AA+PCA-TT: 77.25mg/l vs CC: 55.33mg/l; p-value=0.0123 and TT:44.55mg/l vs CC: 30.73mg/l; p-value=0.0037, respectively). CRP level was comparatively higher among patients with CT genotype (CT:91.43mg/l). Moreover, CRP level of patients with rs3091244-CT and CC genotypes was significantly higher compared to those with TT genotype among AA group (CT: 53.27mg/l, CC: 43.19mg/l vs TT: 22.34mg/l; p-value=0.0089 and 0.0001, respectively). Similarly, patients with rs743777-GA and AA genotype demonstrated significantly higher IL-2R

level compared to those with GG genotype among AA+PCA group (GA: 3345pg/ml, AA: 3050pg/ml, GG: 1120pg/ml; p-value=0.0429, respectively). In case of COMP-rs144778694, patients with GA and GG genotypes showed remarkable increase in COMP level compared to AA genotype within AA+PCA group (GA: 46.55ng/ml and GG: 43.69mg/l vs AA: 27.34ng/ml; p-value=0.0087 and 0.0143, respectively); but this trend was reversed among AA group (AA: 34.07ng/ml vs GG: 21.09ng/ml, GA: 22.45ng/ml; p-value=0.0033, p-value=0.0028).

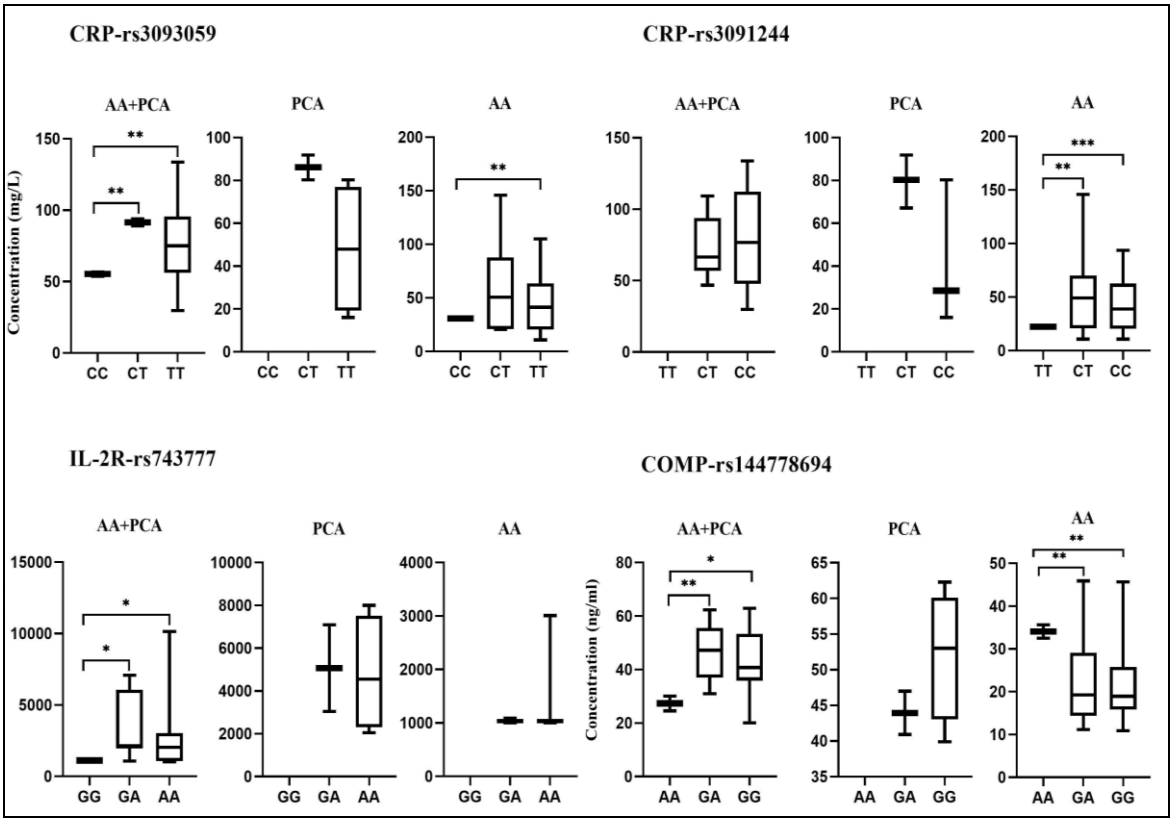


Fig. 4.10: Comparative genotypic distribution pattern of CRP, IL-2R, COMP polymorphisms and their serum levels among different arthritic groups of CHIKV-infected patients.

4.4. DISCUSSION

CHIKV-infection causes prolonged illness among patients, inducing acute and/or chronic polyarthritis/polyarthralgia, which might persist for at least one year, inviting chronic inflammatory rheumatism and musculo-skeletal disease during PCA [39]. Thus, identification of certain biomarkers that might help in differentiating chikungunya patients with only acute arthritis from those who might develop chronic polyarthritis would be useful in pain management of chronic arthritic patients. Serum level and polymorphic genotypes of CRP, Anti-CCP antibody, IL-2R, COMP, RF and hepatic markers were analyzed in this regard.

CRP concentrations significantly increased among all patients with acute and chronic arthralgia compared to those without. Also, CRP level was significantly higher among AA+PCA patients when compared to AA group. Previously, increased level of CRP has been reported among CHIKV-induced arthritic Indian patients; but this is the first study to differentiate CRP level between acute and chronic arthritic patients with 81.13% sensitivity and 72% specificity [12]. Increased CRP might act as inflammatory response to activate innate immune response against viral induced arthritis [40]. Anti-CCP antibody concentration was higher among AA+PCA and PCA groups with respect to NA and AA, but the increase was statistically significant for AA+PCA group. Anti-CCP antibody was previously found among La Reunion island-patients with post-chikungunya chronic arthritis, but its differential concentration among acute and chronic arthritic patients has not been previously demonstrated [15]. Anti-CCP antibody cross-reacting with type II collagen, resulting in proteoglycan depletion and severe arthritis have been previously reported [21]. Similar trend was followed for COMP with its noteworthy higher serum-levels among AA+PCA and PCA groups. Current study indicated that COMP level could be used as differentiating diagnostic factor for chronic arthritis among

CHIKV-infected patients with 86.79% sensitivity and 92% specificity. Previous reports also suggested COMP to be associated with osteoarthritis and rheumatoid arthritis [33, 34]. COMP, found in articular cartilage, ligament, meniscus, synovial membrane, and tendon, have a role in endochondral ossification and its increased level has been linked to cartilage degradation [22,25]. Increased IL-2R level among AA+PCA and PCA compared to NA and AA indicated its differential diagnostic role for chronic arthritic chikungunya patients with 88% sensitivity and 81.13% specificity. Elevated IL-2R concentration has been previously demonstrated among rheumatoid arthritic patients and was reported to be associated with synovitis [23,41]. Thus, according to high sensitivity and specificity scores, current study is the first to demonstrate prognostic relevance of COMP, IL-2R and CRP levels in identifying chronic arthritic chikungunya patients.

Hepatic anomalies have been previously reported among chikungunya patients of northern India, Singapore, Mexico and Sri Lanka; but their differential concentration between arthritic and non-arthritic chikungunya patients has not been studied earlier [13,14, 42, 43]. Increased concentrations of AST, ALT, AST/ALT ratio, bilirubin and ALP were reported among arthritic chikungunya compared to non-arthritic ones.

Principal component analysis segregated AA+PCA and PCA groups from overlapping AA and NA group of patients. There was a stark proximity of non-arthalgic group with healthy controls. The analysis further highlighted association of CRP, anti-CCP antibody, AST, ALT, AST/ALT ratio and bilirubin levels with AA+PCA group, whereas, IL-2R and ALP levels were associated with PCA group. Previous reports also associated high CRP, IL-2R levels with post chikungunya chronic arthritic patients [44, 45].

Genotypic analysis implicated protection of individuals with CRP-rs3091244-TC genotype against CHIKV infection, whereas, those with IL-2R rs743777-GA, G-allele and COMP rs144778694-GA genotypes were susceptible to such infection. Cis-acting CRP-rs3093059 and tri-allelic CRP-rs3091244 has been previously associated with higher CRP levels *in vitro*, thus suggested to have a functional role in transcription factor binding. Various genotypes of CRP-rs3091244 have been previously implicated for susceptibility towards CHIKV/DENV co/mono-infection, ankylosing spondylitis, haemorrhagic and ischaemic stroke among patients of eastern Indian, Turkish, Greek and Chinese origin, respectively [28-32]. Previous report suggested patients with IL-2R-rs743777-G allele to be susceptible to ankylosing spondylitis with peripheral arthritis [36]. Moreover, infected patients with CRP-rs3093059-CT, CRP-rs3091244-TT, IL-2R rs743777-GA and COMP rs144778694-AA genotypes might have been safeguarded from arthralgic manifestations; whereas, those with COMP rs144778694-GA genotype were susceptible to arthralgia. A previous study on Dutch patients indicated IL-2R variants to be protective against severe forms of multiple sclerosis and RA [46].

In this study, patients with various genotypes of CRP-rs3093059 and rs3091244, IL-2R-rs743777 and COMP-rs144778694 demonstrated significantly increased protein levels among chikungunya infected patients suffering from acute arthralgia with/without chronic arthritis. Such elevated CRP levels were previously reported among ankolysing spondylitic patients with certain genotypes of CRP rs3091244 and rs3093059 [29]. Indication for association with increased COMP levels was found with COMP genetic polymorphisms were previously documented among osteo-arthritic patients of Dutch origin [47].

4.5. CONCLUSION

In light of our findings, this study confirmed that both biomarker levels in serum and genetic factors played an important role in development of post-chikungunya chronic arthritis among infected patients. Upon diagnosis of CHIKV infected patients (with/without acute arthralgia), these markers could be used to screen and identify patients who might develop chronic arthritis. This might be helpful for clinical management of PCA patients by a medical specialist.

4.6. REFERENCES (CHAPTER 2)

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CHAPTER 3

Identification of specific genotypes CRP, TLR3, TLR7 & TLR8 polymorphisms associated with chikungunya and dengue co-infection

CHAPTER 3

Objective

- Identification of specific genotypes CRP, TLR3, TLR7 & TLR8 polymorphisms associated with chikungunya and dengue co-infection.

Publication:

- **Sengupta S**, Mukherjee S, Bhattacharya N, Tripathi A. Differential genotypic signatures of Toll-like receptor polymorphisms among dengue-chikungunya mono- and co-infected Eastern Indian patients. **European Journal of Clinical Microbiology & Infectious Diseases.** 2021 Jul;40(7):1369-1381. doi: 10.1007/s10096-020-04125-x. Epub 2021 Jan 25. PMID: 33495940.

- **Sengupta S**, Bhattacharya N, Tripathi A. Association of C-reactive protein polymorphisms with serum-CRP concentration and viral load among dengue-chikungunya mono/co-infected patients. **Antiviral Research.** 2022 Jan;197:105225. doi: 10.1016/j.antiviral.2021.105225. Epub 2021 Dec 13. PMID: 34915091.

5.1. INTRODUCTION

Toll-like receptors (TLRs), a class of proteins located on cell surface or endosome, that acts as first lines of defence in innate immune recognition of single stranded RNA viral genome and as pathogen sensors resulting in secretion of interferons, cytokines and chemokines viz. IFN (interferon)-β, IFN -α, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) [1]. The innate immune system is first line of defence against pathogens and diverse infectious agents and is responsible for cytokine storms promoting chronic inflammation [2]. Innate immunity against RNA viruses involves pattern recognition receptors (PRRs) that recognize structurally conserved molecules from diverse pathogens known as pathogen-associated molecular patterns (PAMPs). PRRs include TLRs (particularly TLR3, TLR7 and TLR8) that detect RNA viruses through their genomic RNA. Activation of TLRs induces downstream anti-viral type I IFN response, which can also occur independently of viral RNA transcription and replication [3]. TLR 3,7,8 specifically recognise presence of dengue and chikungunya viral genomic RNA within infected patient-body [3, 4]. However, genetic variations of these TLR genes might influence viral infectivity, thereby altering disease pathogenesis. TLR3 is located in chromosome number 4, whereas, TLR7 and 8 are located on X chromosome [5, 6]. Several polymorphisms of TLR3,7,8 genes viz. rs3775290 (TLR3), rs179008, rs179010, rs5741880, rs3853839 (TLR7), rs3764879, rs3764880 and rs5744080 (TLR8) have been reported to be associated with Hepatitis-B (HBV), COVID-19, Enterovirus, Influenza A, Hepatitis-C (HCV), Chikungunya, Dengue, HIV, Cytomegalo and Crimean–Congo Hemorrhagic fever virus infectivity among Indian, French, German, Spanish, Brazilian, Chinese, Japanese, Polish, Moroccan and Turkish patient populations [4, 7-23].

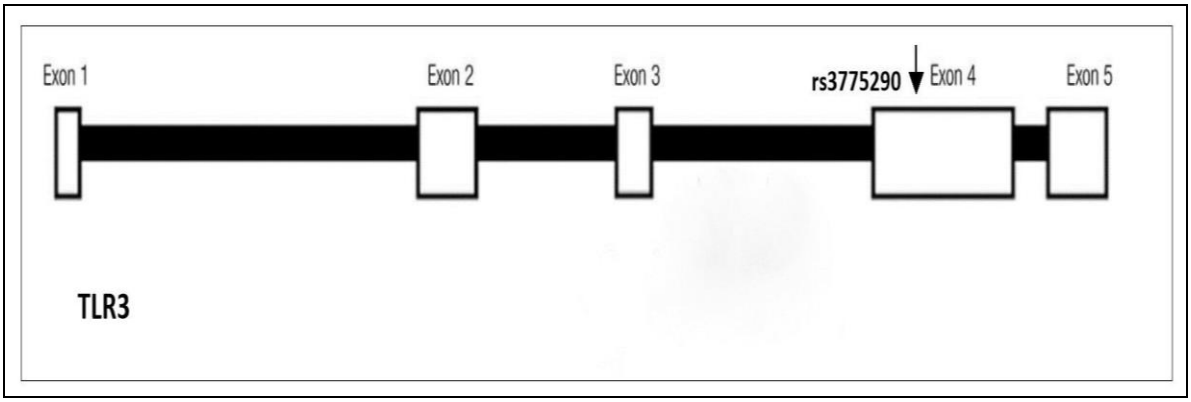

Fig 5.1: Schematic of the TLR3 gene representing the location of rs3775290.

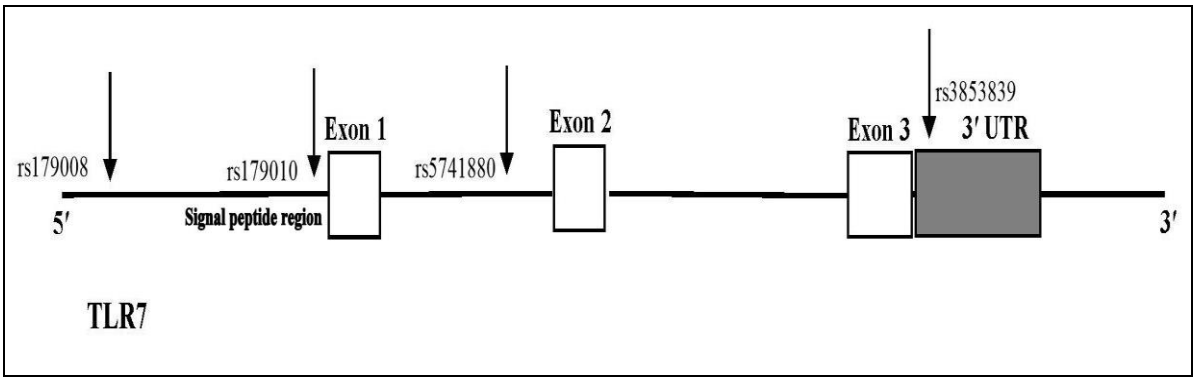

Fig 5.2: Schematic of the TLR7 gene representing the location of rs179008, rs179010, rs5741880 and rs3853839.

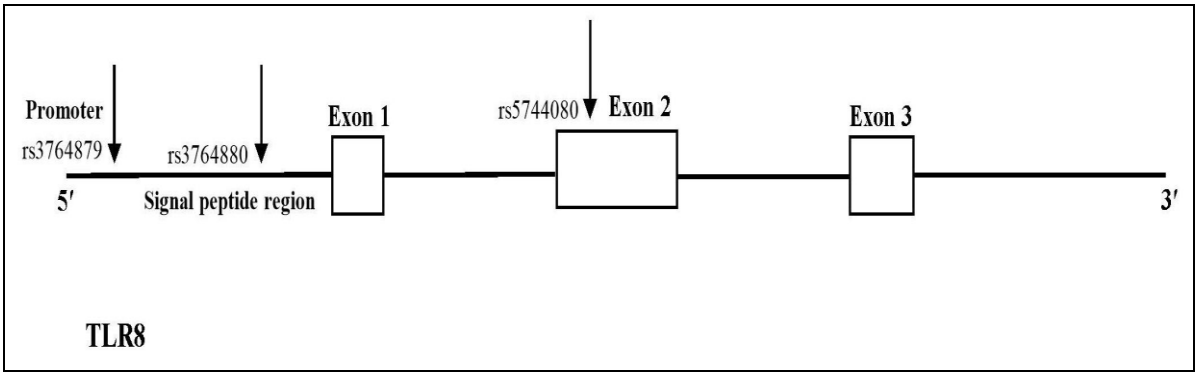

Fig 5.3: Schematic of the TLR8 gene representing the location of rs3764879, rs3764880 and rs5744080.

Non-synonymous polymorphism rs3775290, present in exon-4 of TLR-3 gene altered ecto-domain of the receptor, affecting ligand-receptor interaction [24] [Fig 5.1]. A study conducted by Deng et al. showed polymorphism at 3′UTR (rs3853839) of TLR-7 to be associated with elevated expression of the receptor in Systemic lupus erythematosus [25]. SNP rs179010 located at transcription factor binding site of TLR-7, reported to affect expression of TLR-7, thereby affecting production of associated cytokines among Chinese patients with chronic HBV-infection [21] [Fig 5.2]. Variations in genotype of rs3764879 within promoter region of TLR-8 gene have been reported to modulate patient's immune responses during HCV infection [22] [Fig 5.3]. G allele and A/G genotype of TLR8-rs3764880 has been related to HCV clearance and protection from HIV progression among infected patients [18, 26]. rs179008 and rs3764880 both are non-synonymous SNPs within signal peptide regions of TLR7 and TLR8, respectively, which led to single amino acid change [rs179008: Glycine (Q) 11 Leucine (L); rs3764880: Methionine (M) 1 Valine (V)] within protein sequence that might have damaging or neutral effect [23]. Mutations within signal peptide sequence have been associated with improper cleavage by signal peptidase enzyme which might lead to impaired TLR protein translocation [27, 28]. Genetic variations or SNPs of TLR genes might induce differential innate immune responses towards same pathogen, thereby affecting disease susceptibility and clinical outcome among infected individuals. But role of these TLR SNPs for susceptibility towards DENV and CHIKV co-infection among infected patients has not been previously explored.

C-reactive protein and dengue-chikungunya co-infection:

Upon pathogenic infection, variety of inflammatory cytokines is released, which leads to secretion of C-reactive protein (CRP) by liver [29]. CRP acts as scavenger protein by binding to damaged tissues, nuclear antigens and pathogens, thereby removing toxic

molecules and neutralizing invasive microorganisms [30]. It is a part of the innate immune system where it acts as a pattern recognition molecule to activate the adaptive immune response [29]. IL-6, IL-1β and TNF-α are also stimulated by CRP.

CRP promoter region has three cis-acting polymorphisms that contribute to variance in their CRP level, viz. rs3091244, rs3093059 and rs3093062 [31, 32] [Fig 5.4]. CRP-rs3091244 is a functionally important tri-allelic SNP at position -390 relative to start codon and rs3093062 which is an intronic microsatellite at position -409 are associated with CRP levels [33]. Specific genotypes of rs3091244 and rs3093059 have been associated with HBV-related hepatocarcinoma, stroke, ankylosing spondylitis, osteomyelitis and DENV susceptibility among Chinese, Turkish and Indian patients [30, 34-37]. However, rs3093062 is only CC homozygous in Bengali ethnicity of south Asian population (SAS) [Genome Wide Association Studies (GWAS) database]. Previous evaluation of rs3091244 and rs3093059 among North Indian population indicated maximum contribution of rs3093059 for CRP level variance [32]. CRP rs3091244-TT genotype has been reported to be positively associated with dengue susceptibility, whereas, rs3093059-CT and rs3091244-TT genotypes were found to be correlated with elevated CRP level among dengue infected patients [30].

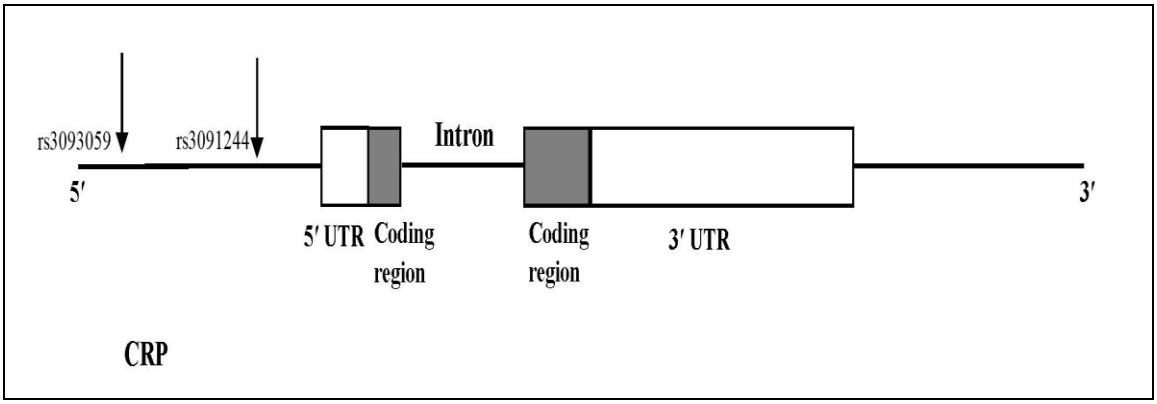

Fig 5.4: Schematic of the C-reactive protein gene representing the location of rs3093059 and rs3091244.

Thus, this study investigated role of TLR-3,7,8 and CRP polymorphisms towards dengue and chikungunya viral co-infection among eastern Indian patients. Moreover, this study elucidated *in silico* interactions between Q11L and M1V polymorphisms, located within TLR-7 and TLR-8 signal peptide regions with signal peptidase, SEC11.

5.2. MATERIALS AND METHODS:

5.2.1. Ethics statement:

Collection of blood from febrile patients and healthy participants as well as experiments were performed in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consents were obtained from patients and healthy individuals before their participation in the study.

5.2.2 Patients and healthy controls:

5ml of blood from all age groups and sexes were collected from each of 641 symptomatic febrile patients (acute phase: 1-3 days, critical phase: 4-6 days late phase: ≥7 days), visiting Calcutta School of Tropical Medicine, West Bengal, India from September 2014 to October 2016. Febrile patients exhibiting any two of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, nausea, vomiting, swollen glands, rash, haemorrhagic manifestation, fatigue were selected as per WHO criteria. Amongst them, 128 were co-infected with DENV and CHIKV, as detected by anti-DENV-IgM ELISA/dengue-NS1 ELISA (NIV, Pune, India)/real-time qRT-PCR and anti-CHIKV IgM (NIV, Pune, India)/real-time qRT-PCR, respectively. To identify secondary DENV/CHIKV infection, anti-DENV and anti-CHIKV IgG ELISA were performed. Patients with IgG/IgM ratio ≥1.1 were considered to be having secondary

infection [38]. To carry out age-matched case control study, blood from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with DENV, CHIKV, Japanese encephalitis, HIV and Hepatitis A/B/C infection - as tested by IgM ELISA/RT-PCR, were collected from same community. Symptomatic patients and healthy controls consisted of Bengali population of eastern India having similar ethnic genetic make up to that of Bengali Bangladeshis (BEB) group of SAS included within 1000 genome project of GWAS.

5.2.3 Extraction of viral RNA and determination of DENV and CHIKV load

Viral RNA was extracted from 140µL of patients' sera, using QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France). Genome presence and viral load of DENV and CHIKV was determined by real-time qRT-PCR using DENV and CHIKV Genesig kit (Primer Design Ltd., UK) respectively, according to manufacturer's protocol. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of positive control templates (provided within kits). Viral load in patient serum was calculated from corresponding Ct values. Limit of detection of both DENV and CHIKV Genesig kits was <100 copies of corresponding target genomes. Real-time PCR was performed on ABI Prism 7500 Fast instrument. Each sample was loaded in triplicate. Viral RNA titre ≥10,000 copies/ml and <10,000 copies/ml was considered as high viral load (HVL) and low viral load (LVL), respectively [4].

5.2.4. TLR and CRP single nucleotide polymorphism selection and genotyping:

SNPs in TLR and CRP genes, TLR3 (rs3775290), TLR7 (rs179008, rs5741880, rs179010, rs3853839), TLR8 (rs5744080, rs3764879, rs3764880) and CRP (rs3093059, rs3091244) of were selected based on their minor allele frequencies (MAF) and

previously reported associations with other viral infections [7, 23, 30]. SNP genotyping was carried out by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Based on sequences available in GenBank database, eleven sets of primer pairs were designed using Primer3 software, to amplify rs3775290 polymorphic region of TLR3; rs179008, rs5741880, rs179010, rs3853839 polymorphisms of TLR7, rs5744080, rs3764879, rs3764880 polymorphisms of TLR8 and rs3093059, rs3091244 of CRP [Table 5.1].

PCR reaction was performed in 20µl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5mM $MgCl_2$ and 20p.moles of previously mentioned primers. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at desired annealing temperature for 30 cycles and PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. Respective PCR products were digested with TaqI (Himedia, India), Bsh1285I, ER1381, MvaI, Eco130I, TaiI, Hin1II (Fermentas, USA), Hpy188I (NEB, UK), Tas I and Bfa I (Fermentas, USA) restriction enzymes accordingly and digested products were visualised on 2.5% agarose gel [Fig 5.6]. Differential RFLP patterns due to genotypic difference were validated by sequencing of respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA) [Fig 5.5].

Table 5.1: List of primers and restriction enzymes used in PCR RFLP

Sr. No.	Gene	SNP Id	5'-Primer-3'	PCR product Size	Restriction Enzymes	Restriction digestion temperature	Restriction digestion pattern
1.	TLR3	rs3775290	F-GGAGCACCTTAACATGGA R-GACCAAGGCAAAGGAGTT	367bp	TaqI	65º C	C : 367 bp T: 275 bp + 92 bp
2.	TLR7	rs5741880	F- CCTACTCAAGTACAAAGGGG R- TCCAGTCTCATGGTCACT	193bp	Hpy188I	37 º C	G : 193 bp T : 128 bp + 65 bp
3.	TLR7	rs179010	F-CCCAGTGTCTGTCATGCTAA R-CTGGCTCTTGTCTTGGATTG	335bp	BstNI	37 º C	T : 335 bp C : 190 bp + 145 bp
4.	TLR7	rs179008	F-CTTCTACCCTCTCGAAAGC R-TAGGAAACCATCTAGCCCC	342bp	ApoI	37 º C	A : 188 bp + 92 bp + 62 bp C : 188 bp + 154 bp
5.	TLR7	rs3853839	F-ACCAATTGCTTCCGTGTC R-TTTCTTCTCCCATCCTCCAG	515bp	Sty I	37 º C	G : 514 bp C : 97 bp + 418 bp
6.	TLR8	rs3764879	F-GTGTGTGTCTGATTTGGG R-TAGGCTCACACCATTTGC	386bp	MaeII	37 º C	G : 386 bp C : 161 bp + 225 bp
7.	TLR8	rs3764880	F-GTGTGTGTCTGATTTGGG R-TAGGCTCACACCATTTGC	386bp	NiaIII	65º C	A :137 bp + 156 bp + 93 bp G : 136 bp + 250 bp
8.	TLR8	rs5744080	F- GTTACCCCAAATACCCTCTG R-AAGCACCACCATCACAAG	416bp	MaeII	65º C	C : 237 bp + 179 bp T : 416 bp
9.	CRP	rs3093059	F-TATCCTGACTCCTGCCTG R-CCCATCTATGAGTGAGAACACG	224bp	TasI	65º C	C: 224bp T: 164bp + 60 bps
10.	CRP	rs3091244	F-AATGTGTCCATGGCTCTG R-AATGGGAAATGGTAACATATTAATC	214bp	Bfa I	37 º C	C/T: 214 bp A: 187 bp + 27 bp
11.	CRP	rs3091244	F- AATGTGTCCATGGCTCTG R- AATGGGAAATGGTAACATATTAATC	214bp	TaqI	65 º C	A/T: 214 bp C: 188 bp + 26 bp

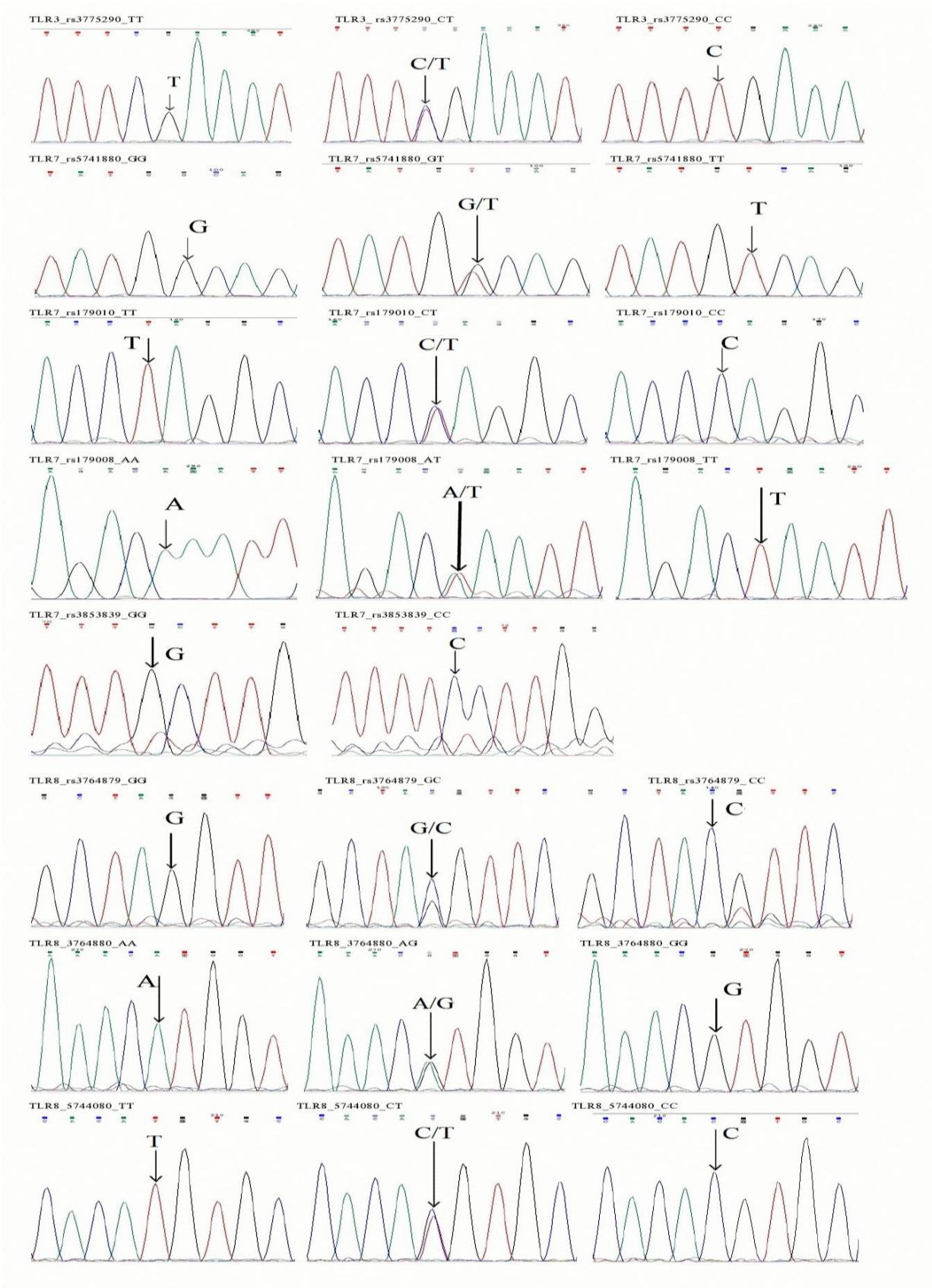

Fig 5.5: DNA sequencing of TLR SNPs.

5.2.5. Statistical analysis:

Association of any particular genotype of respective SNPs with disease susceptibility and specific symptoms (WHO-defined) was analysed using GraphPad Prism 9 software. Allelic and genotypic frequencies were compared between different study groups using Pearson's Chi square test for genotypic associations, *p*-values, odds ratio (OR) & minor allele frequency (MAF). A *p*-value of <0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analysed for SNPs with Haploview [39]. LDlink4.1.0 program of National Institute of Health (NIH) was used for Linkage disequilibrium pair (LDpair) measurements (D′) by using BEB population data (sharing same ethnicity with West Bengal population), present within 1000 genome project of GWAS, the largest public catalogue of human variation and genotype data. This algorithm investigated correlated alleles of TLR7 and TLR8 for a pair of variants in high LD.

5.2.6. Signal Peptide prediction:

The SignalP 5.0 server was used to predict signal peptide sequence and location of their cleavage sites in TLR3 (Accession no:NP_003256.1), TLR7 (NP_057646.1) and TLR8 (NP_619542.1) proteins, extracted from NCBI database [40].

5.2.7. Effects of genotypes on protein stability and pathogenicity:

Effect of amino acid alteration on protein stability was predicted by calculating change in Gibbs free folding energy (ΔG) at mutated site using CUPSAT server [41]. HOPE server was used to analyse effects of amino acid alterations on protein structure [42]. Pathogenic effects of point mutation were analysed using SIFT, PMUT, Polyphen2, MutationTaster, FATHMM, MetaSVM, PROVEAN, DIM, VarsomeClinical and Panther servers [43-48].

5.2.8. 3D-modelling of proteins and protein-protein docking

3D-models of wild type, Q11L and M1V forms of TLR7, TLR8 and Signal peptidase complex catalytic subunit SEC11 (SPC18) proteins were generated using QUARK server, which were used for *ab initio* protein structure prediction and 3D model construction from amino acid sequence only [50, 51]. Resultant structures were viewed using Pymol 2.3.2.

ClusPro 2.0 online server was used to analyze protein-protein interaction and measure binding free energy between wild type, Q11L and M1V forms of TLR7, TLR8 with SPC18. The server utilised electrostatic favoured, hydrophobic favoured and Vander Waal's forces to determine binding free energy; and represented findings in a balanced equation of all three combinations. Cluster scores generated by the server was represented in form of number of members interacting with each other, weighted energy score of the cluster center (structure that has highest number of neighbour structures in the cluster) and binding free energy score between the two proteins [51].

5.3. RESULTS:

In this study, genetic SNPs of CRP, TLR-3, TLR-7 and TLR8 genes were investigated among 128 DENV-CHIKV co-infected patients, collected during dengue-chikungunya outbreaks within 2014-2016, along with 157 age-sex matched healthy control volunteers having same ethnicity of Eastern India. Male to female ratio of co-infected patients was 1.44:1, whereas, that of control group was 1:1.01. Mean age of co-infected patients was 24.28±14.7 years (range: 2-60 years), whereas that of control was 36.2±11.8 years (range: 18–63 years). During blood collection, major symptoms among DENV-CHIKV co-infected patients were myalgia (55.11%), arthralgia (48.81%), headache (33.07%),

rash (14.96%), nausea (18.11%), persistent vomiting (26.77%), abdominal pain (11.02%), joint swelling (17.32%), bleeding (5.51%) and retro-orbital pain (4.72%).

5.3.1. Genotypic association of TLR SNPs with DENV-CHIKV co-infection:

Statistical analysis between co-infected and control populations revealed that for TLR7-rs179010, CC genotype was significantly positively associated (p=0.0346) and TC genotype was significantly negatively associated (p=0.0039) with co-susceptibility against DENV-CHIKV infection [Table 5.2]. Prevalence of TC genotype was more than three-fold among control population compared to infected patients. C-allele was found to be significantly associated with susceptibility towards both viruses. CC genotypes of TLR7-rs179008 and TLR7-rs3853839 were also significantly associated with DENV-CHIKV co-susceptibility. Compared to healthy individuals, frequency of CC genotypes of both rs179008 and rs3853839 was more than 4-8 folds among co-infected patients. C-alleles of rs179008 and rs3853839 were found to be significantly associated with viral susceptibility. Additionally, according to additive model; rs179008, rs179010 and rs3853839 were significantly associated with DENV-CHIKV co-susceptibility ($p < 0.0001$; 0.0052 and < 0.0001, respectively).

Similarly, CC genotypes of TLR8-rs3764879 and TLR8-rs5744080 demonstrated significant association with viral co-susceptibility (p=0.004 and p=0.001, respectively). C-allele of rs3764879 exhibited significant association with co-susceptibility. Conversely, AG genotype of rs3764880 and CT genotype of rs5744080 were significantly related with decreased risk of DENV-CHIKV co-infection [Table 5.2]. Additive model suggested rs3764879, rs5744080 and rs3764880 of TLR8 to be significantly associated with susceptibility against DENV and CHIKV ($p =0.0021$; < 0.0001 and 0.0002, respectively).

Table 5.2: Genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among DENV-CHIKV co-infected patients and healthy controls.

SNP Ref. No.	Chromosome Location	Genotype and allele distribution	Healthy Controls (%)	Dengue-Chikungunya Co-infected patients (%)	OR (95% C.I)	p-value
			n=157	**n=127**		
		CC	5.09	11.02	2.3075 [0.9359-5.6893]	0.07541
		TT	33.75	38.58	1.2327 [0.7575-2.0059]	0.4557
TLR 3 rs3775290	Chr4:186083063 (GRCh38.p12)	CT	61.14	50.39	Ref	
		C allele	35.66	36.22	1.0243 [0.6291-1.6676]	0.9300
		T allele	64.33	63.77	Ref	
		Additive	0.0788			
		MALE	**n=78**	**n=75**		
		CC	6.41	10.67	1.7969 [0.5598-5.7681]	0.3906
		TT	28.20	40	1.7759 [0.9009-3.5008]	0.1230
		CT	65.38	45.33	ref	
		FEMALE	**n=79**	**n=53**		
		CC	3.79	11.32	3.1667 [0.7561-13.2622]	0.1577
		TT	39.24	35.84	0.8406 [0.4099-1.7238]	0.7166
		CT	56.96	54.71	ref	
			n=157	**n=126**		
		TT	12.74	12.69	0.9964 [0.4929-2.014]	1.0000
TLR 7 rs5741880	ChrX:12869297 (GRCh38.p12)	GT	11.46	5.55	0.4542 [0.1834-1.125]	0.0942
		GG	75.79	81.74	0.5426	0.0592

SNP Ref. No.	Chromosome Location	Genotype and allele distribution	Healthy Controls (%)	Dengue-Chikungunya Co-infected patients (%)	OR (95% C.I)	p-value
					[0.2880-1.022]	
		G allele	81.52	84.52	Ref	
		T allele	18.47	15.47	0.7912 [0.4295 to 1.455]	0.5252
		Additive	0.2145			
		MALE	**N=78**	**N=74**		
		T	25.64	12.16	0.5613 [0.2521-1.250]	0.1689
		G	74.35	87.83	Ref	
		FEMALE	**N=79**	**N=52**		
		TT	0	13.46	13.79[1.671-113.7]	**0.0030***
		GT	22.78	13.46	0.3780 [0.141- 1.012]	0.0746
		GG	77.21	73.07	Ref	
TLR 7 rs179010	ChrX:12884766 (GRCh38.p12)		**n=157**	**n=127**		
		CC	30.57	43.30	1.735 [1.059 to 2.779]	**0.0346***
		TC	17.83	6.29	0.3097 [0.1442 to 0.7086]	**0.0039***
		TT	51.5	50.39	Ref	
		C allele	30.89	46.45	1.952 [1.198 to 3.124]	**0.0095***
		T allele	69.10	53.54	Ref	
		Additive				**0.0052***
		MALE	**N=78**	**N=75**		
		C	48.71	38.66	0.6636 [0.3489 to 1.262]	0.2545
		T	51.28	61.33	ref	
		FEMALE	**N=79**	**N= 52**		
		CC	12.65	50	6.900 [2.926 to 16.27]	< **0.0001***
		TC	35.44	15.38	0.3312 [0.1369 to	**0.0159***
		TT	51.89	34.61		

SNP Ref. No.	Chromosome Location	Genotype and allele distribution	Healthy Controls (%)	Dengue-Chikungunya Co-infected patients (%)	OR (95% C.I)	p-value
					0.8011] ref	
TLR 7 rs179008	ChrX:12885540 (GRCh38.p12)		**n=157**	**n=128**		
		CC	4.45	22.66	6.2771 [2.6469-14.8859]	< **0.0001***
		AC	3.82	4.69	1.2377 [0.3894-3.9343]	0.7727
		AA	91.71	72.66	Ref	
		C allele	6.37	25	4.900 [2.381 to 9.927]	< **0.0001***
		A allele	93.63	75	Ref	
		Additive	**< 0.0001***			
		MALE	**N=78**	**N=72**		
		C	3.84	23.61	7.727 [2.157 to 27.68]	**0.0005***
		A	96.15	76.38	ref	
		FEMALE	**N=79**	**N=56**		
		CC	5.06	21.42	5.114 [1.553-16.83]	**0.0058***
		AC	7.59	10.71	1.460 [0.4223 to 5.028]	0.5533
		AA	87.34	67.85	ref	
TLR 7 rs3853839	ChrX:12889539 (GRCh38.p12)		**n=157**	**n=126**		
		CC	22.29 35	36.50 46	0.4989[0.2998 to 0.8442]	**0.0117***
		GC	0	0	-	-
		GG	77.7 122	63.49 80	Ref	
		C allele	13.37	40.87	4.463 [2.498 to 7.914]	< **0.0001***
		G allele	86.62	59.12	Ref	
		Additive	**< 0.0001***			
		MALE	**N=78**	**N=70**		
		C	3.84	45.71	21.05 [6.103 to 67.58]	**<0.0001**
		G	96.15	54.28	ref	
		FEMALE	**N=79**	**N=56**		0.0721
		CC	5.06	25	2.300 [0.9372-5.645]	0.0549

SNP Ref. No.	Chromosome Location	Genotype and allele distribution	Healthy Controls (%)	Dengue-Chikungunya Co-infected patients (%)	OR (95% C.I)	p-value
		GC	35.44	19.64	0.4452 [0.1991-0.9955]	
		GG	59.49	55.35	ref	
TLR8 rs3764879	ChrX:12906578 (GRCh38.p12)		**n=157**	**N=126**		
		CC	22.29	40.47	2.370 [1.419 to 3.916]	**0.0011***
		GC	9.55	11.11	1.183 [0.5714 to 2.624]	0.6969
		GG	68.15	48.41	Ref	
		C allele	27.07	46.03	2.315 [1.393 to 3.800]	**0.0011***
		G allele	72.92	53.96	Ref	
		Additive	**0.0021***			
		MALE	**N=78**	**N=70**		
		C	28.20	42.85	1.909[0.9635-3.783]	0.0843
		G	71.79	57.14	Ref	
		FEMALE	**N=79**	**N=56**		
		CC	16.45	37.5	3.046 [1.363-6.808]	**0.0084***
		GC	18.98	25	1.422 [0.6226-3.249]	0.4054
		GG	64.55	37.5	ref	
TLR8 rs3764880	ChrX:12906707 (GRCh38.p12)		**n=157**	**n=127**		
		AA	28.66	26.77	0.9198 [0.5510 to 1.563]	0.7908
		AG	23.56	6.29	0.2199 [0.1035 to 0.4773]	< **0.0001***
		GG	47.77	66.14	Ref	
		A allele	40.44	30.15	0.6374 [0.3879 to 1.055]	0.0814
		G allele	59.55	69.84	Ref	
		Additive	**0.0002***			
		MALE	**N=78**	**N=70**		
		A	49.36	27.14	0.3725[0.1933 to 0.7582]	**0.0067***

SNP Ref. No.	Chromosome Location	Genotype and allele distribution	Healthy Controls (%)	Dengue-Chikungunya Co-infected patients (%)	OR (95% C.I)	p-value
		G	49.36	72.85	ref	
		FEMALE	**N=79**	**N=57**		
		AA	7.59	26.31	4.345 [1.566-12.05]	**0.0038***
		AG	46.83	14.03	0.1853 [0.07776-0.4417]	< **0.0001***
		GG	45.56	59.64	ref	
TLR8 rs5744080	ChrX:12919685 (GRCh38.p12)		**N=157**	**N=126**		
		CC	0	26.19	57.15 [9.631 to 588.5]	**<0.0001***
		CT	27.38	7.14	0.2039 [0.09504-0.4376]	< **0.0001***
		TT	72.61	66.66	Ref	
		C allele	13.69	29.76	3.945[2.258 to 6.864]	**0.0009***
		T allele	86.30	70.23	Ref	
		Additive	**< 0.0001***			
		MALE	**N=78**	**N=74**		
		C	12.82	31.08	3.067 [1.342-7.009]	**0.0099***
		T	87.17	68.91	ref	
		FEMALE	**N=79**	**N=52**		
		CC	0	19.23	20.25 [2.529-162.1]	**0.0002***
		CT	29.11	17.30	0.5096 [0.2141-1.213]	0.1484
		TT	70.88	63.46	ref	

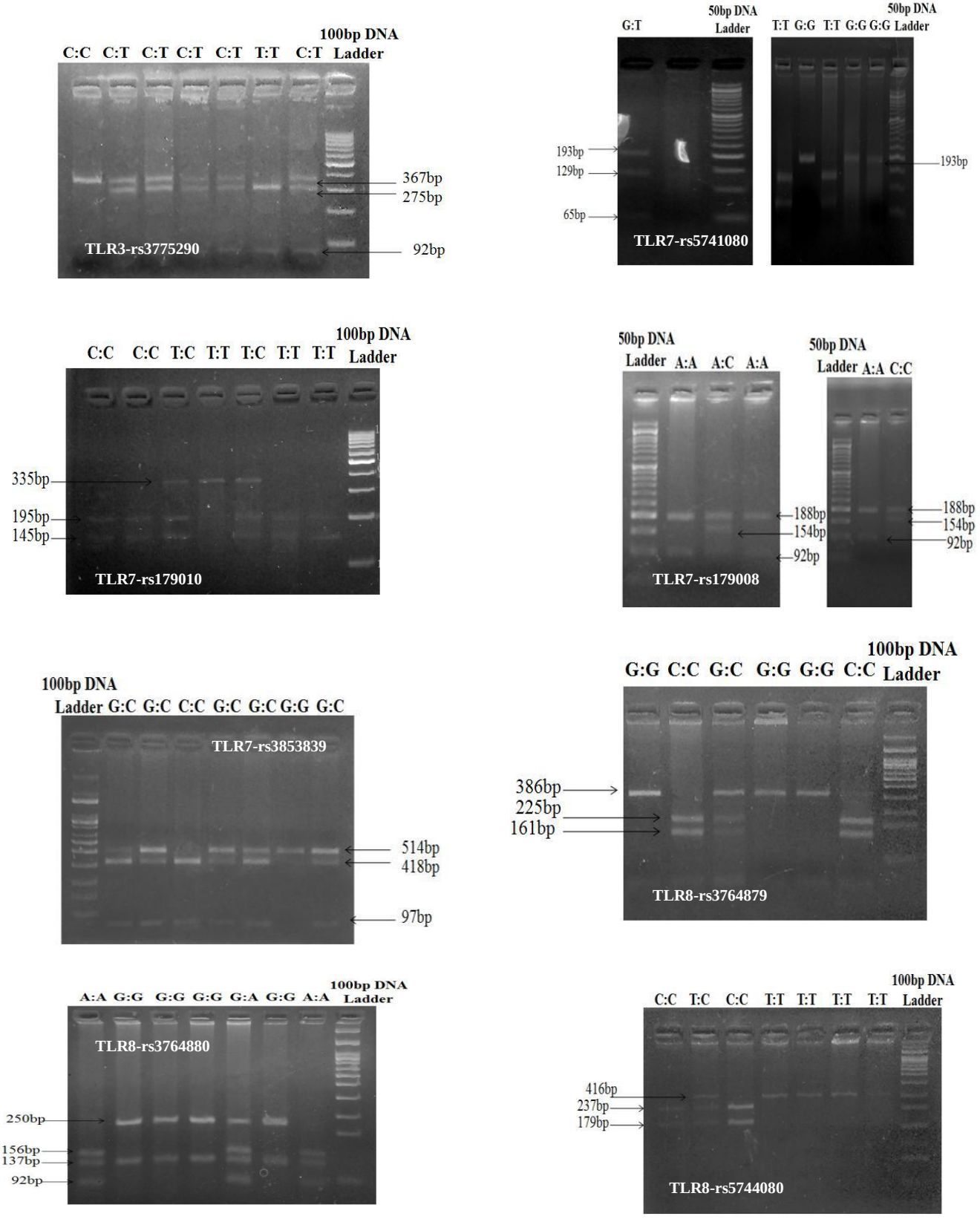

Fig 5.6: Resolution of PCR-RFLP products of TLR 3, 7 and 8.

TLR7,8 genes are located on X chromosome; as a result, there might be gender biasness for these SNPs. In TLR7, TT genotype of rs5741880, CC genotypes of rs179010 and rs179008 were significantly higher among female patients than that among control group; TT genotype of rs5741880 was completely absent among healthy females; CC genotypes of rs179010 and rs179008 were 2-3 times more prevalent among DENV-CHIKV female patients compared to healthy group. On the contrary, TC genotype of rs179010 was 2-fold more prevalent among control females compared to co-infected group [Table 5.2]. C allele of rs179008 was significantly more prevalent (6-fold) among infected males compared to control group. In TLR8, CC genotypes of rs3764879 and rs5744080; AA genotype of rs3764880 were significantly more widespread (2-3 folds) among co-infected females with respect to healthy population. CC genotype of rs5744080 was absent among healthy females. AG genotype of rs3764880 was 3-fold more prevalent among healthy females compared to diseased patients. Among males, A-allele of rs3764880 was significantly higher (5-fold) in control population, whereas, C allele of rs5744080 was more prevalent (2-fold) among co-infected patients.

All SNPs selected in this study followed Hardy-Weinberg equilibrium at $p>0.05$ barring one SNP of TLR7-rs3853839. However, data of rs3853839 was incorporated as its genotypic distribution might arise by chance or could reflect a significant association with various factors. The LDpair measurements among SNPs of TLR7 revealed correlation between alleles of following pairs with p-value:<0.01:rs179010(C) with rs3853839(C); rs179010(T) with rs3853839(G); rs5741880(G) with rs179010(C); rs5741880(T) with rs179010(T) [Table 5.3]. In case of SNPs of TLR8, correlation between alleles of following pairs with p-value:<0.01 were: rs3764879(C) with rs3764880(A); rs3764879(G) with rs3764880(G); rs3764879(C) with rs5744080(C); rs3764879(G) with rs5744080(T); rs3764880(A) with rs5744080(C) and rs3764880(G) with rs5744080(T).

Table 5.3: LDpair measurements among SNPs of TLR7 and TLR8.

GENE	Query SNP's	D' (Linkage disequilibrium)	Pairwise LD (R^2)	P value	Outcome
TLR7	rs179010 and rs179008	0.6389	0.0422	0.0191	rs179010 and rs179008 are in linkage equilibrium
	rs179010 and rs3853839	0.4444	0.1635	**<0.0001**	**rs179010(C) allele is correlated with rs3853839(C) allele rs179010(T) allele is correlated with rs3853839(G) allele**
	rs179008 and rs3853839	0.5	0.0312	0.0438	rs179008 and rs3853839 are in linkage equilibrium
	rs5741880 and rs179010	0.8879	0.1155	**0.0001**	**rs5741880(G) allele is correlated with rs179010(C) allele rs5741880(T) allele is correlated with rs179010(T) allele**
	rs5741880 and rs179008	1.0	0.0152	0.1605	rs5741880 and rs179008 are in linkage equilibrium
	rs5741880 and rs3853839	0.875	0.0928	0.0005	rs5741880 and rs3853839 are in linkage equilibrium
TLR8	rs3764879 and rs3764880	1.0	0.9689	**<0.0001**	**rs3764879(C) allele is correlated with rs3764880(A) allele rs3764879(G) allele is correlated with rs3764880(G) allele**
	rs3764879 and rs5744080	0.6533	0.3081	**<0.0001**	**rs3764879(C) allele is correlated with rs5744080(C) allele rs3764879(G) allele is correlated with rs5744080(T) allele**
	rs3764880 and rs5744080	0.6579	0.3225	**<0.0001**	**rs3764880(A) allele is correlated with rs5744080(C) allele rs3764880(G) allele is correlated with rs5744080(T) allele**

5.3.2. Distribution of dengue and chikungunya viral load among co-infected patients with differential TLR genotypes:

DENV-HVL was significantly more prevalent among co-infected patients with TT genotype of TLR7-rs179010, whereas, significantly higher frequency of CHIKV-HVL was detected among patients with AA genotype of rs179008 [Table 5.4]. On the

contrary, dengue low viral load (LVL) was significantly higher among DENV-CHIKV patients with CC genotypes of rs179010 and rs179008, AA and GC genotypes of rs179008 and rs3853839, respectively. Similarly, CHIKV-LVL was more prevalent among patients with CC genotypes of rs179010 and rs179008.

In TLR8, co-infected patients with GG genotypes of rs3764879 and rs3764880 exhibited CHIKV-HVL. Patients with CC genotypes of rs3764879 and rs5744080 demonstrated CHIKV-LVL, whereas, those with CT genotype of rs5744080 demonstrated DENV-LVL [Table 5.4].

Among DENV-CHIKV co-infected patients, dengue HVL was significantly more prevalent within patients with CRP-rs3093059-TT genotype (p=0.0331), whereas, significant association was observed between CT genotype and occurrence of dengue LVL (p=0.0143). Similarly, dengue HVL was more prevalent among patients with CRP-rs3091244-CT genotype (p=0.0006), whereas, LVL of dengue was significantly associated with TT genotype (p=0.0207). Furthermore, CHIKV-HVL was more prevalent among co-infected patients with rs3091244-CC genotype (p=0.0313), whereas, CHIKV-LVL was significantly high among those with rs3091244-CT genotype (p=0.0278). Percent prevalence of various dengue serotypes among co-infected patients was as follows: DENV2 (60.52%)>DENV1 (26.31%)>DENV4 (10.52%)>DENV3 (2.63%). But, no specific DENV serotype was significantly associated with any rs3093059 and rs3091244 genotypes.

Table 5.4: Distribution of dengue and chikungunya viral load among co-infected patients with different TLR and CRP genotypes.

	Dengue Viral Load				Chikungunya Viral Load			
N=87			**Odds Ratio (O.R.)**	***p*-value at 95% C.I**			**Odds Ratio (O.R.)**	***p*-value at 95% C.I**
TLR 3-rs3775290	HVL	LVL			HVL	LVL		
CC (N=11)	7	4	0.6694 [0.1808-2.478]	0.7477	5	6	1.200[0.3372-4.271]	1.000
TT (N=38)	22	16	0.8211 [0.3499-1.932]	0.6706	20	18	0.7962 [0.3407-1.861]	0.6681
CT (N=38)	19	19	1.450 [0.6173-3.406]	0.5147	18	20	1.157 [0.4955-2.703]	0.8298
TLR 7 rs5741880	HVL	LVL			HVL	LVL		
TT (N=12)	9	3	0.3810 [0.09551-1.520]	0.2159	6	6	0.8750 [0.2585-2.962]	1.0000
GT (N=6)	4	2	0.6250 [0.1082-3.609]	0.6922	1	5	4.878 [0.5452-43.64]	0.2070
GG (N=69)	36	33	2.383 [0.7663-7.413]	0.1827	34	35	0.6551 [0.2272-1.889]	0.5969
TLR 7 rs179010	HVL	LVL			HVL	LVL		
CC (N=18)	4	14	0.1524 [0.04513-0.5146]	**0.0014***	3	15	6.129 [1.625-23.12]	**0.0039***
TC (N=6)	2	6	1.650 [0.4627-5.884]	0.5230	5	3	0.5023[0.1122-2.248]	0.4671
TT (N=61)	43	18	0.1256[0.04326-0.3645]	**<0.0001***	33	28	0.3771 [0.1425-0.9983]	0.0612
TLR 7 rs179008	HVL	LVL			HVL	LVL		
CC (N=24)	4	20	4.265 [1.307-13.92]	**0.0137***	9	15	3.106 [1.171-8.239]	**0.0287***
AC (N=6)	2	4	1.240 [0.2142-7.178]	1.0000	3	3	1.382 [0.2627-7.273]	0.6963
AA (N=57)	27	30	0.2778 [0.09868-0.7819]	**0.0193***	38	19	0.3333 [0.1335-0.8323]	**0.0228***
TLR 7 rs3853839	HVL	LVL			HVL	LVL		
CC (N=21)	13	8	0.7385 [0.2702-2.018]	0.6198	8	13	1.625 [0.5951-4.437]	0.4529
GC (N=11)	2	9	7.293 [1.471-36.16]	**0.0088***	5	6	1.080 [0.3034-3.844]	1.0000
GG (N=55)	34	21	0.5450 [0.2255-1.317]	0.1879	28	27	0.6598 [0.2732-1.593]	0.3817
TLR8 rs3764879	HVL	LVL			HVL	LVL		
CC (N=21)	15	6	0.4800 [0.1657-1.391]	0.2092	3	18	8.667 [2.321-32.36]	**0.0004***
GC (N=12)	5	7	2.221 [0.6436-7.662]	0.2222	5	7	1.363 [0.3969-4.682]	0.7591
GG (N= 54)	31	23	1.141 [0.4722-2.759]	0.8250	34	20	0.1882 [0.07141-0.4962]	**0.0008***
TLR8 rs3764880	HVL	LVL			HVL	LVL		

	Dengue Viral Load				Chikungunya Viral Load			
N=87			**Odds Ratio (O.R.)**	***p*-value at 95% C.I**			**Odds Ratio (O.R.)**	***p*-value at 95% C.I**
AA (N=22)	13	9	1.038 [0.3880-2.779]	1.0000	6	16	3.111 [1.080-8.959]	**0.0471***
AG (N=7)	5	2	0.5697 [0.1041-3.117]	0.6968	1	6	6.000 [0.6903-52.15]	0.1141
GG (N=58)	34	24	1.155 [0.4269-2.882]	0.8194	34	24	0.2246 [0.08275-0.6095]	**0.0030***
TLR8 rs5744080	HVL	LVL			HVL	LVL		
CC (N=15)	9	6	0.8333 [0.2684-2.588]	0.7838	2	13	6.500 [1.367-30.91]	**0.0102***
CT (N=9)	2	7	5.306 [1.033-27.25]	**0.0379***	4	5	0.9659 [0.2408-3.875]	1.0000
TT (N=63)	38	25	0.5567 [0.2156-1.437]	0.2381	32	31	0.3229 [0.1132-0.9209]	0.0517
CRP rs3093059	HVL	LVL			HVL	LVL		
CC	2	0	1.833 [0.2625 to 24.46]	>0.9999	2	0	3.525 [0.5024 to 46.62]	0.3406
CT	11	15	0.2833 [0.1163 to 0.7193]	**0.0143***	12	14	0.9458 [0.3786 to 2.288]	>0.9999
TT	42	17	2.851 [1.167 to 7.339]	**0.0331***	27	32	0.8438 [0.3582 to 1.982]	0.8191
CRP rs3091244	HVL	LVL			HVL	LVL		
TT	3	8	0.1950[0.05346 to 0.7451]	**0.0207***	6	5	1.086 [0.3339 to 3.895]	>0.9999
CT	27	5	6.023[2.120 to 15.76]	**0.0006***	13	19	0.3611 [0.1493 to 0.9020]	**0.0278***
CC	23	21	2.107[0.8752 to 4.901]	0.1248	30	14	2.643 [1.115 to 6.264]	**0.0313***

5.3.3. Functional effects of TLR3-rs3775290, TLR7-rs179008 and TLR8-rs3764880 SNPs:

TLR3-rs3775290, CRP-rs309359 and CRP-rs3091244 polymorphisms does not result in any amino acid changes. VarsomeClinical server predicted all these SNPs to be benign in nature. On the other hand, TLR7-rs179008 and TLR8-rs3764880 were predicted to be pathogenic and damaging in nature, respectively. To determine change in protein stability of polymorphic variants of TLR7-rs179008 and TLR8-rs3764880, ΔΔG values were analysed by CUPSAT. In TLR7-rs179008, structural change due to Q11L alteration was stabilising with favourable torsion angles and ΔΔG (kcal/mol) value of 8.85. But, in TLR8-rs3764880, structural change due to M1V alteration was destabilising in nature

with unfavourable torsion angles and ΔΔG value of -0.71. TLR7-rs179008 SNP was also predicted to be tolerated by SIFT server, whereas, TLR8-rs3764880 was implicated to be damaging. While, MutationTaster application indicated TLR8-rs3764880 to be automatic polymorphism (harmless), Panther server predicted TLR7-rs179008 to be possibly damaging. HOPE server indicated TLR7-rs179008 L variant, lying within signal peptide region to be smaller and more hydrophobic than wild-type (Q); this might lead to loss of interaction, loss of hydrogen bonding, incorrect folding and disturbance in recognition of signal peptide. In case of TLR8-rs3764880, HOPE indicated V variant, lying within signal peptide region to be smaller and thus, might disturb recognition of signal peptide leading to loss of interaction and protein activity disruption. DIM-pred server predicted transition of Q11L SNP to be "order to disorder" in nature (Table 5.5).

Table 5.5: Predicted effect of non-synonymous TLR polymorphisms on protein structure.

Gene	**SNP ref no.**	**AA change**	**Functional effect**						
			CUPSAT	**SIFT prediction**	**Polyphen2 prediction**	**Mutation Taster prediction**	**DIM-Pred**	**PMut**	**Panther**
TLR7	rs179008	Gly(Q)11(L) leu	Stabilizing with favorable torsion angles	Tolerated	Benign	Neutral	Order to Disorder	Neutral	Possibly damaging
TLR8	rs3764880	Met(M)1(V) val	Destabilizing with unfavorable torsion angles	Damaging	Benign	Polymorphism automatic	Order to order	Neutral	Probably benign

5.3.4. Molecular docking of polymorphic variants of TLR3, TLR7, TLR8 with SPC18:

SignalP 5.0 server predicted polymorphisms of TLR3 and CRP to be away from cleavage site leading to no interaction with Signal peptide. As a result, docking with SPC18 was not done. In case of TLR7, SignalP 5.0 server predicted cleavage site between amino acids 26 and 27 for Signal peptidase I (SPI). Correspondingly, for TLR8, the server predicted cleavage site between positions 22 and 23 using SPI [Fig 5.7].

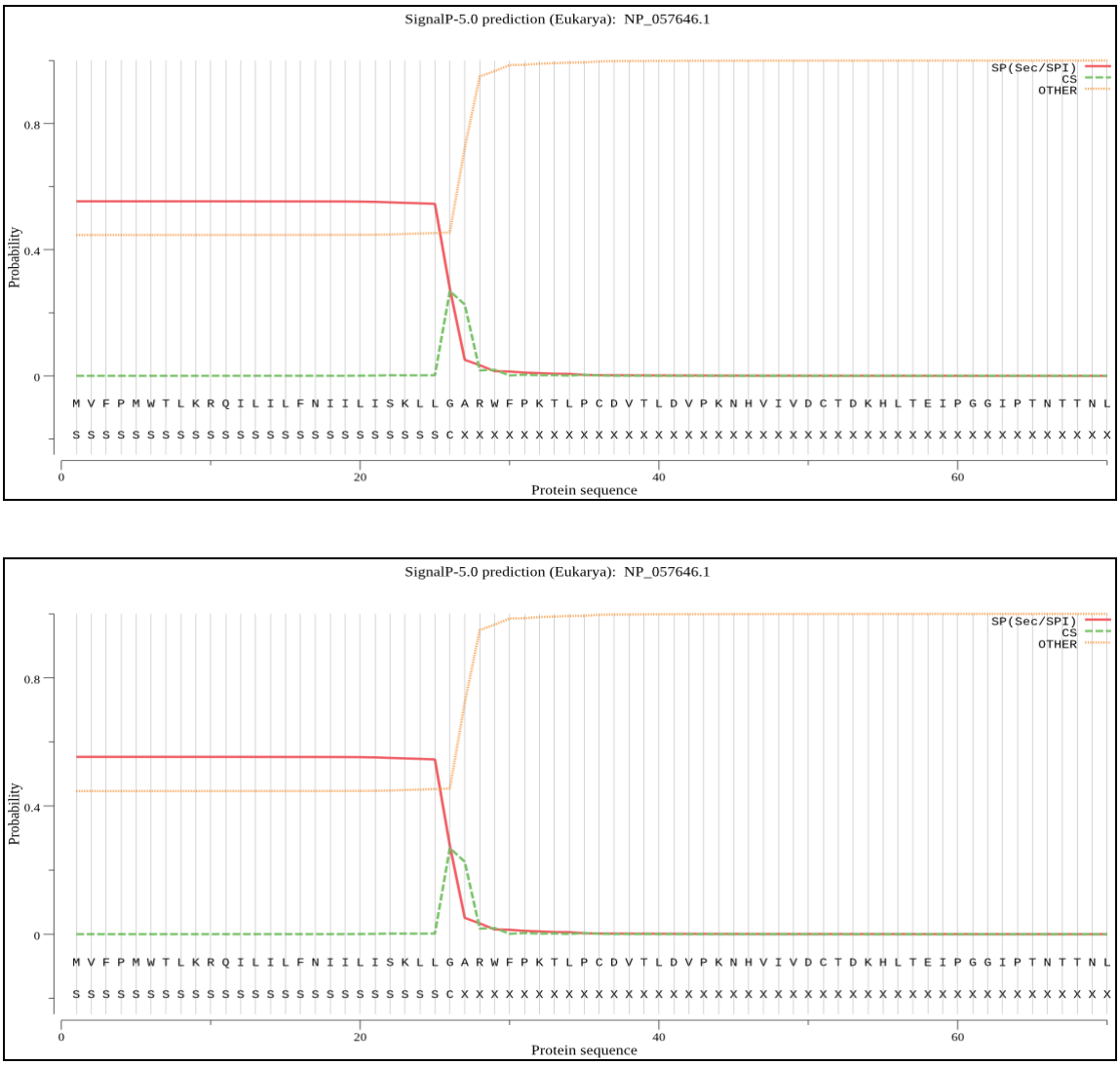

Fig 5.7: Graphical representation of cleavage site of Signal peptidase complex within TLR7 and 8 gene.

3D-models of SPC18 protein, Q and L variants of TLR7-rs179008, M and V variants of TLR8-rs3764880 were generated using QUARK server. Docking of TLR7 Q variant with SPC18 using ClusPro 2.0 generated a model with 104 amino acids interacting with each other along with centred weighted score of -925.2 and overall binding free energy of -1115.7kcal/mol; whereas, for L variant, interaction of 81 amino acids were observed between the two molecules with centre weighted score of -962.2 and overall binding free energy of -1080.8kcal/mol [Fig 5.8]. Congruently, docking of TLR8 M variant with SPC18 involved interaction of 113 amino acids with centred weighted score of -958.9 and binding energy of -975kcal/mol. Whereas, interaction of V variant with SPC18 involved 87 amino acids, yielding a binding free energy of -1014.9kcal/mol and -834.7 weighted score [Table 5.6].

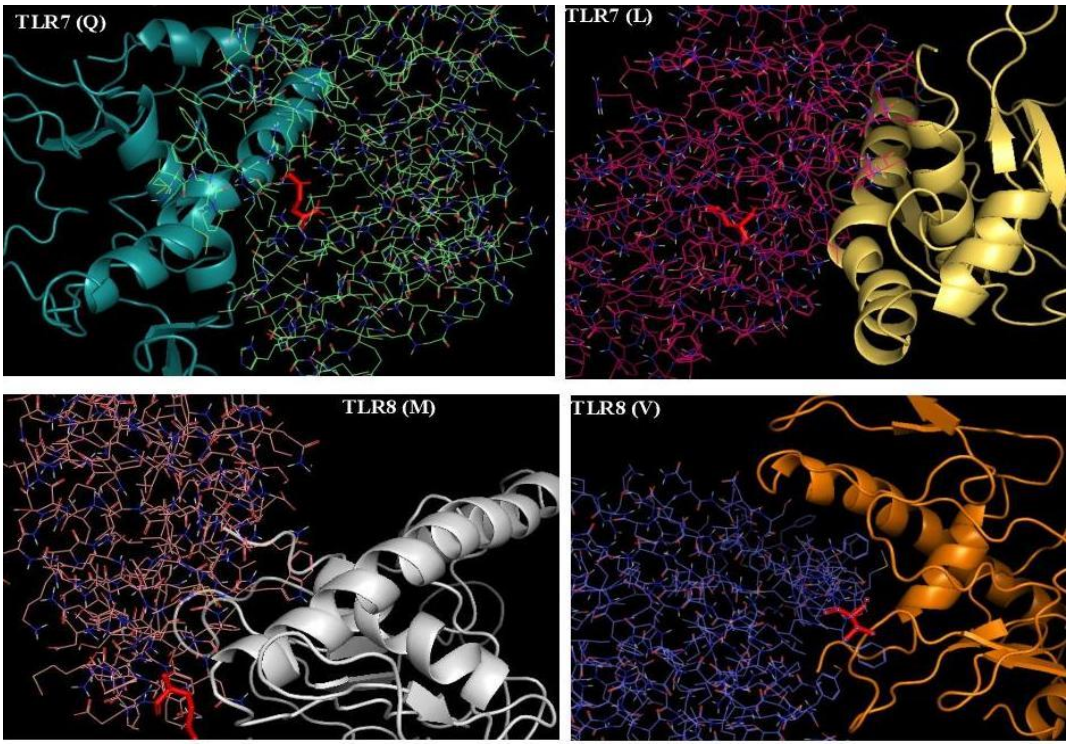

Fig 5.8: Docking results of polymorphic variants of TLR7 and TLR8 with SPC18. TLR7 and 8 proteins are represented by sticks, signal peptidase complex (SPC18) represented by cartoons and red-coloured stick represents variants within the signal peptide region of TLR7 and 8 proteins.

Table 5.6: Docking analysis of polymorphic variants of TLR7 and TLR8 with SPC18.

	Variant	Members involved	Weighted Score	Lowest Energy (kcal/mol)
TLR7-rs179008	Gly(Q)	104	-925.2	-1115.7
	(L)leu	81	-962.2	-1080.8
TLR8-rs3764880	Met(M)	113	-958.9	-975
	(V)val	87	-834.7	-1014.9

5.3.5. Genotypic association of CRP SNPs with DENV-CHIKV co-infection:

Statistical analysis between infected and control populations revealed CRP-rs3093059-CT genotype was positively associated with DENV-CHIKV co-infection (p=0.0378) with >1 OR and relative risk [Table 5.7]. Prevalence of CT genotype was around two-fold more among DENV-CHIKV co-infected patients compared to control population. Furthermore, CRP-rs3091244-TT genotype was significantly associated with both DENV-CHIKV co-infection (p=0.0138), with more than three-fold increase in its frequency among infected groups; >1 OR and relative risk among co-infected groups. T-allele of rs3091244 was found to be significantly associated with co-infectivity (p=0.0221) and >1 OR and relative risk. Additionally, according to additive model, rs3091244 was significantly associated with DENV-CHIKV co-infection and susceptibility (p= 0.0286).

Table 5.7: Genotypic and allelic distribution of CRP polymorphisms among DENV-CHIKV co-infected patients and healthy controls

SNP Ref. No.	Genotype and allele distribution	Healthy Controls	Dengue-Chikungunya Co-infected patients		
		n=102	**n=128**	**OR (95% C.I)**	**Relative risk (95% C.I)**	**p-value**
CRP rs3093059	CC	2	2	0.7937 [0.1228 to 5.142]	0.8850 [0.5015 to 2.969]	>0.9999
	CT	21	43	1.951 [1.081 to 3.599]	1.487 [1.039 to 2.223]	**0.0378***
	TT	79	83	Ref		
	C allele	25	47	0.6211 [0.3605 to 1.038]	0.7526 [0.5284 to 1.025]	0.0927
	T allele	179	209	Ref		
	Additive	0.0915				
CRP rs3091244		**n=102**	**n=128**			
	TT	3	15	4.381 [1.285 to 14.54]	2.802 [1.172 to 8.066]	**0.0138***
	TC	39	51	1.070 [0.6388 to 1.810]	1.038 [0.7757 to 1.412]	0.8039
	TA	0	1			
	CC	60	61	Ref		
	T allele	45	81	1.635 [1.063 to 2.465]	1.333 [1.040 to 1.746]	**0.0221***
	C allele	159	175	Ref		
	Additive	**0.0337***				

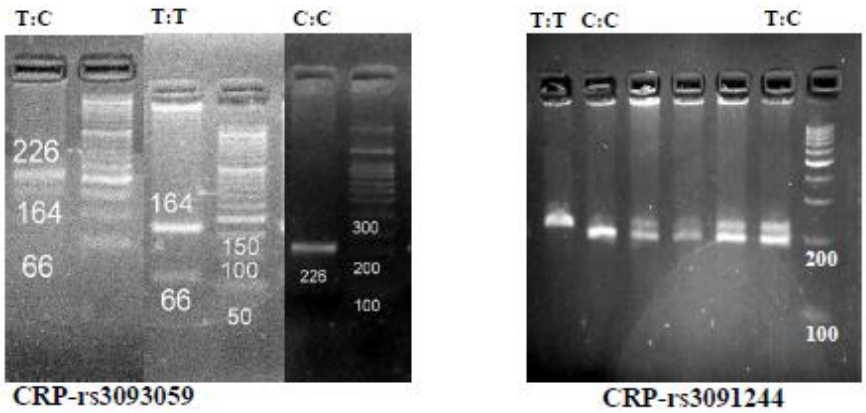

Fig 5.9: Resolution of PCR-RFLP products of CRP SNPs

5.3.6. Genotypic association of CRP polymorphisms of co-infected patients with WHO-defined warning signs and pain:

Among co-infected patients with WHO-defined warning signs, 78.57% had rs3093059-TT genotype (p=0.0051) [Table 5.8]. On the contrary, 74.41% patients with rs3093059-CT did not develop any warning sign (p=0.0044). Approximately, 61% of patients with rs3091244-CT genotype developed warning signs (p=0.0020). In contrast, 71% patients with rs3091244-CC genotype did not develop any warning sign (p=0.0014). Approximately, 47% of co-infected patients with rs3091244-TT genotype were presented during acute phase of illness, whereas, 56% of those with rs3093059-CT genotype were reported during critical phase. Further, 72% co-infected patients with rs3091244-CT genotype demonstrated pain (p=<0.0001), whereas, 64.5% of patients with rs3091244-CC genotype did not show any signs of pain (p=0.0014).

Table 5.8: Genotypic association of CRP polymorphisms of co-infected patients with WHO-defined warning signs and pain.

rs3093059										
	With WHO-defined Warning Signs	**Without WHO-defined Warning Signs**	OR (95% C.I)	Relative risk (95% C.I)	p-value	**PAIN (+)**	**PAIN (-)**	OR (95% C.I)	Relative risk (95% C.I)	p-value
CC	1	1	0.6154 [0.03203 to 11.92]	0.8077 [0.1522 to 1.514]	>0.9999	2	0	1.923 [0.2790 to 25.42]	1.231 [0.4894 to 1.673]	>0.9999
CT	11	32	0.3056 [0.1441 to 0.6677]	0.4832 [0.2732 to 0.8010]	**0.0044***	25	18	1.254 [0.5897 to 2.592]	1.093 [0.8248 to 1.516]	0.5691
TT	44	39	3.103 [1.457 to 7.038]	1.988 [1.221 to 3.434]	**0.0051***	52	31	1.118 [0.5307 to 2.278]	1.044 [0.7924 to 1.430]	0.8495
rs3091244										
TT	7	8	0.8750 [0.2844 to 2.344]	0.9292 [0.5771 to 1.800]	>0.9999	6	9	0.6102 [0.1992 to 1.799]	0.7661 [0.3727 to 1.288]	0.4204
CT	31	20	3.224 [1.521 to 6.761]	1.872 [1.270 to 2.780]	**0.0020***	37	14	4.625 [2.091 to 9.819]	1.995 [1.428 to 2.832]	**<0.0001***
CC	18	44	0.3014 [0.1434 to 0.6307]	0.5042 [0.3202 to 0.7694]	**0.0014***	22	40	0.2942 [0.1435 to 0.6076]	0.5446 [0.3670 to 0.7826]	**0.0014***

5.4. DISCUSSION

TLR family plays an imperative role in recognition of dengue and chikungunya viral genomic RNA and activation of innate immunity against these viruses within infected patient body [7]. TLR3,7,8 activation leads to expression of a plethora of cytokines and thus innate immune system is activated [7, 52]. In this study, patients with CC genotype of TLR7-rs179010 were susceptible to DENV-CHIKV co-infection, whereas, those with TC genotype were protected from co-infection. Previously, this SNP has been associated with CHIKV and enteroviral infections [4, 7]. Furthermore, DENV-CHIKV co-infection susceptibility risk significantly increased among patients with CC genotypes of rs179008 and rs3853839, whereas, patients with GC genotype of rs3853839 impounded a defensive stand towards co-infection among patients. Various genotypes of these SNPs have been previously implicated for susceptibility towards HBV, COVID-19, Enterovirus, Influenza A, HCV, Chikungunya, Dengue, HIV, Cytomegalo and Crimean–Congo Hemorrhagic fever (CCHF) virus [4, 7-23]. Moreover, additive model statistics portrayed a significant association of rs179008, rs179010 and rs3853839 with DENV-CHIKV co-susceptibility. An additive model implies that disease risk is proportional to the number of risk alleles in an individual, i.e., that effect of heterozygous genotype is halfway between two possible homozygous genotypes [53].

In TLR8, rs3764879-CC and rs5744080-CC genotypes demonstrated significant association with co-susceptibility, but patients with AG-rs3764880 and CT-rs5744080 genotypes were protected among studied population against viral co-infection. These genotypes have been previously implicated towards chikungunya, dengue and HCV infections [4, 7]. Additive model analytics indicated significant association between rs3764879, rs5744080, rs3764880 with DENV-CHIKV co-infection.

DENV-high viral load was significantly more prevalent among co-infected patients with TLR7-rs179010-TT genotype. Similarly, patients with AA genotype of TLR7-rs179008, GG genotypes of TLR8-rs3764879 and rs3764880 displayed CHIKV-HVL. Caron *et al.* previously showed that co-infected patients could be subdivided according to their respective CHIKV and DENV viral load levels, suggesting a possible mechanism of competition during viral replication among these patients [54]. Results of the current study suggested probable role of specific genotypes of TLR7,8 SNPs in determining DENV/CHIKV load during such competitive viral replication among co-infected patients.

CRP, an inflammatory biomarker and an acute-phase protein activates innate immune response against infections. It is rapidly synthesized by hepatocytes in response to inflammatory stimuli of viral pathogens. When bound to viral ligands, CRP activates classical complement pathway by involving C3 [55, 56]. Thus, CRP mediated immune enhancement due to DENV/CHIKV co-infection might impact disease pathogenesis. As CRP polymorphic genotypes might affect its protein level, thus in turn, might influence viral infectivity and severity of the disease.

CRP-rs3091244 and rs3093059 are cis-acting genetic variants and tri-allelic rs3091244 has been previously associated with increased CRP levels *in vitro*, thus suggesting to have functional role in transcription factor binding [57]. In this study, patients with rs3093059-CT and rs3091244-TT genotypes were more susceptible to DENV-CHIKV co-infection with both OR and relative risk >1, indicating higher odds of disease outcome and co-infectivity risk of patients. Similarly, rs3093059-CT has been previously associated with DENV infection, HBV-related hepatocarcinoma, HBV infection, haemorrhagic and ischaemic stroke patients from eastern Indian and Chinese origin, respectively [30, 34-37]. Among co-infected patients, DENV-high viral load was

significantly more prevalent among patients with rs3093059-TT and rs3091244-CT genotypes, whereas, patients with rs3093059-CT and rs3091244-TT exhibited low DENV copy number. On the other hand, CHIKV-HVL was prevalent among co-infected patients with rs3091244-CC genotype and CHIKV-LVL among rs3091244-CT genotype. Thus, the results indicated that though rs3093059-CT and rs3091244-TT genotypes favoured DENV-CHIKV co-infection, these genotypes might allow less efficient DENV replication in presence of CHIKV within the system –thereby providing reasonable protection against severe disease development. This was reflected by absence of any WHO-defined warning sign among majority of patients with rs3093059-CT genotype.

Signal peptidase (SPase) enzyme cleaves signal peptide from preprotein, allowing its release from membrane and correct folding of mature protein [58]. SPC18 protein encoded by SEC11 is one of the subunits of signal peptidase complex and has catalytic activity of signal peptidase [59]. rs179008 and rs3764880 lie within signal peptide regions of TLR7 and TLR8, respectively, as predicted by SignalP 5.0 server. Genotypic changes at these two positions might alter stability of TLR receptors. CUPSAT and SIFT servers both predicted Q11L of rs179008 to be stable and tolerable, whereas, M1V of rs3764880 to be disruptive and damaging. Docking of TLR7 and TLR8 variants with SPC18 indicated better binding of Q variant of TLR7-rs179008 and V variant of TLR8-rs3764880 with SPC18 compared to respective L and M variants. However, though Q variant of TLR7-rs179008 interacted with SPC18 via more residues and generated better free binding energy; V variant of TLR8-rs3764880 interacted less efficiently with SPC18 residues, but still generated a better free binding energy. Thus, docking data indicated differential interaction of TLR7 and TLR8 variants with SPC18, which might affect the receptors' improper cleavage, translocation and cellular secretion, leading to disparity in TLR mediated immune signalling against DENV/CHIKV infections [27, 28].

5.5 CONCLUSION

Thus, this study underlined importance of TLR3,7,8 and CRP SNPs towards co-infection of DENV and CHIKV, with certain genotypes imparting a protective role while others associated with susceptibility towards co-infection. Moreover, this study suggested probable role of specific genotypes of TLR7,8 and CRP SNPs towards imparting high dengue/chikungunya viral load during competitive viral replication among co-infected patients.

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CHAPTER 4

Evaluation of analgesic and prophylactic activity of Curcumin against chikungunya-infected acute/chronic arthralgic mice

CHAPTER 4

Objective

- Determination of analgesic effect of curcumin on arbovirus induced arthritis.

Publication:

❖ **Sengupta S**, Tripathi A. Evaluation of analgesic and prophylactic activity of Curcumin against chikungunya-infected acute/chronic arthralgic mice [published online ahead of print, 2023 Mar 10]. **Journal of Medical Virology**. 2023;10.1002/jmv.28661. doi:10.1002/jmv.28661.

6.1. INTRODUCTION

Arthropod-borne viruses (arbovirus) like chikungunya, causes arbovirus-induced arthritogenic pain resulting in eruptive polyarthritis, followed by persistent rheumatologic and disabling symptoms. Thus, chikungunya virus is also known as an arthritogenic virus [1]. It is characterized by extreme patient-morbidity due to extreme incapacitating joint pain, acute or chronic polyarthritis/polyarthralgia, thereby causing their stooped appearance and protracted illness with ankles, knees, hips, wrists, elbows and metacarpo-phalangeal joints being majorly affected [2, 3]. 40–70% of infected patients might develop sub-acute or chronic/post-chikungunya arthritis (PCA) after months or years after initial CHIKV-infection, which mimicked rheumatoid arthritic symptoms [3, 4]. Also, CHIKV-infection may cause relapse of autoimmune arthritis in patients who were in remission prior to infection [2]. Individuals with age more than 45 years, having high viral load during acute phase and severe immunologic response in post-viraemic phase are predictors of development of chronic symptoms [2]. Articular cartilage is a highly specialized connective tissue of diarthrodial joints and provides a smooth, lubricated surface for articulation and load transmission. Articular cartilage is composed of a dense extracellular matrix (ECM) containing cells called chondrocytes. Principal composition of ECM contains water, collagen, and proteoglycans [6]. Thus, injury to articular cartilage is a cause of significant musculoskeletal morbidity. Type II collagen represents 90%-95% of collagen in ECM and forms the fibril network along with proteoglycan aggregates [6]. Type II collagen in the cartilaginous region give joints their structural rigidity [7]. Decreased viability of articular cartilage comprising of type II collagen resulted in deterioration of health and function of bone, thereby, developing arthritis [7-9]. In rheumatoid and osteoarthritis, substantial loss and degradation of type II collagen leads to disease progression [7]. Radiography and MRI have demonstrated

evidence of loss of articular cartilage, bony erosions, joint effusion, marrow oedema, synovial thickening, tendinitis and tenosynovitis in affected joints of patients with histories of CHIKV infection, indicating CHIKV-induced chronic inflammatory arthritis [10, 11]. A healthy bone structure is maintained by proper balance between bone-resorbing osteoclasts and bone-forming osteoblasts [12]. Replication of CHIKV in joint tissue can induce expression of osteoclastogenic cytokines resulting in joint and bone damage. Thus, there is an urgent necessity to identify agents with analgesic property that can alleviate chikungunya induced arthritic pain for palliative care of infected patients. Plant derived compounds with minimal toxicity and ready absorption within bloodstream might be advantageous over chemically synthesized compounds for their therapeutic potential against chikungunya induced arthritis [13, 14]. Currently, no Food and Drug Administration (FDA)- approved specific drug/vaccine is available for this purpose [15]. From 1940 to 2014, 49% of all small molecules approved by FDA were natural products or derivates of natural products [16]. A plant that has been broadly studied for various utilities across several disciplines is turmeric [17]. Curcumin, a natural polyphenol which is isolated from turmeric (*Curcuma longa*) has been recognized as FDA approved drug and belongs to ginger family (*Zingiberaceae*) and natively grows in the Indian sub-continent and Southeast Asia [17, 18]. Its rhizomes contain several secondary metabolites including curcuminoids, sesquiterpenes, and steroids; with curcumin being the principal component and major bioactive substance [17]. Chemically, curcumin is a diferuloylmethane, a diarylheptanoid belonging to natural phenols class. For centuries curcumin has played role in traditional Chinese and Indian medicine, for treatment of different maladies viz. dermatologic ailments, chronic kidney disease, diabetes, allergy, asthma, cardiovascular diseases, neurodegenerative diseases, pancreatitis, infectious diseases, liver complaints, inflammatory bowel disease, osteoarthritis and rheumatoid

arthritis [17-20]. It has been found to be safe and tolerable in human clinical trials and in systematic reviews without any toxic or adverse effects [21]. Various clinical trials among osteoarthritic patients have previously demonstrated efficacy of curcumin in reducing their arthritic manifestation thereby, improving physical function and generating a better quality of life [22]. Curcumin supplementation has been reported to improve walking time, morning stiffness, and joint swelling of rheumatoid arthritic patients [23]. Currently, treatment of chikungunya induced arthritis is being performed using analgesics, viz. steroidal and non-steroidal anti-inflammatory drugs [15]. But, limitations of their long-time use include immune disturbances, serious gastrointestinal and cardiovascular problems of infected patients and better-targeted drugs are needed to treat inflammatory rheumatic disorders. [24]. Curcumin has been described to possess chondro-protective, anti-viral, anti-oxidative and anti-inflammatory effects *in vitro* and *in vivo*, and due to its wide spectrum of biological and pharmacological properties, it is often called colloquially as "cure-cumin." [25, 26]. It has been known to block entry and binding of CHIKV in HeLa and HEK 293T cells, respectively [27]. Previously, incubation of cells with curcumin significantly reduced Dengue virus, Japanese encephalitis virus (JEV) and Zika virus (ZIKV) infection by inhibiting their binding to Vero and HeLa cells [17]. Also, curcumin treatment of DENV and JEV infected vero and neuroblastoma cells resulted in intracellular accumulation of viral proteins and reduction of viral particle production [17]. Another arbovirus, Rift Valley fever virus has been shown to be inhibited by curcumin in human small airway lung epithelial cells [28]. Moreover, curcumin prohibited entry, replication and attachment of HIV, hepatitis, norovirus, Respiratory syncytial virus (RSV), Influenza, herpes simplex virus (HSV), Human papillomavirus (HPV) viruses in both cell culture and murine model [27].

CHIKV infection has been reported to trigger rapid innate immune response by producing pro-inflammatory cytokines viz. IFN (interferon)-β, IFN -α, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) [29, 30]. IL-6 serves as a potent activator of joint pain through sensitizing joint nociceptors and also participates in inflammation-evoked pain through neurons [31]. One study by Shang et al. reported that curcumin inhibited production of osteoclasts in Peripheral blood mononuclear cells (PBMCs) of RA patients [32]. Curcumin can suppress pro-inflammatory pathways linked with many chronic diseases [33]. It can block both production and action of TNFs in *in vitro* models, in animal models and in humans by binding to TNF directly [34]. But, effect of curcumin in alleviating CHIKV induced acute and chronic arthritic pain and its role (if any) in maintaining structural architecture of articular cartilage has not been studied till date. Mice model has been previously implicated in studying cartilage and synovium degradation, fibrosis of skeletal muscles and arthritic-disease progression of CHIKV-infected mice [35, 36]. Thus, this chapter explored analgesic and prophylactic effect of curcumin in alleviating CHIKV induced joint pain of acute/chronic arthritic mouse model, its impact on knee articular cartilaginous type II collagen and pain-induced mice behavioral change.

6.2. MATERIALS AND METHODS

6.2.1 Ethics statement

All mice-based experiments were conducted according to guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India (registration no.: 681/02/a/CPCSEA). Also, approval from animal research ethical board of Calcutta School of Tropical Medicine was obtained, which followed CPCSEA guidelines (AREC-STM/320 dated 09.01.2017).

6.2.2. Animals

Swiss albino mice (*Mus musculus*, pathogen free) were obtained from institutional animal facility, which were housed in polypropylene cages with dust-free rice husk as bedding material at constant temperature of 25°C, with free access to food and water in a 12-hours light/dark cycle. The study was conducted in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [37].

6.2.3 Experimental infection and treatment

6.2.3.1 Virus passaging in mouse:

Chikungunya virus strain was procured from ATCC (ATCC: VR-64, strain S27-African prototype) and cultured in C6/36 cell line using DMEM medium with 10% FBS and antibiotics in 5% CO_2 at 28ºC. CHIKV copy number in culture supernatant was quantified by qRT-PCR and arthralgic model of CHIKV-infected mice was achieved by subcutaneously inoculating 10^6 copies/50µl of culture supernatant in loose skin of mice hind paws, towards ankle. After infection, clinical scoring of mice was done based on behaviour and morphological analysis: 0: no disease signs; 1: ruffled fur; 2: mild hind limb weakness; 3: moderate hind limb weakness; 4: severe hind limb weakness and dragging and 5: moribund. Any deaths were recorded. Clinical score of 5 was set as terminal point.

6.2.3.2. Oral administration of Curcumin:

Curcumin was procured from Sigma Aldrich, India and stored at room temperature without exposure to sunlight. 40 human-days is equivalent to 1 mice-day when correlating their entire lifespan [38]. Hence, CHIKV-infected mouse with arthritic manifestation within 0-1 day was considered as acute CHIKV-infected mouse model; post-CHIKV infected arthritic mouse of 7 days was considered as chronic CHIKV-

infected mouse model. Curcumin treated mouse prior to CHIKV-infection was considered as prophylactic mouse model. A total of seventy-five (75) 6-8 weeks old Swiss albino mice were grouped into Group I: Control, Group II: Infection group, Group III: Daily curcumin treatment for 7days before and after CHIKV infection (14days total) (Pre-treatment group), Group IV: Curcumin treatment along with CHIKV infection (Co-treatment group) and Group V: Daily curcumin treatment for 7days on post-CHIKV infected arthritic mice (Post-treatment group), with fifteen mice in each group. Each group was sub-grouped into High-dose (HD): 2000mg/kg, Mid-dose (MD): 1000mg/kg and Low-dose (LD): 500mg/kg, with 5 mice in each sub-group. Curcumin prepared in 5% DMSO was administered via oral gavage according to OECD guidelines [39] [Fig 6.1]. Oral dosage of curcumin (LD50=2000mg/kg) was fixed according to mice oral toxicity analysis predicted by ProTox-II server [40]. Doses equivalent to LD50 was considered as HD, while half and quarter dilution of LD50 was considered as MD and LD, respectively.

Fig 6.1: Oral administration of curcumin to mice through feeding gavage.

6.2.4. Pain and arthritis related morphological and behavioral tests

Dorsoventral feet swelling of all groups of mice were measured by callipers.

6.2.4.1. von Frey testing

Mice were acclimatized for 30 minutes in individual chambers on top of a wire grid platform prior to von Frey testing. To determine tactile sensitivity of mice hind paws, their plantar surface was stimulated with ascending force intensities of von Frey filaments, having replacement filament range: 1.65-6.65 (Stoelting, USA). A positive response was defined as brisk paw withdrawal, licking or shaking/flicking of the paw when stimulus was applied, and number of positive responses for each stimulus was recorded. Tactile threshold was defined as withdrawal response in ≥5 out of 10 trials to a given stimulus intensity. This threshold was calculated once per animal.

6.2.4.2. Open field test

Mice were acclimated to test room for 30 minutes before open field testing. Mouse was placed in the center of individual plexiglass square chambers (45 cm × 45 cm) and allowed to freely explore the chamber for 5-minutes (test session) [41]. Movement of mouse was recorded with a video camera. Two observers blinded to treatment group assignments manually traced mouse movements to calculate line crossings, Centre Square Entries (CSE), rearing and locomotion time within cage for 5 minutes.

6.2.5. Toxicology and Histology

6.2.5.1. Determination of toxicological effects of curcumin using biochemical parameters

Oral toxicity of high, mid and low-dose curcumin in mice was determined by toxicological studies. Animals of were sacrificed following completion of curcumin treatment according to OECD guidelines. Blood was collected by cardiac puncture method using 26 gauge needles for different biochemical studies. Levels of AST, ALT, ALP, bilirubin, albumin, urea and creatinine were measured by using a standard automated clinical chemistry analyzer (ERBA Model no: EM360) according to manufacturer's instructions.

6.2.5.2 Histology of liver and kidney

Liver and kidneys were cut into small pieces and kept in paraformaldehyde (10% at room temperature) fixative for 24 hours and processed for histological study. After fixation, tissues were dehydrated through increasing ethanol grade series (30%, 50%, 70%, 90% and 100%), then tissues were embedded in paraffin (Merck). Sections were photographed with a light microscope.

6.2.5.3 Safranin O staining, OARSI/SMASH score, and histo-morphological evaluation of articular cartilage

After sacrifice, mice hind limbs were fixed in formalin for 3 days, decalcified in formic acid, embedded in paraffin following standard protocol, and sectioned for histological and immunohistochemical analysis. Briefly, after removal of soft tissue, bones were

submerged in 5% formic acid for decalcification. Each sample was weighed and placed in decalcifying solution and regularly monitored. Formic acid was replaced with fresh solution after observations according to decalcification rate of bones. 10-12 days were required for complete decalcification of bones. After decalcification, bones were fixed in 10% formalin at 4°C. This cross-linked fixative gave good accessibility and retention of chondrocytes. Fixed tissues were rinsed thoroughly in distilled water and dehydrated through ethanol grade series (30%, 50%, 70%, 90% and 100%) - 1hour for each gradation. Dehydrated tissues were cleared by xylene treatment for 30 minutes; then tissues were embedded in paraffin blocks (Merck, U.S.A.). 7 mm thick paraffin sections were cut in Leica RM2235 microtome (Leica Biosystems, India).

Safranin O-fast green staining was used to visualize proteoglycans in knee articular cartilage. Tissues sections were deparaffinized (kept in xylene for 5mins) and rehydrated through ethanol grade series (100% for 3mins, 90% for 5 mins, 70% for 5 mins, 50% for 5 mins and 30% for 5mins). Slides were hydrated in distilled water for 10mins. Slides were placed in a glass staining jar containing 40 ml Weigert's iron hematoxylin for 10mins. Then, slides were washed in tap water and placed in 40 ml fast green solution for 5mins. After that, they were rinsed in 1% acetic Acid for 15 seconds. Thereafter, slides were placed in 40 ml safranin O solution for 5mins. Slide was then dehydrated through ethanol grade series (95% for 2mins, 100% for 3mins) and in xylene for 15-30 seconds to fix the stain. Finally, slides were covered with coverslip with DPX mount and visualized under light microscope at 4x and 10x magnification.

Severity of arthritis was evaluated in medial compartment of the knee for each mouse using Osteoarthritis Research Society International (OARSI) 2010 scoring and Standardized Microscopic Arthritis Scoring of Histological sections (‘SMASH’) 2020 recommendations for standardized processing and microscopic scoring of characteristic

histopathological features of arthritis [42-44]. OARSI scoring was analyzed on a scale of 0-6, which is calculated on basis of articular damage grade scale (0: cartilage/surface intact or normal, 0.5: loss of Safranin-O without structural changes, 1: small fibrillations without cartilage loss, 2: vertical clefts down to layer immediately below the superficial layer and some surface lamina loss, 3: vertical clefts/erosion to calcified cartilage extending to <25% of articular surface, 4: vertical clefts/erosion to calcified cartilage extending to 25–50% of articular surface, 5: vertical clefts/erosion to calcified cartilage extending to 50–75% of articular surface and 6: vertical clefts/erosion to calcified cartilage extending >75% of articular surface) and stage (0: no arthritic activity, 1: <10% activity, 2: 10-25% activity, 3: 25-50% activity and 4: >50% activity), along with scoring of loss of proteoglycan based on a scale of 0-6. SMASH based severity of arthritis was scored on arbitrary scale of 0–3 for two different parameters (Loss of cartilage proteoglycan, where, 0: no pathology or healthy intact smooth surface of articular cartilage; 1: mild loss of staining in 1/3 of superficial cartilage zone, superficial cartilage layer is still predominantly reddish; 2: moderate loss of red staining up to 2/3 of superficial cartilage zone and 3: complete loss of red staining of superficial cartilage zone) and (Cartilage erosion, where, 0: healthy intact cartilage; 1: minor roughening or mild loss of superficial non-calcified cartilage layer affecting up to 1/3 of cartilage area; 2: moderate loss of superficial non-calcified cartilage layer affecting up to 2/3 of cartilage area; 3: complete loss of superficial non-calcified cartilage).

6.2.5.4 Immunohistochemical analysis

Immunohistochemical analysis of type II collagen was performed using Mouse specific HRP/DAB Detection IHC Kit (Abcam, USA), according to manufacturer's protocol. In brief, after deparaffinization, tissue sections were blocked for endogenous peroxidases using hydrogen peroxide for 10 minutes. Blockage of unspecific binding was done by

incubating sections with protein block for 10 minutes. Between each step, washing with PBST buffer (137mM/L NaCl, 2.7mM KCl, 10mM/L Na_2HPO_4, 1.8mM/L KH_2PO_4. and 0.1% (v/v) Tween20) was performed. Incubation with anti-mouse type II collagen (COL2A1) primary antibody (Santa Cruz Biotechnology, USA) was done overnight (12-14 hours) at 4°C. After washing, sections were incubated with biotinylated goat anti-mouse IgG secondary antibody (Abcam, USA) for 10 minutes. The reactions were visualized after incubation with 3,3-diaminobenzidine (DAB) chromogen for 10 minutes. Counterstaining with haematoxylin was done and slides were visualized under light microscope at 4x and 10x magnification. Immunostaining intensity for type II collagen epitopes was quantified by determining "reciprocal intensity" of stained articular cartilage matrix; briefly, intensity value of six random locations within MFC (Medial femoral condyle) and MTP (Medial tibial plateau) regions of three sections per mouse was measured using ImageJ version 1.53t.

6.2.6 Determination of pro-inflammatory biomarker levels (CRP, IL-6 and TNF-α) and viral load

CRP, IL-6 and TNF-α quantification in mice sera was performed by immunoturbidimetry method (CRP) using AUTOSPAN turbi gold kit (SPAN diagnostics, India) and sandwich ELISA (IL-6 and TNF-α) using KINESISDx kits (CA, USA), according to manufacturer's protocols. Absorbance was measured at 450nm wavelength. Standard-curve was drawn using GraphPad prism9 and concentrations of each protein were analyzed.

Viral load of CHIKV in mice sera post-sacrifice, was determined by real-time qRT-PCR using CHIKV Genesig kit (Primer Design Ltd., UK), according to manufacturers' protocol. A standard curve of cycle threshold (Ct) values was constructed using six 10-

fold dilution series of positive control template (provided within the kit). Viral load was calculated from corresponding Ct values. Limit of detection of CHIKV Genesig kit was <100 copies of corresponding target genomes.

6.2.7 Statistical analysis

Group sizes per experiment were based on a "resource equation" method [45]. For all experiments, data were expressed as mean±SD of five animals per sub-group. Level of significance was set at $p<0.05$. All experiments were repeated at least twice. Significance analysis and representations was determined using Student's t test (Welch's correction), one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using GraphPad Prism 9.

6.3. RESULTS

This study investigated possible effectiveness of curcumin in alleviating CHIKV-induced joint pain of acute/chronic arthritic mouse model and their impact on maintaining articular cartilaginous type II collagen, a major component of knee and other joints. A total of 75, 6-8 weeks old Swiss albino mice were acclimatized for 7 days and grouped into Group I: Control (CO), Group II: CHIKV-infected group (IN), Group III: Curcumin treatment 7 days prior and 7 days post CHIKV-infection (Pre-treatment group: PT), Group IV: Curcumin treatment along with CHIKV-infection for 7 days (Co-treatment group: CT) and Group V: Curcumin treatment starting 7 days after CHIKV-infected arthritic manifestation, for 7 days (Post-treatment group: Post-T), with 5 mice in each sub-group. A schematic diagram representing timeline of experiments for infection, curcumin treatment, behavioural, histo-morphological and immunohistochemical study

on mice groups has been shown [Fig 6.2]. No death of Swiss-albino mice was observed during experimental timeline.

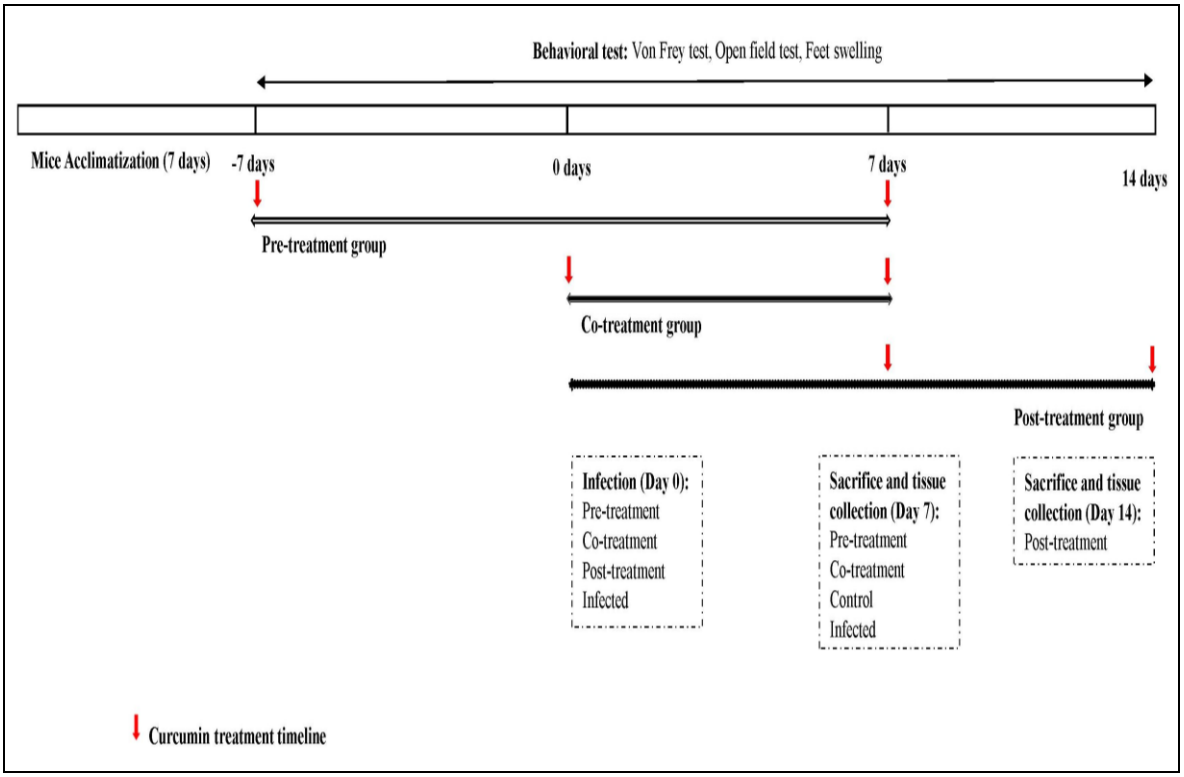

Fig 6.2: **Schematic representation of timeline of experiments for study on healthy, infected, and curcumin- treated mice groups.**

6.3.1 Toxicology and histopathology of liver and kidney

There was no significant difference in the AST, ALT, ALP, bilirubin, albumin, urea and creatinine serum levels of curcumin-treated low, mid and high-dose mice groups when compared to control mice group [Table 6.1].

Microscopic investigation of stained tissue sections of liver and kidney revealed no abnormal changes in gross morphology during necropsy [Fig 6.3]. Kidney histopathological features of control and curcumin treated mice was similar with normal renal glomeruli and tubules having intact epithelial cells. Liver sections of control group

as well as curcumin treated mice showed normal arrangement of hepatocytes and proper central vein with no hepatotoxicity.

Table 6.1: Distribution of serum biological parameters for toxicological study of curcumin doses.

Animal groups	AST (IU/L) [Mean±SEM]	ALT (IU/L) [Mean±SEM]	ALP (IU/L) [Mean±SEM]	Bilirubin (mg/dL) [Mean±SEM]	Albumin (gm/L) [Mean±SEM]	Urea (mg/dL) [Mean±SEM]	Creatinine [Mean±SEM]
Control	17.41±1.030	31.39±0.9722	0.6067±0.04485	3.810±0.09	4.250±0.07	19.52±1.32	3.21±0.56
Low	19.07±0.621	29.56±0.5503	0.6833±0.01764	2.190±0.02	4.450±0.15	20.24±1.56	3.35±0.11
Mid	18.13±0.3789	33.47±0.7311	0.5500±0.0321	1.300±0.78	4.430±0.13	21.81±0.98	3.41±0.34
High	17.98±0.5927	35.14±0.5724	0.5340±0.4532	2.740±0.67	4.120±0.03	22.40±1.23	4.1±0.14

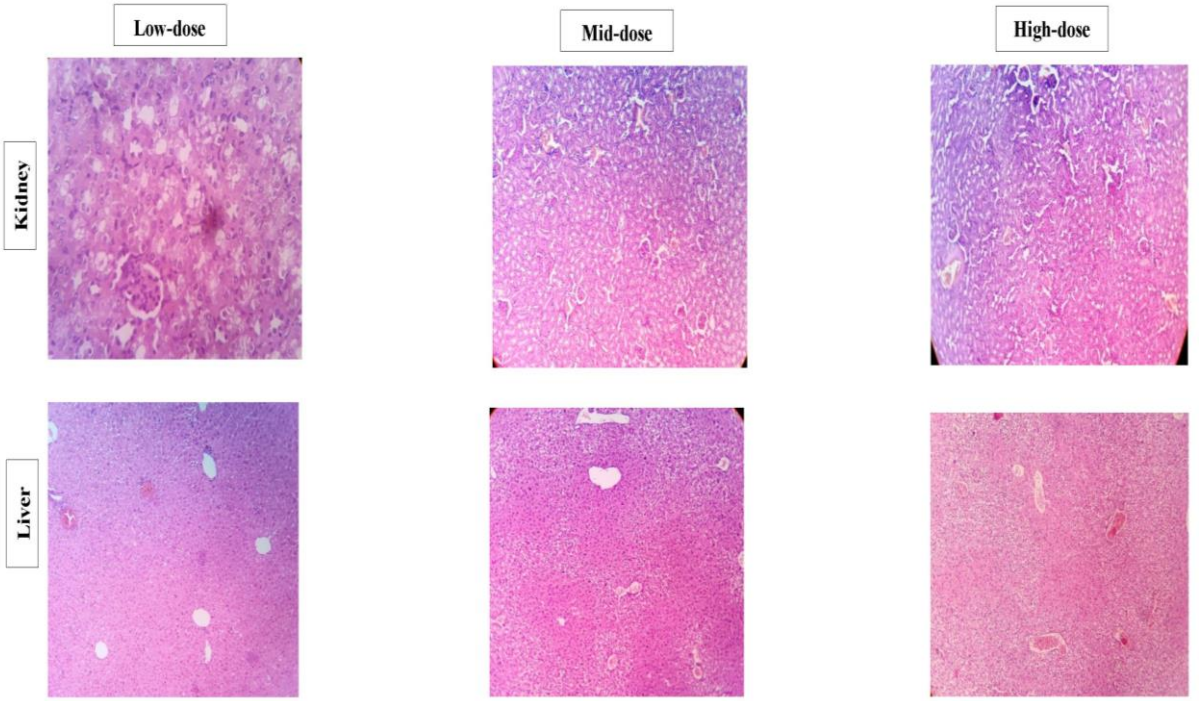

Fig 6.3: Histological features of Kidney & liver stained with H&E for toxicological study of curcumin doses.

6.3.2 Effect of curcumin on mice nociceptive behaviour

6.3.2.1 Von Frey analysis

Nociceptive thresholds of mice hind paws in each group were observed using von Frey filaments from day of infection (day 0) to sacrifice (PT and CT: 7^{th}day; post-T: 14^{th}day of curcumin treatment). Mean pain-threshold of left and right leg of control group (CO) was significantly higher compared to that of infected (IN) ($p<0.0001$) [Fig. 6.4]. Average pain-threshold of both legs of Pre-treated high-dose sub-group (PT_{HD}) was significantly higher than that of IN group (For left leg: $p=0.0053$) [Fig 6.4a]. In case of Co-treated high and mid-dose sub-groups (CT_{HD} and CT_{MD}), pain-threshold of their left leg significantly increased compared to that of IN ($p=0.0009$ and $p=0.0256$, respectively) [Fig 6.4b]. Similar trend was followed for right leg of CT_{HD} sub-group ($p=0.0035$). For post-treated mid-dose sub-group (post-T_{MD}), pain-threshold of both the legs significantly increased compared to IN ($p=0.0005$; $p= 0.0271$) [Fig 6.4c].

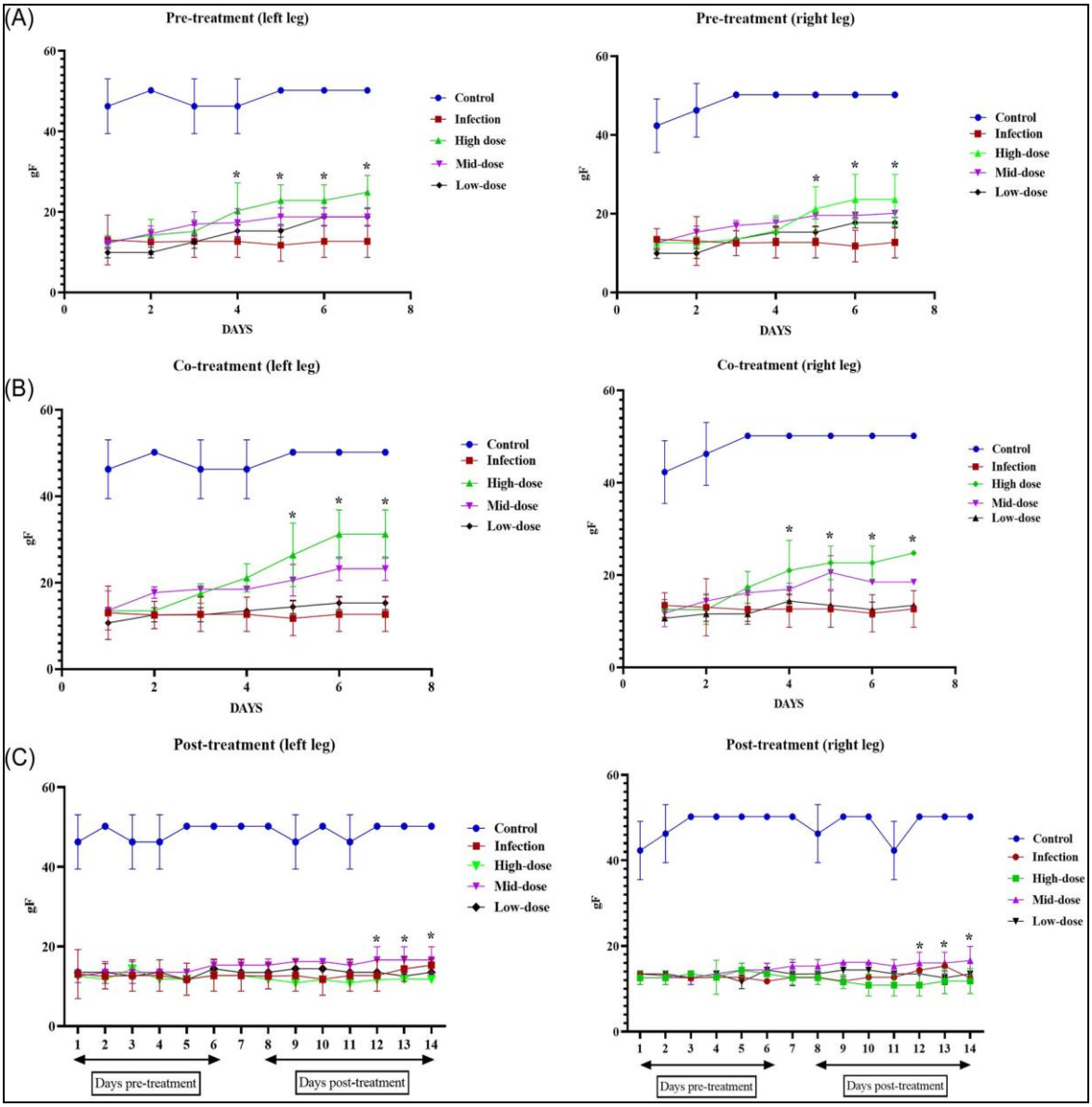

Fig 6.4: Effect of curcumin on mice nociceptive behavior using von Frey filaments

6.3.2.2 Feet swelling

To evaluate anti-inflammatory efficacy of curcumin administration, day-wise dorsoventral feet measurement of each mice group was performed [Fig 6.5]. Reduction in feet swelling on curcumin treatment (PT, CT and post-T) was observed for all sub-groups [Fig. 6.6 a, b, c]. Significant reduction in both left and right feet swelling of PT_{HD} sub-group from 5^{th} and 6^{th} day onwards post CHIKV-infection compared to IN was observed, respectively ($p<0.05$). For both CT_{HD} and MD sub-groups, swelling reduced from 5^{th} and 6^{th} day onwards for left and right leg ($p<0.05$), respectively. But, degree of swelling reduction was more than 1-fold in HD sub-group compared to MD. For post-T group, only MD was significantly effective for reduction of day-wise left feet swelling ($p= 0.0330$).

Feet swelling post-CHIKV-infection

Dragging of hind limbs post-CHIKV -infection

Control

Post-curcumin treatment

Fig 6.5: Representative photograph of control, infected and curcumin treated mice

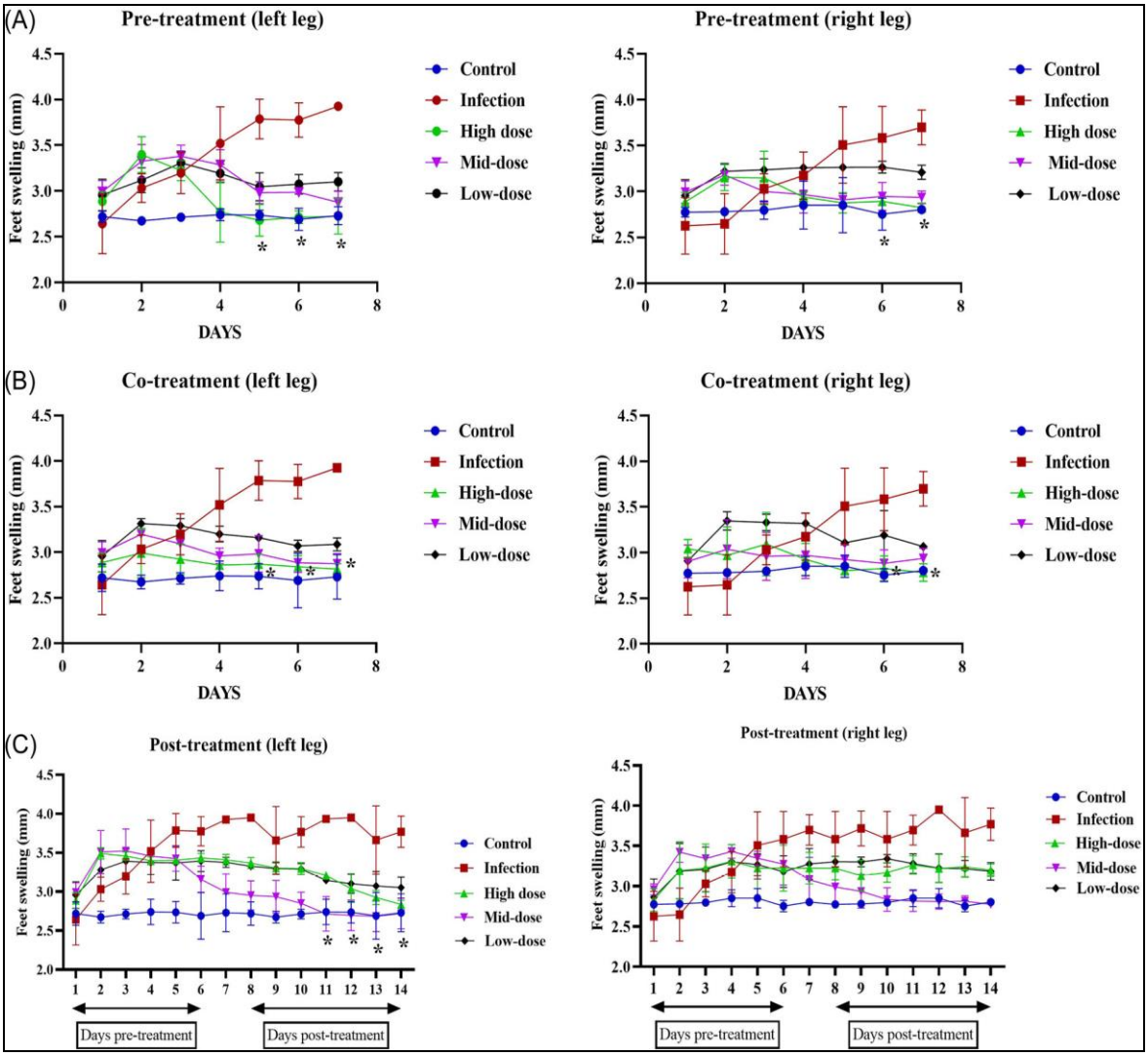

Fig 6.6: Feet measurement of healthy, infected, and curcumin- treated mice groups using calipers

6.3.3 Histo-morphological observations: Safranin O stain, OARSI and SMASH score

Structural integrity of mice knee articular cartilage following CHIKV-infection and curcumin treatment was microscopically studied by Safranin O staining and implementing OARSI and SMASH scores. OARSI and SMASH scoring of control group negated any loss of cartilage proteoglycan or Safranin O (Score: 0) [Fig. 6.7, Table 6.2]. OARSI scoring of IN group's knee articular cartilage region exhibited severe Safranin O loss, appearance of vertical fissures, cartilage fibrillation, loss of cartilage proteoglycan (Score: 3) and cartilage erosion in both MTP and MFC regions (Grade:3.5, Stage:3, Score:10.5). SMASH analysis of IN group revealed a moderate loss of Safranin O and cartilage erosion of MTP and MFC region (Score: 2). Upon oral administration of curcumin, PT_{HD} and PT_{MD} sub-groups exhibited less Safranin O loss and cartilage fibrillation compared to IN group, implicated by their significantly lower OARSI and SMASH scores. Comparing the two doses, PT_{HD} had lower OARSI and SMASH scores with respect to PT_{MD} sub-group. In case of CT group, all three doses showed significantly lower OARSI and SMASH scores compared to IN. But, OARSI and SMASH scores were lower in CT_{HD} compared to CT_{MD} and CT_{LD} sub-groups. Finally, in case of Post-T group, all curcumin doses showed reduced OARSI and SMASH scores, with Post-T_{MD} sub-group exhibiting lowest OARSI and SMASH scores.

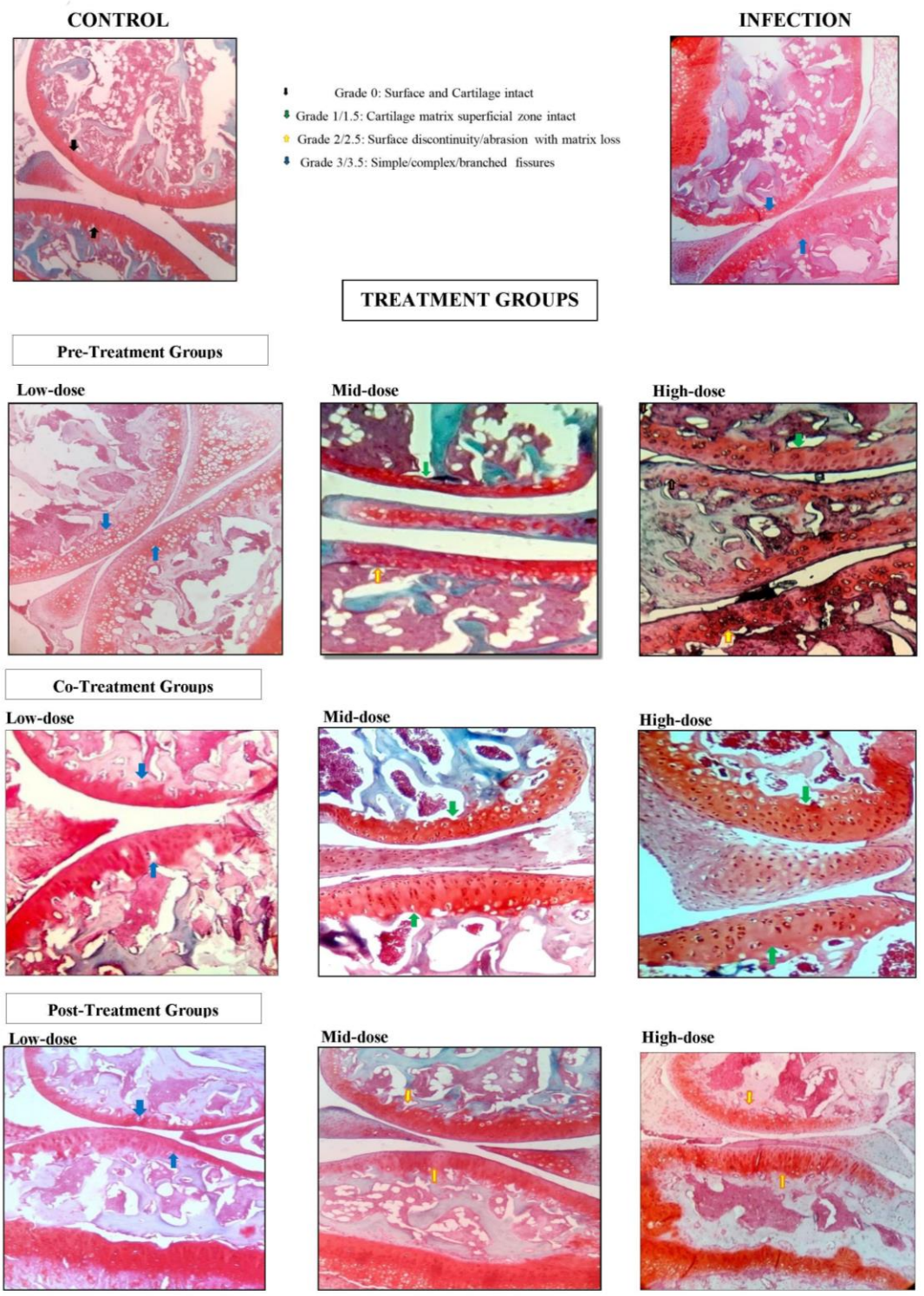

Fig 6.7: Safranin O staining of knee articular cartilaginous region of healthy, infected, and curcumin- treated mice groups.

Table 6.2: OARSI and SMASH scores of Safranin-O-stained knee articular cartilage

		OARSI scoring								SMASH scoring	
		Arthritic damage						Loss of cartilage proteoglycan		Loss of cartilage proteoglycan	Cartilage erosion
		Grade		Stage		Score					
Groups		Medial femoral condyle (MFC)	Medial tibial plateau (MTP)	Medial femoral condyle (MFC)	Medial tibial plateau (MTP)	Medial femoral condyle (MFC)	Medial tibial plateau (MTP)	Medial femoral condyle (MFC)	Medial tibial plateau (MTP)		
Control		0	0	0	0	0	0	0	0	0	0
Infection		3.5	3.5	3	3	10.5	10.5	3	3	2	2
Pre-treatment	High	1	1	1	1	1	1	1	1	0	0
	Mid	1.5	2	2	3	3	6	2	2	2	1
	Low	3	3.5	3	3	9	10.5	3	3	2	1
Co-treatment	High	1	1.5	1	1	1	1.5	1	1	1	0
	Mid	1.5	1.5	1	1	1.5	1.5	2	2	2	1
	Low	3	3.5	1	1	3	3.5	2	2	2	1
Post-treatment	High	2	2	3	3	6	6	3	3	2	1
	Mid	2.5	2	1	1	2.5	2	3	3	1	0
	Low	3.5	3.5	2	2	7	7	1	1	2	1

6.3.4 Immunohistochemistry

Immunostaining intensities of type II collagen of knee articular cartilage showed its decreased presence in only CHIKV-infected mice, whereas, upon curcumin treatment, collagen concentration increased among all groups compared to infected ones. In PT_{HD} sub-group, collagen intensity significantly increased by 3-fold, while there was 2-fold elevation among PT_{MD} and PT_{LD} sub-groups at MFC region ($p < 0.0001$, < 0.0001 and 0.0006, respectively) [Fig. 6.8, Fig. 6.9 a, b, c]. Similar trend was followed at MTP region with increased intensity by 2-fold and 1-fold among PT_{HD} and PT_{MD}, PT_{LD} sub-groups, respectively ($p=0.0003$, 0.0016 and 0.0029). Thus, PT_{HD} dosage was most effective for preventing collagen degradation. Among CT_{HD}, CT_{MD} and CT_{LD} sub-groups type II collagen intensity increased significantly 2-3-fold at MFC and MTP regions when compared to IN, with CT_{HD} sub-group showing highest intensity (MFC vs IN; $p<0.0001$; MTP vs IN; $p=0.0001$, 0.0003 and 0.0003, respectively). However, type II collagen intensity was highest among Post-T_{MD} sub-group at MFC and MTP regions (1-fold) (MFC vs IN: $p<0.0001$; MTP vs IN $p= 0.0029$). Overall, type II collagen intensity was comparable between high and mid-dose curcumin treated and control mice, except MFC region of Post-T group.

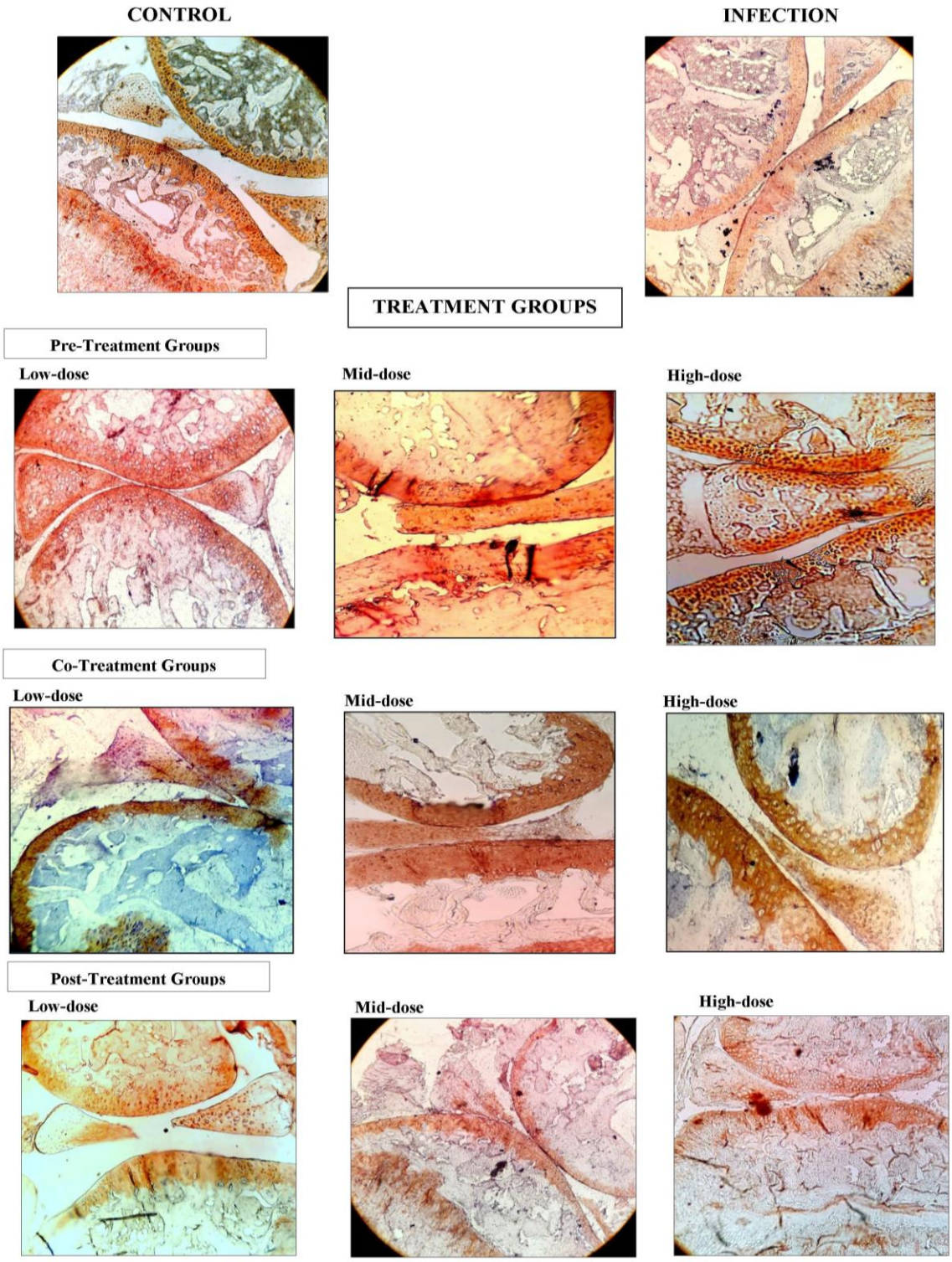

Fig 6.8: Immunohistochemically stained type II collagen of knee articular cartilaginous region of healthy, infected, and curcumin- treated mice groups

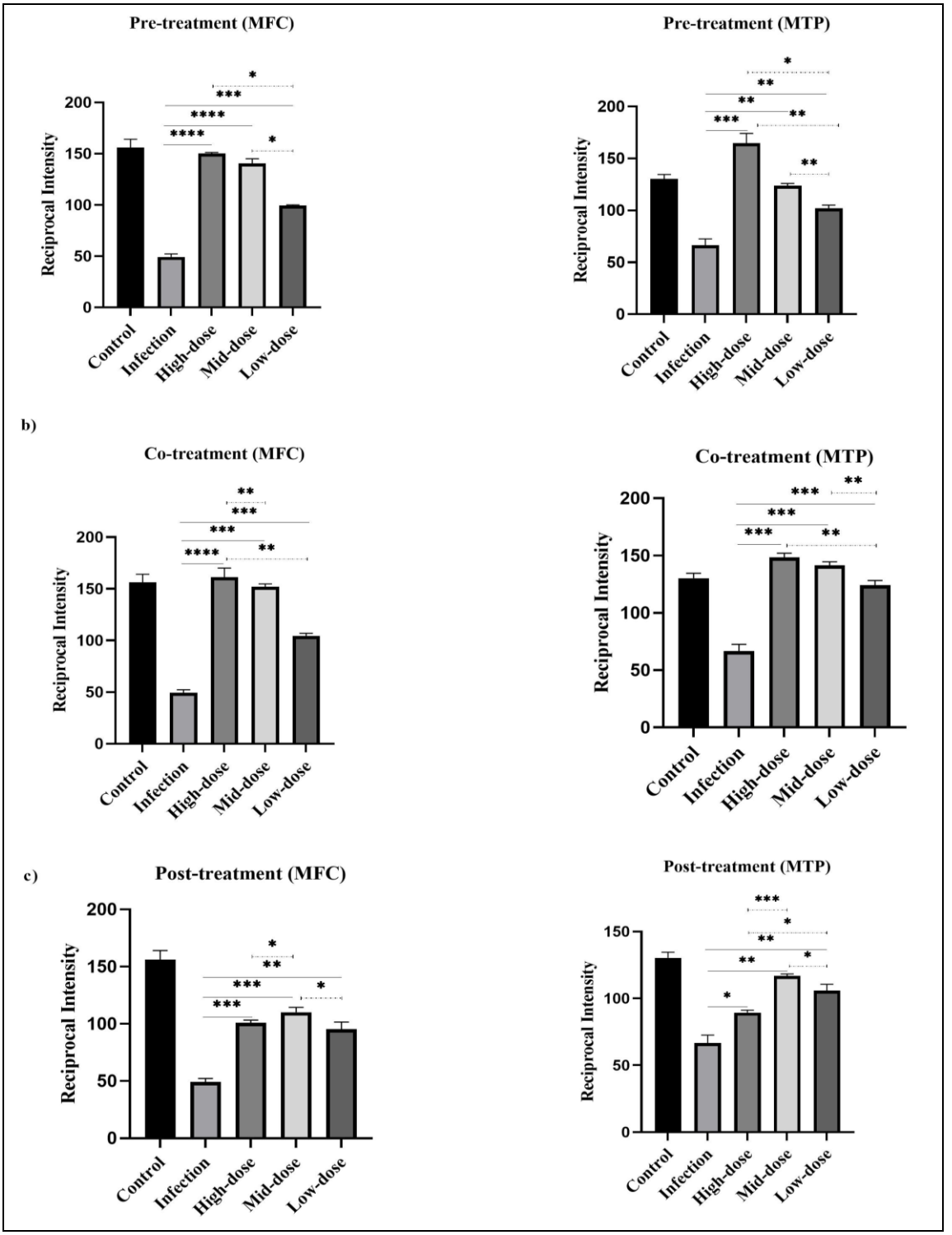

Fig 6.9: Reciprocal intensity of type II collagen in knee articular cartilaginous region of healthy, infected, and curcumin- treated mice groups.

6.3.5. Open field analysis

Several movements viz. line crossings, rearing, centre square entries and locomotion time were observed of all mice groups by open field test, which delved into their pain related behavioural patterns [6.10]. Significantly increased line crossings were observed in PT_{HD} mice sub-group compared to IN group ($p < 0.0001$) (Fig. 6.11a). However, significantly increased line crossings were observed among CT and Post-T group-mice receiving all three doses ($p<0.05$), with CT_{HD} and Post-T_{MD} demonstrating highest line crossing.

Furthermore, increased number of rearing was observed in all doses of PT and CT mice groups compared to IN (PT: $p<0.0001$, 0.0012 and 0.0245, respectively; CT: $p=0.0002$, 0.0119 and 0.0026, respectively) [Fig 6.11b]. Moreover, in both groups HD mice displayed 1-2-fold enhanced rearing compared to MD and LD. In Post-T sub-group, only MD mice rearing significantly increased with respect to IN ($p=0.0001$).

Additionally, day-wise mice centre square entries (CSE) were observed among all groups. In comparison to IN group, CSE increased for PT_{HD} and PT_{MD} (Day 3-7), CT_{HD} (Day 4-6) and Post-T_{MD} (Day 9-14) sub-groups.

Finally, mice locomotion time was calculated to analyse duration of nociceptive pain following CHIKV-infection and curcumin treatment [Fig 6.11c]. Significantly higher locomotor activity was observed following treatment with all three curcumin doses for PT, CT and Post-T groups compared to IN ($p<0.0001$). However, degree of increased locomotion time was more than 1-fold in PT_{HD}, CT_{HD} and Post-T_{MD} sub-groups. Furthermore, day-wise analysis revealed a gradual increase in locomotion time for all

doses between day 4-7 for PT and CT mice groups ($p<0.05$), while, in case of Post-T, time of increased locomotion varied for each sub-group: HD: 9^{th}-12^{th} day, MD: 8^{th}-14^{th} day and LD: 8^{th}-12^{th} day.

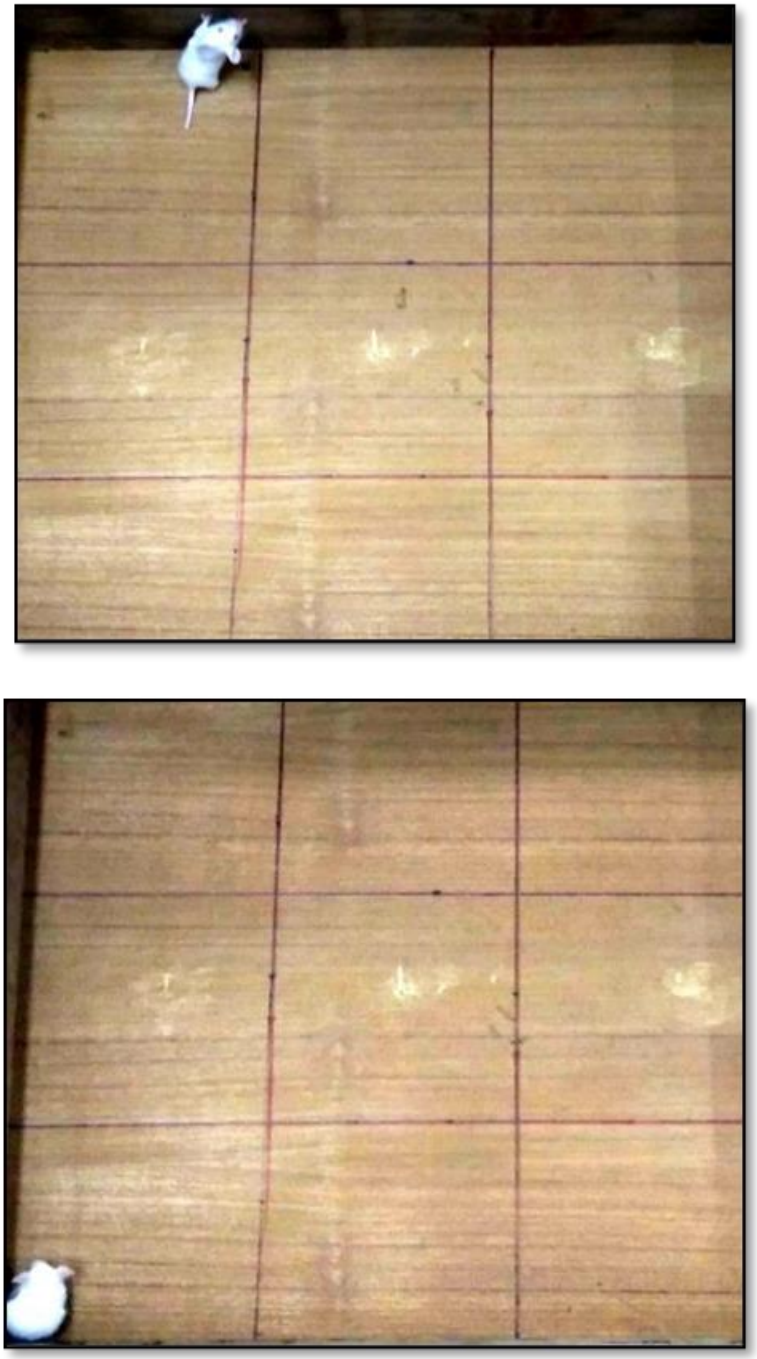

Fig 6.10: Representative picture of open-field test

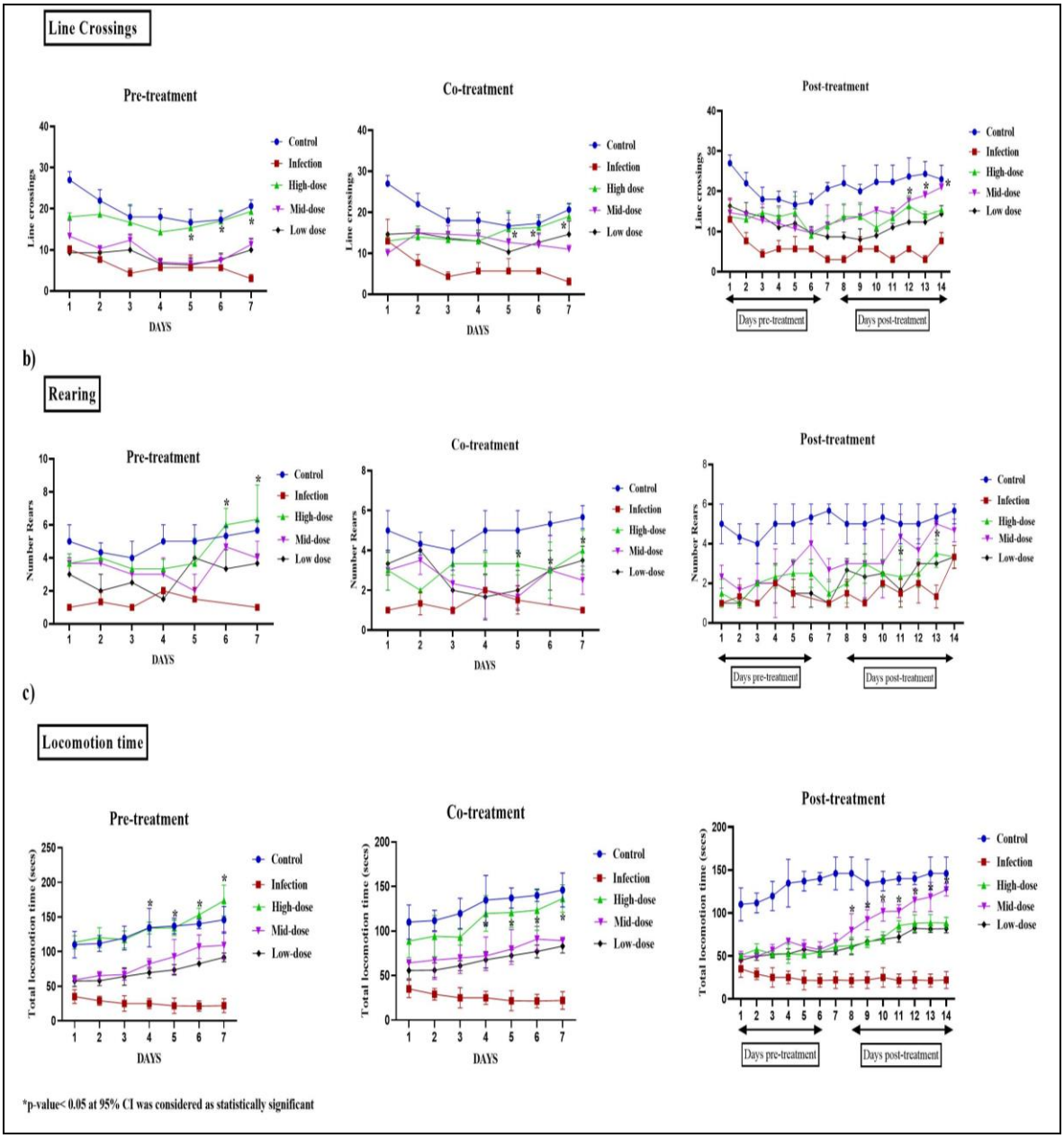

Fig 6.11: Effect of curcumin treatment on locomotory behavior using open- field test of healthy, infected, and curcumin- treated mice groups

6.3.6 Effect of curcumin on Pro-inflammatory biomarkers and viral load

Since, mice treated with PT_{HD}, CT_{HD} and Post-T_{MD} curcumin doses exhibited most effective observations in von Frey, feet swelling, safranin O staining, immunohistochemistry and open field tests, effect of these doses on CHIKV-induced release of murine pro-inflammatory cytokines was evaluated. Serum levels of pro-inflammatory cytokines, IL-6, TNF-α and inflammatory biomarker, CRP were significantly reduced among PT_{HD}, CT_{HD} and Post-T_{MD} treated sub-groups [$p<0.005$] compared to infected mice [Fig 6.12]. Also, significant reduction in CHIK viral load was detected among PT_{HD}, CT_{HD} and Post-T_{MD} treated murine blood compared to that of infected mice [$p<0.005$].

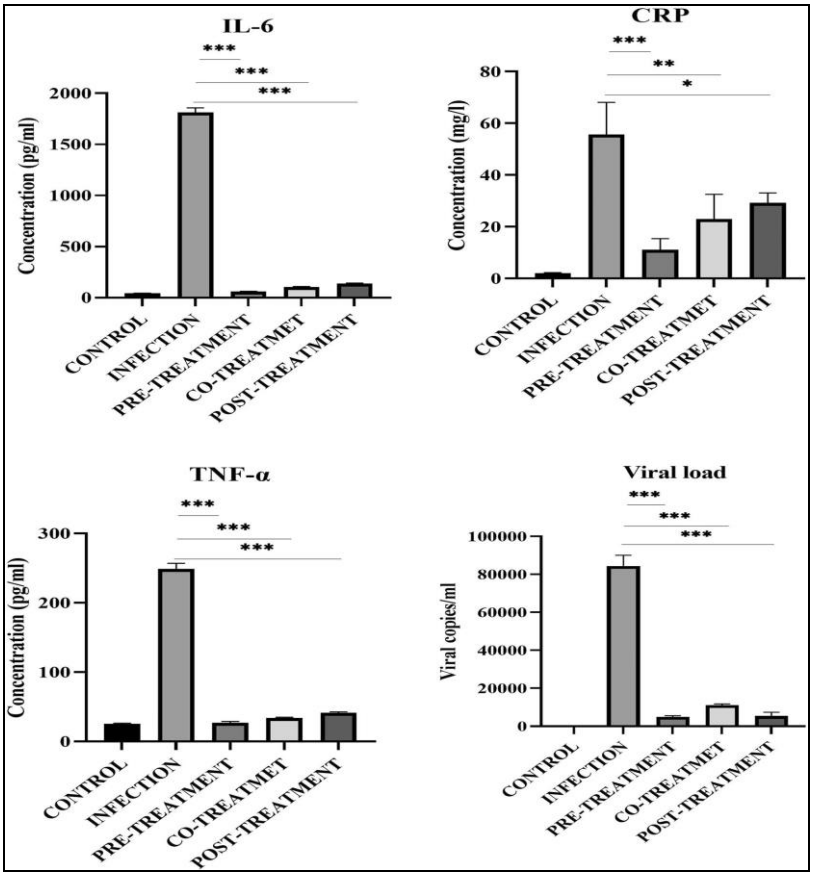

Fig 6.12: Effect of curcumin on pro- inflammatory biomarkers and viral load determination

6.4. DISCUSSION

There is no specific treatment for alleviating CHIKV-induced arthritis till date. Currently, supportive treatment using ibuprofen, naproxen, corticosteroid or other non-steroidal anti-inflammatory agent (NSAID) is the only alternative therapy used in clinical practice [15]. But, long-term use of these drugs might induce bleeding, gastro-intestinal complications, renal disturbances, cardiovascular events, osteoporosis, aseptic joint necrosis and hepatic effects [29, 46, 47]. Curcumin, a plant- derived FDA- approved drug with low toxicity has been shown to be beneficial in improving DAS28, american college of rheumatology/european league against rheumatism (ACR/EULAR), visual analog scale (VAS), western ontario and mcMaster universities arthritis index (WOMAC) scoring of rheumatoid arthritic and osteoarthritic patients [48].

This study is the first to demonstrate analgesic activity of curcumin also in alleviating CHIKV-induced acute/chronic arthritis within mouse model Treatment (pre, co, and post) with curcumin significantly increased the leg pain-threshold level and decreased the feet-swelling of CHIKV-infected mice, demonstrating its analgesic, prophylactic, and anti-inflammatory effects against CHIKV-induced acute/chronic arthritic manifestation. However, its effect in reducing nociceptive pain was more prominent for pre and co-treatment groups compared to post-treatment ones. High-dose of 2000mg/kg curcumin was most effective against alleviating pain and feet-swelling. CHIKV-infection triggers a rapid innate immune response by producing pro-inflammatory cytokines that might lead to articular cartilage loss and bony erosion in affected joints, as documented among Thai and Indian patients [29, 49, 50]. CHIKV replication in joint tissues induced expression of pro-osteoclastogenic cytokines, IL-6, NF-κB, Tumor necrosis factor-alpha (TNF-α), IL-1β and receptor activator of nuclear factor kappa-B ligand (RANKL) among Singaporean and Thai patients that promoted bone resorption and arthritic manifestation [29, 51, 52].

There were previous reports of release of pro-inflammatory cytokines upon CHIKV-infection, along with raised CRP levels, among patients of Singaporean origin [53]. Curcumin has been reported to down-regulate CRP, IL-6, NF-κB, IL-1β and TNF-α production, thereby, possibly inhibiting osteoclastogenic mechanisms and bone destruction [29, 54]. This study also demonstrated both anti-CHIKV and anti-inflammatory effects of curcumin among CHIKV-infected arthritic murine models. Previous studies have also demonstrated protective and anti-inflammatory effects of curcumin in Balb/c mice against CMV and RSV infection [55, 56]. Curcumin treatment has been previously reported to decrease histopathological changes, paw swelling rate, arthritis score, and neutrophil activity among rheumatoid arthritic mice models [32].

Lower OARSI and SMASH arthritic scores of $PT_{HD,}$ CT_{HD} and Post-T_{MD} mice sub-groups indicated less cartilage and proteoglycan loss in knee articular cartilage tissues of curcumin-treated mice compared to infection group. CHIKV-infection has been previously associated with IL-1β production [57]. Over-production of this cytokine was reported to stimulate matrix metalloproteinases (MMPs) activation leading to excessive cartilage matrix degradation in rheumatoid and osteo-arthritic patients [7]. Protection of type II collagen producing chondrocytes from catabolic effect of IL-1β and MMPs was previously reported following curcumin treatment among cultured condrocytes and osteoarthritic mouse model [41]. Increase in intensity of type II collagen of MFC and MTP knee joints following curcumin treatment within immunohistochemical analysis of present study corroborated with histological scoring. This was also evident following curcumin treatment on chondrocyte, synovial fibroblast cultures *in vitro* and in collagen-induced arthritic, osteoarthritic and rheumatoid arthritic mouse models [41, 32].

Effect of curcumin treatment on locomotory behaviour of arthralgic mice model was also evident in open field tests. Compared to infected mice, line crossings, CSE, rearing and

locomotion time of curcumin PT_{HD}, CT_{HD} and Post-T_{MD} sub-groups improved significantly and was comparable to control group. This finding was consistent with previous report of increased locomotory behaviour of curcumin nanoparticle treated osteoarthritic mice with destabilised medial meniscus [41]. Similar finding of reduction in Lequesne's pain functional index score was noted among osteoarthritic patients receiving curcuminoid therapy, a bioactive constituent of turmeric [58].

6.5. CONCLUSION

Thus, the present study confirmed anti-inflammatory, analgesic and chondroprotective role of curcumin against chikungunya induced arthritic mice, which needs to be validated among viral infected arthritic patients. This might help physicians in managing severe arthritic pain of chikungunya infected patients without prescribing ibuprofen, naproxen or NSAIDs.

6.6. REFERENCES (CHAPTER 4)

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CONCLUSION

7. GENERAL CONCLUSION

Chapter 1 confirmed emergence and spread of CHIKV mono-infection and DENV-CHIKV co-infection in West Bengal, eastern India during the period of 2014-2016. In this study a significant presence of joint pain and joint swelling among chikungunya patients confirmed the severity of arthralgic manifestation, which required prioritized attention for proper management of these patients. Also, arthralgia, myalgia and bleeding were significant clinical manifestations among co-infected patients which may turn severe if untreated. Thus, the health authorities and community should, therefore, keep a strict vigilance for early diagnosis of CHIKV mono-infection and DENV-CHIKV co-infection.

Chapter 2 elucidated diagnostic potential of serum and genetic markers during early stage of infection to identify patients who might develop post-chikungunya chronic arthritis. Upon diagnosis of CHIKV-infected patients (with/without acute arthralgia), these markers could be used to screen and identify patients who might develop chronic arthritis. This study was the first to demonstrate prognostic relevance of CRP, COMP and IL-2R level in identifying chronic arthritic chikungunya patients. Furthermore, it was found out that serum level of anti-CCP antibody, IL-2R, COMP was high during 0-7 days of symptomatic onset among patients developing post-chikungunya chronic arthritis. Also, CHIKV patients with COMP-rs144778694-GA were susceptible to arthralgic manifestation, whereas, patients with certain genotypes of CRP, IL-2R and COMP were protected from arthralgia. Thus, this study would be helpful to medical specialists for clinical management of chikungunya patients who would develop post-chikungunya chronic arthritis.

Chapter 3 underlined the importance of TLR3,7,8 and CRP SNPs towards co-infection of DENV and CHIKV, with certain genotypes imparting a protective role while others

associated with susceptibility towards co-infection. Several genotypes of TLR7-rs179008, TLR7-rs179010, TLR8-rs3853839, TLR8-rs3764879, TLR8-rs5744080, CRP-rs3093059 and CRP-rs3091244 polymorphisms was susceptible to co-infection. Moreover, this study suggested probable role of specific genotypes of TLR7,8 and CRP SNPs towards imparting high dengue/chikungunya viral load during competitive viral replication among co-infected patients. Co-infected patients with TLR7-rs179010-TT, CRP-rs3093059-TT and CRP-rs3091244-CT exhibited DENV-HVL, whereas CHIKV-HVL was detected among patients with other genotypes. These variants might also act as potential prognostic biomarkers for predicting co-susceptibility among uninfected individuals. *In silico* protein-protein docking evaluated interactive effect of TLR variants with signal peptidase complex and it was found that TLR7-rs179008 Q variant and TLR8-rs3764880 V variant generated better free binding energy.

Chapter 4 explored and established the anti-inflammatory, prophylactic, analgesic and chondroprotective role of curcumin against chikungunya-induced arthritic mice. Mice were administered high (HD: 2000 mg/kg), mid (MD: 1000mg/kg), and low (LD: 500mg/kg) curcumin doses, before (PT: pre-treatment), during (CT: co-treatment) and after (Post-T: post-treatment) CHIKV-infection. Curcumin treatment using PT-HD, CT-HD, and Post-T-MD significantly alleviated CHIKV-induced arthritic pain by improving pain-threshold, locomotory behavior and reduced feet swelling of infected mice. Also, decreased proteoglycan loss and cartilage erosion with lower OARSI, SMASH scores were observed among these three subgroups compared to infected ones. Compared to infected ones, one to two fold increased intensity of type II collagen in knee medial femoral condyle and medial tibial plateau regions of these subgroups were also observed by immunohistochemical staining. Thus, these findings might help physicians in managing severe arthritic pain of chikungunya-infected patients.

8. LIST OF PUBLICATIONS DURING PH.D. TENURE

1. **Sengupta S,** Mukherjee S, Haldar SK, Bhattacharya N, Tripathi A. Re-emergence of Chikungunya virus infection in Eastern India. **Brazilian Journal of Microbiology**. 2020 Mar;51(1):177-182. doi: 10.1007/s42770-019-00212-0. Epub 2020 Jan 2. PMID: 31898249; PMCID: PMC7058808.

2. **Sengupta S**, Mukherjee S, Bhattacharya N, Tripathi A. Differential genotypic signatures of Toll-like receptor polymorphisms among dengue-chikungunya mono- and co-infected Eastern Indian patients. **European journal of clinical microbiology & infectious diseases.** 2021 Jul;40(7):1369-1381. doi: 10.1007/s10096-020-04125-x. Epub 2021 Jan 25. PMID: 33495940.

3. **Sengupta S**, Bhattacharya N, Tripathi A. Association of C-reactive protein polymorphisms with serum-CRP concentration and viral load among dengue-chikungunya mono/co-infected patients. **Antiviral Research.** 2022;197:105225. doi:10.1016/j.antiviral.2021.105225

4. **Sengupta S,** Bhattacharya N, Tripathi A. Increased CRP, anti-CCP antibody, IL-2R, COMP levels in prognosis of post-chikungunya chronic arthritis and protective role of their specific genotypes against arthritic manifestation. **Virus Research.** 2022 Nov 7:198998. doi: 10.1016/j.virusres.2022.198998. Epub ahead of print. PMID: 36356677

5. **Sengupta S**, Tripathi A. Evaluation of analgesic and prophylactic activity of Curcumin against chikungunya-infected acute/chronic arthralgic mice [published online ahead of print, 2023 Mar 10]. **Journal of Medical Virology**. 2023;10.1002/jmv.28661. doi:10.1002/jmv.28661.

6. Mukherjee S., Dutta S. K., **Sengupta S**, & Tripathi A.. Evidence of dengue and chikungunya virus co-infection and circulation of multiple dengue serotypes in a recent Indian outbreak. **European journal of clinical microbiology & infectious diseases** 2017: official publication of the European Society of Clinical Microbiology, 36(11), 2273–2279. https://doi.org/10.1007/s10096-017-3061-1

7. Dutta, S. K., **Sengupta S**., & Tripathi, A. In silico and in vitro evaluation of silibinin: a promising anti-Chikungunya agent. **In vitro cellular & developmental biology.** 2022 Animal, 58(3), 255–267. https://doi.org/10.1007/s11626-022-00666-x

APPENDIX

Publications

Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-019-00212-0

CLINICAL MICROBIOLOGY - RESEARCH PAPER

Re-emergence of Chikungunya virus infection in Eastern India

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Received: 12 April 2019 / Accepted: 11 December 2019
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Abstract

Chikungunya fever is a major public health issue in India. Re-emergence of chikungunya virus (CHIKV) in West Bengal was detected after 32 years in 2006. After 2010, this infection was in apparent decline, but in 2016 a massive outbreak affected the country. Present study was carried out to understand CHIKV infection dynamics during recent outbreaks in West Bengal, Eastern India and its implication on disease manifestations. Blood was collected from 641 symptomatic patients. Patients' sera were serologically diagnosed to detect presence of anti-chikungunya-IgM antibodies. Viral RNA was extracted; presence of CHIKV genome and its respective viral load was determined by real time quantitative reverse transcription-PCR (real-time qRT-PCR). Statistical analysis was performed using EPI INFO software. CHIKV infection was detected in 24.64% of symptomatic patients. Middle-aged patients (31–40 years) were predominantly affected; clinically, both arthralgia and joint-swelling were significantly prevalent among CHIKV-infected patients. Myalgia, joint-swelling, and arthralgic manifestation were found in significantly higher frequency among patients with high chikungunya viral load (> 10,000 copies/ml). Thus, this study clearly indicated the re-emergence of CHIKV in Eastern India. Significant presence of myalgia, joint swelling, and arthralgia among chikungunya patients with high viral load implied association of disease severity with viral load; requiring vigilance for proper management of infected patients as this disease is highly morbid in nature. However, in addition to chikungunya virus, other viral, bacterial, and protozoal infections also occur during post-monsoon season in India, having overlapping symptoms. Hence, continuous monitoring of these infections is required for better clinical management of patients.

Keywords Chikungunya · Viral load · qRT-PCR · IgM ELISA · Myalgia · Arthralgia

Introduction

Chikungunya is an arthropod-borne neglected tropical disease caused by chikungunya virus (CHIKV), which is a single-stranded positive sense RNA virus, belonging to *Alphavirus* genus of *Togaviridae* family [1, 2]. While, *Aedes albopictus* serves as major vector in Indian Ocean islands, *Aedes aegypti* is the principal vector for CHIKV in mainland India [3, 4]. In Africa, CHIKV is maintained in sylvatic cycle involving non-human primates and a number of forest-dwelling mosquitoes (*Aedes furcifertaylori, Aedes luteocephalus*, and *Aedes africanus*) [5]. Since the first detection of chikungunya infection during late 2013 in Saint Martin island, CHIKV has alarmingly infected a million people in Caribbean, Latin America, Mexico and parts of South America viz. Brazil, Bolivia, Colombia, Ecuador, Paraguay, and Venezuela having both imported from Asia-Africa and autochthonous cases [6]. Currently, chikungunya is a re-emerging vector-borne disease of high public health significance in South-East Asia Region viz., India, Indonesia, Maldives, Myanmar, Sri Lanka, and Thailand [7]. In India, CHIKV was first isolated in 1963 at Kolkata (Calcutta) and continued imparting several epidemics at different regions of India, viz. Chennai, Pondicherry, Vellore, Nagpur, and Mumbai between 1964 and 1975 [8, 9]. In the following years, CHIKV seemed to have

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Responsible Editor: Mauricio Nogueira

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42770-019-00212-0) contains supplementary material, which is available to authorized users.

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Published online: 02 January 2020

disappeared from India, eventually making a sudden re-emergence in 2005 after a gap of 32 years causing an explosive outbreak [10, 11]. Kerala was the worst affected state in 2007 with approximately 3.6 million fever cases [12]. Subsequently, recurring mass infection and disease spread was reported from 13 different states of India, resulting in 1.4–6.5 million estimated cases across the country [13]. In 2006, West Bengal had witnessed a CHIKV outbreak [14]. Though 2010 onwards chikungunya fever has been in the decline, until date, there has been several sporadic cases of CHIKV infection reported from different parts of India necessitating implementation of surveillance programs in clinical settings [15–17]. CHIKV is responsible for an acute infection, characterized by abrupt onset of high fever, joint pain, myalgia, headache, and rash [18, 19]. Joint pain, persisting for months, or even years, is a typical clinical symptom of CHIKV infection [20]. In some patients, maculopapular rashes are found mainly on trunks or exterior surfaces of limbs, and rashes are usually accompanied by secondary rise in temperature [21].

The present study has been aimed to detect CHIKV among symptomatic patients from Eastern India and to review current status of chikungunya infection in West Bengal during 2013–2016. The study was also aimed to correlate the appearance of characteristic clinical symptoms with CHIKV infection.

Materials and methods

Inclusion criteria

As per World Health Organization (WHO) criteria, an acute febrile illness with history of headache, body ache, myalgia, arthralgia, and rash, with or without hemorrhagic manifestation from all age groups and both sexes was selected.

Exclusion criteria

Patients with hematological malignancies, bleeding disorders, chronic liver disease, diabetes mellitus, renal diseases, shock, meningoencephalitis, and neuropathy were excluded from the study.

Specimen collection

After obtaining the institutional ethical committee approval (CREC-STM/53 dated 26.09.2013), 5 ml of blood were collected from each of the 641 symptomatic febrile patients visiting Calcutta School of Tropical Medicine, Kolkata, West Bengal, India, from September 2014 to October 2016. At the time of blood collection, all patients exhibited fever. Sera were separated from the collected blood samples through density gradient centrifugation and stored at − 80 °C and − 20 °C freezer for molecular and serological tests, respectively.

Molecular techniques

Viral RNA isolation and detection of CHIK virus using real-time qRT-PCR

Viral RNA was extracted from 140 μL of patient serum by using QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France) [22]. Presence of CHIKV genome and their respective viral loads (copies/ml) was determined through real-time qRT-PCR in a total volume of 20 μl using 8 μl viral RNA, One Step qRT-PCR Master Mix and components of CHIKV Genesig standard kit (PrimerDesign Ltd., UK), according to the manufacturer's protocol. Real-time qRT-PCR was performed in 7500 fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). Cloned CHIKV non-structural protein 2 gene, provided in the kit, was used as positive control [16]. Steps for one step qRT-PCR were reverse transcription (55 °C for 10 min), activation (95 °C for 8 min), amplification (50 cycles at 95 °C for 10 s, 60 °C for 60 s and 72 °C for 12 s), and cooling (40 °C for 30 s). A standard curve of cycle threshold (C_T) values was constructed using six 10-fold dilution series of a positive control template. Copy number of CHIKV in patient serum was calculated from the corresponding C_T values. Each sample was loaded in triplicate.

Determination of chikungunya viral load

Chikungunya viral load was determined using real-time qRT-PCR in total volume of 20 μl, using 5 μl of patient-extracted viral RNA, one step qRT-PCR master mix, and other components of CHIKV Genesig kit (Primer Design Ltd., UK). Real-time qRT-PCR was performed in 7500 fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). A standard curve of cycle threshold (C_T) values was constructed using six 10-fold dilution series of the positive control template (provided in the kit). Copy number was calculated from the corresponding C_T values. Each sample was analyzed in triplicate, according to the manufacturer's protocol. CHIKV load of $\geq$ 10,000 copies/ml and < 10,000 copies/ml were identified as high viral load (HVL) and low viral load (LVL), respectively [15].

Serological techniques

Detection of anti-CHIKV IgM antibody using ELISA

Sera samples of patients were subjected to serological diagnosis for presence of anti-chikungunya-IgM antibodies using NIV CHIKV MAC ELISA Kits (National Institute of

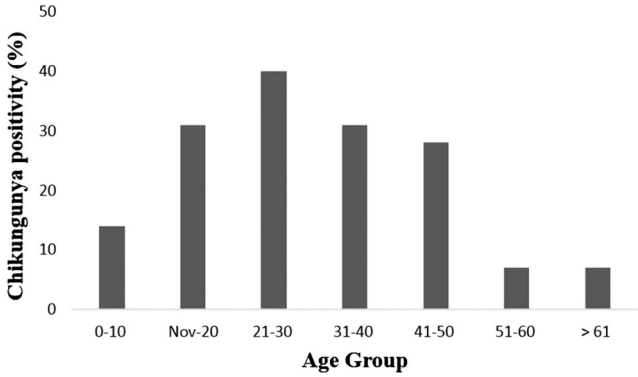

Fig. 1 Age-group wise distribution of CHIKV-infected patients. Infected age group (divided in ranges from 0–10 to above 60) has been plotted on *X*-axis and percentage of chikungunya-infected patients has been plotted on *Y*-axis

Virology, Pune, India), following the manufacturer's instructions. Briefly, 100 μL of 100 times diluted serum samples, negative control, positive control, and calibrator were added to respective wells and incubated for 1 h at 37 °C. The wells were washed five times and were incubated with 100 μL of 10 times diluted enzyme conjugate for 1 h at 37 °C. Following incubation, wells were washed five times and further incubated at room temperature with 100 μL of TMB (3,3′,5,5′-Tetramethylbenzidine). After 30 min of incubation, reaction was terminated by adding 50 μL of stop solution and absorbance was measured at 450 nm wavelength with reference filter of 600–650 nm within 30 min in Infinite M200 (Tecan Group, M nnedorf, Switzerland). Results were interpreted as negative or positive according to assay manual.

Statistical analyses

A 2 × 2 contingency Chi square and odds ratio analysis were performed to find association between CHIKV infections or

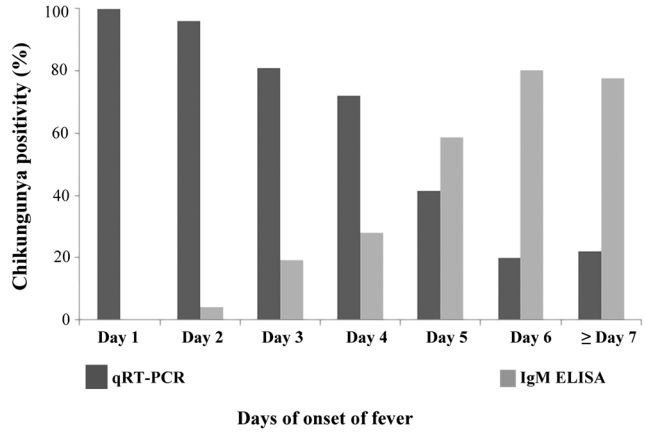

Fig. 3 Percentage of CHIKV positivity by real-time qRT-PCR and IgM ELISA according to the days of collection from symptomatic onset. Days of blood collection from symptomatic onset (plotted on *X*-axis) along with percentage of CHIKV positivity detected using real-time qRT-PCR (■) and anti-Chikungunya-IgM ELISA (■) (plotted on *Y*-axis)

CHIKV load with different clinical symptoms using CDC Epi-Info software (version 3, CDC, Atlanta, GA, USA). Probability values of $P < 0.05$ were considered as statistically significant. Odds ratio values > 1 indicated that a condition/event was more likely to occur in a particular disease group.

Results

During 2014–2016, incidence of chikungunya infection was investigated among the 641 symptomatic patients from various parts of West Bengal. Among them, 24.64% (158/641) were found to be chikungunya-infected through real-time qRT-PCR and anti-chikungunya-IgM ELISA (Table S1). Among these CHIKV infected patients, 90 were within acute stage (0–5 days of symptomatic onset). Male to female ratio of

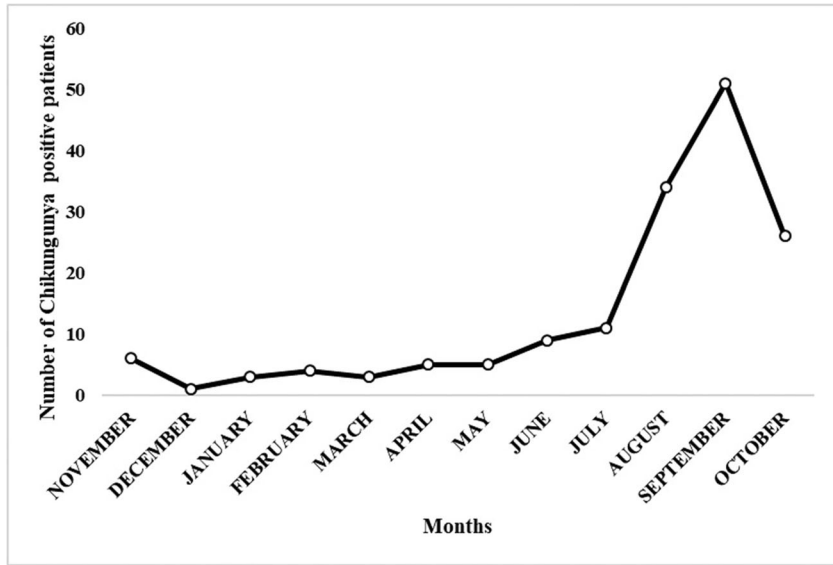

Fig. 2 Cumulative distribution of CHIKV positivity across monsoon and post-monsoon seasons of 2014–2016. Monthly demography (plotted on *X*-axis) and total number of chikungunya-positive patients identified per month (plotted on *Y*-axis)

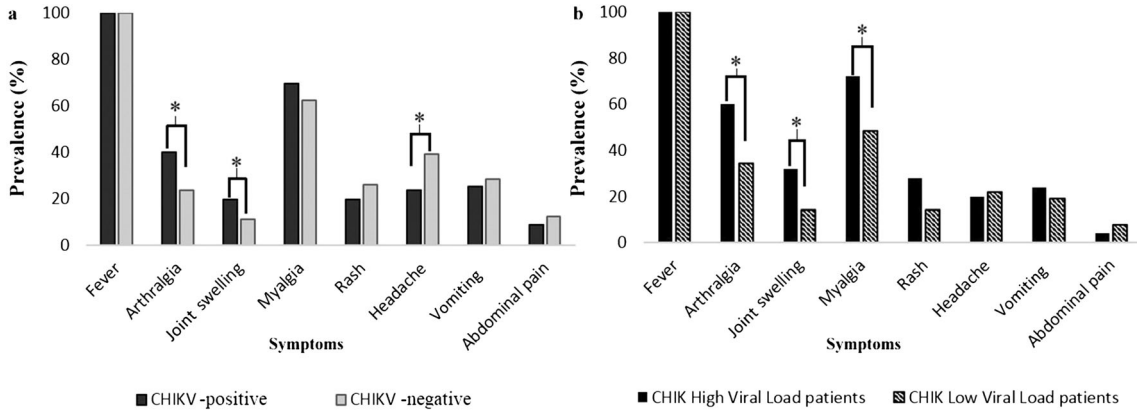

Fig. 4 Comparison of clinical symptoms between **a** CHIKV infected and uninfected symptomatic patients, **b** HVL and LVL group of CHIKV infected patients. **a** Clinical symptoms exhibited by CHIKV-positive (■) and CHIKV-negative patients (□) (plotted on *X*-axis) and percentage prevalence exhibited by both groups (plotted on *Y*-axis). **b** Clinical symptoms exhibited by CHIKV HVL (■) and CHIKV LVL patients (▚)(plotted on *X*-axis) and percentage prevalence exhibited by both groups (plotted on *Y*-axis) asterisk (✱) indicated χ^2 p value < 0.05 as statistically significant at 95% confidence interval

chikungunya patients was 1.36:1 and mean age group was 33.06 ± 14.7 years (range 7–72 years).

Frequency of CHIKV infection was highest among patients 21–30-year age group, and gradually decreased with increasing age (Fig. 1). Similar to other mosquito-borne infections, CHIKV infection peaked up after July, which corresponds to the onset of monsoon in India. Maximum percentage of CHIKV infection was reported during September ((51/158) = 32.27%) (Fig.2). Depending upon day of sample collection after symptomatic onset, real-time qRT-PCR assay could detect highest percentage of CHIKV genome on the first day of infection, which gradually decreased with increasing number of days after symptomatic onset (Fig.3). On the contrary, percentage prevalence of anti-chikungunya-IgM followed the reverse trend. Anti-chikungunya-IgM ELISA could detect highest percentage of anti-chikungunya-IgM antibody in patients' sera on the sixth day of symptomatic onset (Fig. 3).

Clinically, arthralgia (OR = 2.14, 95% CI 1.46–3.14, p = 0.0001) and joint swelling (OR = 1.67, 95% CI 1.03–2.70, p = 0.03) were more frequent among chikungunya-positive patients than that of chikungunya-negative symptomatic patients (Fig. 4a). Approximately 23.8% PCR-positive chikungunya patients exhibited HVL of CHIKV, whereas, remaining 76.2% PCR-positive chikungunya patients showed LVL. Interestingly, compared with chikungunya LVL group, arthralgia, joint swelling, and myalgia were significantly

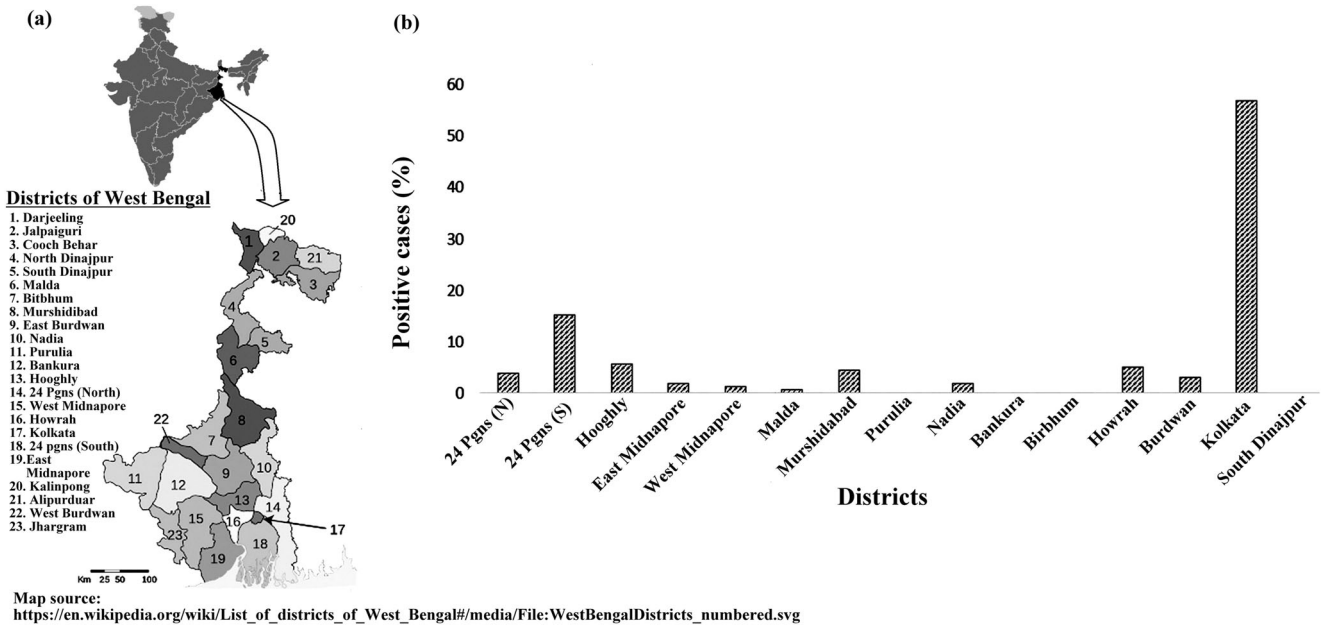

Fig. 5 District-wise prevalence of CHIKV infected patients in West Bengal, Eastern India. **a** Maps of India and West Bengal. **b** Districts of West Bengal has been plotted on *X*-axis and percentage of chikungunya-positive cases has been plotted on *Y*-axis

higher among chikungunya HVL patients (Fig. 4b). According to symptomatic history accounted by patients, pain started on the first or second day of fever. It then involved both small and big joints of both the extremities with no upper/lower limb predilection. Vomiting and abdominal pain were the other associated minor symptoms. Majority of the symptoms subsided within 3–5 days, whereas, joint pain persisted for more than 30 days. All the patients were treated symptomatically and no death was observed.

Burden of chikungunya infection was markedly noticed within Kolkata metropolitan, West Bengal, India and its adjoining districts, viz. South 24 Parganas, Howrah, Hooghly, and North 24 Parganas (Fig. 5).

Discussion

In this cross-sectional study, multi-diagnostic approach viz. real-time qRT-PCR and IgM ELISA were carried out for laboratory confirmation of chikungunya infection. Similar to an earlier study by Dutta et al. 2014, the present study also confirmed that real-time qRT-PCR is the most sensitive diagnostic tool for detecting chikungunya infection during early stage of infection as it confirmed the presence of CHIKV genome among acute stage patients' blood—indicating greater efficacy of molecular diagnosis compared with serology-based techniques for early detection [16]. Maximum number of CHIKV infection was reported during September, the first post-monsoon month in India. Furthermore, a higher rate of CHIKV infection was found among middle age groups [17, 23, 24]. In this study, arthralgic manifestation and joint swelling were significantly more prevalent among CHIKV infected patients compared with that of symptomatic uninfected patients which have been mentioned in several earlier studies [17, 23]. Chikungunya patients with HVL were found to demonstrate arthralgia, joint swelling, and myalgia at significantly greater frequency compared with patients with LVL. HVL has been characterized previously by higher production of pro-inflammatory cytokines, viz. IL15, which might be associated with development of joint inflammation [25]. Prevalence of joint swelling among chikungunya patients with HVL has been previously reported by Dutta et al. 2014 [16].

Since 2010, CHIKV infection had been declining in India until the current outbreak in West Bengal and Delhi depicted its reemerging path [26]. As both dengue and chikungunya are transmitted by the same vectors and having similar disease manifestation, CHIKV has been reported as a co-infection in almost 10% of dengue virus (DENV) cases in recent studies [27, 28]. In the present study, approximately 7% (45/641) of the chikungunya cases were also found to be dengue-infected using real-time qRT-PCR, anti-dengue IgM, and dengue NS1 ELISA. However, the present study highlighted the re-emergence of chikungunya in Eastern India, West Bengal, especially surrounding Kolkata [15, 17].

In this study, chikungunya infection was detected among 24.64% of symptomatic patients. However, in addition to chikungunya, other viral, bacterial, and protozoal infections having similar symptoms might have occurred during post-monsoon season in India and hence might be considered for remaining other febrile patients. Hence, continuous monitoring of these infections is essential for better clinical management of patients.

Conclusion

This study confirmed the emergence and spread of CHIKV infection in West Bengal, India. Though in this study no mortality was reported among patients infected with CHIKV, a significant presence of joint swelling among chikungunya patients implied the severity of arthralgia, which required prioritized attention for proper management of these patients—as this disease is highly morbid in nature. However, in addition to chikungunya virus, other viral, bacterial, and protozoal infections also occur during post-monsoon season in India, which has overlapping symptoms. Thus, detailed analyses of outbreak will be important to understand the actual impact of the current outbreak. The health authorities and community should, therefore, keep a strict vigilance for early diagnosis of illness.

Acknowledgments The authors are extremely grateful to the Director, Calcutta School of Tropical Medicine, Kolkata, India, for his support, inspiration, and providing necessary facilities for this study.

Funding information This study was partly funded by the Department of Science and Technology, Government of West Bengal, India (615(sanc.)/ST/P/S& T/9G-14/2010). The authors are grateful to the Indian Council of Medical Research, India, for granting fellowship of the first author (5/3/8/6/ITR-F/2018-ITR).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval and informed co1)nsents All procedures performed in this study involving human participants were in accordance with the ethical standards at the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. The study was approved by the Clinical Research Ethics Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26 September .09.2013). Informed consent was obtained from all individual participants included in the study.

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Virus Research 323 (2023) 198998

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Increased CRP, anti-CCP antibody, IL-2R, COMP levels in prognosis of post-chikungunya chronic arthritis and protective role of their specific genotypes against arthritic manifestation

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ARTICLE INFO

Keywords:
Chikungunya
Post-chikungunya chronic arthritis
Arthritic biomarkers
Hepatic biomarkers
Principal component analysis

ABSTRACT

Chikungunya infection leads to acute/chronic polyarthritis/polyarthralgia causing long-term morbidity among patients. Prognosis of post-chikungunya chronic arthritis (PCA) is of utmost necessity for proper disease management. Arthritic and hepatic biomarkers were evaluated among chikungunya patients without arthritis, with acute arthritis and with post-chikungunya chronic arthritis in the study. Serum levels of arthritic [CRP (C-reactive protein), anti cyclic-citrullinated-peptide (anti-CCP) antibody, soluble interleukin-2 receptor (sIL-2R), cartilage oligomeric matrix protein (COMP)] and hepatic [ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), albumin and bilirubin] biomarkers of 167 chikungunya positive patients were determined by sandwich-ELISA/immunoturbidimetry/auto-analyser. 167 chikungunya-patients and 102 healthy controls were genotyped to understand role of CRP-rs3093059/rs3091244, IL-2R-rs743777 and COMP-rs144778694 polymorphisms towards chikungunya virus (CHIKV) infectivity and arthralgic manifestation. CRP, anti-CCP antibody, IL-2R and COMP levels significantly increased among PCA patients. Concentrations of AST, ALT, AST/ALT-ratio, bilirubin and ALP increased among arthritic chikungunya patients. Principal component analysis differentiated PCA groups from acute (AA) and non-arthritic groups. Patients with IL-2R-rs743777-GA, G-allele and COMP-rs144778694-GA genotypes were susceptible to chikungunya infection. Moreover, patients with CRP-rs3093059-CT, rs3091244-TT, IL-2R-rs743777-GA and COMP-rs144778694-AA genotypes were significantly protected from arthralgia, whereas, COMP-rs144778694-GA genotype was susceptible towards it. Patients with certain genotypes of CRP, IL-2R and COMP demonstrated significantly higher biomarker serum-levels among patients suffering from AA with/without PCA. Thus, both serum biomarker levels and polymorphic genotypes of infected patients play decisive role in development of PCA.

1. Introduction

Epidemics caused by chikungunya virus (CHIKV) have been frequently occurring in India 2005 onwards, after its disappearance for more than three decades (Naresh Kumar and Sai Gopal, 2010; Sengupta et al., 2020). This disease is characterized by abrupt onset of fever, frequently accompanied with joint pain, acute or chronic arthritis, causing stooped appearance of patients (Mohan et al., 2010). Acute arthritis might lead to development of devastating oligo- or polyarthritis involving 4 or more joints of both arms and legs (Alpay-Kanıtez et al., 2018). Approximately, 88-100% of infected patients might also experience post-chikungunya sub-acute arthritis (6 weeks) and 40–60% might demonstrate chronic arthritis even after 1.5 to 5 years of infection, indicating role of host genetics in determining chronic arthritis among infected patients (Javelle et al., 2015; Tritsch et al., 2020). There were reports of development of chronic inflammatory rheumatism and musculo-skeletal disease during post-chikungunya arthritis, which could be relapsing or unremitting (Tritsch et al., 2020). Clinical presentation of long term/post-chikungunya infection might mimic autoimmune rheumatological conditions viz. rheumatoid arthritis (Krutikov and Manson, 2016; Gauri et al., 2016). Hence, early identification of infected patients who might develop chronic arthralgia in long term could help clinicians in their pain management. Both DNA and serum level biomarkers might help in early differentiation between acute (AA) and

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https://doi.org/10.1016/j.virusres.2022.198998
Received 18 August 2022; Received in revised form 21 October 2022; Accepted 4 November 2022
Available online 7 November 2022
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post-chikungunya arthritic (PCA) patients (Flögel et al., 1998; Booth et al., 1998).

CHIKV primarily infects skin cells, thereby replicating in skin, liver, muscle and bone joints and simultaneously invading monocytes and blood vessels – resulting in bone erosion, degradation of extracellular matrix and release of pro-inflammatory cytokines (Srivastava et al., 2020). Increased level of C-reactive protein (CRP), an acute-phase hepatic protein has been reported among viraemic and arthritic infected patients (Gauri et al., 2016; Solanke and Karmarkar, 2014). Similarly, elevated ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) levels has been reported among both viraemic and post-viraemic chikungunya patients – indicating involvement of hepatic anomalies in CHIKV infection (Danis-Lozano et al., 2017; Ng et al., 2009). Arthralgic manifestation during CHIKV infection resembles that of rheumatoid arthritis (RA), characterized by increased rheumatoid factor (RF) and anti cyclic-citrullinated-peptide (anti-CCP) antibody related articular damage (Imai et al., 2016). On the other hand, cartilage oligomeric matrix protein (COMP), an extracellular matrix protein, has been associated with cartilage turnover and joint destruction related to osteoarthritis and rheumatoid arthritis (Tseng et al., 2009). Soluble Interleukin-2 receptor (sIL-2R), a pro-inflammatory cytokine related to chikungunya infection, has also been implicated in development of synovitis and exacerbation of disease severity of rheumatoid arthritis (Wood et al., 1988). Moreover, RF, anti-CCP antibody and CRP has been recognized as serological and acute phase response markers for rheumatoid arthritis, according to 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria (Aletaha et al., 2010).

Besides protein levels, polymorphic genotypes of these genes might contribute to development of chikungunya induced arthritis among infected patients. Specific genotypes of cis-acting polymorphisms of CRP, viz. rs3091244, rs3093059, have been reported to contribute to variance in protein level among dengue, chikungunya, ankylosing spondylitic and osteomyelitic patients (Sengupta et al., 2022). Previously, mutations within COMP gene have been strongly associated with pseudoachondroplasia, multiple epiphyseal dysplasia and osteoarthritis (Deere et al., 1999; Mishra et al., 2019). Similarly, specific polymorphic genotypes of rs743777 (IL-2R), localized 6kb upstream of transcription start site (5′near gene region) and were associated with RA and peripheral arthritis of ankylosing spondylitic patients (Ruyssen-Witrand et al., 2014; Polo et al., 2019; Pál et al., 2010). But, contribution of these polymorphic variants towards development of chikungunya induced arthritis has not been studied. Moreover, prognostic value of these polymorphic genotypes and protein levels has not been evaluated in case of CHIKV induced arthritis.

Thus, this study explores prognostic values of these serum proteins and their genetic polymorphisms in determining acute and chronic arthritis among CHIKV infected patients.

2. Materials and methods

2.1. Ethics statement

Collection of blood from each of the febrile patients and healthy participants was performed in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine, Kolkata, India (CREC-STM/53 dated 26.09.2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consents were obtained from patients and healthy individuals.

2.2. Inclusion criteria

Patients with acute febrile illness and history of headache, body ache, myalgia, arthralgia, rash, with or without haemorrhagic manifestation of all age-groups and both sexes were reviewed by physician according to WHO criteria of chikungunya infection.

2.3. Exclusion criteria

Haematological malignancies, bleeding disorders, chronic liver disease, diabetes mellitus, and renal diseases.

2.4. Patients and healthy controls

Around 2ml of blood of all age groups and sexes were collected from each of 641 symptomatic patients during their first visit at Calcutta School of Tropical Medicine (CSTM), West Bengal, India from September 2014 to October 2016, within acute phase of infection. Febrile patients exhibiting any two of the following symptoms: headache, myalgia, arthralgia, nausea, vomiting, rash, fatigue was selected as per WHO criteria. Amongst them, 167 were CHIKV infected, as detected by anti-CHIKV IgM ELISA (NIV, Pune, India)/real-time qRT-PCR. To carry out age-matched case control study, blood from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with DENV, CHIKV, Japanese encephalitis, HIV and Hepatitis A/B/C infection - as tested by IgM and IgG ELISA/RT-PCR, were collected from same community. Symptomatic patients and healthy controls consisted of Bengali population of eastern India having similar ethnic genetic make up to that of Bengali Bangladeshis (BEB) group of SAS included within 1000 genome project of GWAS. Control population size was calculated using EpiInfo™ version 7.2 software of CDC, with 95% confidence interval and 24.64% CHIKV infection rate among eastern Indian patients (Sengupta et al., 2020).

All the following biochemical and genotypic experiments were performed on patient-blood collected during their first visit at CSTM. Detailed follow up study of arthralgic manifestations of CHIKV infected patients was performed both during their first visit and after 2 years from date of collection of blood and patients were grouped into acute and chronic chikungunya cases according to criteria of World Health Organization (WHO) (WHO, 2015). Patients were categorised into four groups: NA: Patients without any arthralgia; AA: patients with only acute arthralgia; AA+PCA: patients with both acute and post chikungunya arthralgia and PCA: patients developing only post-chikungunya arthralgia. Depending upon rheumatoid arthritic outcome, CHIKV-infected patients were also categorised according to 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria (Aletaha et al., 2010).

2.5. Extraction of viral RNA and determination of CHIKV

Viral RNA was extracted from 140μL of patients' sera, using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France). Genome presence of CHIKV was determined by real-time qRT-PCR using CHIKV Genesig kit (Primer Design Ltd., UK), according to manufacturers' protocol. Limit of detection (LOD) of CHIKV Genesig kit was <100 copies of corresponding target genomes. Real-time PCR was performed on ABI Prism 7500 fast instrument. Each sample was loaded in triplicate.

2.6. Quantification of CRP

Sera collected from infected patients were subjected to CRP quantification. CRP level was determined by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics, India) according to manufacturer's protocols. Briefly, 3μl of patient serum/calibrator was mixed with 500μl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and absorbance was measured at 550nm wavelength after 10 s (A1) and 120 s (A2). CRP concentration (mg/l) in serum was measured using following formula:

Serum concentration of CRP (mg/l) = [(A2-A1) Serum sample/(A2-A1) Calibrator] x Concentration of calibrator

2.7. Quantification of serum COMP, Anti-CCP, IL-2R

Serum levels of COMP (LOD:0.085ng/ml, Invitrogen, MA, USA), Anti-CCP (LOD:0.582pg/ml, KINESISDx, CA, USA) and IL-2R (LOD:29pg/ml, Invitrogen, MA, USA) were determined by sandwich ELISA kits, according to manufacturers' instructions. Absorbance was measured at 450nm wavelength. Standard-curve was drawn using GraphPad prism9 and levels of each protein were analysed.

2.8. Detection of RF

RF detection was done using RF-latex agglutination slide test kit (AUTOSPAN, UK), where patient serum was placed within circled area on special slide (provided with kit). One drop of Reagent-1 was added to it and mixed well. Agglutination was observed macroscopically under direct light source.

2.9. Quantification of biochemical parameters

Levels of AST, ALT, ALP, bilirubin and albumin were measured by using a standard automated clinical chemistry analyzer (ERBA Model no: EM360) according to manufacturer's instructions.

2.10. Identification of CRP, IL-2R and COMP genotypes susceptible to chikungunya related arthritis

To understand role of CRP, IL-2R and COMP polymorphisms (if any) towards CHIKV infectivity and arthralgic manifestation compared to healthy controls, genotyping of CRP (rs3093059 and rs3091244), IL-2R (rs743777) and COMP (rs144778694) polymorphisms was performed among 167 CHIKV infected and 102 healthy controls, based on their minor allele frequencies (MAF).

SNP (Single nucleotide polymorphism) genotyping was carried out by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Based on sequences available in GenBank database, four primer pairs were designed using Primer3 software, to amplify polymorphic region of CRP (rs3093059 and rs3091244), IL-2R (rs743777) and COMP (rs144778694) genes. PCR reaction was performed in 20μl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5mM $MgCl_2$ and 20p. moles of previously mentioned primers (Table S1). Respective PCR products were digested with Tas I, BfaI, StuI, PspFI (Fermentas, USA) and Taq I (Himedia, India) accordingly. Different RFLP patterns were validated by sequencing of the respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

2.11. Statistical analysis

Associations of protein levels and their SNP genotypes with CHIKV infectivity and arthralgic manifestations were analyzed and represented using GraphPad Prism 9. Allelic and genotypic frequencies were compared between different study groups using Pearson's Chi-square test, unpaired Welch's t-test and multi-variant analysis, using one way ANOVA. For genotypic associations; p-values, odds ratio (OR) and risk ratio were calculated. A p-value of <0.05 was considered statistically significant. Among healthy controls, Hardy–Weinberg equilibrium was analyzed for all the polymorphisms using Haploview program. Cut-off values, sensitivity and specificity of serum-markers associated with post-chikungunya chronic arthritis (PCA) were analyzed by ROC curve. A principal component analysis was conducted to identify the markers in different samples' groups.

3. Results

In this study, male to female ratio of CHIKV infected patients:1.49:1 and mean age:33.06±14.7years, whereas, that of the control group was 1:1.01 and 36.2±11.8 years, respectively (Table 1). Major WHO-defined symptoms of febrile chikungunya patients were arthralgia, myalgia, persistent vomiting and joint swelling. According to arthralgic manifestation, as mentioned in materials and methods section, approximately, 14.10% (n = 24) of infected patients were NA (mean age:42.5 ±15.5years), 53.84% (n = 89) were AA (mean age:37.57±10.2years), 24.35% (n = 41) were AA+PCA (mean age:40.61±14.2years) and

Table 1
Comparative demographics, symptomatic diversity and 2010 ACR/EULAR classification of CHIKV infected patients.

		Chikungunya infected patients (N = 167)	**Healthy Controls (N = 102)**
Mean age (in years)		33.06 ± 14.7 (range: 4-74 years)	36.2 ± 11.8 (range: 18–63 years)
Sex	Male	59.88%	50.98%
	Female	40.11%	49.02%
	Male: Female ratio	1.49:1	1:1.01
Diagnostic Tools	Anti-CHIKV-IgM ELISA	44.31%	
	Real time RT-PCR	82.03%	
	Both anti-CHIKV-IgM ELISA and Real time RT-PCR	26.34%	
Symptomatic prevalence	Fever	100%	
	Myalgia	53.89%	
	Arthralgia	67.06%	
	Headache	22.15%	
	Rash	19.76%	
	Nausea	2.39%	
	Persistent Vomiting	25.14%	
	Abdominal Pain	8.98%	
	Joint Swelling	21.55%	
	Bleeding	0%	
	Retro-Orbital Pain	0%	
Patient's arthralgic manifestation	No arthralgia (NA)	14.10%	
	Mean age (in years)	42.5±15.5years (range 7-62years)	
	Acute arthralgia (AA)	53.84%	
	Mean age (in years)	37.57±10.2years (range 12-72years)	
	Acute arthralgia + Post-chikungunya arthritis (AA+PCA)	24.35%	
	Mean age (in years)	40.61±14.2years (range 10-72years)	
	Post-chikungunya arthritis (PCA)	7.69%	
	Mean age (in years)	33.5±19.1years (range 11-46years)	
% prevalence of patients with post-chikungunya rheumatoid arthritis according to 2010 ACR/EULAR scoring (score ≥6/10)*	NA	0%	
	AA	2.43%	
	PCA	83.33%	
	AA+PCA	95.23%	
Mean ACR/EULAR classification score	NA	3.09	
	AA	4.66	
	PCA	6	
	AA+PCA	6.21	

*2010 American College of Rheumatology/EuropeanLeague Against Rheumatism (ACR/EULAR) classification criteria

7.69% ($n = 13$) were PCA (mean age:33.5±19.1years) groups. Younger and older patients were equally affected eliminating age biasness or any underlying medical condition. In addition, there was no significant difference in male-female distribution among the above mentioned groups. PCA patients complained about persistent joint pain even after 2 years of first visit blood collection. Whereas, AA+PCA patients reported arthralgia both during first visit and after 2 years of blood collection. 2010 ACR/EULAR scoring classification indicated majority of PCA and AA+PCA patients qualified for rheumatoid arthritis (score ≥6/10), with average score of 6 and 6.21, respectively. Principal component analysis of 2010 ACR/EULAR scoring parameters of CHIKV-infected patients indicated similar clustering of PCA and AA+PCA groups (Fig. S1).

3.1. CRP, anti-CCP antibody, COMP and IL-2R levels among chikungunya patients with acute and chronic arthritis

Mean serum CRP concentrations significantly increased in AA (46.03mg/ml, p-value= 0.0291), PCA (60.71mg/ml, p-value=0.0212) and AA+PCA (77.05mg/ml, *p*-value<0.0001) groups when compared to NA which had a mean level of 19.60mg/ml (Fig. 1a). Moreover, CRP level of AA+PCA group was significantly higher than AA patients (*p*-value=0.0004). Anti-CCP antibody level was significantly high only among AA+PCA group when compared to both NA and AA patients (NA:133pg/ml, AA:190.1pg/ml, AA+PCA:419.7pg/ml, *p*-value=0.0003 and *p*-value<0.0001, respectively). On a similar trend, COMP level was significantly higher among AA+PCA and PCA groups when compared to both NA and AA groups (AA+PCA:40.35ng/ml, PCA:49.35ng/ml, AA:21.88ng/ml, NA:21.18ng/ml; *p*-value<0.0001). AA+PCA and PCA groups also showed higher IL-2R level compared to NA (AA+PCA:2421.22pg/ml and PCA:4882.45pg/ml vs NA:969.78pg/ml; *p*-value=0.0202 and *p*-value<0.0001, respectively). Also, PCA group showed highest mean IL-2R level compared to AA+PCA and AA groups (AA: 1128.96pg/ml; *p*-value<0.0001 and p-value=0.0005). RF was detected among 13.17% (22/167) CHIKV patients which was insignificant when compared to healthy individuals.

Level of all four arthritic biomarkers was higher among chikungunya patients with chronic arthritis (PCA, AA+PCA) compared to those with only acute or no arthritis among all age groups (Fig. S2). Similar trend was followed among male and female chikungunya patients (Fig. S3).

3.2. AST, ALT, ALP, bilirubin and albumin levels among chikungunya patients with acute and chronic arthritis

Amongst hepatic markers, gradual increase of AST, ALT, albumin level and AST/ALT ratio was found in the groups according to following order: AA+PCA>AA>PCA>NA (Fig. 1b). AST levels were significantly elevated among AA+PCA (269.6IU/L) compared to NA (NA:41.50IU/L; p-value<0.0001), AA (AA: 102.3IU/L; p-value<0.0001) and PCA (PCA:82.36IU/L; p-value<0.0001) groups. Similar trend was followed for ALT among AA+PCA (AA+PCA:143.3IU/L) compared to all the other groups (NA:39.97IU/L, p-value<0.0001; AA:89.57IU/L, p-value=0.0016; PCA:71.49, p-value=0.0217). AST/ALT ratio was significantly high among AA+PCA (2.001) with respect to NA (1.102; p-value=0.0043) and AA (1.257; p-value=0.0008) groups. Also, AA+PCA

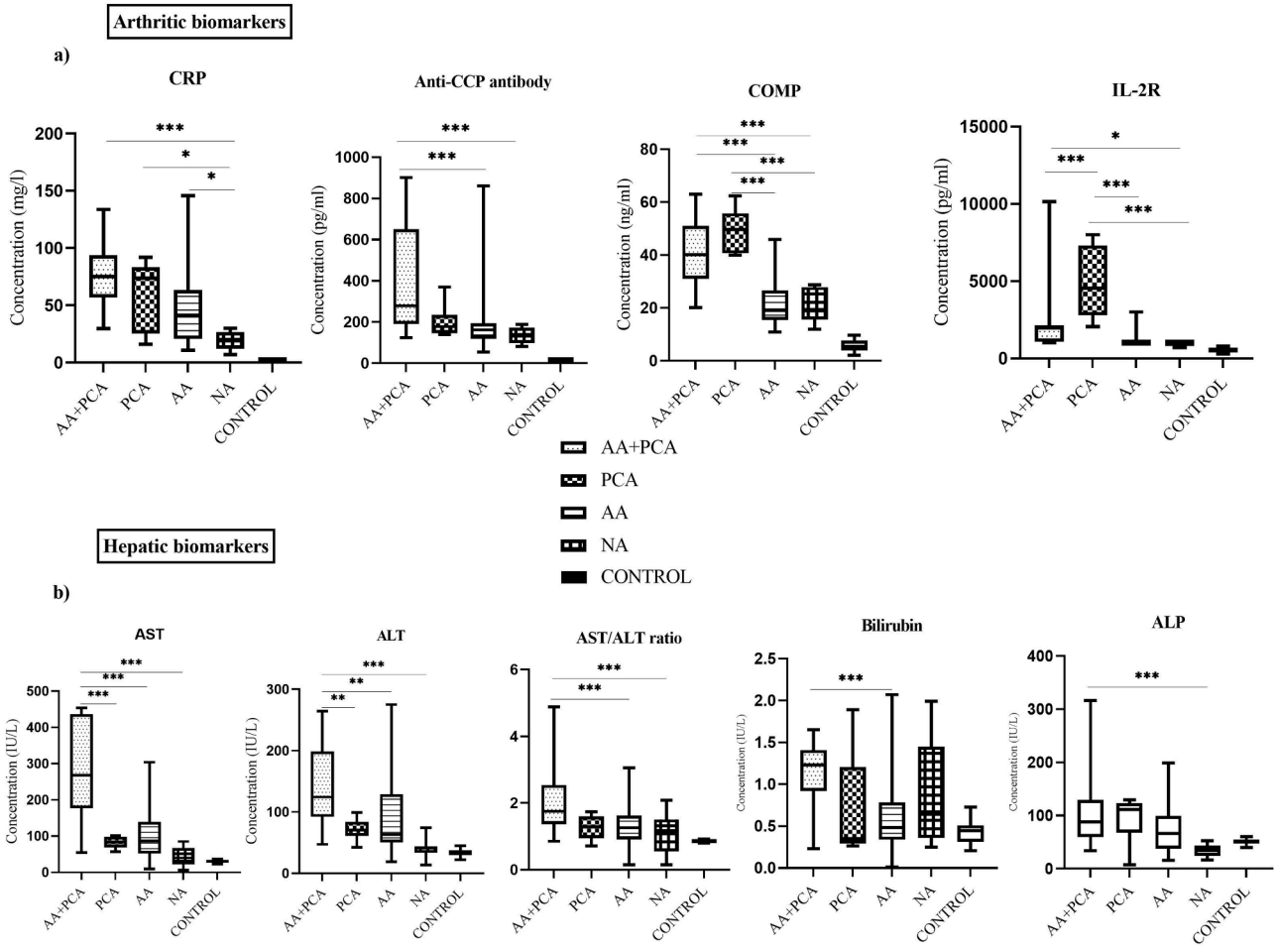
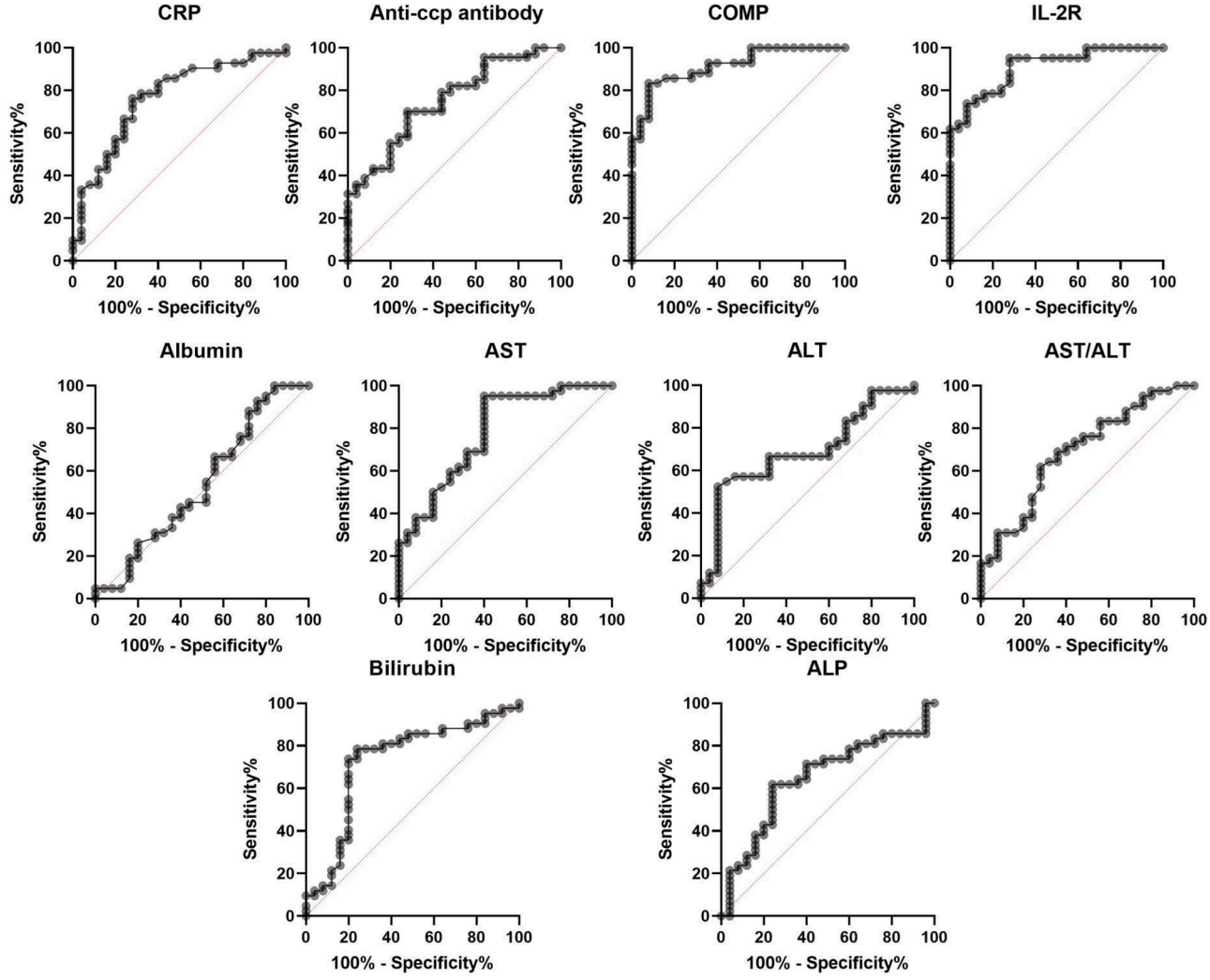


Fig. 1. (a) Comparative analysis of serum concentration of arthritic biomarkers among AA+PCA, PCA, AA and NA groups of CHIKV-infected patients. **(b)** Comparative analysis of serum concentration of hepatic biomarkers among AA+PCA, PCA, AA and NA groups of CHIKV-infected patients.

had significantly higher bilirubin levels compared to AA (AA+PCA:1.173IU/L vs AA:0.6350IU/L; p-value=0.0004) and ALP compared to NA (AA+PCA=97.74IU/L, NA:34.12IU/L; p-value=0.0051). Single sample for each of AA and AA+PCA groups were exceptions with "maximum outlier" value for CRP, Anti-CCP antibody, COMP, IL-2R, AST, ALT, AST/ALT ratio, bilirubin and ALP. Among AA+PCA group, a single 72years old male patient with fever for 2 months exhibited these outlier values.



	Area	Sensitivity %	Specificity %	Cut-off value	p-value
CRP	0.8057	81.13	72	> 63.95	<0.0001*
Anti-CCP antibody	0.7826	75.47	72.00	> 186.0	<0.0001*
COMP	0.9257	86.79	92	>28.88	<0.0001*
IL-2R	0.9257	88.00	81.13	> 1066	<0.0001*
Albumin	0.5585	49.06	56	>3.095	NS
AST	0.8219	69.81	72	>133.6	0.0001*
ALT	0.7442	73.58	68.00	> 90.75	0.0005*
AST/ALT	0.7023	67.92	64.00	> 1.46	0.0041*
Bilirubin	0.6974	75.47	76	> 0.83	0.0051*
ALP	0.7026	69.81	76	>70.43	0.004*

Fig. 2. Receiver operating characteristic curve (ROC curve) analysis of arthritic and hepatic biomarkers with their sensitivity, specificity and cut-off value of chikungunya induced chronic arthritis.

3.3. ROC curve analysis of biomarkers among chikungunya patients with and without chronic arthritis

A cut-off value of chikungunya induced chronic arthritis was analyzed by ROC curve to differentiate between patients with (PCA, AA+PCA) and without (AA, NA) chronic arthritis, using markers significantly altered amongst these two groups (Fig. 2). Cut off values of arthritic and hepatic biomarkers that significantly increased in case of chronic arthralgia were: CRP: >63.95mg/ml, Anti-CCP antibody: >186.0pg/ml, COMP: >28.88ng/ml, IL-2R: >1066pg/ml, AST: >133.6U/L, ALT: >90.75U/L, AST/ALT: >1.46U/L, Bilirubin: >0.83U/L, ALP: >70.43U/L (p-value: 0.0001-0.0051). High sensitivity and specificity values of COMP and IL-2R indicated their diagnostic potential for chronic arthralgia among infected patients. On the contrary, hepatic markers demonstrated lower sensitivity level compared to arthritic ones.

3.4. Heatmap, correlation and principal component analysis of biomarkers among chikungunya patients with and without chronic arthritis

Heatmap analysis of arthritic and hepatic biomarkers revealed increase of CRP, anti-CCP antibody as well as COMP levels among four groups of infected patients compared to healthy controls (Fig. 3a). Serum albumin level increase was negligible. Principal component analysis of all analyzed biomarkers indicated gradual segregation of AA+PCA and PCA groups from AA and NA, which in turn overlapped with each other; NA was in proximity to healthy controls (Fig. 3b). Anti-CCP antibody, CRP, AST, ALT, AST/ALT ratio and bilirubin levels were linked with AA+PCA group, whereas, ALP and IL-2R levels were associated with PCA group. Highest proportion of variance of PC1 was 37.99%, PC2 was 51.72% and PC3 was 63.75%. Correlation studies of these biomarkers among these groups indicated a strong positive significant correlation between Anti-CCP antibody vs. CRP of AA+PCA (p-value=0.044), Anti-CCP antibody vs. CRP (p-value<0.001), COMP vs. CRP (p-value=0.023), COMP vs. Anti-CCP antibody (p-value=0.029) and COMP vs. IL-2R (p-value=0.004) of AA groups (Fig. 3a). For CRP and anti-CCP antibody, negative correlation was found between AA and NA, PCA and NA groups, respectively.

3.5. Genotypic association of CRP, IL-2R, COMP polymorphisms with chikungunya susceptibility

Genotypic and allelic distribution of CRP, IL-2R and COMP polymorphisms was analyzed among 167 CHIKV infected patients and 102 healthy controls (Table 2a). Statistical analysis revealed individuals with CRP-rs3091244-TC genotype were less susceptible to CHIKV infection (p-value=0.0126). Additionally, according to additive model, rs3091244 was significantly associated with infection susceptibility (p-value=0.0286). Furthermore, patients with IL-2R-rs743777-GA genotype and G-allele were significantly susceptible to CHIKV infection (p-value=0.0198 and p-value=0.0079, respectively). Additive model suggested this IL-2R polymorphism to be significantly associated to CHIKV infection susceptibility (p-value=0.0179). Subsequently, COMP-rs144778694-GA genotype, A-allele and its additive model were positively associated with CHIKV infection when compared to healthy control population (p-values= 0.0032, 0.0020 and 0.0051, respectively).

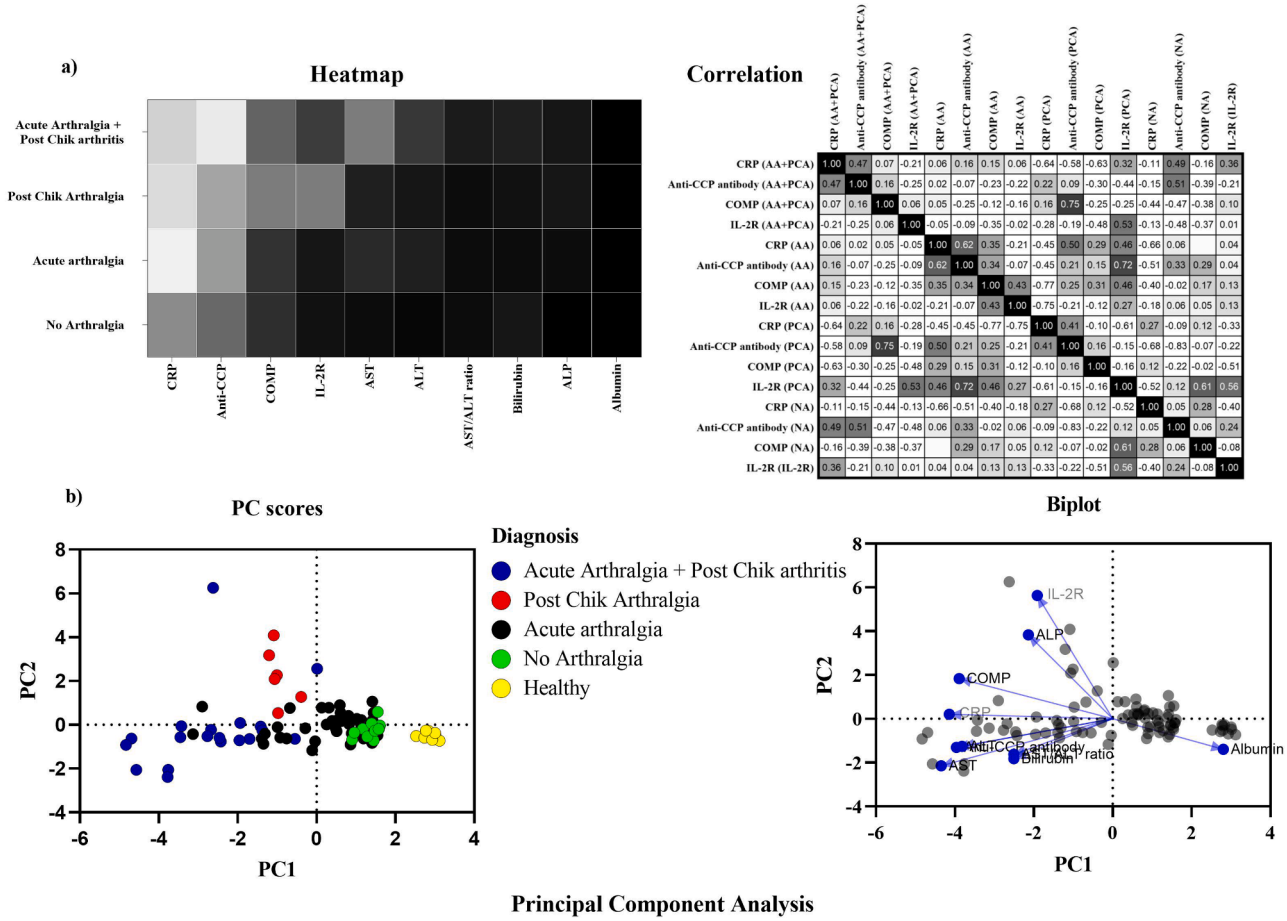


Fig. 3. **(a)** Differential heatmap and correlation analysis of arthritic and hepatic biomarkers with AA+PCA, PCA, AA and NA groups of CHIKV-infected patients. **(b)** Principal component analysis representing linkage and variance among AA+PCA, PCA, AA, NA of CHIKV-infected patients and healthy groups, with arthritic and hepatic biomarkers.

Table 2

(a) Genotypic and allelic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV infected patients and healthy controls. **(b)** Genotypic and allelic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV infected patients with or without arthralgia.

SNP Ref. No.	Genotype and allele distribution	Healthy Controls (%)	Chikungunya infected patients (%)	OR (95% C.I)	Relative risk (95% C.I)	*p*-value
		***n* = 102**	***n* = 167**			
	CC	2	3	1.093 [0.1913 to 5.429]	1.056 [0.3082 to 2.098]	>0.9999
	CT	21	43	0.7476 [0.4144 to 1.374]	0.8304 [0.5526 to 1.196]	0.3776
	TT	79	121	ref		
	C allele	25	49	0.8123 [0.4783 to 1.377]	0.8757 [0.6125 to 1.199	0.5191
CRP rs3093059	T allele	179	285	ref		
	Additive	0.628				
		***n* = 102**	***n* = 167**			
	TT	3	11	2.327 [0.6932 to 7.942]	1.812[0.7990 to 5.176]	0.1915
	TC	39	40	1.965 [1.166 to 3.323]	1.489 [1.092 to 1.996]	**0.0126***
CRP rs3091244	CC	60	116	Ref		
	T allele	45	62	1.242 [0.8138 to 1.907]	1.140 [0.8736 to 1.452]	0.3243
	C allele	159	272	Ref		
	Additive	**0.0286***				
		***n* = 102**	***n* = 167**			
	GG	0	3	2.512 [0.4077 to 31.01]	1.929 [0.6057 to 10.69]	0.6523
IL-2R	GA	22	59	1.987 [1.139 to 3.422]	1.567 [1.079 to 2.353]	**0.0198***
rs743777	AA	80	105	Ref		
	G allele	22	65	1.999 [1.195 to 3.323]	1.596 [1.119 to 2.364]	**0.0079***
	A allele	182	269	Ref		
	Additive	**0.0179***				
		***n* = 102**	***n* = 167**			
	AA	0	3	2.452 [0.3981 to 30.27]	1.900 [0.5966 to 10.54]	0.4103
COMP	GA	11	42	2.848 [1.409 to 5.897]	2.058 [1.241 to 3.630]	**0.0032***
rs144778694	GG	91	122	Ref		
	Aallele	11	46	2.822 [1.444 to 5.524]	2.088 [1.268 to 3.655]	**0.0020***
	G allele	193	286	Ref		
	Additive	**0.0051***				

(b): Genotypic and allelic distribution of CRP, COMP, IL-2R polymorphisms among CHIKV infected patients with or without arthralgia

SNP Ref. No.	Genotype and allele distribution	With Arthralgia (%)	Without Arthralgia (%)	OR (95% C.I)	Relative risk (95% C.I)	*p*-value
		***n* = 112**	***n* = 55**			
	CC	3	0	2.036[0.3239 to 25.32]	1.207[0.5633 to 1.531]	0.6419
	CT	8	35	0.04396[0.01826 to 0.1106]	0.2218[0.1157 to 0.3914]	**<0.0001***
	TT	101	20	Ref		
	C allele	14	35	0.1429[0.07313 to 0.2746]	0.3878[0.2411 to 0.5797]	**<0.0001***
CRP rs3093059	T allele	210	75	ref		
		***n* = 112**	***n* = 55**			
	TT	2	9	0.09293 [0.01981 to 0.3832]	0.2579 [0.07262 to 0.6821]	**0.0004***
	TC	22	18	0.5025 [0.2510 to 1.029]	0.7761 [0.5532 to 1.012]	0.0626
CRP rs3091244	CC	88	28	Ref		
	T allele	26	36	0.2699 [0.1519 to 0.4839]	0.5761 [0.4156 to 0.7555]	**<0.0001***
	C allele	198	74	Ref		
		***n* = 112**	***n* = 55**			
	GG	1	2	0.2387 [0.01635 to 2.108]	0.4925[0.09066 to 1.187]	0.2097
IL-2R	GA	24	35	0.1558[0.07779 to 0.3111]	0.4992 [0.3534 to 0.6680]	**<0.0001***
Rs743777	AA	87	18	Ref		
	G allele	26	39	0.2391 [0.1356 to 0.4223]	0.5434[0.3908 to 0.7166]	**0.0017***
	A allele	198	71	Ref		
		***n* = 112**	***n* = 55**			
	AA	0	3	0.1184 [0.009611 to 0.7509]	0.2947 [0.05328 to 0.9292]	**0.0255***
COMP	GA	32	10	2.343 [1.024 to 5.225]	1.256 [1.003 to 1.516]	**0.0471***
rs144778694	GG	80	42	Ref		
	Aallele	32	16	0.9792 [0.5266 to 1.925]	0.9931 [0.7738 to 1.196]	0.9493
	G allele	192	94	Ref		

*$p<0.05$ at 95% CI was considered as statistically significant.
"Ref"= reference genotype

3.6. Genotypic association of CRP, IL-2R, COMP polymorphisms with chikungunya induced arthritis

Role of these CRP, IL-2R and COMP polymorphisms was analyzed between patients with (n = 112) and without arthralgia (n = 55) (Table 2b). Analysis revealed CRP-rs3093059-CT, CRP-rs3091244-TT, IL-2R-rs743777-GA and COMP-rs144778694-AA genotypes were significantly associated with patients without any arthralgic manifestations (p-value<0.0001, p-value=0.0004, p-value<0.0001 and p-value=0.0255, respectively). In contrast, COMP rs144778694-GA genotype was significantly linked to patients with arthralgia (p-value=0.0471). Also, CRP-rs3093059-C, CRP-rs3091244-T and IL-2R-rs743777-G alleles were associated with patients without arthralgia (p-value<0.0001, p-value=0.0004 and p-value=0.0017, respectively). Interestingly, CRP-rs3093059-CT genotype was significantly more prevalent among patients who never developed PCA (p-value=0.0317) (Table S2). However, genotyping associations should be analysed upon larger patient cohorts for better clarity.

3.7. Association of CRP, IL-2R, COMP polymorphic genotypes with serum concentration among patients with and without chronic arthritis

Association between polymorphic genotypes and serum level of CRP, IL-2R and COMP was analyzed among AA, PCA and AA+PCA groups (Fig. 4). Serum level of CRP-rs3093059-TT genotype was significantly higher among patients of AA+PCA and AA groups when compared to CC genotype (AA+PCA-TT:77.25mg/l vs CC:55.33mg/l; p-value=0.0123 and TT:44.55mg/l vs CC:30.73mg/l; p-value=0.0037, respectively). CRP level was comparatively higher among patients with CT genotype (CT:91.43mg/l). Moreover, CRP level of rs3091244-CT and CC genotypes was significantly higher compared to patients with TT genotype among AA group (CT:53.27mg/l, CC:43.19mg/l vs TT:22.34mg/l; p-value=0.0089 and 0.0001, respectively). Similarly, patients with rs743777-GA and AA genotype demonstrated significantly higher IL-2R level compared to GG genotype among AA+PCA group (GA:3345pg/ml, AA:3050pg/ml, GG:1120pg/ml; p-value=0.0429, respectively). In case of COMP-rs144778694, patients with GA and GG genotypes showed remarkable increase in COMP level compared to AA genotype within AA+PCA group (GA:46.55ng/ml and GG:43.69mg/l vs AA:27.34ng/ml; p-value=0.0087 and 0.0143, respectively); but this trend was reversed among AA group (AA:34.07ng/ml vs GG:21.09ng/ml GA:22.45ng/ml; p-value=0.0033, p-value=0.0028).

4. Discussion

CHIKV infection has been known to cause protracted illness among patients inducing acute and/or chronic polyarthritis/polyarthralgia, which might persist for at least one year, inviting chronic inflammatory rheumatism and musculo-skeletal disease during PCA (Imad et al., 2021). Thus, identification of certain biomarkers that might help in differentiating chikungunya patients with only acute arthritis from those who might develop chronic polyarthralgia would be useful in pain management of chronic arthritic patients. Both serum level and polymorphic genotypes of CRP, Anti-CCP antibody, IL-2R, COMP, RF and hepatic markers were analyzed in this regard.

CRP concentrations significantly increased among all patients with arthralgia (both acute and chronic) compared to those without. Also, its level was significantly high among AA+PCA patients compared to AA group. Increased level of CRP has been previously reported among chikungunya induced arthritic patients of Indian origin; but this is the

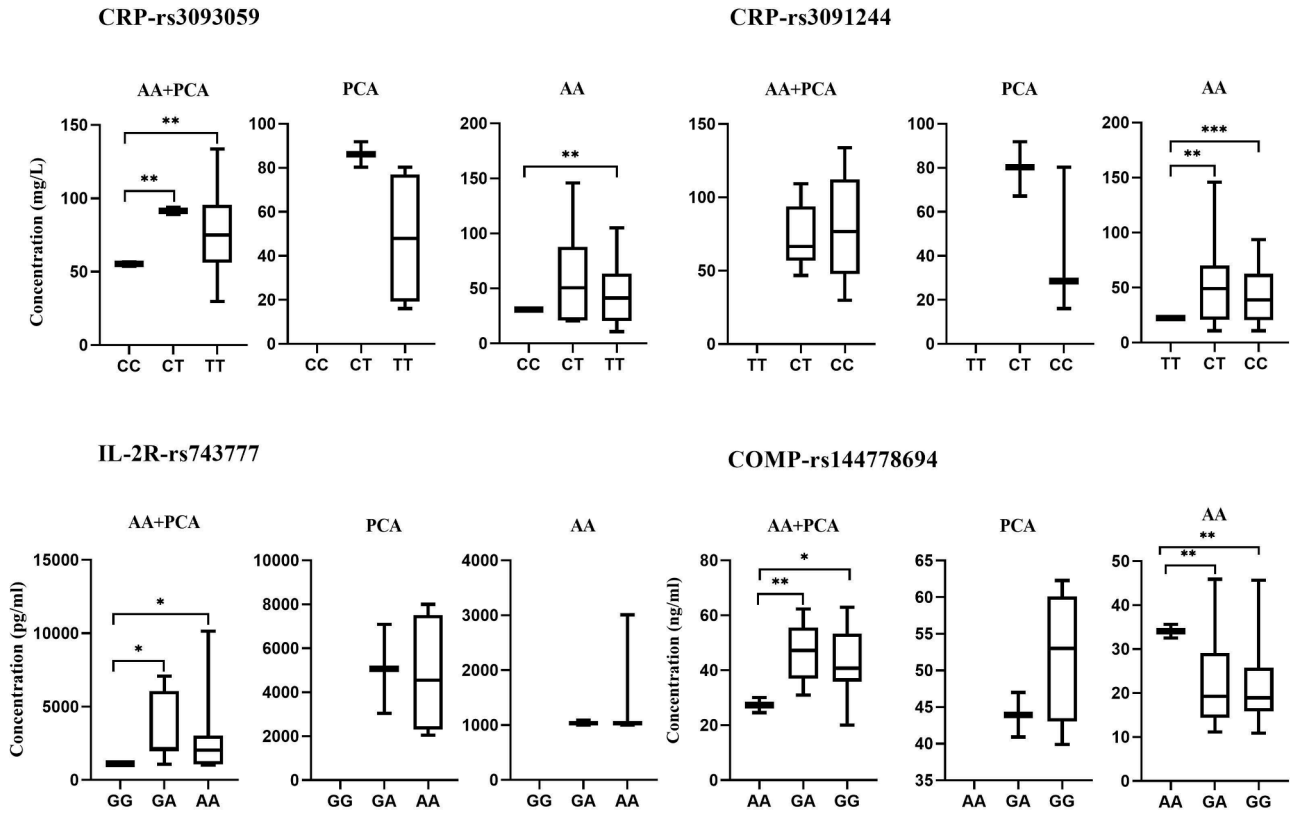


Fig. 4. Comparative genotypic distribution pattern of CRP, IL-2R, COMP polymorphisms and their serum levels among different arthritic groups of CHIKV-infected patients.

first study to differentiate CRP level between acute and chronic arthritic patients with 81.13% sensitivity and 72% specificity (Solanke and Karmarkar, 2014). Increased CRP might act as inflammatory response to activate innate immune response against viral induced arthritis (Sprosston and Ashworth, 2018). Anti-CCP antibody concentration was higher among AA+PCA and PCA groups with respect to NA and AA, but the increase was statistically significant for AA+PCA. Anti-CCP antibody was previously detected among La Reunion island-patients with post-chikungunya chronic arthritis, but its differential concentration among acute and chronic arthritic patients has not been previously demonstrated (Imai et al., 2016). Anti-CCP antibody was reported to cross-react with type II collagen, resulting in proteoglycan depletion and severe arthritis (Wu et al., 2020). Similar trend was followed for COMP with its noteworthy increase among AA+PCA and PCA. Current study indicated that COMP level could be used as differentiating diagnostic factor for chronic arthritis among CHIKV-infected patients with 86.79% sensitivity and 92% specificity. Previous reports also suggested COMP to be associated with osteoarthritis and rheumatoid arthritis (Deere et al., 1999; Mishra et al., 2019). COMP, found in articular cartilage, ligament, meniscus, synovial membrane, and tendon, have a role in endochondral ossification and its increased level has been linked to cartilage degradation (Tseng et al., 2009; Arellano et al., 2017). Increased IL-2R level among AA+PCA and PCA compared to NA and AA indicated its differential diagnostic role for chronic arthritic chikungunya patients with 88% sensitivity and 81.13% specificity. Elevated IL-2R concentration has been previously demonstrated among rheumatoid arthritic patients and was reported to be associated with synovitis (Wood et al., 1988; Symons et al., 1988). Thus, according to high sensitivity and specificity scores, current study is the first to demonstrate prognostic relevance of COMP, IL-2R and CRP levels in identifying chronic arthritic chikungunya patients (Fig. 2).

Hepatic anomalies have been previously reported among chikungunya patients of northern India, Singapore, Mexico and Sri Lanka; but their differential concentration between arthritic and non-arthritic chikungunya patients has not been studied earlier (Danis-Lozano et al., 2017; Ng et al., 2009; Singh et al., 2018; Premaratna et al., 2011). Increased concentrations of AST, ALT, AST/ALT ratio, bilirubin and ALP were reported among arthritic chikungunya compared to non-arthritic ones.

Principal component analysis segregated AA+PCA and PCA groups from overlapping AA and NA group of patients. There was a stark proximity of NA group with healthy controls. It further highlighted association of CRP, anti-CCP antibody, AST, ALT, AST/ALT ratio and bilirubin levels with AA+PCA group, whereas, IL-2R and ALP levels were associated with PCA group. Previous reports also associated high CRP, IL-2R levels with post chikungunya chronic arthritic patients (Patel et al., 2019; Teng et al., 2015).

Genotypic analysis implicated protection of individuals with CRP-rs3091244-TC genotype against CHIKV infection, whereas, those with IL-2R rs743777-GA, G-allele and COMP rs144778694-GA genotypes were susceptible to such infection. Cis-acting rs3093059 and tri-allelic rs3091244 has been previously associated with increased CRP levels *in vitro*, thus suggested to have functional role in transcription factor binding (Sengupta et al., 2022). Various genotypes of CRP-rs3091244 have been previously implicated for susceptibility towards CHIKV/-DENV co/mono-infection, ankylosing spondylitis, haemorrhagic and ischaemic stroke among patients of eastern Indian, Turkish, Greek and Chinese origin, respectively. Previous report suggested patients with IL-2R rs743777-G allele to be susceptible to ankylosing spondylitis with peripheral arthritis (Polo et al., 2019). Moreover, infected patients with CRP-rs3093059-CT, CRP-rs3091244-TT, IL-2R rs743777-GA and COMP rs144778694-AA genotypes might have been safeguarded from arthralgic manifestations; whereas, those with COMP rs144778694-GA genotype were susceptible to arthralgia. CRP-rs3091244 variants have been previously reported to give protection against onset of pain among DENV-CHIKV co-infected Indian patients (Sengupta et al., 2022). A previous study on Dutch patients indicated IL-2R variants to be protective against severe forms of multiple sclerosis and RA (van Steenbergen et al., 2015).

In this study, patients with various genotypes of CRP-rs3093059 and rs3091244, IL-2R-rs743777 and COMP-rs144778694 manifested significantly higher protein levels among chikungunya patients suffering from acute arthralgia with/without chronic arthritis. Such elevated CRP levels were previously reported among ankolysing spondylitic and DENV-CHIKV co-infected patients with certain genotypes of CRP rs3091244 and rs3093059 (Sengupta et al., 2022). Indication for association with COMP levels was found with COMP genetic polymorphisms among osteo-arthritic patients of Dutch origin (Ramos et al., 2014).

5. Conclusion

In light of our findings, this study confirmed that both biomarker levels in serum and genetic factors played an important role in development of post-chikungunya chronic arthritis among infected patients. Upon diagnosis of CHIKV infected patients with/without acute arthralgia, these markers could be used to screen patients to identify their potential of developing chronic arthritis. This might be helpful for clinical management of PCA patients by a medical specialist. However, these factors should be validated among larger patient cohorts.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Availability of data and material

All data generated and analysed during this study are included in this article.

Ethics approval

All procedures performed in this study involving collection of blood from all human participants were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013). Written consents were received from patients and healthy control individuals prior to participation in the study.

Fig. S1: Principal component analysis representing linkage and variance among AA+PCA, PCA, AA, NA of CHIKV-infected patients according to 2010 ACR/EULAR classification.

Fig. S2: Comparative analysis of age groups and serum concentration of arthritic biomarkers among chronic arthritic, acute arthritic and non-arthritic groups of CHIKV-infected patients.

Fig. S3: Comparative analysis of gender and serum concentration of arthritic biomarkers among chronic arthritic, acute arthritic and non-arthritic groups of CHIKV-infected patients.

CRediT authorship contribution statement

Siddhartha Sengupta: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Nemai Bhattacharya:** Resources. **Anusri Tripathi:** Conceptualization, Formal analysis, Investigation, Resources, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgements

The authors are extremely grateful to the Director, Calcutta School of Tropical Medicine, Kolkata, India, for his support, inspiration and providing necessary facilities for this study. The authors are grateful to Indian Council of Medical Research, India, for granting fellowship to the first author [5/3/8/6/ITR-F/2018-ITR].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198998.

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European Journal of Clinical Microbiology & Infectious Diseases
https://doi.org/10.1007/s10096-020-04125-x

ORIGINAL ARTICLE

Differential genotypic signatures of Toll-like receptor polymorphisms among dengue-chikungunya mono- and co-infected Eastern Indian patients

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Received: 27 July 2020 / Accepted: 13 December 2020
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Abstract

Dengue (DENV) and chikungunya (CHIKV) viral infections trigger high patient morbidity and mortality. Mono-/co-infection of these viruses activates innate immune response, triggering Toll-like receptor (TLR) pathways. The present study investigated the differential role of TLR3, 7 and 8 single-nucleotide polymorphisms (SNPs) between mono- and co-infected Eastern Indian patients. Interaction of TLR polymorphic variants with signal peptidase complex (SPC18) was explored which might affect immune signalling against DENV/CHIKV infections. Out of 550 febrile symptomatic patients, 128 DENV-CHIKV co-infected samples were genotyped for eight SNPs of TLR3 (rs3775290-chr4:186083063), TLR7 (rs179008-chrX:12885540, rs5741880-chrX:12869297, rs179010-chrX:12884766, rs3853839-chrX:12889539) and TLR8 (rs5744080-chrX:12919685, rs3764879-chrX:12906578, rs3764880-chrX:12906707) by PCR-RFLP along with 157 healthy individuals. Statistical analysis established genotypic association of TLR SNPs with DENV-CHIKV co-infection, and difference between mono- and co-infected patients and their role in determining high viral load (HVL) during competitive viral replication among co-infected patients. In silico protein-protein docking evaluated interactive effect of TLR variants with SPC18. The findings revealed patients with CC genotypes of TLR7 and 8 SNPs were significantly susceptible towards co-infection, whereas specific genotypes of TLR7 and 8 imparted protection against co-infection. Differential analysis between mono-/co-infected patients revealed distinct genotypic distribution of TLR3, 7 and 8 SNPs. Co-infected patients with TT-rs179010 exhibited DENV-HVL, whereas CHIKV-HVL was detected among patients with other genotypes. Molecular docking of TLR7-rs179008 Q variant and TLR8-rs3764880 V variant with SPC18 generated better free binding energy. This study underlined the importance of TLR7 and 8 SNPs towards mono-/co-infection of DENV/CHIKV, with certain genotypes associated with co-infection susceptibility. Moreover, it suggested a probable role of specific genotypes of TLR7 and 8 polymorphisms imparting high dengue/chikungunya viral load among co-infected patients.

Keywords Dengue · Chikungunya · Co-infection · TLR · Single-nucleotide polymorphism · Signal peptide · Docking

Introduction

One of the most serious emerging threats to global health is caused by various arthropod-borne viruses viz. dengue and chikungunya. Recurrent infection with dengue and chikungunya viruses leads to high morbidity and mortality, making them one of the most important public health problems in India. India is a dengue-endemic region with seroprevalence of 70% or higher, whereas chikungunya outbreak in India was reported in 1973 after which it disappeared for three decades only to re-emerge during 2005 and then through 2014–2016 causing severe pan-Indian outbreak [1–3]. Hence, 2005 onwards, dengue-endemic areas of India overlapped with chikungunya-affected regions [4]. Though DENV and CHIKV viruses belong to different families, both are transmitted by the same vector, i.e., *Aedes aegypti* [5]. The transmission period of both viruses in India mostly occurs during the

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Published online: 25 January 2021

post-monsoon period (September–November) favouring mosquito breeding [4]. Vomiting, abdominal pain, headache, maculopapular rash, myalgia and arthralgia are common symptoms in these regions of co-circulation; and thus, chikungunya is often misdiagnosed as dengue [6]. Arthralgia (79%) and thrombocytopenia (77%) among DENV-CHIKV co-infected North Indian patients and prevalence of both joint swelling and WHO-defined dengue warning signs (abdominal pain, retro-orbital pain and haemorrhagic manifestation) among co-infected East Indian patients revealed the overlapping nature of DENV-CHIKV co-infection [4, 7].

Host innate immune system receptors, like endosomally localised Toll-like receptors TLR3, 7 and 8, specifically recognise the presence of dengue and chikungunya viral genomic RNA within an infected patient body. A single-nucleotide polymorphism, or SNP, is a variation at a single position in a DNA sequence present among more than 1% of a population [8]. SNP is one of the genetic susceptibility factors that contribute to many human disease pathogeneses [9]. Multiple SNPs (rs5741880, rs179010, rs3853839) of TLR7 gene were reported to be associated with CHIKV susceptibility, whereas numerous SNPs (rs3764880, rs5744080) of TLR8 gene were associated with DENV susceptibility [10, 11]. Genetic variations or SNPs of TLR genes might induce differential innate immune responses towards the same pathogen, thereby affecting disease susceptibility and clinical outcome among infected individuals. But the role of these TLR SNPs for susceptibility towards both DENV and CHIKV among co-infected patients has not been previously explored.

Both the rs179008 and rs3764880 are non-synonymous SNPs in signal peptide regions of TLR7 and TLR8, respectively, which leads to single amino acid change (rs179008: Q11L; rs3764880: M1V) in protein sequence that may have damaging or neutral effect [11]. The signal peptidase (SPase) enzyme is responsible for cleavage of signal peptide from pre-protein, allowing its release from cytoplasmic membrane and leading to correct folding of mature protein [12]. SPC18 protein encoded by SEC11 is one of the subunits of the signal peptidase complex and has catalytic activity of signal peptidase [13]. Mutations in human signal peptides have been associated with improper cleavage by signal peptidase and defective secretion that might lead to impaired protein translocation [12, 14].

The main objective of this study is to understand the role of TLR3, 7 and 8 SNPs towards co-susceptibility of Eastern Indian patients for both dengue and chikungunya viruses and also to elucidate in silico interactions between Q11L (TLR7) and M1V (TLR8) with signal peptidase.

Materials and methods

Ethics statement

All procedures performed in this study involving collection of blood from human participants as well as healthy controls were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26 September 2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consent was received from patients and healthy control individuals prior to participation in the study.

Patients and healthy controls

After obtaining institutional ethical committee approval, 5 ml of blood from all age groups and both sexes was collected from each of the 550 symptomatic febrile patients (within 0–7 days of symptomatic onset), visiting Calcutta School of Tropical Medicine, West Bengal, India, from September 2014 to October 2016. Patients exhibiting fever along with any two of the symptoms headache, retro-orbital pain, myalgia, arthralgia, nausea, vomiting, swollen glands, rash, haemorrhagic manifestation and fatigue were selected as per WHO criteria [15]. Among them, 128 were co-infected with both DENV and CHIKV as detected by anti-dengue-IgM ELISA/dengue-NS1ELISA (NIV, Pune, India)/real-time qRT-PCR and anti-CHIKV IgM ELISA (NIV, Pune, India)/real-time qRT-PCR, respectively. To carry out age-matched case control study, blood from 157 healthy unrelated individuals of the same ethnicity, who neither had any signs and history of other infections nor were detected with DENV, CHIKV, Japanese encephalitis, HIV and hepatitis A/B/C infection as tested by IgM ELISA/quantitative RT-PCR, was collected from the same community, as described previously [10]. Viral load of DENV and CHIKV was determined by real-time qRT-PCR using DENV and CHIKV Genesig kit (Primer Design Ltd., UK), respectively, according to the manufacturer's protocol [4]. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided within the kit). Viral load in patient serum was calculated from the corresponding Ct values. Limit of detection of both DENV and CHIKV Genesig kits was < 100 copies of corresponding target genomes. To understand the differential role of TLR SNPs towards DENV-CHIKV co-infectivity compared to infectivity against DENV or CHIKV alone, genotyping data of these TLR SNPs from 201 DENV mono-infected and 173 CHIKV mono-infected patients were collected from our earlier studies and compared with those of co-infected patients [10, 16].

SNP selection and genotyping

SNPs in TLR genes, TLR3 (rs3775290), TLR7 (rs179008, rs5741880, rs179010, rs3853839) and TLR8 (rs5744080, rs3764879, rs3764880), were selected based on their minor allele frequencies (MAF) and previously reported associations with other viral infections [10, 11, 16]. SNP genotyping was carried out by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Based on sequences available in the GenBank database, seven primer pairs were designed using Primer3 software, to amplify rs3775290 polymorphic region of TLR3; rs179008, rs5741880, rs179010, and rs3853839 polymorphisms of TLR7; and rs5744080, rs3764879, and rs3764880 polymorphisms of TLR8 [7, 12]. PCR reaction was performed in 20 μl volume, using 1× PCR buffer (Fermentas, USA), 1 mM of each dNTP, 1 unit of Taq DNA polymerase (Fermentas, USA), 1.5 mM $MgCl_2$ and 20 p·moles of previously mentioned primers. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at the desired annealing temperature for 30 cycles and the PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. Respective PCR products were digested with TaqI (Himedia, India), Bsh1285I, ER1381, MvaI, Eco130I, TaiI, Hin1II (Fermentas, USA) and Hpy188I (NEB, UK) accordingly and digested products were visualised on 2.5% agarose gel. Different RFLP patterns were validated by sequencing of the respective PCR products using ABI Prism BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA) (Fig. S1).

Statistical analysis

The association of any particular genotype of respective SNPs with disease susceptibility and specific symptoms (WHO-defined) was calculated by using GraphPad Prism 7. Allele and genotype frequencies were compared between different study groups using Pearson's chi-square test. For genotypic associations, p values, odds ratio (OR) and minor allele frequency (MAF) were calculated. A p value of < 0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analysed for eight SNPs with Haploview [17]. LDlink4.1.0 program of National Institute of Health (NIH) was used for Linkage disequilibrium pair (LDpair) measurements (D′) by using Bengalis in Bangladesh (BEB) population data (sharing the same ethnicity with West Bengal population), present within 1000 genome project of genome-wide association studies (GWAS), the largest public catalogue of human variation and genotype data. This algorithm investigated correlated alleles of TLR7 and TLR8 for a pair of variants in high LD.

Signal peptide prediction

The SignalP 5.0 server was used to predict signal peptide sequence and location of their cleavage sites in TLR3 (Accession no: NP_003256.1), TLR7 (NP_057646.1) and TLR8 (NP_619542.1) proteins, extracted from NCBI database [18].

Effects of TLR3, 7 and 8 SNPs on protein stability and pathogenicity

Effect of amino acid alteration on protein stability was predicted by calculating change in Gibbs free folding energy (ΔG) at mutated site using the CUPSAT server [19]. The HOPE server was used to analyse effects of amino acid alterations on protein structure [20]. Pathogenic effects of point mutation were analysed using SIFT, PMUT, Polyphen2, MutationTaster, FATHMM, MetaSVM, PROVEAN, DIM, VarSomeClinical and Panther servers [21–26].

3D modelling of proteins and protein-protein docking

3D models of wild-type, Q11L and M1V forms of TLR7, TLR8 and signal peptidase complex catalytic subunit SEC11 (SPC18) proteins were generated using the QUARK server, which were used for ab initio protein structure prediction and 3D model construction from amino acid sequence only [27, 28]. Resultant structures were viewed using PyMOL 2.3.2.

ClusPro 2.0 online server was used to analyse protein-protein interaction and measure binding free energy between wild-type, Q11L and M1V forms of TLR7 and TLR8 with SPC18. The server utilised electrostatic-favoured, hydrophobic-favoured and Van der Waals forces to determine binding free energy, and represented findings in a balanced equation of all three combinations. Cluster scores generated by the server were represented in the form of the number of members interacting with each other, weighted energy score of the cluster centre (structure that has the highest number of neighbour structures in the cluster) and binding free energy score between the two proteins [29].

Results

In this study, genetic SNPs of TLR7 and TLR8 genes were investigated among 128 DENV-CHIKV co-infected patients, collected from dengue and chikungunya outbreaks during 2014–2016, along with 157 age-sex-matched healthy control volunteers with the same ethnicity of Eastern India. Male to

female ratio of co-infected patients was 1.44:1, whereas that of the control group was 1:1.01. Mean age of infected patients was 24.28 ± 14.7 years (range 2–60 years), whereas that of the control was 36.2 ± 11.8 years (range 18–63 years). During blood collection, the major symptoms among DENV-CHIKV co-infected patients were myalgia (55.11%), arthralgia (48.81%), headache (33.07%), rash (14.96%), nausea (18.11%), persistent vomiting (26.77%), abdominal pain (11.02%), joint swelling (17.32%), bleeding (5.51%) and retro-orbital pain (4.72%).

Genotypic association of TLR SNPs with DENV-CHIKV co-infection

Statistical analysis between infected and control populations revealed that in TLR7-rs179010, CC genotype was significantly positively associated ($p = 0.0346$) and TC genotype was significantly negatively associated ($p = 0.0039$) with co-susceptibility against DENV-CHIKV (Table 1). Prevalence of TC genotype was more than threefold among control population compared to infected patients. C allele was found to be significantly associated with susceptibility towards both viruses. CC genotypes of TLR7-rs179008 and TLR7-rs3853839 were also significantly associated with DENV-CHIKV susceptibility. Compared to healthy individuals, frequency of CC genotypes of both rs179008 and rs3853839 was more than 4–8-fold among co-infected patients. C alleles of rs179008 and rs3853839 were found to be significantly associated with viral susceptibility. Additionally, according to additive model, rs179008, rs179010 and rs3853839 were significantly associated with DENV-CHIKV co-susceptibility ($p < 0.0001$, 0.0052 and < 0.0001, respectively).

Similarly, CC genotypes of TLR8-rs3764879 and TLR8-rs5744080 demonstrated significant association with viral co-susceptibility ($p = 0.004$ and $p = 0.001$, respectively). C allele of rs3764879 exhibited significant association with co-susceptibility. Conversely, AG genotype of rs3764880 and CT genotype of rs5744080 were significantly related with decreased risk of DENV-CHIKV co-infection (Table 1). Additive model suggested rs3764879, rs5744080 and rs3764880 of TLR8 to be significantly associated with susceptibility against DENV and CHIKV ($p = 0.0021$, < 0.0001 and 0.0002, respectively).

TLR7 and 8 genes are located on the X chromosome; as a result, there might be gender biasness among these SNPs. In TLR7, TT genotype of rs5741880 and CC genotypes of rs179010 and rs179008 were significantly higher among female patients than that among the control group; TT genotype of rs5741880 was completely absent among healthy females; CC genotypes of rs179010 and rs179008 were 2–3 times more prevalent among DENV-CHIKV female patients compared to the healthy group. On the contrary, TC genotype of rs179010 was 2-fold more prevalent among control females compared to the co-infected group (Table 1). C allele of rs179008 was significantly more prevalent (6-fold) among infected males compared to the control group. In TLR8, CC genotypes of rs3764879 and rs5744080 and AA genotype of rs3764880 were significantly more widespread (2–3-fold) among co-infected females with respect to healthy population. CC genotype of rs5744080 was absent among healthy females. AG genotype of rs3764880 was 3-fold more prevalent among healthy females compared to diseased patients. Among males, A allele of rs3764880 was significantly higher (5-fold) in control population, whereas C allele of rs5744080 was more prevalent (2-fold) among co-infected patients.

Comparative analysis of prevalent TLR genotypes among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients

Statistical analysis between DENV-CHIKV co-infected and CHIKV mono-infected patients revealed that patients with TT genotype of TLR3-rs3775290 exhibited significant susceptibility towards co-infection. Similarly, CC genotypes of TLR7-rs179008 and rs3853839 were significantly related with DENV-CHIKV co-infection (1–3-fold). Conversely, GC genotype of TLR7-rs3853839 was more susceptible (3-fold) towards only CHIKV infection. In TLR8, CC genotypes of rs3764879 and rs5744080 were significantly more susceptible (1–2-fold) towards DENV-CHIKV co-infection. On the contrary, GC-rs3764879 (3-fold) and AG-rs3764880 (1–2-fold) were significantly more prevalent among only CHIKV-infected patients compared to patients with co-infection (Table 2).

Comparison between DENV-CHIKV co-infected patients and DENV mono-infected patients revealed significantly more prevalence of CC (2-fold) and AC genotypes of TLR7-rs179008 among DENV-CHIKV co-infected patients ($p = 0.0131$ and 0.0079). Similarly, GC genotype of TLR7-rs3853839 was only present among DENV-CHIKV co-infected patients ($p = 0.0002$). Furthermore, CC and AG genotypes of rs3764879 and rs3764880 of TLR8 were more prevalent among co-infected patients (Table 2).

All SNPs selected in this study followed Hardy-Weinberg equilibrium at $p > 0.05$ barring one SNP of TLR7-rs3853839. However, data of rs3853839 was incorporated as its genotypic distribution might arise by chance or could reflect a significant association with various factors. The LDpair measurements among SNPs of TLR7 revealed correlation between alleles

Table 1 Genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among DENV-CHIKV co-infected patients and healthy controls

SNP ref. no.	Chromosome location	Genotype and allele distribution	Healthy controls (%)	Dengue-chikungunya co-infected patients (%)	OR (95% CI)	*p* value
TLR 3 rs3775290	Chr4:186083063 (GRCh38.p12)		*n* = 157	*n* = 127		
		CC	5.09	11.02	2.3075 (0.9359–5.6893)	0.07541
		TT	33.75	38.58	1.2327 (0.7575–2.0059)	0.4557
		CT	61.14	50.39	Ref.	
		C allele	35.66	36.22	1.0243 (0.6291–1.6676)	0.9300
		T allele	64.33	63.77	Ref.	
		Additive	0.0788			
		Male	*n* = 78	*n* = 75		
		CC	6.41	10.67	1.7969 (0.5598–5.7681)	0.3906
		TT	28.20	40	1.7759 (0.9009–3.5008)	0.1230
		CT	65.38	45.33	Ref.	
		Female	*n* = 79	*n* = 53		
		CC	3.79	11.32	3.1667 (0.7561–13.2622)	0.1577
		TT	39.24	35.84	0.8406 (0.4099–1.7238)	0.7166
		CT	56.96	54.71	Ref.	
TLR 7 rs5741880	ChrX:12869297 (GRCh38.p12)		*n* = 157	*n* = 126		
		TT	12.74	12.69	0.9964 (0.4929–2.014)	1.0000
		GT	11.46	5.55	0.4542 (0.1834–1.125)	0.0942
		GG	75.79	81.74	0.5426 (0.2880–1.022)	0.0592
		G allele	81.52	84.52	Ref.	
		T allele	18.47	15.47	0.7912 (0.4295–1.455)	0.5252
		Additive	0.2145			
		Male	*N* = 78	*N* = 74		
		T	25.64	12.16	0.5613 (0.2521–1.250)	0.1689
		G	74.35	87.83	Ref.	
		Female	*N* = 79	*N* = 52		
		TT	0	13.46	13.79 (1.671–113.7)	*0.0030**
		GT	22.78	13.46	0.3780 (0.141–1.012)	0.0746
		GG	77.21	73.07	Ref	
TLR 7 rs179010	ChrX:12884766 (GRCh38.p12)		*n* = 157	*n* = 127		
		CC	30.57	43.30	1.735 (1.059–2.779)	*0.0346**
		TC	17.83	6.29	0.3097 (0.1442–0.7086)	*0.0039**
		TT	51.5	50.39	Ref	
		C allele	30.89	46.45	1.952 (1.198 to 3.124)	*0.0095**
		T allele	69.10	53.54	Ref	
		Additive				*0.0052**
		Male	*N* = 78	*N* = 75		0.2545
		C	48.71	38.66	0.6636 (0.3489–1.262)	
		T	51.28	61.33	Ref	
		Female	*N* = 79	*N* = 52		
		CC	12.65	50	6.900 (2.926–6.27)	*< 0.0001**
		TC	35.44	15.38	0.3312 (0.1369–0.8011)	*0.0159**
		TT	51.89	34.61	Ref	
TLR 7 rs179008	ChrX:12885540 (GRCh38.p12)		*n* = 157	*n* = 128		
		CC	4.45	22.66	6.2771 (2.6469–14.8859)	< 0.0001*
		AC	3.82	4.69	1.2377 (0.3894–3.9343)	0.7727
		AA	91.71	72.66	Ref	

Table 1 (continued)

SNP ref. no.	Chromosome location	Genotype and allele distribution	Healthy controls (%)	Dengue-chikungunya co-infected patients (%)	OR (95% CI)	*p* value
		C allele	6.37	25	4.900 (2.381–9.927)	*< 0.0001**
		A allele	93.63	75	Ref	
		Additive	< 0.0001*			
		Male	*N* = 78	*N* = 72		
		C	3.84	23.61	7.727 (2.157–27.68)	*0.0005**
		A	96.15	76.38	Ref	
		Female	*N* = 79	*N* = 56		
		CC	5.06	21.42	5.114 (1.553–16.83)	*0.0058**
		AC	7.59	10.71	1.460 (0.4223–5.028)	0.5533
		AA	87.34	67.85	Ref	
TLR 7 rs3853839	ChrX:12889539 (GRCh38.p12)		*n* = 157	*n* = 126		
		CC	22.29 35	36.50 46	0.4989 (0.2998–0.8442)	*0.0117**
		GC	0	0	–	–
		GG	77.7122	63.49 80	Ref	
		C allele	13.37	40.87	4.463 (2.498–7.914)	*< 0.0001**
		G allele	86.62	59.12	Ref	
		Additive	*< 0.0001**			
		Male	*N* = 78	*N* = 70		
		C	3.84	45.71	21.05 (6.103–67.58)	*< 0.0001**
		G	96.15	54.28	Ref	
		Female	*N* = 79	*N* = 56		
		CC	5.06	25	2.300 (0.9372–5.645)	0.0721
		GC	35.44	19.64	0.4452 (0.1991–0.9955)	0.0549
		GG	59.49	55.35	Ref	
TLR8 rs3764879	ChrX:12906578 (GRCh38.p12)		*n* = 157	*N* = 126		
		CC	22.29	40.47	2.370 (1.419–3.916)	*0.0011*
		GC	9.55	11.11	1.183 (0.5714–2.624)	0.6969
		GG	68.15	48.41	Ref	
		C allele	27.07	46.03	2.315 (1.393–3.800)	*0.0011*
		G allele	72.92	53.96	Ref	
		Additive	0.0021*			
		Male	*N* = 78	*N* = 70		
		C	28.20	42.85	1.909 (0.9635–3.783)	0.0843
		G	71.79	57.14	Ref	
		Female	*N* = 79	*N* = 56		
		CC	16.45	37.5	3.046 (1.363–6.808)	*0.0084**
		GC	18.98	25	1.422 (0.6226–3.249)	0.4054
		GG	64.55	37.5	Ref	
TLR8rs3764880	ChrX:12906707 (GRCh38.p12)		*n* = 157	*n* = 127		
		AA	28.66	26.77	0.9198 (0.5510–1.563)	0.7908
		AG	23.56	6.29	0.2199 (0.1035–0.4773)	*< 0.0001**
		GG	47.77	66.14	Ref	
		A allele	40.44	30.15	0.6374 (0.3879–1.055)	0.0814
		G allele	59.55	69.84	Ref	
		Additive	*0.0002**			
		Male	*N* = 78	*N* = 70		
		A	49.36	27.14	0.3725 (0.1933–0.7582)	*0.0067**
		G	49.36	72.85	Ref	

Table 1 (continued)

SNP ref. no.	Chromosome location	Genotype and allele distribution	Healthy controls (%)	Dengue-chikungunya co-infected patients (%)	OR (95% CI)	*p* value
		Female	*N* = 79	*N* = 57		
		AA	7.59	26.31	4.345 (1.566–12.05)	*0.0038**
		AG	46.83	14.03	0.1853 (0.07776–0.4417)	*< 0.0001**
		GG	45.56	59.64	Ref	
TLR8 rs5744080	ChrX:12919685 (GRCh38.p12)		*n* = 157	*N* = 126		
		CC	0	26.19	57.15 (9.631–588.5)	*< 0.0001**
		CT	27.38	7.14	0.2039 (0.09504–0.4376)	*< 0.0001**
		TT	72.61	66.66	Ref.	
		C allele	13.69	29.76	3.945 (2.258–6.864)	*0.0009**
		T allele	86.30	70.23	Ref	
		Additive	*< 0.0001**			
		Male	*N* = 78	*N* = 74		
		C	12.82	31.08	3.067 (1.342–7.009)	*0.0099**
		T	87.17	68.91	Ref	
		Female	*N* = 79	*N* = 52		
		CC	0	19.23	20.25 (2.529–162.1)	*0.0002**
		CT	29.11	17.30	0.5096	0.1484
		TT	70.88	63.46	(0.2141–1.213) Ref	

*$p < 0.05$ at 95% CI was considered as statistically significant and italized

Ref, reference genotype

of the following pairs with p value < 0.01: rs179010(C) with rs3853839(C), rs179010(T) with rs3853839(G), rs5741880(G) with rs179010(C) and rs5741880(T) with rs179010(T).

Distribution of dengue and chikungunya viral load among co-infected patients with different TLR genotypes

DENV-HVL was significantly more prevalent among co-infected patients with TT genotype of TLR7-rs179010, whereas significantly high frequency of CHIKV-HVL was detected among patients with AA genotype of rs179008. On the contrary, dengue low viral load (LVL) was significantly higher among DENV-CHIKV patients with CC genotypes of rs179010 and rs179008 and AA and GC genotypes of rs179008 and rs3853839, respectively. Similarly, CHIKV-LVL was more prevalent among patients with CC genotypes of rs179010 and rs179008.

In TLR8, co-infected patients with GG genotypes of rs3764879 and rs3764880 exhibited CHIKV-HVL. Patients with CC genotypes of rs3764879 and rs5744080 demonstrated CHIKV-LVL, whereas those with CT genotype of rs5744080 demonstrated DENV-LVL (Table 3).

Functional effect of TLR3-rs3775290, TLR7-rs179008 and TLR8-rs3764880 SNPs

TLR3-rs3775290 does not undergo any amino acid changes; and as a result, there is no apparent functional effect of its polymorphic variants. The VarSomeClinical server predicts this SNP to be benign in nature. On the other hand, the same server predicts TLR7-rs179008 to be pathogenic and TLR8-rs3764880 to be damaging in nature. To determine change in protein stability of polymorphic variants of TLR7-rs179008 and TLR8-rs3764880, $\Delta\Delta G$ values were analysed by CUPSAT. In TLR7-rs179008, Q11L structural change was stabilising with favourable torsion angles and $\Delta\Delta G$ (kcal/mol) value of 8.85. But, in TLR8-rs3764880, M1V structural alteration was destabilising with unfavourable torsion angles and $\Delta\Delta G$ value of −0.71. TLR7-rs179008 SNPs were also predicted to be tolerated by SIFT server, whereas TLR8-rs3764880 was implicated to be damaging. While MutationTaster

Table 2 Comparative genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among DENV-CHIKV co-infected and DENV/CHIKV mono-infected infected patients

SNP ref. no.	Genotype and allele distribution	Dengue-chikungunya co-infected patients	Dengue-infected patients			Chikungunya-infected patients		
TLR3 rs3775290		*N*=127	*N*=192	Odds ratio (OR)	*p* value at 95% C.I	*N*=173	Odds ratio (OR)	*p* value at 95% CI
	CC	11.02	18.22	0.5558 (0.2857–1.081)	0.0839	9.24	1.216 (0.5702–2.592)	0.6979
	TT	38.58	31.77	1.349 (0.8439–2.157)	0.2298	25.43	1.842 (1.123–.3.021)	*0.0167**
	CT	50.39	50	Ref		65.31	Ref	
TLR7 rs5741880		*N*=126	*N*=196					
	TT	12.69	13.26	0.9510 (0.4948–1.859)	1.0000	9.82	1.335 (0.6463–2.756)	0.4590
	GT	5.55	5.61	0.9893 (0.3832–2.594)	1.0000	4.04	1.395 (0.4766–4.083)	0.5870
	GG	81.74	81.12	Ref		86.12	Ref	
TLR7 rs179010		*N*=127	*N*=196			*N*=173		
	CC	43.30	34.18	1.471 (0.9297–2.327)	0.1018	35.26	1.403 (0.8769–2.243)	0.1869
	C T	6.29	9.69	0.6263 (0.2655–1.477)	0.3112	9.82	0.6169 (0.2575–1.478)	0.2993
	TT	50.39	56.12	Ref		54.91	Ref	
TLR7 rs179008		*N*=128	*N*=193			*N*=173		
	C:C	22.66	11.91	2.165 (1.187–3.949)	*0.0131**	5.78	4.775 (2.231–10.22)	*< 0.0001**
	A:C	4.69	0	11.04 (1.341–90.88)	*0.0079**	2.89	1.652 (0.4929–5.540)	0.5373
	A:A	72.66	88.08	Ref		91.32	Ref	
TLR7 rs3853839		N=126	*N*=175			*N*=173		
	CC	36.50	35.42	1.083 (0.6669–1.742)	0.8068	23.12	1.912 (1.152–3.173)	*0.0139**
	GC	0	0	18.21 (2.335–142.0)	*0.0002**	27.16	0.02083 (0.002035–0.1187)	*< 0.0001**
	GG	63.49	64.57	Ref		49.71	Ref	
TLR8 rs3764879		*N*=126	*N*=196			*N*=173		
	CC	40.47	27.55	1.788 (1.113–2.873)	*0.0205**	28.32	1.721 (1.059–2.797)	*0.0347**
	GC	11.11	8.16	1.406 (0.6608–2.993)	0.4333	22.54	0.4295 (0.2219–0.8313)	*0.0136**
	GG	48.41	64.28	Ref.		49.13	Ref	
TLR8 rs3764880		*N*=126	*N*=190			*N*=173		
	AA	26.77	18.94	1.581 (0.9256–2.700)	0.0985	32.36	0.7721 (0.4654–1.281)	0.3717
	AG	6.29	1.05	6.373 (1.330–30.54)	*0.0165**	19.07	0.2876 (0.1279–0.6469)	*0.0019**
	GG	66.14	80.00	Ref		46.82	Ref	
TLR8 rs5744080		*N*=126	*N*=191			*N*=173		
	CC	26.19	26.70	0.9741 (0.5846–1.623)	1.000	13.29	2.314 (1.280–4.183)	*0.0066**
	CT	7.14	13.08	0.5108 (0.2300–1.134)	0.0994	14.45	0.4554 (0.2047–1.013)	0.0642
	TT	66.66	60.20	Ref		72.25	Ref	

*$p < 0.05$ at 95% CI was considered as statistically significant and italicized

p value and odds ratio calculated between co-infected with respect to mono-infected

Ref, reference genotype

application indicated TLR8-rs3764880 to be automatic polymorphism (harmless), the Panther server predicted TLR7-rs179008 to be possibly damaging. The HOPE server indicated TLR7-rs179008L variant, which lies in the signal peptide region to be smaller and more hydrophobic than the wild type (Q), which might lead to loss of interactions, loss of hydrogen bonding, incorrect folding and disturbance in recognition of signal peptide. In the case of TLR8-rs3764880, HOPE indicated V variant, lying within signal peptide region to be smaller and, thus, might disturb recognition of signal peptide leading to loss of interactions and protein activity disruption. The DIM-pred server predicted transition of Q11L SNPs to be from "order to disorder" in nature (Table 4).

Molecular docking of polymorphic variants of TLR3, TLR7 and TLR8 with SPC18

The SignalP 5.0 server predicted a cleavage site between amino acids 22 and 23 for TLR3, and TLR3-rs3775290 lies 435 amino acids away from the cleavage site leading to no interaction with the signal peptide. As a result, docking with SPC18 was not done. In the case of TLR7, the SignalP 5.0 server predicted a cleavage site between amino acids 26 and 27 for signal peptidase I (SPI). Correspondingly, for TLR8, the server predicted a cleavage site between positions 22 and 23 using SPI.

3D models of SPC18 protein, Q and L variants of TLR7-rs179008 and M and V variants of TLR8-rs3764880 were

Table 3 Distribution of dengue and chikungunya viral load among co-infected patients with different TLR genotypes

N=87	Dengue viral load				Chikungunya viral load			
			Odds ratio (OR)	*p* value at 95% CI			Odds ratio (OR)	*p* value at 95% CI
TLR 3-rs3775290	HVL	LVL			HVL	LVL		
CC (*N*=11)	7	4	1.494 (0.4141–4.844)	0.7477	5	6	0.8333 (0.2573–3.038)	1.000
TT (*N*=38)	22	16	1.216 (0.5208–2.928)	0.6706	20	18	1.256 (0.5413–2.967)	0.6681
CT (*N*=38)	19	19	1.450 (0.6173–3.406)	0.5147	18	20	0.8640 (0.3671–2.006)	0.8298
TLR 7 rs5741880	HVL	LVL			HVL	LVL		
TT (*N*=12)	9	3	2.625 (0.7467–9.478)	0.2159	6	6	0.8750 (0.2585–2.962)	1.0000
GT (*N*=6)	4	2	1.600 (0.3541–8.738)	0.6922	1	5	0.2050 (0.01703 to 1.657)	0.2070
GG (*N*=69)	36	33	2.383 (0.7663–7.413)	0.1827	34	35	0.6551 (0.2272–1.889)	0.5969
TLR 7 rs179010	HVL	LVL			HVL	LVL		
CC (*N*=18)	4	14	0.1524 (0.04513–0.5146)	*0.0014**	3	15	0.1632 (0.04764–0.5662)	*0.0039**
TC (*N*=6)	2	6	0.2270 (0.04509–0.9989)	0.5230	5	3	1.991 (0.4700–7.866)	0.4671
TT (*N*=61)	43	18	7.963 (2.811–23.61)	*< 0.0001**	33	28	2.652 (1.057–7.140)	0.0612
TLR 7 rs179008	HVL	LVL			HVL	LVL		
CC (*N*=24)	4	20	0.2345 (0.08056–0.7572)	*0.0137**	9	15	0.3220 (0.1254–0.8328)	*0.0287**
AC (*N*=6)	2	4	0.8065 (0.1470–3.642)	1.0000	3	3	1.382 (0.2627–7.273)	0.6963
AA (*N*=57)	27	30	0.2778 (0.09868–0.7819)	*0.0193**	38	19	3.000 (1.163–7.580)	*0.0228**
TLR 7 rs3853839	HVL	LVL			HVL	LVL		
CC (*N*=21)	13	8	1.056 (0.3777–3.007)	1.0000	8	13	0.5792 (0.2054–1.597)	0.3242
GC (*N*=0)	–	–	–	–	–	–	–	–
GG (*N*=66)	40	26	1.056 (0.3777–3.007)	1.0000	34	32	0.5792 (0.2054–1.597)	0.3242
TLR8 rs3764879	HVL	LVL			HVL	LVL		
CC (*N*=21)	15	6	2.083 (0.7182–6.153)	0.2092	3	18	0.1154 (0.03412–0.4415)	*0.0004**
GC (*N*=12)	5	7	0.4503 (0.1449–1.501)	0.2222	5	7	0.7336 (0.2368–2.419)	0.7591
GG (*N*=54)	31	23	1.141 (0.4722–2.759)	0.8250	34	20	5.313 (1.967–14.30)	*0.0008**
TLR8 rs3764880	HVL	LVL			HVL	LVL		
AA (*N*=22)	13	9	1.038 (0.3370–9.191)	1.0000	6	16	0.3214 (0.1100–0.9135)	*0.0471**
AG (*N*=7)	5	2	1.755 (0.1041–3.117)	0.6968	1	6	0.1667 (0.01421–1.132)	0.1141
GG (*N*=58)	34	24	1.155 (0.4269–2.882)	0.8194	34	24	4.452 (1.574–11.10)	*0.0030**
TLR8 rs5744080	HVL	LVL			HVL	LVL		
CC (*N*=15)	9	6	1.200 (0.3790–3.915)	0.7838	2	13	0.1538 (0.03321–0.6983)	*0.0102**
CT (*N*=9)	2	7	0.1884 (0.03830–0.9763)	*0.0379**	4	5	0.9659 (0.2408–3.875)	1.0000
TT (*N*=63)	38	25	1.796 (0.7035–4.833)	0.2381	32	31	3.097 (1.121–8.923)	0.0517

*$p < 0.05$ at 95% CI was considered statistically significant and italicized *p* value and odds ratio calculated between HVL with respect to LVL

generated using the QUARK server. Docking of TLR7 Q variant with SPC18 using ClusPro 2.0 generated a model with 104 amino acids interacting with each other along with centre-weighted score of − 925.2 and overall binding free energy of − 1115.7 kcal/mol, whereas in L variant interaction 81 amino acids were observed between the two molecules with centre-weighted score of − 962.2 and overall binding free energy of − 1080.8 kcal/mol (Fig. 1). Congruently, docking of TLR8 M variant with SPC18 involved interaction of 113 amino acids with centre-weighted score of − 958.9 and binding energy of − 975 kcal/mol, whereas interaction of V variant with SPC18 involved 87 amino acids, yielding a binding free energy of − 1014.9 kcal/mol and − 834.7 weighted score (Table 4).

Discussion

TLR family plays an imperative role in recognition of dengue and chikungunya viral genomic RNA and innate immunity activation against them within an infected patient body [10]. TLR activation leads to expression of cytokines; TLR3 initiates production of IFN (interferon)-β and immunoregulatory

Table 4 (a) Predicted effect of non-synonymous TLR polymorphisms on protein structure and (b) docking analysis of polymorphic variants of TLR7 and TLR8 with SPC18

(a)

Gene	SNP ref. no.	AA change		Functional effect						
			CUPSAT	SIFT prediction	Polyphen2 prediction	MutationTaster prediction	DIM-Pred	PMut	VarSomeClinical	Panther
TLR3	rs3775290	Phe(F)-Phe(F)	NA	NA	NA	NA	NA	NA	Benign	NA
TLR7	rs179008	Gly(Q)11(L)leu	Stabilising with favourable torsion angles	Tolerated	Benign	Neutral	Order to disorder	Neutral	Pathogenic	Possibly damaging
TLR8	rs3764880	Met(M)1(V)val	Destabilising with unfavourable torsion angles	Damaging	Benign	Polymorphism automatic	Order to order	Neutral	Damaging	Probably benign

(b)

	Variant	Members involved	Weighted score	Lowest energy (kcal/mol)
TLR7 rs179008	Gly(Q)	104	−925.2	−1115.7
	(L)leu	81	−962.2	−1080.8
TLR8 rs3764880	Met(M)	113	−958.9	−975
	(V)val	87	−834.7	−1014.9

cytokines while TLR7 and 8 initiate IFN-α production, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) [10, 30]. TLR7 stimulation produces proinflammatory cytokines through the phosphorylation of interferon regulatory factor 7 and liberation of nuclear factor-kB, respectively [31]. Genetic variations of these innate immunity genes are likely to influence DENV-CHIKV co-infection susceptibility and disease pathogenesis.

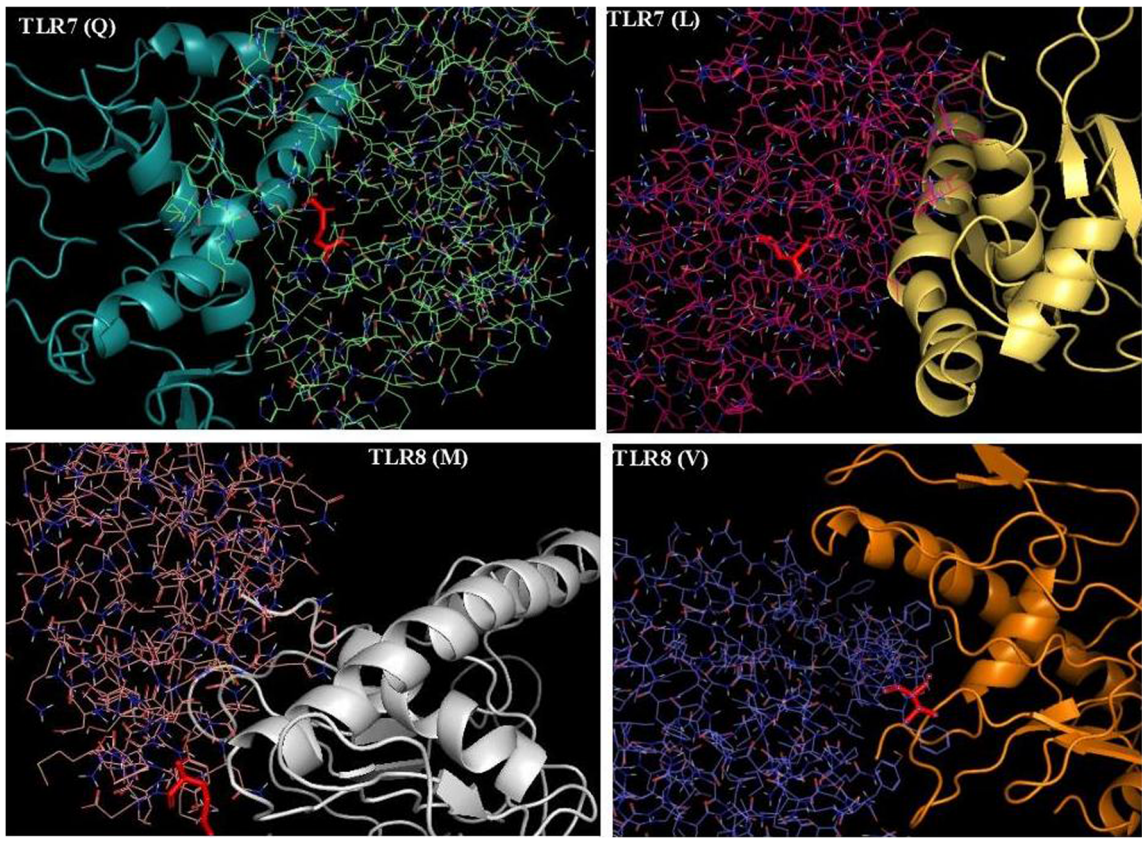

Fig. 1 Docking results of polymorphic variants of TLR7 and TLR8 with SPC18. TLR7 and 8 proteins are represented by sticks, signal peptidase complex (SPC18) represented by cartoons and red-coloured stick represents variants within the signal peptide region of TLR7 and 8 proteins

This study embodies a comprehensive analytical role of TLR3, 7 and 8 SNPs towards DENV-CHIKV co-infection among Eastern Indian patients.

In this study, patients with CC genotype of TLR7-rs179010 were susceptible to DENV-CHIKV co-infection, whereas those with TC genotype were protected from co-infection. Similarly, this SNP has been previously associated with chikungunya and enteroviral infections [10, 16]. Furthermore, DENV-CHIKV co-infection susceptibility risk significantly increased among patients with CC genotypes of rs179008 and rs3853839, whereas GC genotype of rs3853839 impounded a defensive stand towards co-infection among patients. Various genotypes of these SNPs have been previously implicated for susceptibility towards dengue, chikungunya, hepatitis C (HCV) and enteroviral infections [10, 16, 32]. Moreover, additive model statistics portrayed a significant association of rs179008, rs179010 and rs3853839 with DENV-CHIKV co-susceptibility.

In TLR8, CC genotypes of rs3764879 and rs5744080 demonstrated significant association with viral co-susceptibility, but AG-rs3764880 and CT-rs5744080 protected the studied population against viral co-infection. These genotypes have been previously implicated towards chikungunya, dengue and HCV infections [10, 16]. Additive model analytics indicated significant association between rs3764879, rs5744080 and rs3764880 with DENV-CHIKV co-infection.

Furthermore, the differential role of TLR3, 7 and 8 SNPs against CHIKV/DENV mono-infection and DENV-CHIKV co-infections was investigated. Patients with TT genotype of TLR3-rs3775290 were susceptible towards viral co-infection compared to CHIKV mono-infection. Similarly, patients with CC genotypes of rs179008 and rs3853839 (TLR7) and rs3764879 and rs5744080 (TLR8) were susceptible towards DENV-CHIKV co-infection. On the contrary, patients with GC genotypes of TLR7-rs3853839 and TLR8-rs3764879 and AG-rs3764880 were more susceptible towards CHIKV mono-infection. Similarly, patients with TLR7-rs179008 CC, AC and TLR7-rs3853839 GC, CC and AG genotypes of rs3764879 and rs3764880 genotypes were more susceptible towards viral co-infection compared to those with DENV mono-infection. Similar to the difference in genetic signature of DENV/CHIKV mono- and co-infected patients observed in this study, global profiling of serum metabolites was also found to be distinct among co-infected patients compared to mono-infected cases [32]. Similarly, glutamine levels were previously noted to increase only in co-infected patients compared to DENV/CHIKV mono-infected ones [33, 34]. Also, Shrinet et al. reported downregulation of amino acid biosynthesis pathway exclusively among DENV-CHIKV co-infected *Aedes aegypti* which was absent among mono-infected mosquitoes [35].

DENV-HVL was significantly more prevalent among co-infected patients with TT genotype of TLR7-rs179010. Similarly, patients with AA genotype of TLR7-rs179008, and GG genotypes of TLR8-rs3764879 and rs3764880 displayed CHIKV-HVL. Caron et al. previously showed that co-infected patients could be subdivided according to their respective CHIKV and DENV viral load levels, suggesting a possible mechanism of competition during viral replication among these patients [35]. The results of the current study suggested the probable role of specific genotypes of TLR7 and 8 SNPs in determining DENV/CHIKV load during such competitive viral replication among co-infected patients.

rs179008 and rs3764880 lie within signal peptide regions of TLR7 and TLR8, respectively, as predicted by the SignalP 5.0 server; hence, genotypic changes at these two positions might alter stability of TLR receptors. CUPSAT and SIFT both predicted Q11L of rs179008 to be stable and tolerable, whereas M1V of rs3764880 to be disruptive and damaging. Docking of TLR7 and TLR8 variants with SPC18 indicated better binding of Q variant of TLR7-rs179008 and V variant of TLR8-rs3764880 with SPC18 compared to respective L and M variants. However, though Q variant of TLR7-rs179008 interacted with SPC18 via more residues and generated better free binding energy, V variant of TLR8-rs3764880 interacted less efficiently with SPC18 residues, but still generated a better free binding energy. SPase enzyme is responsible for cleavage of signal peptide from pre-protein, allowing its release from membrane and correct folding of mature protein [12]. Thus, docking data indicated differential interaction of TLR7 and TLR8 variants with SPC18, which might affect the receptors' improper cleavage, translocation and cellular secretion, leading to disparity in TLR-mediated immune signalling against DENV/CHIKV infections [12, 14].

Conclusion

Thus, this study underlined the importance of TLR3, 7 and 8 SNPs towards mono- or co-infection of DENV and CHIKV, with certain genotypes imparting a protective role while others associated with susceptibility towards co-infection. It further indicated the differential role of TLR polymorphic genotypes towards DENV-CHIKV mono- and co-infection suggesting a shift in TLR-mediated immune responses. Moreover, this study suggested the probable role of specific genotypes of TLR7 and 8 SNPs towards imparting high dengue/chikungunya viral load during competitive viral replication among co-infected patients. However, the findings of this study have to be validated by a cross-sectional study on a larger number of DENV-CHIKV co-infected patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-020-04125-x.

Acknowledgements The authors are extremely grateful to the Director, Calcutta School of Tropical Medicine, Kolkata, India, for his support, inspiration and providing of necessary facilities for this study. The authors are grateful to Indian Council of Medical Research, India, for granting fellowship to the first author [5/3/8/6/ITR-F/2018-ITR]. The authors also acknowledge Mr. Advaita Chakraborty, Lab Manager, Department of Biomedical Sciences, College of Health Sciences, Marquette University, Milwaukee, WI, for providing his email ID for using QUARK server and ClusPro 2.0 server.

Authors' contributions Siddhartha Sengupta: Experimental work, formal analysis and investigation, original draft preparation, review and editing. Saikat Mukherjee: Dengue detection methodology, review and editing. Nemai Bhattacharya: Sample resources. Anusri Tripathi: Conceptualization, formal analysis and investigation, review and editing, consumable and chemical resources and supervision

Data availability All data generated and analysed during this study are included in this article and supplementary file.

Compliance with ethical standards

Ethical approval All procedures performed in this study involving collection of blood from human participants as well as healthy controls were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26 September 2013).

Consent to participate Written consent was received from patients and healthy control individuals prior to participation in the study.

Consent to publish Not applicable.

Competing interest The authors declare that they have no competing interests.

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Antiviral Research 197 (2022) 105225

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Association of C-reactive protein polymorphisms with serum-CRP concentration and viral load among dengue-chikungunya mono/co-infected patients

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ARTICLE INFO

Keywords:
Dengue
Chikungunya
Co-infection
CRP
Single-nucleotide polymorphism
Viral load

ABSTRACT

India being endemic to Dengue (DENV) and chikungunya (CHIKV) infections faces high patient-mortality and morbidity with overlapping clinical features. C-reactive protein (CRP) acts as early defence system in response to these infections. This study investigated role of CRP single-nucleotide polymorphism (SNP) genotypes and protein levels towards DENV/CHIKV mono and co-infection among eastern Indian patients. 128 DENV-CHIKV co-infected, 206 DENV and 167 CHIKV mono-infected patients were subjected to genotyping of two CRP SNPs by PCR-RFLP along with 102 healthy individuals. CRP levels were determined by immunoturbidimetry. Statistical correlation of CRP genotypes with CRP concentration, DENV-CHIKV mono/co-infection and viral load was performed. Patients with rs3093059-CT and rs3091244-TT were more susceptible to DENV-CHIKV co-infection, whereas, rs3091244-CT might have imparted protection against CHIKV mono-infection. DENV-HVL was more prevalent within rs3093059-TT and rs3091244-CT co-infected patients, whereas, CHIKV-HVL among rs3091244-CC. Acute phase co-infected patients had significantly higher CRP level compared to mono-infections. Both mono and co-infected patients with aches/pain exhibited 2-3-fold higher CRP levels compared to those without. rs3093059-CT and rs3091244-CT co-infected patients had higher CRP concentration compared to rs3093059-TT and rs3091244-CC, respectively. Co-infected patients with WHO-defined warning signs had higher anti-dengue IgG/IgM ratio and serum CRP level compared to those without warning signs. Thus, patient's CRP genotype might play significant role in determining serum-CRP concentration, viral load and DENV-CHIKV mono/co-infection.

1. Introduction

Over the last decade, arthropod-borne emerging and re-emerging tropical diseases viz. dengue, chikungunya is responsible for critical health hazards including high mortality and morbidity, respectively globally and in Indian subcontinent [Mukherjee et al., 2017; Sengupta et al., 2020; Sengupta et al., 2021]. Overlapping clinical features such as aches/pain often led to chikungunya being mis-diagnosed as dengue [Furuya-Kanamori et al., 2016]. Due to same vector and similar post-monsoon transmission period, DENV-CHIKV co-infection has emerged as potential health hazard among eastern Indian patients [Sengupta et al., 2021].

Upon pathogenic infection C-reactive protein (CRP) secreted by liver acts in response to variety of inflammatory cytokines [Du Clos 2000]. It acts as scavenger protein by binding to damaged tissues, nuclear antigens and pathogens, thereby removing toxic molecules and neutralizing invasive microorganisms [Mukherjee and Tripathi 2020]. CRP levels increase very rapidly during inflammation, infection and subsequently decrease swiftly when condition resolves [Du Clos 2000]. CRP has been implicated in assisting complement binding to foreign infection and damaged cells, thereby activating innate immune response as early defence system [Sproston and Ashworth 2018].

There have been reports of CRP being used as a prognostic marker for chikungunya infection, where CRP levels were significantly elevated in viraemic group compared to non-viraemic group [Solanke et al., 2014; Anfasa et al., 2017]. In a recent study, CRP level significantly increased among acute phase DENV infected patients, patients with WHO-defined warning signs and among patients with high DENV copy number

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https://doi.org/10.1016/j.antiviral.2021.105225
Received 3 June 2021; Received in revised form 25 September 2021; Accepted 11 December 2021
Available online 13 December 2021
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[Mukherjee and Tripathi 2020]. Elevated CRP level was previously reported among high percentage (95.24%) of chikungunya patients with persistent arthralgia [Gauri et al., 2016]. CRP promoter region has three cis-acting polymorphisms that contribute to variance in CRP level, viz. rs3091244, rs3093059 and rs3093062 [Szalai et al.,2005; Mahajan et al., 2011](Fig. S1). Among them, rs3093062 is only CC homozygous in Bengali ethnicity of south Asian population (SAS) [Genome Wide Association Studies (GWAS) database]. Moreover, specific genotypes of rs3091244 and rs3093059 have been associated with stroke, ankylosing spondylitis, osteomyelitis and DENV susceptibility [Shen et al., 2013, Mukherjee and Tripathi 2020, Kasapoğlu Aksoy et al., 2020, Wang et al., 2020]. Previous evaluation of rs3091244 and rs3093059 among North Indian population indicated maximum contribution of rs3093059 for CRP level variance [Mahajan et al., 2011]. CRP rs3091244-TT genotype has been reported to be positively associated with dengue susceptibility, whereas, rs3093059-CT genotype and rs3091244-TT genotype were found to correlate with elevated CRP level [Mukherjee and Tripathi 2020]. Role of CRP polymorphisms and CRP levels for susceptibility towards both DENV and CHIKV among co-infected patients has not been previously explored. Thus, this study investigated role of CRP polymorphisms and protein levels towards mono and co-infection of dengue and chikungunya viruses among eastern Indian patients.

2. Materials and methods

2.1. Ethics statement

Collection of blood from febrile patients and healthy participants as well as experiments were performed in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated September 26, 2013) and with that of 1964 Helsinki Declaration and its later amendments. Written consents were obtained from patients and healthy individuals before their participation in the study.

2.2. Patients and healthy controls

5 ml of blood from all age groups and sexes were collected from each of 550 symptomatic febrile patients (acute phase: 1–3 days, critical phase: 4–6 days late phase: ≥7 days), visiting Calcutta School of Tropical Medicine, West Bengal, India from September 2014 to October 2016. Febrile patients exhibiting any two of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, nausea, vomiting, swollen glands, rash, haemorrhagic manifestation, fatigue were selected as per WHO criteria. Amongst them, 128 were co-infected with DENV and CHIKV, as detected by anti-DENV-IgM ELISA/dengue-NS1 ELISA (NIV, Pune, India)/real-time qRT-PCR and anti-CHIKV IgM (NIV, Pune, India)/real-time qRT-PCR, respectively. To identify secondary DENV/CHIKV infection, anti-DENV and anti-CHIKV IgG ELISA were performed. Patients with IgG/IgM ratio ≥1.1 were considered to be having secondary infection [Changal et al., 2016]. To carry out age-matched case control study, blood from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with DENV, CHIKV, Japanese encephalitis, HIV and Hepatitis A/B/C infection - as tested by IgM ELISA/RT-PCR, were collected from same community, as described previously [Dutta and Tripathi 2017]. Symptomatic patients and healthy controls consisted of Bengali population of eastern India having similar ethnic genetic make up to that of Bengali Bangladeshis (BEB) group of SAS included within 1000 genome project of GWAS.

2.3. Extraction of viral RNA and determination of DENV and CHIKV load

Viral RNA was extracted from 140 μL of patients' sera, using QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France). Genome presence and viral load of DENV and CHIKV was determined by real-time qRT-PCR using DENV and CHIKV Genesig kit (Primer Design Ltd., UK) respectively, according to manufacturer's protocol. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided within kit). Viral load in patient serum was calculated from corresponding Ct values. Limit of detection of both DENV and CHIKV Genesig kits was <100 copies of corresponding target genomes. Real-time PCR was performed on ABI Prism 7500 fast instrument. Each sample was loaded in triplicate. Viral RNA titre ≥10,000 copies/ml and <10,000 copies/ml was considered as high viral load (HVL) and low viral load (LVL), respectively [Mukherjee et al., 2017].

2.4. Quantification of CRP

Sera collected from infected patients were subjected to CRP quantification. CRP level was determined by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics, India) according to manufacturer's protocols. Briefly, 3 μl of patient serum/calibrator was mixed with 500-μl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and absorbance was measured at 550 nm wavelength after 10 s (A1) and 120 s (A2). Serum concentration (mg/l) of CRP was measured using following formula:

Serum concentration of CRP (mg/l) = [(A2-A1) Serum sample/(A2-A1) Calibrator] x Concentration of calibrator.

2.5. SNP genotyping

To understand role of CRP polymorphisms (if any) towards DENV-CHIKV co-infectivity compared to infectivity against DENV/CHIKV alone, genotyping of CRP-rs3093059 and rs3091244 was done among 167 CHIKV mono-infected and 128 DENV-CHIKV co-infected patients. Data of these polymorphisms from 201 DENV mono-infected patients were collected from our earlier study [Mukherjee and Tripathi 2020].

Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) was done for SNP genotyping. QIAamp DNA BloodMini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from patient blood, according to manufacturer's protocol. Polymorphic regions of rs3093059 and rs3091244 were identified using dbSNP (Single Nucleotide Polymorphism Database) and based on sequences available in GenBank database, three primer pairs were designed using Primer3 software, to amplify these regions [Mukherjee and Tripathi 2020].

PCR reaction was carried out in 20-μl volume, using 1 × PCR buffer (Fermentas, USA),1 mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5 mM $MgCl_2$ and 20p.moles of previously mentioned primers. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at desired annealing temperature for 30 cycles and PCR products were electrophoresed on 2% agarose gel at 5V/cm. Respective PCR products were digested with Tas I, Bfa I (Fermentas, USA) and Taq I (Himedia, India) and digested products were visualized on 2.5% agarose gel [Mukherjee and Tripathi, 2020]. Different RFLP patterns were validated by sequencing of respective PCR products using ABI Prism Big Dye Terminatorv3.1 Cycle sequencing kit (Applied Biosystems, USA) in ABI-Prism 3100 Avant Genetic Analyzer (Applied Biosystems, USA).

2.6. Statistical analysis

Genotypic associations of respective SNPs with DENV-CHIKV co-infected and mono-infected disease susceptibility, their CRP levels, viral loads, course of illness and specific symptoms was analysed and represented using GraphPad Prism 9 (Los Angeles, USA). Allelic and genotypic frequencies were compared between different study groups using Pearson's Chi square test, unpaired Welch's *t*-test and multi-variant analysis using one way ANOVA. For genotypic associations, p-values,

odds ratio (OR) and risk ratio were calculated. A p-value of <0.05 was considered statistically significant. For controls, Hardy–Weinberg equilibrium was analysed for two polymorphisms with Haploview programme.

3. Results

In this study, blood from 550 febrile symptomatic patients was collected during post-monsoon seasons of 2014–2016, out of which 128 were found to be DENV-CHIKV co-infected, 201 were DENV mono-infected and 167 were CHIKV mono-infected. CRP levels and genetic polymorphisms were investigated amongst co-infected and mono-infected patients along with 102 age-sex matched healthy control volunteers. Clinico-pathological history of the patients and healthy cohorts is mentioned in Table 1. Majority of DENV-CHIKV co-infected and DENV mono-infected patients were in acute (co-infected: 30.46% and DENV mono-infected: 33.49%) and critical phase (co-infected: 46.87% and DENV mono-infected: 53.88%) of illness, whereas, bulk of CHIKV mono-infected patients were in late phase (52.09%). During blood collection, myalgia, headache, rash, nausea and abdominal pain were most prevalent among DENV infected patients, whereas, arthralgic manifestation and joint swelling were most prevalent among CHIKV infected patients.

3.1. Genotypic association of CRP polymorphisms with susceptibility towards DENV/CHIKV mono-infection and DENV-CHIKV co-infection

Statistical analysis between infected and control populations revealed CRP-rs3093059-CT genotype was positively associated with DENV-CHIKV co-infected patients ($p = 0.0378$) with >1 OR and relative risk [Table 2]. Prevalence of CT genotype was around two-fold more among DENV-CHIKV co-infected patients compared to control population. Furthermore, CRP-rs3091244-TT genotype was significantly associated with both DENV-CHIKV co-infection ($p = 0.0138$) and DENV mono-infection ($p = 0.03$), with more than three-fold increase in its frequency among infected groups; >1 OR and relative risk among co-infected and DENV mono-infected groups. T-alleles of rs3091244 were found to be significantly associated with co-infectivity ($p = 0.0221$) and >1 OR and relative risk. Furthermore, rs3091244-TC genotype was negatively associated with CHIKV mono-infection compared to healthy individuals ($p = 0.0126$). Additionally, according to additive model, rs3091244 was significantly associated with DENV-CHIKV co-infection and CHIKV mono-infection susceptibility ($p = 0.0337$ and 0.0286, respectively).

3.2. Anti-DENV/CHIKV antibody titres among co-infected patients and their association with WHO-defined warning signs, pain and CRP levels

Approximately, 66% (84/128) of co-infected patients were anti-DENV IgM+ and 14% (18/128) were anti-DENV IgG+ (secondary dengue). Furthermore, about 30% (38/128) of them were anti-CHIKV IgM+ and 34% (43/128) were anti-CHIKV IgG+ (secondary chikungunya). Co-infected patients exhibited average anti-DENV IgM titre: 12.71units, anti-DENV IgG titre: 3.76units and average anti-CHIKV IgM titre: 6.05units, anti-CHIKV IgG titre: 1.71units. Average anti-DENV IgG/IgM ratio of co-infected patients having WHO-defined warning signs was 4.44units, whereas that among patients without warning signs was 2.03units [Table 4]. Similarly, anti-CHIKV IgG/IgM ratio among these groups was 8.33units and 7.01units. Co-infected patients with pain had one-fold higher average anti-DENV IgG/IgM ratio when compared to those without any pain (3.28units vs. 2.68units). Co-infected patients having anti-DENV IgG/IgM ratio≥1.1 (secondary dengue) had mean CRP level of 62.57 mg/l, higher than those with primary infection (mean CRP level: 40.047 mg/l) (p value $= 0.06$). Inadvertently, patients with anti-CHIKV IgG/IgM ratio≥1.1 had mean CRP level of 47.17 mg/l, whereas that among primary infection was 63.86 mg/l (p value $= 0.02$).

3.3. Distribution of dengue, chikungunya viral load and DENV serotypes among co-infected patients with different CRP genotypes

Among DENV-CHIKV co-infected patients, dengue HVL was significantly more prevalent within patients with CRP-rs3093059-TT genotype ($p = 0.0331$), whereas, significant association was observed between CT genotype and occurrence of dengue LVL ($p = 0.0143$) [Table 3]. Similarly, dengue HVL was more prevalent among patients with CRP-rs3091244-CT genotype ($p = 0.0006$), whereas, LVL of dengue was significantly associated with TT genotype ($p = 0.0207$). Furthermore, CHIKV-HVL was more prevalent among co-infected patients with rs3091244-CC genotype ($p = 0.0313$), whereas, CHIKV-LVL was significantly high among those with rs3091244-CT genotype ($p = 0.0278$). Percent prevalence of various dengue serotypes among co-infected patients was as follows: DENV2 (60.52%)>DENV1 (26.31%)>DENV4 (10.52%)>DENV3 (2.63%). But, no specific DENV serotype was significantly associated with any rs3093059 and rs3091244 genotypes.

Table 1
Comparative demographics and symptomatic diversity of DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients.

		Dengue-Chikungunya co-infected patients (N = 128)	Dengue infected patients (N = 206)	Chikungunya infected patients (N = 167)	Healthy Controls (N = 102)
Mean age (in years)		24.28 ± 14.7 (range: 2–60 years)	32.01 ± 14.7 (range: 4–65 years)	34.64 ± 13.8 (range: 4–74 years)	36.2 ± 11.8 (range: 18–63 years)
Sex	Male	58.59% (75)	53.39% (110)	59.88% (100)	50.98% (52)
	Female	41.40% (53)	46.60% (96)	40.11% (67)	49.02% (50)
	Male:female ratio	1.44:1	1.14:1	1.19:1	1:1.01
Course of illness	Acute phase	30.46% (39)	33.49% (69)	12.06% (20)	NA
	Critical phase	46.87% (60)	53.88% (111)	36.20% (60)	
	Late phase	22.65% (29)	12.62% (26)	52.09% (87)	
Symptomatic diversity	Myalgia	55.11% (71)	79.61% (164)	53.89% (90)	
	Arthralgia	48.81% (62)	40.29% (83)	67.06% (112)	
	Headache	33.07% (42)	44.17% (91)	22.15% (37)	
	Rash	14.96% (19)	28.15% (58)	19.76% (33)	
	Nausea	18.11% (23)	33.49% (69)	2.39% (4)	
	Persistent Vomiting	26.77% (34)	29.12% (60)	25.14% (42)	
	Abdominal Pain	11.02% (14)	31.06% (64)	8.98% (15)	
	Joint Swelling	17.32% (22)	12.62% (26)	21.55% (36)	
	Bleeding	5.51% (7)	8.73% (18)	0%	
	Retro-Orbital Pain	4.72% (6)	8.25% (17)	0%	
Viral titre	High viral load (HVL: ≥ 10,000 copies/ml)	Dengue 56.32% (49) Chikungunya 47.12% (41)	56.14% (32)	46.87% (15)	
	Low viral load (LVL: < 10,000 copies/ml)	Dengue 43.67% (38) Chikungunya 52.87% (46)	43.85% (25)	53.12% (17)	

Table 2
Genotypic and allelic distribution of CRP SNPs among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients with healthy controls.

SNP Ref. No.	Genotype and allele distribution	Healthy Controls	Dengue-Chikungunya Co-infected patients				Dengue mono- infected patients				Chikungunya mono-infected patients			
		n = 102	n = 128	OR (95% C.I)	Relative risk (95% C.I)	p-value	n = 206	OR (95% C. I)	Relative risk (95% C.I)	p-value	n = 167	OR (95% C.I)	Relative risk (95% C.I)	p-value
CRP rs3093059	CC	2	2	0.7937 [0.1228 to 5.142]	0.8850 [0.5015 to 2.969]	>0.9999	1	0.24 [0.02 to 2.72]	0.4918 [0.3276 to 1.592]	0.25	3	1.093 [0.1913 to 5.429]	1.056 [0.3082 to 2.098]	>0.9999
	CT	21	43	1.951 [1.081 to 3.599]	1.487 [1.039 to 2.223]	**0.0378***	59	1.54 [0.87 to 2.79]	1.353 [0.9200 to 2.060]	0.16	43	0.7476 [0.4144 to 1.374]	0.8304 [0.5526 to 1.196]	0.3776
	TT	79	83	Ref			146	Ref			121	Ref		
	C allele	25	47	0.6211 [0.3605 to 1.038]	0.7526 [0.5284 to 1.025]	0.0927	61	1.24 [0.75 to 2.04]	1.162 [0.8361 to 1.676]	0.45	49	0.8123 [0.4783 to 1.377]	0.8757 [0.6125 to 1.199]	0.5191
	T allele	179	209	Ref			351	Ref			285	Ref		
	Additive	0.0915					0.1627				0.6280			
CRP rs3091244		**n = 102**	**n = 128**				**n = 206**				**n = 167**			
	TT	3	15	4.381 [1.285 to 14.54]	2.802 [1.172 to 8.066]	**0.0138***	20	3.54 [1.02 to 12.23]	2.663 [1.063 to 7.731]	**0.03***	11	2.327 [0.6932 to 7.942]	1.812[0.7990 to 5.176]	0.1915
	TC	39	51	1.070 [0.6388 to 1.810]	1.038 [0.7757 to 1.412]	0.8039	80	1.21 [0.74 to 1.99]	1.017 [0.7383 to 1.418]	0.45	40	1.965 [1.166 to 3.323]	1.489 [1.092 to 1.996]	**0.0126***
	TA	0	1											
	CC	60	61	Ref			106	Ref			116	Ref		
	T allele	45	81	1.635 [1.063 to 2.465]	1.333 [1.040 to 1.746]	**0.0221***	120	1.45 [0.97 to 2.15]		0.06	62	1.242 [0.8138 to 1.907]	1.140 [0.8736 to 1.452]	0.3243
	C allele	159	175	Ref			292	Ref			272	Ref		
	Additive	**0.0337***					0.0873				**0.0286***			

*$p < 0.05$ at 95% CI was considered as statistically significant.
"Ref" = reference genotype.

Table 3
Distribution of dengue and chikungunya viral load among co-infected patients with different CRP genotypes.

	Dengue Viral Load				Chikungunya Viral Load			
N = 87			Odds Ratio (O.R.)	*p*-value at 95% C.I			Odds Ratio (O.R.)	*p*-value at 95% C.I
CRP rs3093059	HVL	LVL			HVL	LVL		
CC	2	0	1.833 [0.2625 to 24.46]	>0.9999	2	0	3.525 [0.5024 to 46.62]	0.3406
CT	11	15	0.2833 [0.1163 to 0.7193]	**0.0143***	12	14	0.9458 [0.3786 to 2.288]	>0.9999
TT	42	17	2.851 [1.167 to 7.339]	**0.0331***	27	32	0.8438 [0.3582 to 1.982]	0.8191
CRP rs3091244	HVL	LVL			HVL	LVL		
TT	3	8	0.1950[0.05346 to 0.7451]	**0.0207***	6	5	1.086 [0.3339 to 3.895]	>0.9999
CT	27	5	6.023[2.120 to 15.76]	**0.0006***	13	19	0.3611 [0.1493 to 0.9020]	**0.0278***
CC	23	21	2.107[0.8752 to 4.901]	0.1248	30	14	2.643 [1.115 to 6.264]	**0.0313***

*$p < 0.05$ at 95% CI was considered as statistically significant.

Table 4
Genotypic association of CRP polymorphisms and anti-DENV/CHIKV IgG/IgM titre of co-infected patients with WHO-defined warning signs and pain.

	With WHO-defined Warning Signs	**Without WHO-defined Warning Signs**	OR (95% C.I)	Relative risk (95% C.I)	p-value	**PAIN (+)**	**PAIN (−)**	OR (95% C.I)	Relative risk (95% C.I)	p-value
rs3093059										
CC	1	1	0.6154 [0.03203 to 11.92]	0.8077 [0.1522 to 1.514]	>0.9999	2	0	1.923 [0.2790 to 25.42]	1.231 [0.4894 to 1.673]	>0.9999
CT	11	32	0.3056 [0.1441 to 0.6677]	0.4832 [0.2732 to 0.8010]	**0.0044***	25	18	1.254 [0.5897 to 2.592]	1.093 [0.8248 to 1.516]	0.5691
TT	44	39	3.103 [1.457 to 7.038]	1.988 [1.221 to 3.434]	**0.0051***	52	31	1.118 [0.5307 to 2.278]	1.044 [0.7924 to 1.430]	0.8495
rs3091244										
TT	7	8	0.8750 [0.2844 to 2.344]	0.9292 [0.5771 to 1.800]	>0.9999	6	9	0.6102 [0.1992 to 1.799]	0.7661 [0.3727 to 1.288]	0.4204
CT	31	20	3.224 [1.521 to 6.761]	1.872 [1.270 to 2.780]	**0.0020***	37	14	4.625 [2.091 to 9.819]	1.995 [1.428 to 2.832]	< **0.0001***
CC	18	44	0.3014 [0.1434 to 0.6307]	0.5042 [0.3202 to 0.7694]	**0.0014***	22	40	0.2942 [0.1435 to 0.6076]	0.5446 [0.3670 to 0.7826]	**0.0014***
Average anti-DENV IgG/IgM titre	4.44	2.03	–	–	-	3.28	2.68	–	–	-
Average anti-CHIKV IgG/IgM titre	8.33	7.01	–	–	-	5.99	9.64	–	–	-

*$p < 0.05$ at 95% CI was considered as statistically significant.

3.4. Genotypic association of CRP polymorphisms of co-infected patients with WHO-defined warning signs and pain

Among co-infected patients with WHO-defined warning signs, 78.57% had rs3093059-TT genotype (p = 0.0051) [Table 4]. On the contrary, 74.41% patients with rs3093059-CT did not develop any warning sign (p = 0.0044). Approximately, 61% of patients with rs3091244-CT genotype developed warning signs (p = 0.0020). In contrast, 71% patients with rs3091244-CC genotype did not develop any warning sign (p = 0.0014). Approximately, 47% of co-infected patients with rs3091244-TT genotype were presented during acute phase of illness, whereas, 56% of those with rs3093059-CT genotype were reported during critical phase. Further, 72% co-infected patients with rs3091244-CT genotype demonstrated pain (p=<0.0001), whereas, 64.5% of patients with rs3091244-CC genotype did not show any signs of pain (p = 0.0014).

3.5. Comparative analysis of CRP levels among co-infected and mono-infected patients

Similar to co-infected patients, DENV/CHIKV mono-infected patients were also divided into three groups according to course of illness and their CRP levels were measured. During acute phase, mean CRP level was significantly higher among co-infected patients compared to DENV/CHIKV mono-infected groups (Co-infected vs DENV: 82.58 mg/l vs 46.80 mg/l, p = 0.000036; Co-infected vs CHIKV: 82.58 mg/l vs 45.54 mg/l, p = 0.000011) [Fig. 1(a)]. Critical phase co-infected patients had higher mean CRP level of 47.52 mg/l, compared to DENV mono-infected (27.05 mg/l, p = 0.000014) but, comparatively lower than CHIKV mono-infected group (71.90 mg/l, p = 0.000970). CRP levels of co-infected and DENV mono-infected groups decreased progressively from acute to late phase, whereas, CRP levels was highest during critical phase of CHIKV mono-infected patients.

Serum CRP concentration was analysed with development of WHO-defined warning signs among co-infected and DENV/CHIKV mono-infected patients. Mean CRP level of co-infected patients with warning sign increased by 1-fold when compared to patients who did not develop any warning signs (68.11 mg/l vs 51.28 mg/l) [Fig. 1(b)]. Mean CRP concentration among DENV mono-infected patients with warning sign was 25.6 mg/l, whereas, that among patients without warning sign was 11.07 mg/l. Only two CHIKV mono-infected patients developed abdominal pain and vomiting – considered as WHO-defined warning signs.

CRP concentration was also analysed for any association with prevalence of aches/pains among these three patient groups. CRP level was significantly higher among patients with aches/pain compared to those

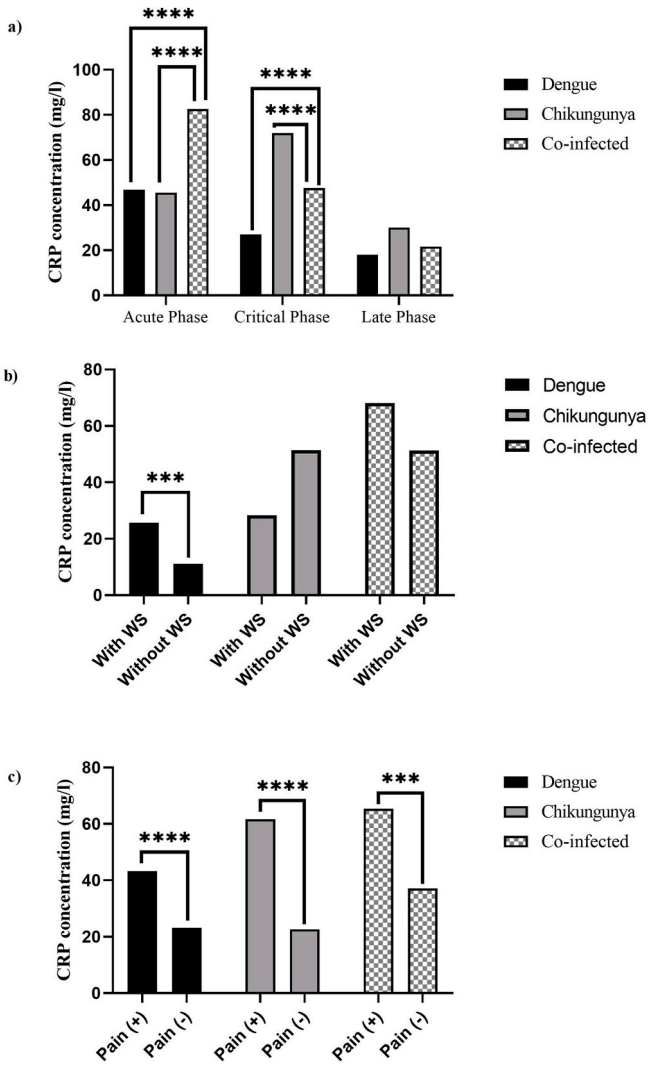

Fig. 1. Comparative serum CRP concentration (a) among acute, critical and late phase DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients (b) among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients having WHO-defined warning signs (WS) and without WS (c) among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients having aches/pain and without aches/pain.

without, among co-infected and DENV/CHIKV mono-infected groups. Among co-infected, DENV/CHIKV mono-infected patients with aches/pain, mean CRP level increased significantly by 2-fold and 3-fold compared to those without any aches/pain (co-infected: pain + vs pain-:65.35 mg/l vs 37.10 mg/l; $p = 0.0002$; DENV mono-infected: pain + vs pain-: 43.28 mg/l vs 23.17 mg/l; $p < 0.0001$ and CHIKV mono-infected: pain + vs pain-: 61.66 mg/l vs 22.61 mg/l; $p < 0.0001$) [Fig. 1(c)].

3.6. Genotypic association of CRP polymorphisms with their CRP levels among co-infected patients and DENV/CHIKV mono-infected patients

DENV-CHIKV co-infected patients having rs3093059-CT genotype had average CRP value of 79.04 mg/l, which was significantly higher when compared to co-infected patients having TT genotype with 40.87 mg/l ($p < 0.0001$) [Fig. 2]. Patients with CC genotype had an average of 36.47 mg/l CRP. Patients with rs3091244-CT and TT genotypes had slightly higher mean CRP concentration compared to those with CC genotype (CT: 61.15 mg/l; TT: 70.93 mg/l; CC: 51.50 mg/l).

Co-infected patients with rs3093059-CT genotype had significantly

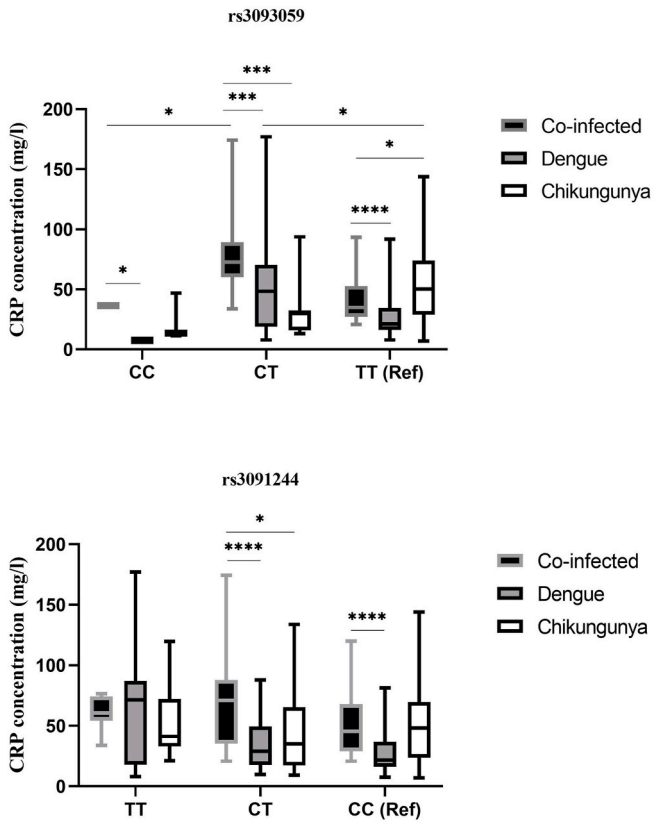

Fig. 2. Genotypic distribution pattern of polymorphic variants of CRP-rs3093059 and rs3091244 among DENV-CHIKV co-infected patients and DENV/CHIKV mono-infected with serum CRP levels.

higher mean CRP level compared to mono-infected patients of same genotype [Co-infected:79.04 mg/l; DENV: 49.28 mg/l; CHIKV: 30.95 mg/l; ($p = 0.000441$; $p = 0.000027$)] [Fig. 2]. Co-infected patients with rs3093059-CC and TT genotypes followed same trend of mean CRP level compared to DENV mono-infected patients [CC genotype: Co-infected: 36.47 mg/l; DENV: 7.51 mg/l ($p = 0.029112$); TT genotype: Co-infected: 40.87 mg/l; DENV: 27.13 mg/l; ($p = 0.000898$)]. However, CHIKV mono-infected patients with TT genotype had higher mean CRP level than co-infected patients [Co-infected: 40.87 mg/l; CHIKV: 55.51 mg/l ($p = 0.0279$)]. Similarly, co-infected patients with rs3091244-CT and CC genotypes had higher mean CRP level compared to DENV mono-infected patients of respective genotypes [CT: Co-infected: 70.93 mg/l; DENV: 34.87 mg/l ($p < 0.0001$); CC: Co-infected: 51.50 mg/l; DENV: 27.57 mg/l ($p < 0.0001$)]. Likewise, co-infected patients with CT genotype had higher mean CRP level than CHIKV mono-infected patients [Co-infected: 70.93 mg/l; CHIKV: 46.19 mg/l; ($p = 0.019624$)].

4. Discussion

CRP being an inflammatory biomarker and an acute-phase protein, it activates innate immune response against infections. It is rapidly synthesized by hepatocytes in response to inflammatory stimuli of viral pathogens. When bound to viral ligands, CRP activates classical complement pathway by involving C3 and might also regulate alternative pathway amplification by providing viral secondary binding site to factor H [Vuong et al., 2020, Pepys and Hirschfield, 2003]. Thus, CRP mediated immune enhancement by DENV/CHIKV might impact disease pathogenesis. As CRP polymorphisms might affect protein level, presence of specific CRP genotypes might influence viral infectivity and disease severity.

Alteration in CRP level with dengue and chikungunya disease

severity has been studied previously, but associative response with DENV-CHIKV co-infection has not been explored yet. This study exemplifies a comprehensive analytical and differential role of CRP polymorphisms and serum-levels towards DENV-CHIKV co-infection and DENV/CHIKV mono-infection among Eastern Indian patients.

Both rs3091244 and rs3093059 are cis-acting genetic variants and tri-allelic rs3091244 has been previously associated with increased CRP levels *in vitro*, thus suggested to have functional role in transcription factor binding [Szalai et al., 2005]. In this study, patients with rs3093059-CT and rs3091244-TT genotypes were more susceptible to DENV-CHIKV co-infection with both OR and relative risk >1, indicating higher odds of disease outcome and co-infectivity risk of patients with these genotypes. Similarly, rs3093059-CT has been previously associated with DENV infection, Hepatitis B virus (HBV) infection, haemorrhagic and ischaemic stroke patients from eastern Indian and Chinese origin, respectively [Shen et al., 2013, Peng et al., 2014, Xue *et al.* 2017, Mukherjee and Tripathi 2020]. On the contrary, rs3091244-CT genotype might have imparted some protection against CHIKV mono-infection. Various genotypes of this polymorphism have been previously implicated for susceptibility towards DENV infection, ankylosing spondylitis, abdominal aortic aneurysm, haemorrhagic and ischaemic stroke among patients of eastern Indian, Turkish, Greek and Chinese origin, respectively [Shen et al., 2013, Saratzis et al., 2014, Xue *et al.* 2017, Mukherjee and Tripathi 2020, Kasapoğlu Aksoy et al., 2020]. Similarly, earlier studies indicated differential role of CD195 receptor genetic variants towards HBV-HIV co-infection and TLR polymorphic genotypes towards DENV-CHIKV co-infection and DENV/CHIKV mono-infection [Ellwanger et al., 2020; Sengupta et al., 2021].

Co-infected patients with WHO-defined warning signs or pain had higher anti-DENV and anti-CHIKV IgG/IgM ratio. Similarly, Ngwe Tun et al. and Garcia et al. had also previously reported that dengue patients with higher IgG and lower IgM titres demonstrated more clinical severity [Ngwe Tun et al., 2013 and García et al., 2011]. Significant increase in CRP level among secondary dengue co-infected patients observed in this study was also previously reported by Mukherjee et al. among dengue infected patients [Mukherjee and Tripathi, 2020]. Increased CRP level was also reported in an anti-dengue IgG positive perinatal patient [Manzano Núñez et al., 2017].

Among co-infected patients, DENV-HVL was significantly more prevalent among patients with rs3093059-TT and rs3091244-CT genotypes, whereas, patients with rs3093059-CT and rs3091244-TT exhibited low DENV copy number. On the other hand, CHIKV-HVL was prevalent among co-infected patients with rs3091244-CC genotype and CHIKV-LVL among rs3091244-CT. Thus, the results indicated that though rs3093059-CT and rs3091244-TT have favoured DENV-CHIKV co-infection, these genotypes might allow less efficient DENV replication in presence of CHIKV in the system –thereby providing reasonable protection against severe dengue development. This was also reflected by absence of any WHO-defined warning sign among majority of the patients with rs3093059-CT genotype. Similar observation was noted with specific genotypes of TLR7,8 polymorphisms in determining high/low copy number of DENV/CHIKV during such competitive viral replication among co-infected patients [Sengupta et al., 2021]. Caron et al. also showed that DENV-CHIKV co-infected patients could be subdivided according to their respective CHIKV and DENV viral loads suggesting a possible mechanism of competition during viral replication [Caron et al., 2012].

Acute phase DENV-CHIKV co-infected patients had significant high mean CRP level compared to DENV/CHIKV mono-infections, whereas, critical phase CHIKV patients possessed significantly higher level of CRP concentrations compared to co-infected and DENV mono-infected patients. Similarly, significant difference in cytokine levels was observed between DENV-CHIKV co-infected, DENV mono-infected (IL-2 and IL-12) and CHIKV mono-infected (IL-2, IL-8, IFNα, IFN γ, and IL-12) patients and that of CRP level between HCV-HIV co-infected and HCV mono-infected patients of India and China, respectively [Salter et al., 2013; Krishnan et al., 2021].

Based on symptomatic presentation of aches/pain, CRP levels increased significantly by 2-3-fold among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients with aches/pain compared to those without. Previous studies also reported significantly increased CRP level among chikungunya, dengue patients with persistent arthralgia/myalgia, juvenile rheumatoid arthritis patients and liver cirrhosis patients demonstrating aches/pain [Harada et al., 1991; Rogal et al., 2015; Gauri et al., 2016; Mukherjee and Tripathi 2020]. Recently, increased CRP level was reported among COVID-19 infected patients with myalgia and arthralgia [Tuzun et al., 2021].

Genotypic association of CRP polymorphisms with their mean CRP levels among DENV-CHIKV co-infected patients revealed patients with rs3093059-CT and rs3091244-CTgenotypes had significantly higher serum-CRP concentration compared to those with rs3093059-TT and rs3091244-CC, respectively. Similar observation was earlier noted among dengue patients and patients with ischaemic stroke and cardiovascular disease [Wang et al., 2006; Shen et al., 2013].

Thus, this study explored possible role of CRP polymorphisms with serum CRP concentration and viral load among DENV-CHIKV co-infected and DENV/CHIKV mono-infected patients. CRP concentration was higher among co-infected patients compared to mono-infected ones and patients with rs3093059-CT and rs3091244-TT genotypes were more susceptible to co-infection. However, these observations should be validated among larger group of patient cohorts.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

All procedures performed in this study involving collection of blood from all human participants were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated September 26, 2013). Written consents were received from patients and healthy control individuals prior to participation in the study.

Availability of data

All data generated and analysed during this study are included in this article.

Declaration of competing interests

The authors declare that they have no conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Acknowledgements

The authors are extremely grateful to the Director, Calcutta School of Tropical Medicine, Kolkata, India, for his support, inspiration and providing necessary facilities for this study. The authors are grateful to Indian Council of Medical Research, Government of India, for granting fellowship to the first author [5/3/8/6/ITR-F/2018-ITR].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.antiviral.2021.105225.

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Received: 6 December 2022 | Accepted: 9 March 2023

DOI: 10.1002/jmv.28661

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Evaluation of analgesic and prophylactic activity of curcumin against chikungunya-infected acute/chronic arthralgic mice

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Abstract

Chikungunya virus (CHIKV) infection, a global public health problem, might lead to acute/chronic polyarthritis causing long-term morbidity among infected patients. But, except nonsteroidal anti-inflammatory drugs (NSAIDs) with gastrointestinal, cardiovascular, and immune-related side-effects, no Food and Drug Administration (FDA)-approved analgesic drug is available till date for the treatment of CHIKV-induced arthritis. Curcumin, a plant product with minimal toxicity has been FDA-approved as a Generally Recognized As Safe drug. This study aimed to determine the analgesic and prophylactic effect of curcumin, if any, among CHIKV-induced arthralgic mice. Arthritic pain was evaluated by von Frey assay, locomotory behavior by open-field test, and feet swelling by calipers. Cartilage integrity and proteoglycan loss were evaluated by Safranin O staining followed by Osteoarthritis Research Society International (OARSI), Standardized Microscopic Arthritis Scoring of Histological sections (SMASH) score, and type II collagen loss by immunohistochemistry. Mice were administered high (HD), mid (MD), and low (LD) curcumin doses, before (PT: pretreatment), during (CT: cotreatment) and after (Post-T: posttreatment) CHIKV-infection. Curcumin treatment using PT_{HD} (2000 mg/kg), CT_{HD}, and Post-T_{MD} (1000 mg/kg) significantly alleviated CHIKV-induced arthritic pain by improving pain-threshold, locomotory behavior and reducing feet swelling of infected mice. Also, decreased proteoglycan loss and cartilage erosion with lower OARSI, SMASH scores were observed among these three subgroups compared to infected ones. Compared to infected ones, one- to twofold increased intensity of type II collagen in knee medial femoral condyle and medial tibial plateau regions of these subgroups was observed by immunohistochemical staining. Thus, this study highlighted both the analgesic (CT, Post-T), and prophylactic (PT) activity of curcumin in alleviating CHIKV-induced acute/chronic arthritis within mouse model.

KEYWORDS

chikungunya, chikungunya-induced arthritis, chondroprotection, curcumin, knee articular region, type II collagen

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1 | INTRODUCTION

Chikungunya viral (CHIKV) infection has re-emerged as one of the major global public health concerns with regional occurrence of its frequent epidemics.[1] It is characterized by extreme patient-morbidity due to joint pain, acute or chronic polyarthritis/polyarthralgia, thereby causing their stooped appearance.[2] 40%–70% of infected patients might develop subacute or chronic/postchikungunya arthritis (PCA) after months or years after the initial CHIKV-infection, which mimicked rheumatoid arthritic symptoms.[3,4] Decreased viability of articular cartilage comprising of type II collagen resulted in deterioration of health and function of bone, thereby, developing arthritis.[5–7] Radiography and magnetic resonance imaging have demonstrated evidence of loss of articular cartilage and bony erosions in affected joints of patients with histories of CHIKV infection.[8] Thus, there is an urgent necessity to identify agents with analgesic property that can alleviate chikungunya-induced arthritic pain for palliative care of infected patients. Currently, no Food and Drug Administration (FDA)-approved specific drug/vaccine is available for this purpose.[9] Plant-derived compounds with minimal toxicity and ready absorption within bloodstream might be advantageous over chemically synthesized compounds for their therapeutic potential against chikungunya-induced arthritis.[10,11] Curcumin, a natural polyphenol isolated from turmeric (*Curcuma longa*) has been recognized as FDA approved drug.[12] It has been found to be safe and tolerable in human clinical trials and systematic reviews without any toxic or adverse effects.[13] Various clinical trials among osteoarthritic patients have previously demonstrated efficacy of curcumin in reducing their arthritic manifestation thereby, improving physical function and quality of life.[14] Curcumin supplementation has been reported to improve walking time, morning stiffness, and joint swelling of rheumatoid arthritic patients.[15] Currently, treatment of chikungunya-induced arthritis is being performed using analgesics, viz. steroidal and nonsteroidal anti-inflammatory drugs.[9] But, limitations of their long-time use are immune disturbances, serious gastrointestinal and cardiovascular problems of infected patients.[16] Curcumin has been described to possess chondroprotective, antiviral, antioxidative, and anti-inflammatory effects in vitro and in vivo.[17] It has been known to block entry and binding of CHIKV in HeLa and HEK 293 T cells, respectively.[18] Moreover, curcumin prohibited entry, replication, and attachment of Dengue, human immunodeficiency virus, Zika, respiratory syncytial virus (RSV), influenza, and herpes simplex virus in both cell culture and murine model.[18] But, effect of curcumin in alleviating CHIKV-induced acute and chronic arthritic pain and its role (if any) in maintaining the structural architecture of articular cartilage has not been studied till date. Mice model has been previously implicated in studying cartilage and synovium degradation, fibrosis of skeletal muscles, and arthritic-disease progression of CHIKV-infected mice.[19,20] Thus, the current study explored the analgesic and prophylactic effect of curcumin in alleviating CHIKV-induced joint pain of acute/chronic arthritic mouse model, its impact on knee articular cartilaginous type II collagen and pain-induced mice behavioral change.

2 | MATERIALS AND METHODS

2.1 | Animals

Swiss albino mice (*Mus musculus*, pathogen free) were used for the studies. Animals obtained from the institutional animal facility, were housed in polypropylene cages with dust-free rice husk as bedding material at constant temperature of 25°C with free access to food and water in a 12-h light/dark cycle. The study was conducted in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[21]

2.2 | Experimental infection and treatment

2.2.1 | Virus passaging in mouse

Chikungunya virus strain was procured from ATCC (ATCC: VR-64, strain S27-African prototype) and cultured in C6/36 cell line using Dulbecco's modified eagle medium with 10% fetal bovine serum) and antibiotics in 5% CO_2 at 28°C. CHIKV copy number in the culture supernatant was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and arthralgic model of CHIKV-infected mice was achieved by subcutaneously inoculating 10^6 copies/50 µL of culture supernatant in the loose skin of mice hind paws, toward the ankle. After infection, clinical scoring of mice was done based on behavior and morphological analysis: 0: no disease signs; 1: ruffled fur; 2: mild hind limb weakness; 3: moderate hind limb weakness; 4: severe hind limb weakness and dragging and 5: moribund. Any deaths were recorded. Clinical score of 5 was set as the terminal point.

2.2.2 | Oral administration of curcumin

Curcumin was procured from Sigma Aldrich, India, and stored at room temperature without exposure to sunlight. Forty human days is equivalent to 1 mice-day when correlating their entire lifespan.[22] Hence, a CHIKV-infected mouse with arthritic manifestation within 0–1 day was considered as acute CHIKV-infected mouse model; post-CHIKV-infected arthritic mouse of 7 days was considered as chronic CHIKV-infected mouse model. Curcumin-treated mouse before CHIKV-infection was considered as prophylactic mouse model. A total of 75 6–8 weeks old Swiss albino mice were grouped into Group I: control, Group II: infection group, Group III: daily curcumin treatment for 7 days before and after CHIKV infection (14 days total) (pretreatment group), Group IV: Curcumin treatment along with CHIKV infection (cotreatment group) and Group V: Daily curcumin treatment for 7days on post-CHIKV infected arthritic mice (posttreatment group), with 15 mice in each group. Each group was subgrouped into high-dose (HD): 2000 mg/kg, mid-dose (MD): 1000 mg/kg, and low-dose (LD): 500 mg/kg, with 5 mice in each subgroup. Curcumin prepared in 5% dimethyl sulfoxide was

administered via oral gavage according to The Organization for Economic Cooperation and Development (OECD) guidelines.[23] Oral dosage of curcumin (LD50 = 2000 mg/kg) was fixed according to mice oral toxicity analysis predicted by ProTox-II server.[24] Doses equivalent to LD50 were considered as HD, while half and quarter dilution of LD50 was considered as MD and LD, respectively.

2.3 | Pain- and arthritis-related morphological and behavioral tests

Dorsoventral feet swelling of all groups of mice were measured by callipers.

2.3.1 | von Frey testing

Mice were acclimatized for 30 min in individual chambers on top of a wire grid platform before von Frey testing. To determine tactile sensitivity of mice hind paws, their plantar surface was stimulated with ascending force intensities of von Frey filaments, having replacement filament range: 1.65–6.65 (Stoelting). A positive response was defined as brisk paw withdrawal, licking, or shaking/flicking of the paw when the stimulus was applied, and the number of positive responses for each stimulus was recorded. Tactile threshold was defined as a withdrawal response in ≥5 out of 10 trials to a given stimulus intensity. This threshold was calculated once per animal.

2.3.2 | Open field test

Mice were acclimated to the test room for 30 min before open field testing. Mouse was placed in the center of individual plexiglass square chambers (45 cm × 45 cm) and allowed to freely explore the chamber for 5-min (test session).[25] Movement of mouse was recorded with a video camera. Two observers blinded to treatment group assignments manually traced mouse movements to calculate line crossings, center square entries (CSE), rearing, and locomotion time within the cage for 5 min.

2.4 | Toxicology and histology

2.4.1 | Determination of toxicological effects of curcumin using biochemical parameters

Oral toxicity of HD, MD, and LD curcumin in mice was determined by toxicological studies. Animals of were killed following completion of curcumin treatment according to OECD guidelines. Blood was collected by cardiac puncture method using 26 gauge needles for different biochemical studies. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, albumin, urea, and creatinine were measured by using a standard automated clinical chemistry analyzer (ERBA Model no: EM360) according to the manufacturer's instructions.

2.4.2 | Histology of liver and kidney

Liver and kidneys were cut into small pieces and kept in paraformaldehyde (10% at room temperature) fixative for 24 h and processed for histological study. After fixation, tissues were dehydrated through increasing ethanol grade series (30%, 50%, 70%, 90%, and 100%), then tissues were embedded in paraffin (Merck). Sections were photographed with a light microscope.

2.4.3 | Safranin O staining, Osteoarthritis Research Society International (OARSI)/Standardized Microscopic Arthritis Scoring of Histological sections (SMASH) score, and histomorphological evaluation of articular cartilage

After sacrifice hind limbs were fixed in formalin for 3 days, decalcified in formic acid, embedded in paraffin following standard protocol, and sectioned for histological and immunohistochemical analysis. Safranin O-fast green staining was used to visualize proteoglycans in knee articular cartilage. The severity of arthritis was evaluated in medial compartment of the knee for each mouse using OARSI 2010 scoring and SMASH sections 2020 recommendations for standardized processing and microscopic scoring of characteristic histopathological features of arthritis.[26-28] OARSI scoring was analyzed on a scale of 0–6, which is calculated on the basis of the articular damage grade scale (0: cartilage/surface intact or normal, 0.5: loss of Safranin O without structural changes, 1: small fibrillations without cartilage loss, 2: vertical clefts down to layer immediately below the superficial layer and some surface lamina loss, 3: vertical clefts/erosion to calcified cartilage extending to <25% of articular surface, 4: vertical clefts/erosion to calcified cartilage extending to 25%–50% of articular surface, 5: vertical clefts/erosion to calcified cartilage extending to 50%–75% of articular surface, and 6: vertical clefts/erosion to calcified cartilage extending >75% of articular surface) and stage (0: no arthritic activity, 1: <10% activity, 2: 10%–25% activity, 3: 25%–50% activity, and 4: >50% activity), along with scoring of loss of proteoglycan based on a scale of 0–6. SMASH-based severity of arthritis was scored on arbitrary scale of 0–3 for two different parameters (loss of cartilage proteoglycan, where, 0: no pathology or healthy intact smooth surface of articular cartilage; 1: mild loss of staining in one-third of superficial cartilage zone, superficial cartilage layer is still predominantly reddish; 2: moderate loss of red staining up to two-thirds of superficial cartilage zone and 3: complete loss of red staining of superficial cartilage zone) and (cartilage erosion, where, 0: healthy intact cartilage; 1: minor roughening or mild loss of superficial noncalcified cartilage layer affecting up to one-third of cartilage area; 2: moderate loss of superficial noncalcified cartilage layer affecting up to two-thirds of cartilage area; 3: complete loss of superficial noncalcified cartilage).

2.4.4 | Immunohistochemical analysis

Immunohistochemical analysis of type II collagen was performed using Mouse specific horseradish peroxidase/DAB Detection IHC Kit (Abcam), according to the manufacturer's protocol. In brief, after deparaffinization, tissue sections were blocked for endogenous peroxidases using hydrogen peroxide for 10 min. Blockage of unspecific binding was done by incubating sections with protein block for 10 min. Between each step, PBST buffer (137 mM/L NaCl, 2.7 mM KCl, 10 mM/L Na_2HPO_4, 1.8 mM/L KH_2PO_4, and 0.1% (v/v) Tween20) washing was performed. Incubation with anti-mouse type II collagen (COL2A1) primary antibody (Santa Cruz Biotechnology) was done overnight (12–14 h) at 4°C. After washing, sections were incubated with biotinylated goat anti-mouse immunoglobulin G secondary antibody (Abcam) for 10 min. The reactions were visualized after incubation with 3,3-diaminobenzidine (DAB) chromogen for 10 min. Counterstaining with hematoxylin was done and slides visualized under light microscope at ×4 and ×10 magnification. Immunostaining intensity for type II collagen epitopes was quantified by determining "reciprocal intensity" of stained articular cartilage matrix; briefly, intensity value of six random locations within medial femoral condyle (MFC) and medial tibial plateau (MTP) of three sections per mouse was measured using ImageJ version 1.53t.

2.5 | Determination of pro-inflammatory biomarker levels (C-reactive protein [CRP], interleukin (IL)-6, and tumor necrosis factor-alpha [TNF-α]) and viral load

CRP, IL-6, and TNF-α quantification in mice sera was performed by immunoturbidimetry method (CRP) using AUTOSPAN turbi gold kit (SPAN diagnostics) and sandwich enzyme-linked immunosorbent assay (IL-6 and TNF-α) using KINESISDx kits according to manufacturer's protocols. Absorbance was measured at 450 nm wavelength. Standard curve was drawn using GraphPad prism9 and concentrations of each protein were analyzed.

Viral load of CHIKV in mice sera postsacrifice was determined by real-time qRT-PCR using CHIKV Genesig kit (Primer Design Ltd.), according to the manufacturer's protocol. A standard curve of cycle threshold (C_t) values was constructed using six 10-fold dilution series of a positive control template (provided within the kit). Viral load was calculated from corresponding C_t values. Limit of detection of CHIKV Genesig kit was <100 copies of corresponding target genomes.

2.6 | Statistical analysis

Group sizes per experiment were based on a "resource equation" method.[29] For all experiments, data were expressed as mean ± SD of five animals per subgroup. Level of significance was set at $p < 0.05$. All experiments were repeated at least twice. Significance analysis and representations were determined using Student's t test (Welch's correction), one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using GraphPad Prism 9.

3 | RESULTS

This study investigated possible effectiveness of curcumin in alleviating CHIKV-induced joint pain of acute/chronic arthritic mouse model and their impact on maintaining articular cartilaginous type II collagen, a major component of knee and other joints. A total of 75, 6–8 weeks old Swiss albino mice were acclimatized for 7 days and grouped into Group I: control (CO), Group II: CHIKV-infected group (IN), Group III: curcumin treatment 7 days prior and 7 days post-CHIKV-infection (pretreatment group: PT), Group IV: Curcumin treatment along with CHIKV-infection for 7 days (cotreatment group: CT) and Group V: Curcumin treatment starting 7 days after CHIKV-infected arthritic manifestation, for 7 days (posttreatment group: Post-T), with five mice in each subgroup. A schematic diagram representing timeline of experiments for infection, curcumin treatment, behavioral, histomorphological, and immunohistochemical study on mice groups has been shown (Figure 1). No death of Swiss-albino mice was observed during the experimental timeline.

3.1 | Toxicology and histopathology of liver and kidney

There was no significant difference in the AST, ALT, ALP, bilirubin, albumin, urea, and creatinine serum levels of curcumin-treated low, mid, and HD mice groups when compared to the control mice group (Supporting Information: Figure S1).

Microscopic investigation of stained tissue sections of liver and kidney revealed no abnormal changes in gross morphology during necropsy. Histopathological features of kidney of control and curcumin-treated mice were similar with normal renal glomeruli and tubules having intact epithelial cells. Liver sections from the control group as well as curcumin-treated mice showed normal arrangement of hepatocytes and proper central vein with no hepatotoxicity (Supporting Information: Figure S1).

3.2 | Effect of curcumin on mice nociceptive behavior

3.2.1 | von Frey analysis

Nociceptive thresholds of mice hind paws in each group were observed using von Frey filaments from day of infection (Day 0) to sacrifice (PT and CT: 7th day; Post-T: 14th day of curcumin treatment). Mean pain threshold of left and right leg of control group (CO) was significantly higher compared to that of infected (IN) ($p < 0.0001$) (Figure 2). Average pain threshold of both legs of pretreated HD subgroup (PT_{HD}) was significantly higher than that of

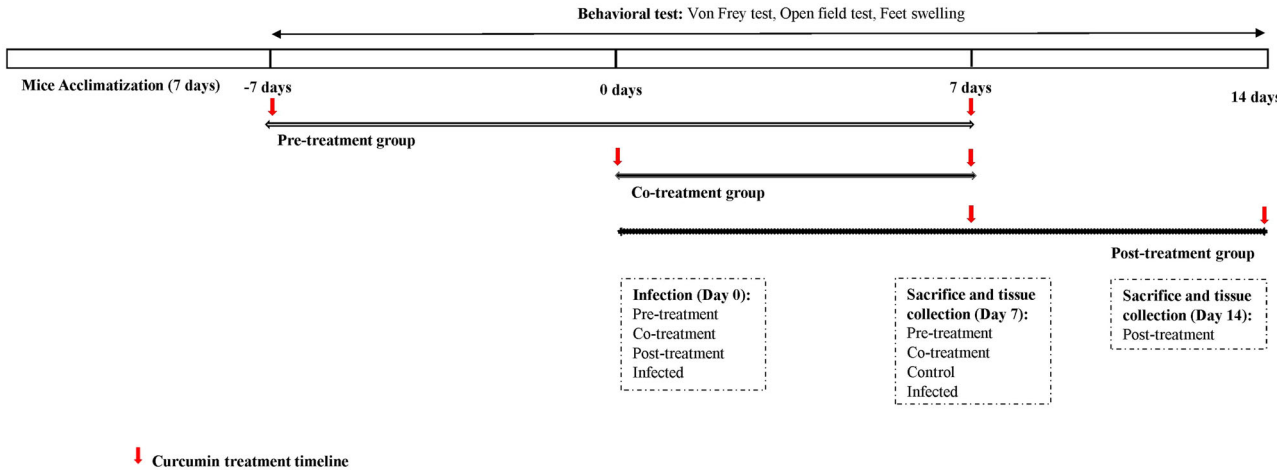

FIGURE 1 Schematic representation of timeline of experiments for study on healthy, infected, and curcumin-treated mice groups: Mice were grouped into pretreatment (7 days prior infection), cotreatment (0 days), posttreatment (7 days postinfection), control, and infection groups. ↓, curcumin treatment timeline, along with behavioral observations. Sacrifice was made and respective tissues were collected for further analysis.

IN (For left leg: $p = 0.0053$) (Figure 2A). In case of cotreated high and MD subgroups (CT_{HD} and CT_{MD}), pain threshold of their left leg significantly increased compared to that of IN ($p = 0.0009$ and $p = 0.0256$, respectively) (Figure 2B). Similar trend was followed for right leg of CT_{HD} subgroup ($p = 0.0035$). For the posttreated MD subgroup (Post-T_{MD}), pain threshold of both legs significantly increased compared to IN ($p = 0.0005$; $p = 0.0271$) (Figure 2C).

3.2.2 | Feet swelling

To evaluate the anti-inflammatory efficacy of curcumin administration, day-wise dorsoventral feet measurement of each mice group was performed. Reduction in feet swelling on curcumin treatment (PT, CT, and Post-T) was observed for all subgroups (Figure 3A–C). Significant reduction in both left and right feet swelling of PT_{HD} subgroup from 5th and 6th day onwards post-CHIKV-infection compared to IN, respectively, was observed ($p < 0.05$). For both CT_{HD} and MD subgroups, swelling reduced from 5th and 6th day onwards for left and right leg ($p < 0.05$), respectively. But, the degree of swelling reduction was more than onefold in HD subgroup compared to MD. For Post-T group, only MD was significantly effective for the reduction of day-wise left feet swelling ($p = 0.0330$).

3.3 | Histomorphological observations: Safranin O stain, OARSI, and SMASH score

The structural integrity of mice knee articular cartilage following CHIKV infection and curcumin treatment was microscopically studied by Safranin O staining and implementing the OARSI and SMASH scores. OARSI and SMASH scoring of control group negated any loss of cartilage proteoglycan or Safranin O (Score: 0) (Figure 4, Table 1). OARSI scoring of IN group knee articular cartilage region exhibited severe Safranin O loss, the appearance of vertical fissures, cartilage fibrillation, loss of cartilage proteoglycan (Score: 3), and cartilage erosion in both MTP and MFC regions (Grade: 3.5, Stage: 3, Score: 10.5). SMASH analysis of IN group revealed a moderate loss of Safranin O and cartilage erosion of MTP and MFC region (Score: 2). Upon oral administration of curcumin, PT_{HD} and PT_{MD} subgroups exhibited less Safranin O loss and cartilage fibrillation compared to IN implicated by their significantly lower OARSI and SMASH scores. Comparing the two doses, PT_{HD} had lower OARSI and SMASH scores with respect to PT_{MD} subgroup. In case of CT group, all three doses showed significantly lower OARSI and SMASH scores compared to IN. But, OARSI and SMASH scores were lower in CT_{HD} compared to CT_{MD} and CT_{LD} subgroups. Finally, in case of Post-T group, all curcumin doses showed reduced OARSI and SMASH score, with Post-T_{MD} subgroup exhibiting lowest OARSI and SMASH scores.

3.4 | Immunohistochemistry

Immunostaining intensities of type II collagen of knee articular cartilage showed its decreased presence in only CHIKV-infected mice, whereas, upon curcumin treatment, collagen concentration increased among all groups compared to infected ones. In PT_{HD} subgroup, collagen intensity significantly increased by threefold, while there was twofold elevation among PT_{MD} and PT_{LD} subgroups at MFC region ($p < 0.0001$, <0.0001, and 0.0006, respectively) (Figures 5 and 6A–C). Similar trend was followed at MTP region with increased intensity by two- and onefold among PT_{HD} and PT_{MD}, PT_{LD} subgroups, respectively ($p = 0.0003$, 0.0016, and 0.0029). Thus, PT_{HD} dosage was most

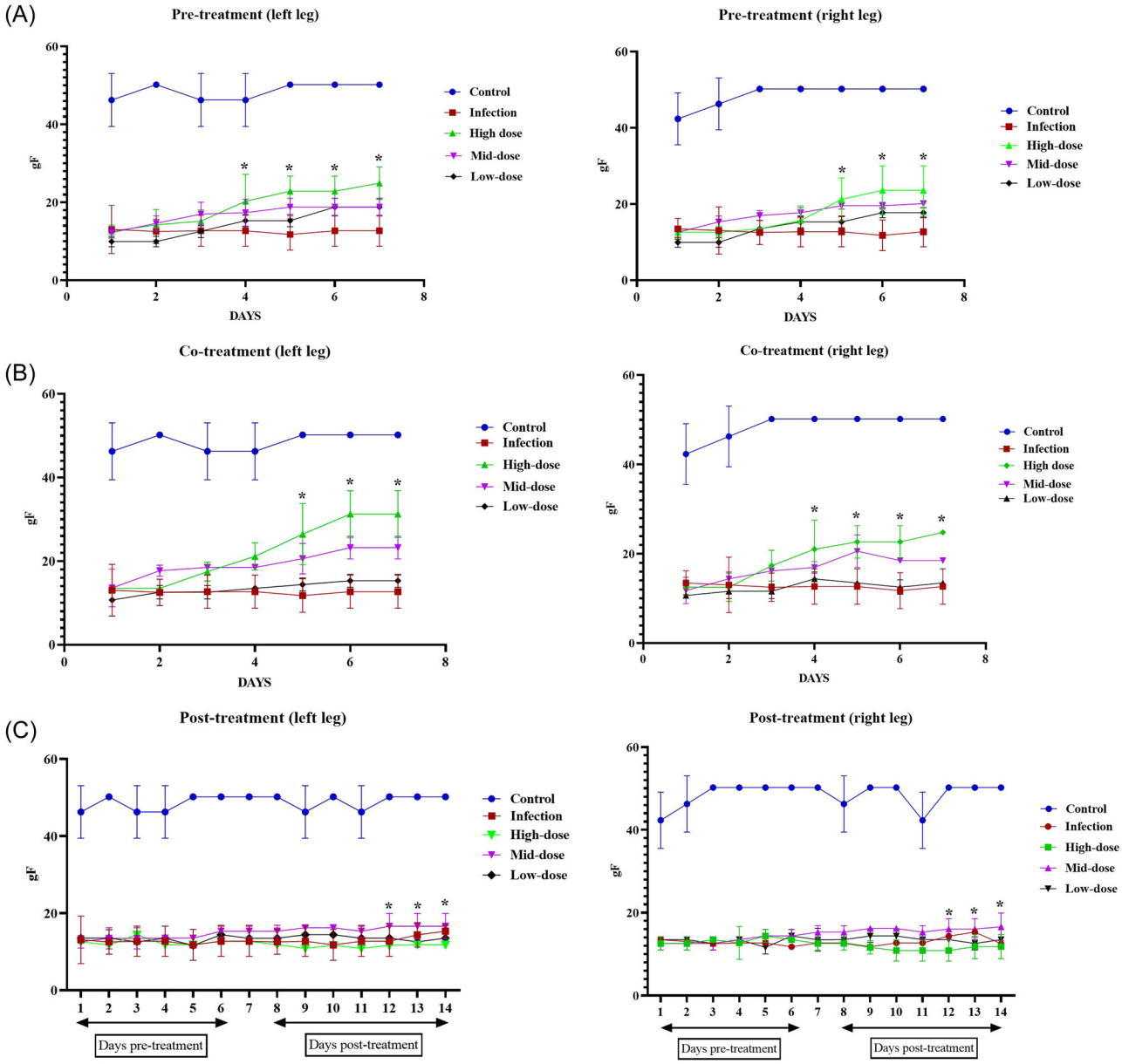

FIGURE 2 Effect of curcumin on mice nociceptive behavior using von Frey filaments: Mice hind paw withdrawal threshold in each group was measured by von Frey filaments (range: 1.65–6.65 gF) from day of infection (Day 0) to sacrifice. $p < 0.05$ at 95% confidence interval was considered as statistically significant and denoted by *. (A) Paw withdrawal threshold of pretreatment subgroup (left and right paws). (B) Paw withdrawal threshold of cotreatment subgroup (left and right paws). (C) Paw withdrawal threshold of posttreatment subgroup (left and right paws).

effective for preventing collagen degradation. Among CT_{HD}, CT_{MD}, and CT_{LD} subgroups type II collagen intensity increased significantly two- to threefold at MFC and MTP regions when compared to IN, with CT_{HD} subgroup showing highest intensity (MFC vs. IN; $p < 0.0001$; MTP vs. IN; $p = 0.0001$, 0.0003, and 0.0003, respectively). However, type II collagen intensity was highest among Post-T_{MD} subgroup at MFC and MTP regions (onefold) (MFC vs. IN: $p < 0.0001$; MTP vs. IN $p = 0.0029$). Overall, type II collagen intensity was comparable between high and MD curcumin-treated and control mice, except MFC region of Post-T group.

3.5 | Open field analysis

Several movements viz. line crossings, rearing, CSE, and locomotion time were observed of all mice groups by open field test, which delved into their pain-related behavioral patterns. Significantly increased line crossings were observed in PT_{HD} mice subgroup compared to IN group ($p < 0.0001$) (Figure 7A). However, significantly increased line crossings were observed among CT and Post-T group-mice receiving all three doses ($p < 0.05$), with CT_{HD} and Post-T_{MD} demonstrating the highest line crossing.

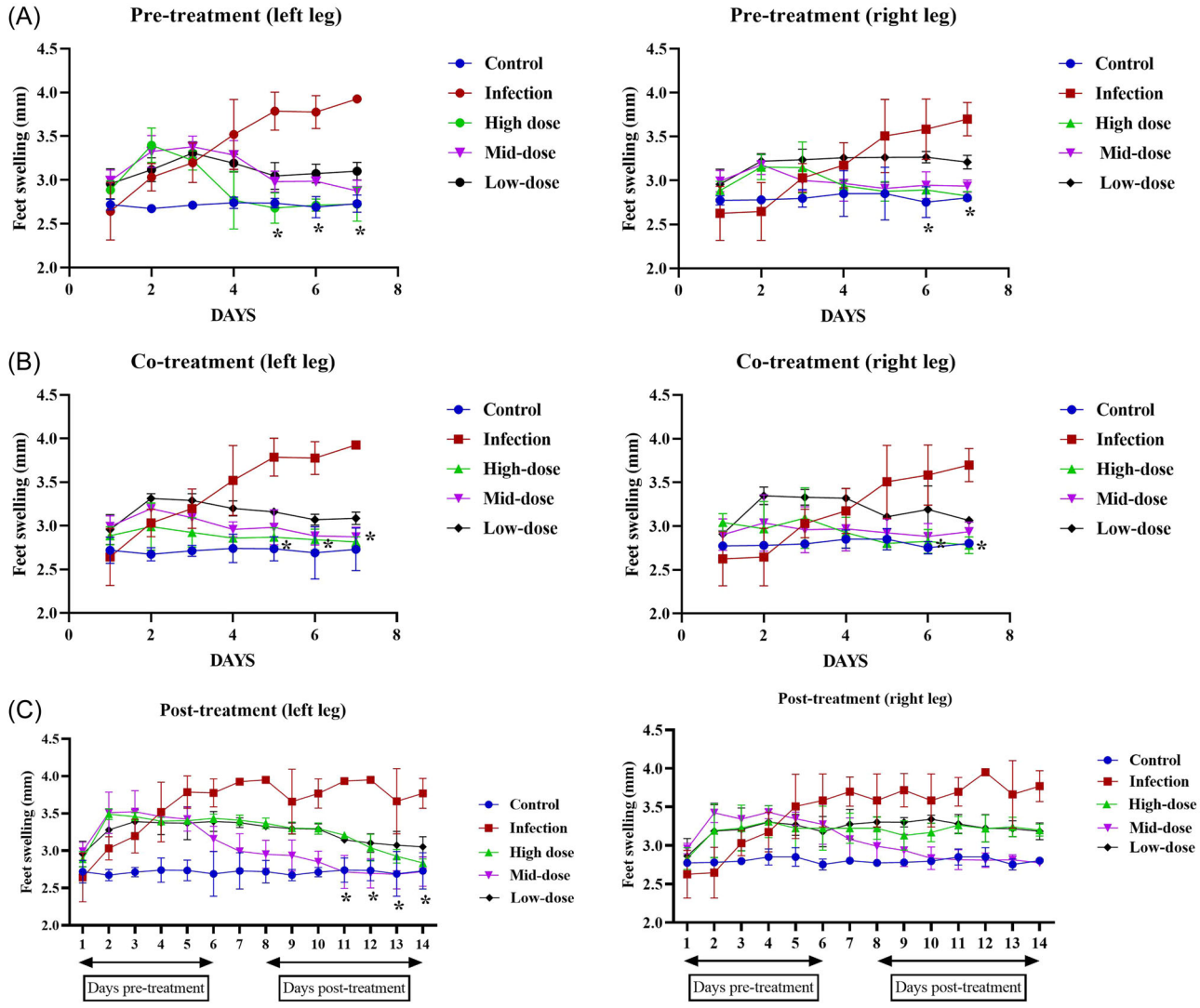

FIGURE 3 Feet measurement of healthy, infected, and curcumin-treated mice groups using calipers: Dorsoventral feet swelling and reduction of each mice group were measured using calipers from day of infection (Day 0) to sacrifice. $p < 0.05$ at 95% confidence interval was considered as statistically significant and denoted by *. (A) Feet swelling/reduction of pretreatment subgroup (left and right paws). (B) Feet swelling/reduction of cotreatment subgroup (left and right paws). (C) Feet swelling/reduction of posttreatment subgroup (left and right paws).

Furthermore, an increased number of rearing was observed in all doses of PT and CT mice groups compared to IN (PT: $p < 0.0001$, 0.0012, and 0.0245, respectively; CT: $p = 0.0002$, 0.0119, and 0.0026, respectively) (Figure 7B). Moreover, in both groups HD mice displayed one- to twofold enhanced rearing compared to MD and LD. In Post-T subgroup, only MD mice rearing significantly increased with respect to IN ($p = 0.0001$).

Additionally, day-wise mice CSE were observed among all groups. In comparison to IN group, CSE increased for PT_{HD} and PT_{MD} (Days 3–7), CT_{HD} (Days 4–6), and Post-T_{MD} (Days 9–14) subgroups.

Finally, mice locomotion time was calculated to analyze the duration of nociceptive pain following CHIKV infection and curcumin treatment (Figure 7C). Significantly higher locomotor activity was observed following treatment with all three curcumin doses for PT, CT, and Post-T groups compared to IN ($p < 0.0001$). However, the degree of increased locomotion time was more than onefold in PT_{HD}, CT_{HD}, and Post-T_{MD} subgroups. Furthermore, the day-wise analysis revealed a gradual increase in locomotion time for all doses between Days 4–7 for PT and CT mice groups ($p < 0.05$), while, in case of Post-T, time of increased locomotion varied for each subgroup: HD: 9th–12th day, MD: 8th–14th day, and LD: 8th–12th day.

3.6 | Effect of curcumin on pro-inflammatory biomarkers and viral load

Since, mice treated with PT_{HD}, CT_{HD}, and Post-T_{MD} curcumin doses exhibited most effective observations in von Frey, feet swelling, Safranin O staining, immunohistochemistry, and open field tests, the effect of these doses on CHIKV-induced release of murine pro-inflammatory cytokines was evaluated. Serum levels of

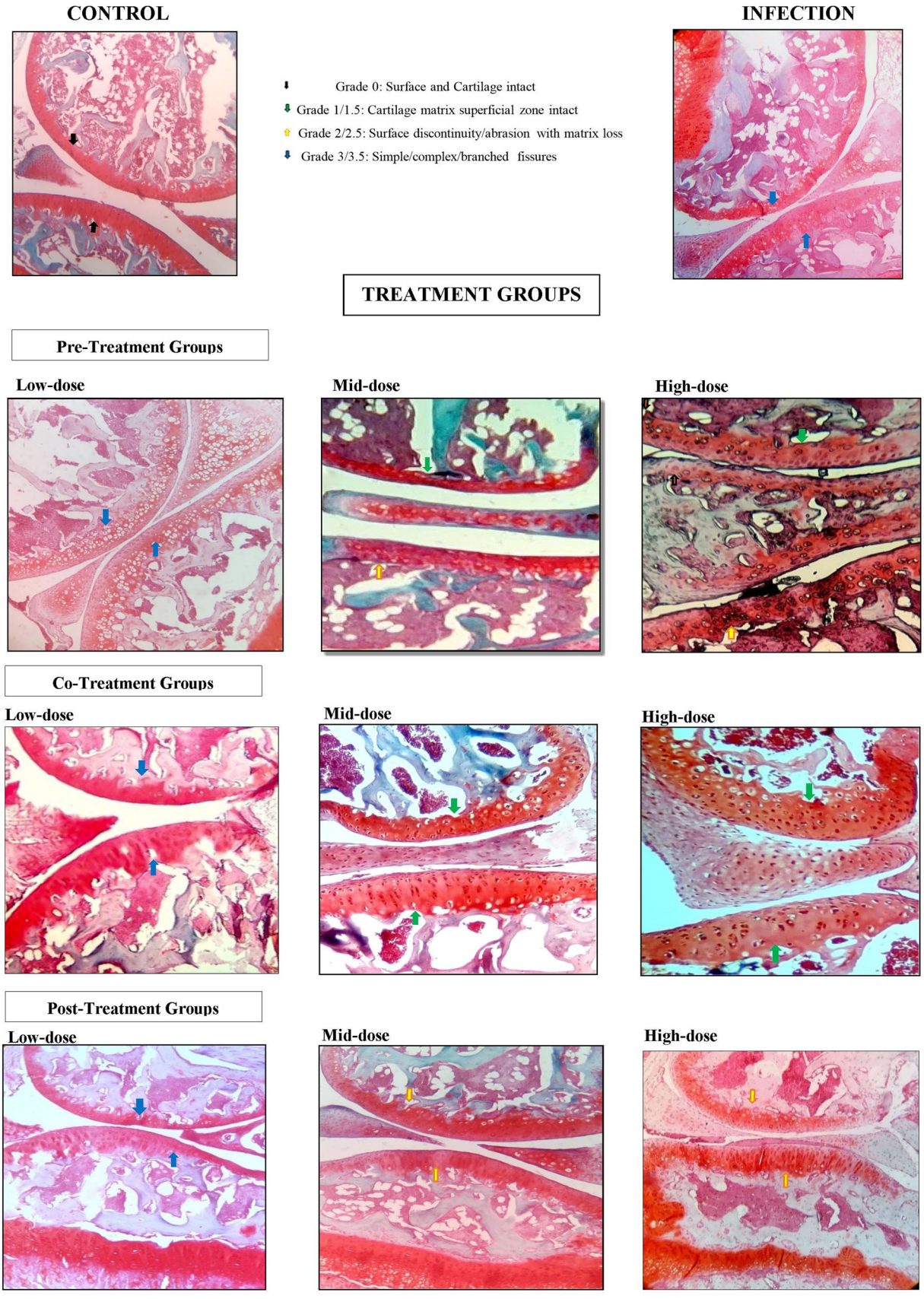

FIGURE 4 Safranin O staining of knee articular cartilaginous region of healthy, infected, and curcumin-treated mice groups: Postsacrifice hind limbs of each mice group were collected, fixed, decalcified, sectioned, and stained using Safranin O staining protocol to visualize and observe the degree of damage and recovery of proteoglycans in knee articular cartilage using OARSI and SMASH scoring/grading. Colored arrows indicated grade scores. OARSI, Osteoarthritis Research Society International; SMASH, Standardized Microscopic Arthritis Scoring of Histological sections.

TABLE 1 OARSI and SMASH scores of Safranin O-stained knee articular cartilage.

	OARSI scoring								SMASH scoring	
	Arthritic damage						Loss of cartilage proteoglycan			
	Grade		Stage		Score				Loss of cartilage proteoglycan	Cartilage erosion
Groups	MFC	MTP	MFC	MTP	MFC	MTP	MFC	MTP		
Control	0	0	0	0	0	0	0	0	0	0
Infection	3.5	3.5	3	3	10.5	10.5	3	3	2	2
Pretreatment										
High	1	1	1	1	1	1	1	1	0	0
Mid	1.5	2	2	3	3	6	2	2	2	1
Low	3	3.5	3	3	9	10.5	3	3	2	1
Cotreatment										
High	1	1.5	1	1	1	1.5	1	1	1	0
Mid	1.5	1.5	1	1	1.5	1.5	2	2	2	1
Low	3	3.5	1	1	3	3.5	2	2	2	1
Posttreatment										
High	2	2	3	3	6	6	3	3	2	1
Mid	2.5	2	1	1	2.5	2	3	3	1	0
Low	3.5	3.5	2	2	7	7	1	1	2	1

Abbreviations: MFC, medial femoral condyle; MTP, medial tibial plateau; OARSI, Osteoarthritis Research Society International; SMASH, Standardized Microscopic Arthritis Scoring of Histological sections.

pro-inflammatory cytokines, IL-6, TNF-α, and inflammatory biomarker, CRP were significantly reduced among PT_{HD}, CT_{HD}, and Post-T_{MD} treated subgroups ($p < 0.005$) compared to infected mice (Figure 8). Also, significant reduction in CHIK viral load was detected among PT_{HD}, CT_{HD}, and Post-T_{MD} treated murine blood compared to that of infected mice ($p < 0.005$).

4 | DISCUSSION

Since there is no specific treatment for alleviating CHIKV-induced arthritis till date, supportive treatment using ibuprofen, naproxen, corticosteroid, or other nonsteroidal anti-inflammatory agent (NSAID) is the only alternative therapy currently used in clinical practice.[9] But, long-term use of these drugs might induce bleeding, gastrointestinal complications, renal disturbances, cardiovascular events, osteoporosis, aseptic joint necrosis, and hepatic effects.[30-32] Curcumin, a plant-derived FDA-approved drug with low toxicity has been proved to be beneficial in improving DAS28, american college of rheumatology/european league against rheumatism, visual analog scale, western ontario and mcMaster universities osteoarthritis index) scoring of rheumatoid arthritic and osteoarthritic patients.[33]

Present study is the first to highlight the analgesic activity of curcumin also in alleviating CHIKV-induced acute/chronic arthritis within mouse model. Significant increase in leg pain-threshold level and decrease in feet-swelling of CHIKV-infected mice after treatment (pre, co, and post) with curcumin demonstrated its analgesic, prophylactic, and anti-inflammatory effect against CHIKV-induced acute/chronic arthritic manifestation. However, its effect in reducing nociceptive pain was more prominent for pre- and cotreatment groups compared to posttreatment ones. HD of 2000 mg/kg curcumin was most effective against alleviating pain and feet-swelling. CHIKV infection has been reported to trigger rapid innate immune response by producing pro-inflammatory cytokines that might lead to articular cartilage loss and bony erosion in affected joints, as documented among Thai and Indian patients.[30,34,35] CHIKV replication in joint tissues induced expression of pro-osteoclastogenic cytokines, IL-6, nuclear factor kappa B (NF-κB), TNF-α, IL-1β, and receptor activator of nuclear factor kappa-B ligand (RANKL) among Singaporean and Thai patients that promoted bone resorption and arthritic manifestation.[30,36,37] Release of pro-inflammatory cytokines upon CHIKV-infection, in turn, raised CRP levels, which was significantly higher among acute and chronic arthralgic patients of eastern Indian origin.[2] Curcumin has been reported to down-regulate CRP, IL-6, NF-κB, IL-1β, and TNF-α production, thereby, possibly inhibiting osteoclastogenic mechanisms and bone destruction.[30,38] Present study also demonstrated both anti-CHIKV and anti-inflammatory effect of curcumin among CHIKV-infected arthritic murine model. Previous studies have also demonstrated protective and anti-inflammatory effects of curcumin in Balb/c mice against CMV and RSV infection.[39,40] Curcumin treatment has been previously reported to decrease histopathological changes, paw swelling

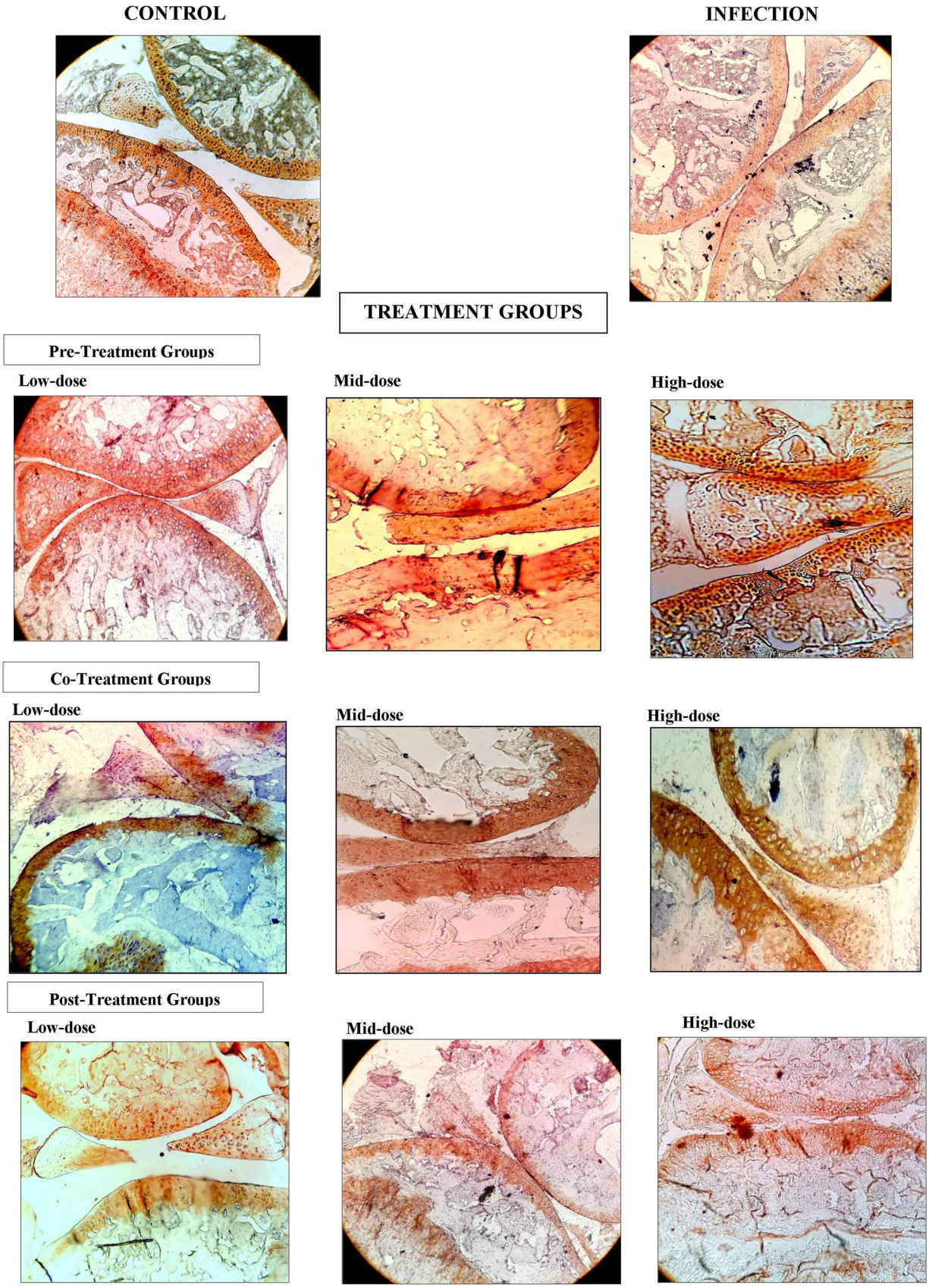

FIGURE 5 Immunohistochemically stained type II collagen of knee articular cartilaginous region of healthy, infected, and curcumin-treated mice groups: Postsacrifice hind limbs of each mice group were collected, fixed, decalcified, sectioned, and immunohistochemically stained to visualize and measure the intensity of type II collagen in medial femoral condyle and medial tibial plateau regions of knee articular cartilage.

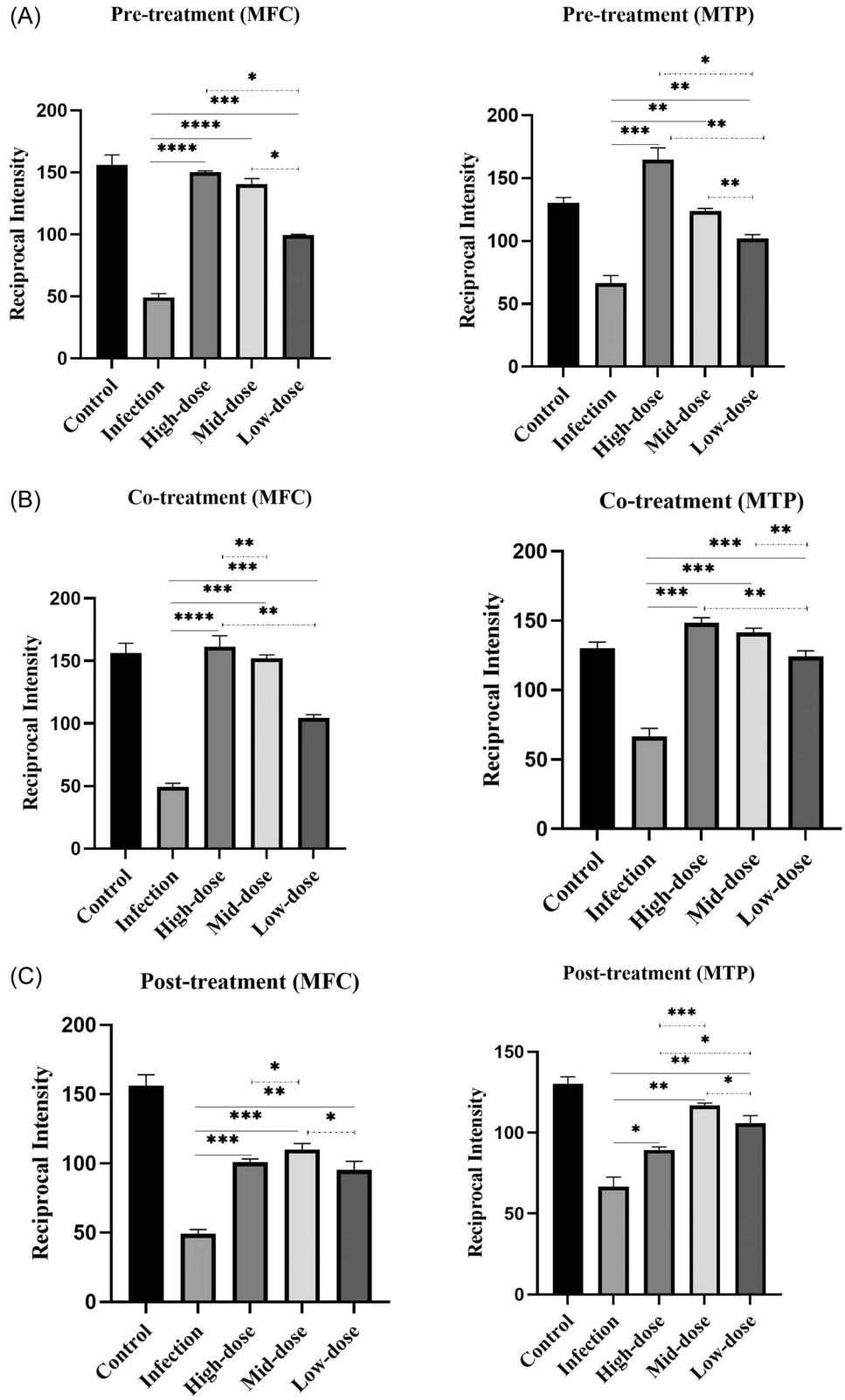

FIGURE 6 Reciprocal intensity of type II collagen in knee articular cartilaginous region of healthy, infected, and curcumin-treated mice groups: Comparative bar graphs of mice groups for depicting mean and SD of type II collagen intensity using ImageJ software. $p < 0.05$, <0.005, <0.001, and <0.0001 at 95% CI were considered as statistically significant and denoted by *,**, ***, and ****, respectively. (A) Type II collagen intensity of pretreatment subgroup (MFC and MTP regions). (B) Type II collagen intensity of cotreatment subgroup (MFC and MTP regions). (C) Type II collagen intensity of posttreatment subgroup (MFC and MTP regions). CI, confidence interval; MFC, medial femoral condyle; MTP, medial tibial plateau; SD, standard deviation.

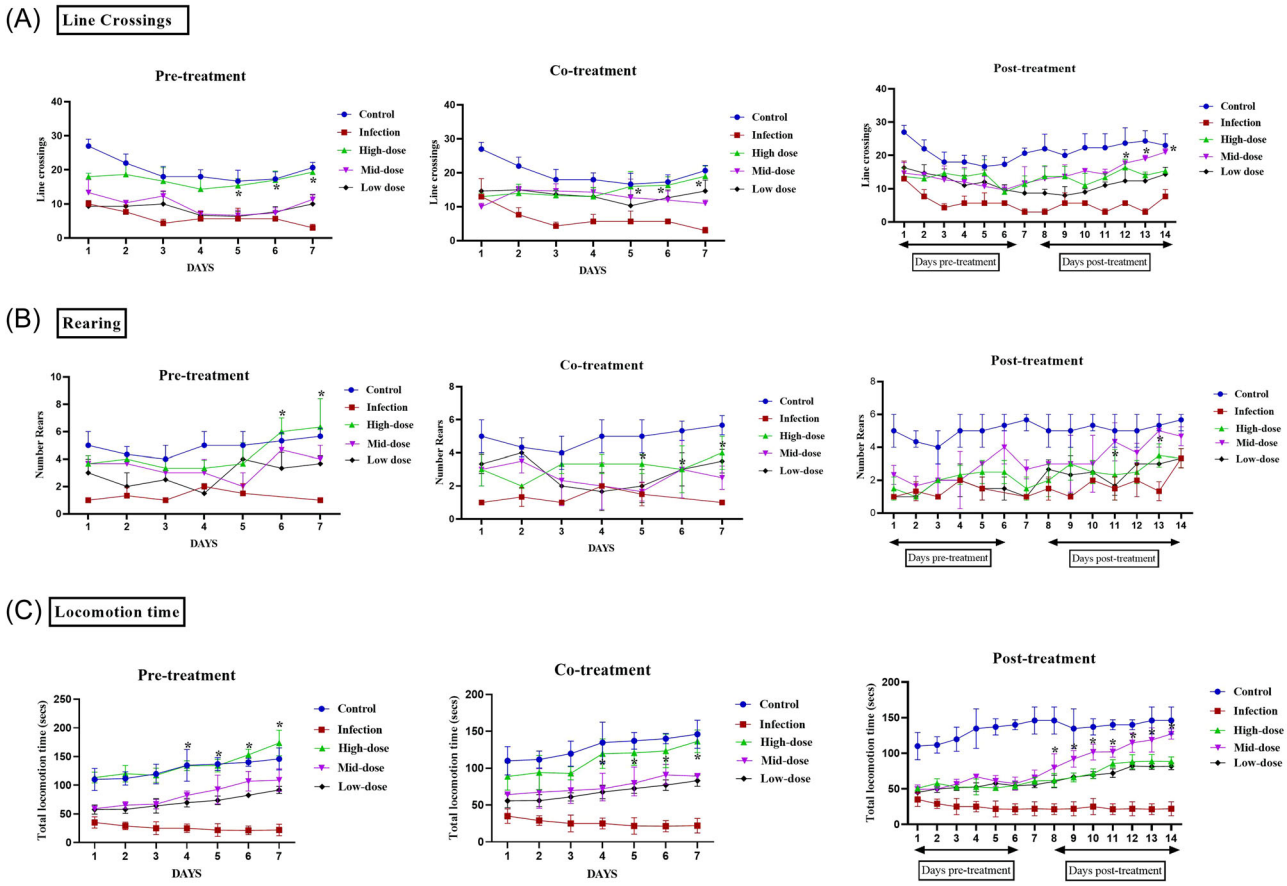

FIGURE 7 Effect of curcumin treatment on locomotory behavior using open-field test of healthy, infected, and curcumin-treated mice groups: Group-wise mice movements were traced to calculate line crossings, rearing, and locomotion time in an open-field test chamber. (A) Line crossings: Comparative graph of each mice group, depicting number of times mice crossed line grids within the chamber to analyze improved or reduced locomotion. (B) Rearing: Comparative graph of each mice group, depicting number of rearing made by them within open-field chamber to analyze improved or reduced body movement. (C) Locomotion time: Comparative graph of each mice group, depicting total locomotion time made by them within open-field chamber to analyze improved or reduced body movement.

rate, arthritis score, and neutrophil activity among rheumatoid arthritic mice models.[41]

Lower OARSI and SMASH arthritic scores of PT_{HD}, CT_{HD}, and Post-T_{MD} mice subgroups indicated less cartilage and proteoglycan loss in knee articular cartilage tissues of curcumin-treated mice compared to IN group. CHIKV-infection has been previously associated with IL-1β production.[42] Overproduction of this cytokine was reported to stimulate matrix metalloproteinases (MMPs) activation leading to excessive cartilage matrix degradation in rheumatoid and osteoarthritic patients.[7] Protection of type II collagen producing chondrocytes from catabolic effect of IL-1β and MMPs was previously reported following curcumin treatment among cultured condrocytes and osteoarthritic mouse model.[25] Increase in intensity of type II collagen of MFC and MTP knee joints following curcumin treatment within the immunohistochemical analysis of present study corroborated with histological scoring. This was also evident following curcumin treatment on chondrocyte, synovial fibroblast cultures in vitro and in collagen-induced arthritic, osteoarthritic, and rheumatoid arthritic mouse models.[25,41]

Effect of curcumin treatment on locomotory behavior of arthralgic mice model was also evident in open field tests. Compared to infected mice, line crossings, CSE, rearing, and locomotion time of curcumin PT_{HD}, CT_{HD}, and Post-T_{MD} subgroups improved significantly and was comparable to control group. This finding was consistent with previous report of increased locomotory behavior of curcumin nanoparticle-treated osteoarthritic mice with destabilized medial meniscus.[25] Similar finding of reduction in Lequesne's pain functional index score was noted among osteoarthritic patients receiving curcuminoid therapy, a bioactive constituent of turmeric.[43]

Thus, the present study confirmed anti-inflammatory, analgesic, and chondroprotective role of curcumin against chikungunya-induced arthritic mice, which needs to be validated among viral-infected arthritic patients. This might help physicians in managing severe arthritic pain of chikungunya-infected patients without prescribing ibuprofen, naproxen, or NSAIDs.

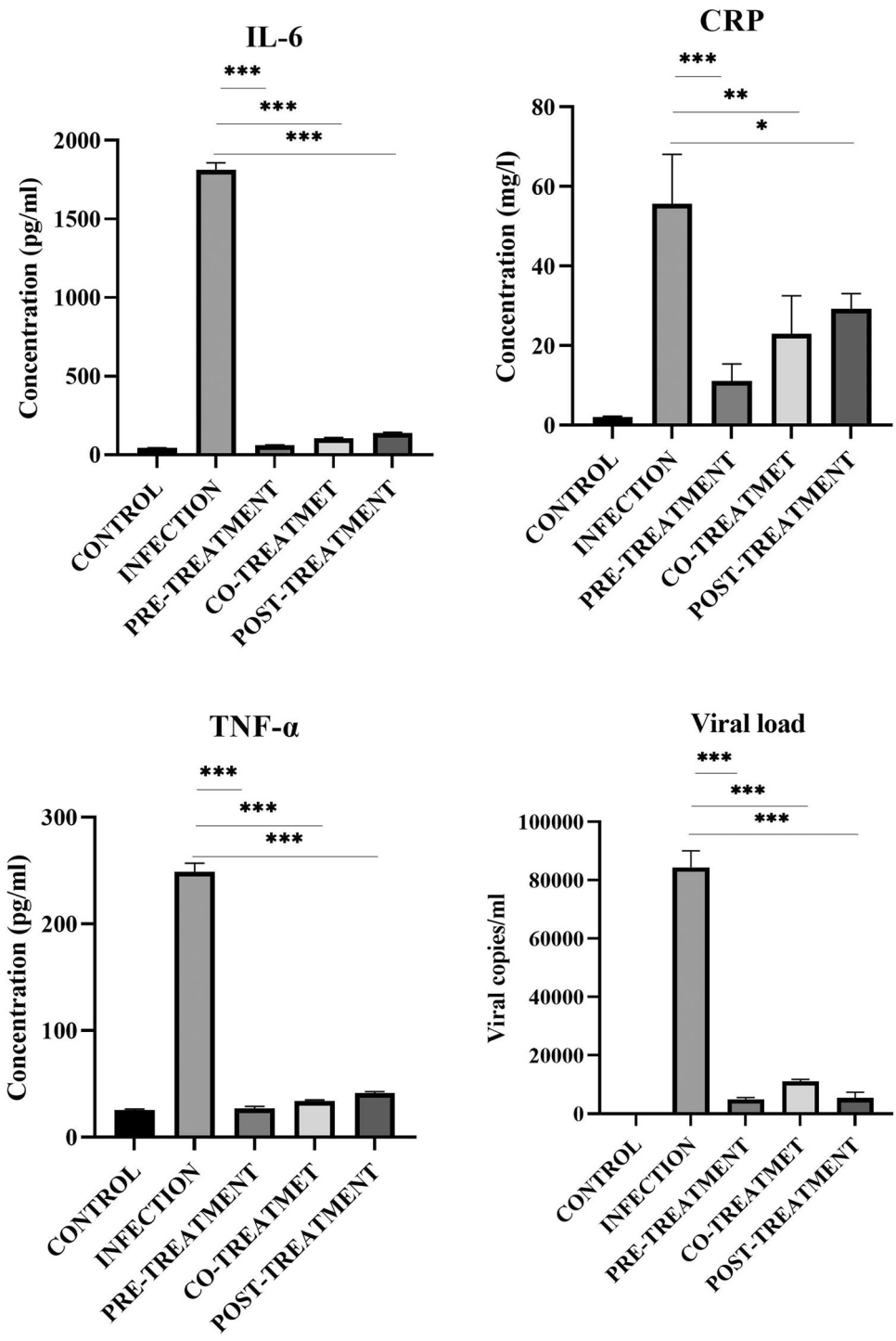

FIGURE 8 Effect of curcumin on pro-inflammatory biomarkers and viral load determination: Comparative bar-graph of each mice groups depicting serum levels of IL-6, CRP, and TNF-α; along with CHIK viral load determination of each group postsacrifice. $p < 0.05$, <0.005, <0.001 at 95% CI was considered as statistically significant and denoted by *,**, ***, respectively. CI, confidence interval; CHIK, Chikungunya virus; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha.

ACKNOWLEDGMENTS

The authors are extremely grateful to the Director, Calcutta School of Tropical Medicine, Kolkata, India, for his support, inspiration, and providing necessary facilities for this study. The authors are grateful to Indian Council of Medical Research, India, for granting fellowship to the first author (5/3/8/6/ITR-F/2018-ITR). Authors acknowledge kind help of Mr. Akash Shaw, R&D virologist, Panacea Biotech, for recording data related to behavioral experiments, respectively. This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated and analyzed during this study are included in this article. The data that supports the findings of this study are available in the supplementary material of this article.

ETHICS STATEMENT

All mice-based experiments were conducted according to guidelines from Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India (registration no.: 681/02/a/CPCSEA). Also, approval from animal research ethical board of Calcutta School of Tropical Medicine was obtained, which followed CPCSEA guidelines (AREC-STM/320 dated 09.01.2017).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Sengupta S, Tripathi A. Evaluation of analgesic and prophylactic activity of curcumin against chikungunya-infected acute/chronic arthralgic mice. *J Med Virol*. 2023;95:e28661. doi:10.1002/jmv.28661