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Gender based dimorphism in host response to viral infection: Two interesting epidemiological trends observed during Chikungunya outbreak in Kerala

Rajan J Payyappilly,[1] Usha Karunakaran,[2] NP Kumar[3]

Abstract:

Introduction: Males and females respond differently to infection, which again is modified by the 'stage of life cycle' they belong to. This knowledge however is often neglected during surveys/ studies or while deliberating protocols for disease control.

Materials and methods: Data collected without segregation of sexes in separate age groups may thus be biased, confounding conclusions and disease control strategies formulated based on them.

We analyzed results of IgM ELISA tests for chikungunya virus (CHIKV) released by Kerala State Institute for Virology and Infectious Diseases (KSIVID) done in 2007 during last epidemic caused by the virus in Kerala, and found two interesting epidemiological trends relating to sex based dimorphism in host response to CHIKV.

Results: The '*proportion*' of seropositive females over males increased steadily from near puberty, to become one of clear female predominance in reproductive age group, and then waning through middle age, reverting to original proportions, by menopause. From a situation where IgM positive males and females about equally represented, during the early epidemic, the proportion of positive females increased through mid-epidemic period, almost 'eclipsing' the male segment and then waned during post-epidemic period, the ratio tending to revert to original proportions.

Conclusions: We seek to emphasize the importance of collecting and analyzing data separately for males and females categorized in different stages of life cycle (as per guidelines of W.H.O.) in studies/ epidemiological surveys.

Key words: Chikungunya, dimorphism, gender, infection, IgM, epidemic

Introduction:

Chinkungunya is a febrile illness associated with severe polyarthralgia, + / - rash, caused by the arbovirus CHIKV, an 'old world' alphavirus in the Togaviridae family, transmitted by mosquitoes belonging to Aedes (Stegomyia)genus[1,2,3]- *Ae. aegypti* and *Ae.albopictus*.

The disease was described in 1952[4] and the virus isolated in 1953 from febrile patient in Tanzania.[5] The name 'Chikungunya' is derived from the word '*kunganyala*', in Makonde dialect meaning ' shriveled leaf', alluding to contorted posture of patients due to joint pain.[1,2,6]

A conspicuous feature of Chikungunya, is its propensity to spread to fresh geographical regions - the virus mutating and adapting to ubiquitous vectors with ability to adjust to a wide range of ecological conditions.

E1-A226V mutation in CHIKV for instance [2,7,8,9,10] has enhanced competence/ transmission fitness of *Ae. albopictus*, promoting it from a 'secondary' role to become a major vector transmitting the virus, today. *Ae.albopictus* is ubiquitous, and better endowed in survival, than *Ae aegypti.* [11,12,13] The mosquito has an uncanny ability to adapt to new environmental plights - the temperate biotype of *Ae. albopictus*, laying photoperiod sensing, diapausing, 'cold hardy' eggs, shows how this species can adapt to new environmental conditions. They have stretched their habitat up to

latitude 42 N, in America where they were introduced only recently.

Human travel and movement of cargo provide free transport - the former carrying the virus; the 'mosquito' [egg, larva or adult (with or without virus)], moving with the cargo, to distant destinations, even across international borders, where they may find niche.   Chikungunya today, is a malady of global concern.[14]

Sex and gender influencing CHIKV infection:

Males and females respond differently to CHIKV infection,[9] which further is modified by the stage of life cycle they belong.

A. Gender roles predispose man/ woman to CHIKV infection mainly by 'occupational' exposure.[13,14,15]

B. Biological differences in males and females in different age groups influencing response to CHIKV infection may be hormonal, immunological, genetic or related to lipid metabolism.

1. Sex hormones comprise androgens, estrogens and progesterone. They exert their effect, via specific receptors expressed in/on immunocytes[16,17,18], that become transcription factors, to produce cytokines and chemokines regulating proliferation, differentiation, maturation and function of cells of innate and adaptive immune system. The effect thus, is decided by differential distribution of the receptors on different cell types and serum levels of the hormones, which differs between sexes, and are subject to 'biological fluctuations' during different stages of life cycle.

Estrogen is a 'pro-inflammatory', hormone. Its effect however is dependent on the 'dose' and cell type involved.[15,16,17] In concentrations found in males and females in luteal phase or past menopause, it induces production of IFNγ by CD4+ Th1 lymphocytes, promoting a type 1 Th response. It has however the opposite effect on 'macrophages' during innate immune response[15,18]. Besides, it promotes

expansion of TREG clone, that inhibits both Th1 and Th2 responses.[15,16,18]

At high levels (as in pregnancy and pre-ovulatory periods), estrogen generates an anti-inflammatory, Th2 type response.[15,16,17]   CD4+Th2 cells so activated, induce proliferation of B cells and their differentiation into plasma cells, producing antibodies (auto antibodies as well)[17,18]. Estradiol also increases somatic hyper mutation and class-switch recombination, leading to high-affinity Ig-producing cells.[18] These effects contribute to the robust humoral response to infections, in women.[19,20]

Estrogens acts on cells of the innate immune system, promoting proliferation and function of neutrophils, maturation of dendritic cells and modifying macrophage function.[15,16,17,21]

Estradiol inhibits 'apoptosis' of defective/auto reactive B cells, thereby allowing 'forbidden clones' to proliferate,[15,16,21,22] which plays a role in pathogenesis of 'chronic' Chikungunya. Progesterone is anti-inflammatory.[15,16,20,22]

Pregnancy-induced expression of progesterone receptors in activated T lymphocytes, leads to production of 'pregnancy induced blocking factors' that inhibit pro-inflammatory cytokines and NK cell activity, which is a natural response that evolved to prevent allo rejection of the growing fetus.[15,16]

Estrogen and progesterone levels fall with advancing age, dropping low during peri-menopause. Androgens promote Th1 type immune response with production of pro-inflammatory cytokines viz. IL2, IFNγ and CTL activity.[15,16] Both progesterone and androgens inhibit 'class switching' of antibodies and promote apoptosis of T and B cells.[15,20,22] Androgen secretion also wanes with age, dropping steeply after 50-60 years.

2. Immune Response:

Innate immune response forms first line of defense against invading CHIKV.

Virions released by mosquito into

capillaries of the dermis, may gain entry and multiply in the dermal fibroblasts, which is the susceptible cell type for CHIKV.[15]

Uncapped 5' ppp RNA, and 'lengthy' stretches of uncapped ssRNA, generated during viral replication are detected by pattern recognition receptors, RIGI (Retinoic acid inducible gene) and MDA5 (Melanoma differentiation associated gene), respectively.[15,19,23] They utilize the 'mitochondrial anti-viral signaling proteins' (MAVS) in signal cascades leading to activation of 'interferon response factors' (IRF 3 and 7) or NFkB.[15,19,23] 'Autophagy' of infected cells may make available CHIKV RNA to toll-like receptors in the endosomal compartment (i.e.TLR3, TLR 7,TLR8 and TLR-9), as well.[15]

Macrophages, albeit non-permissive to 'direct entry' of CHIKV, may acquire the virus by phagocytosis of virions contained in apoptotic blebs[15], or being carried in by antibodies via Fc receptors.

M1 M2 paradigm: Classically activated M1 phenotype, stimulated by IFNγ, predominates in males responding to infection, which secretes 'pro-inflammatory' cytokines viz. IL-2, IL-6 and IL-13 and IFNγ. (T1 type response).[24,25]

In females who produce antibodies prolifically, M2 is the preferred phenotype, in particular M2b, which is stimulated by virions bound to antibodies via Fc receptors on them. The response is of T2 type, which is 'anti-inflammatory' in nature, with secretion of cytokines viz., IL-4, IL-10.[24,25] Over stimulation of M2b, however may lead to 'anergy', allowing 'persistence' of the virus in host cells.[16,17,25]

Adaptive Immunity: Activated B lymphocytes produce protective specific antibodies that are essential in neutralizing the virus. The very process disregulated however, may give rise to 'autoantibodies'.[16,17,23,25] T cell mediated immune response in CHICKV infection, is mediated by CD8+ and CD4+ T lymphocytes.

Naive CD8+ T cells, upon activation by viral antigen on infected cells belonging to MHC class I, become cytotoxic T cells (CTLs).[16,25]

Naive CD4+Th0 cells on antigen activation, become T helper cells, and release 'signal' molecules that may amplify or dampen immune response. Paradigm of 'Th1' and 'Th2' phenotypes of T helper cells, each requiring different conditions of activation and secreting cytokines in discrete combination patterns, forms bed rock for sex based dimorphism in immune responses to viral infections.[16,17,18,19,24,25]

Male response for instance, is T1 type, with production of pro-inflammatory cytokines IL2-, IL-12 and IFNγ and CD8+ cytotoxic T lymphocytes (a cell mediated immune response, basically.)[15,16] Females on the other hand, mount a T2 type response, involving CD4+ Th2 lymphocytes producing 'anti-inflammatory' cytokines like IL-4,IL-5,IL-6,IL-9, IL-10 and IL-13, with suppression of phagocytosis. Plasma B cells are induced to produce 'antibodies' prolifically (humoral immune response, predominantly).[16,24,25] High levels of antibody effectively clear the virus, however it also carries increased inherent risk of 'auto antibodies' being formed. T regulatory cells, 'nTREGs' and 'iTREGs' downregulates both Th1 and Th2 responses which serve to protect host against auto immunopathology.[16,17,18,19,25]

3. Genetic / Epigenetic Factors: X chromosome carries many genes deciding innate and adaptive immune responses viz. TLRs, cytokine/ hormone receptors, and several transcriptional / translational regulators of T and B cell function; while Y chromosome encodes a number of 'inflammatory pathway genes' expressed exclusively in males.

X chromosome diploidy in females, may possibly contribute to sexual dimorphism

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in response to infection. Other idiosyncracies viz. gene dose, microchimerisms etc. may be involved e.g. in 'rheumatic' joint afflictions ' associated with chronic form of the disease [16,17,20], which albeit falls outside scope of our study.

4. Lipids in CHIKV entry:

Cholesterol in plasma membrane is required for fusion and release by budding of CHIKV. Level of cholesterol modulates the curvature and plasticity of membranes, a process controlled by Sterol regulatory element binding proteins'(SREBPS) in the ER. Viruses are known to hijack these mechanisms, for entry and forming replication complexes in the cell. Differences in lipid metabolism, pertaining to expression of LDL receptors on the cells in particular, could translate to differences in 'permissiveness' between male and female cells.[26,27,28,29,30,31,32,33]

Systematic, statewide serosurveillance of chikungunya in reactive response to an ongoing epidemic, was the aim of the institute, for early identification of 'geographical areas' with virus activity, where vector control activity may be concentrated. KSIVID was an apex institute in the national network for arboviral surveillance, and the only centre in Kerala at that time, doing serodiagnosis of chikungunya. Sex based dimorphism in host response to CHIKV infection in different age groups, were our objectives.

Materials and Methods:

Serum samples of clinically suspected cases (with h/o fever and arthralgia, with or without rash) were being received from all districts, statewide. It comprised patients attending public and private hospitals belonging to all age groups in both sexes, from all occupational/ socioeconomic classes and religious/educational background.

Samples tested were selected at random, from properly collected specimens of sufficient quantity, with 3 or more days of clinical history. (Acute samples with < 2 days history were reserved for virus isolation.). Priority however, was given to sera coming from 'newer' geographical areas, for obvious reasons.

A total of 7246 specimens were received during the year 2007. However only 1524 of them could be tested owing to constraints viz. non availability of test kits, manpower and time.

Of the 1524 samples tested ,773 (50.8%) were males and 751 (49.2%) were females. IgM capture ELISA for CHIKV antibody was done, using test kits manufactured by the National Institute of Virology, India, which claimed sensitivity to the tune of 95% and 97.22% specificity. Tests were performed as per manufacturer's instructions. Samples giving more than fourfold OD obtained with negative control was taken as positive.

Analysis was done with descriptive statistics like frequencies, proportions and percentages and inferential statistics namely Chi square test using Epi Info version 7. A 'P' value of less than 0.05 was taken to be significant.

Results:

Of the 1524 samples tested, (751 males and 773 females), 528 (34.64%) sera were positive for Chikungunya IgM antibody. Males accounted for 240 and females 288 (**Figure I**). Male: female ratio was 1 :1.2.

Figure I: **Number of Chikungunya IgM positive in males and females**
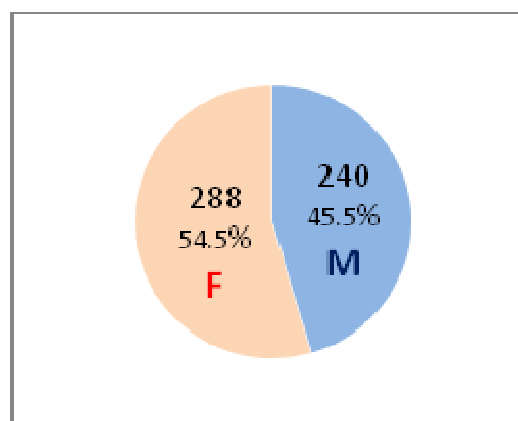
Notwithstanding, we analyzed the distribution of IgM positives in males and females separately, in different age groups they belonged and also in the different phases of the epidemic (month wise) which gave results as follows:

1. Dimorphism in 'stages of life cycle':

Significant variation was found, in male / female distribution in the different age groups (**Figure IIA**). IgM positive males and females where about equally distributed in the extreme age groups. The 'proportion' of seropositive females over males increased steadily from near

puberty, to become one of clear female predominance in reproductive age group, and then waning through middle age, reverting to original proportions, by menopause.

There was a significant difference between numbers of males and females in the different age groups. Chi square value is 17.781 and 'p' value 0.0013. The same was analysed in different 'stages of life cycle', pre-pubertal, adult/reproductive and old/post menopausal age groups, the findings were as given in **Figure IIB** and **C.**

Figure IIA: Age wise distribution of IgM positive males and females
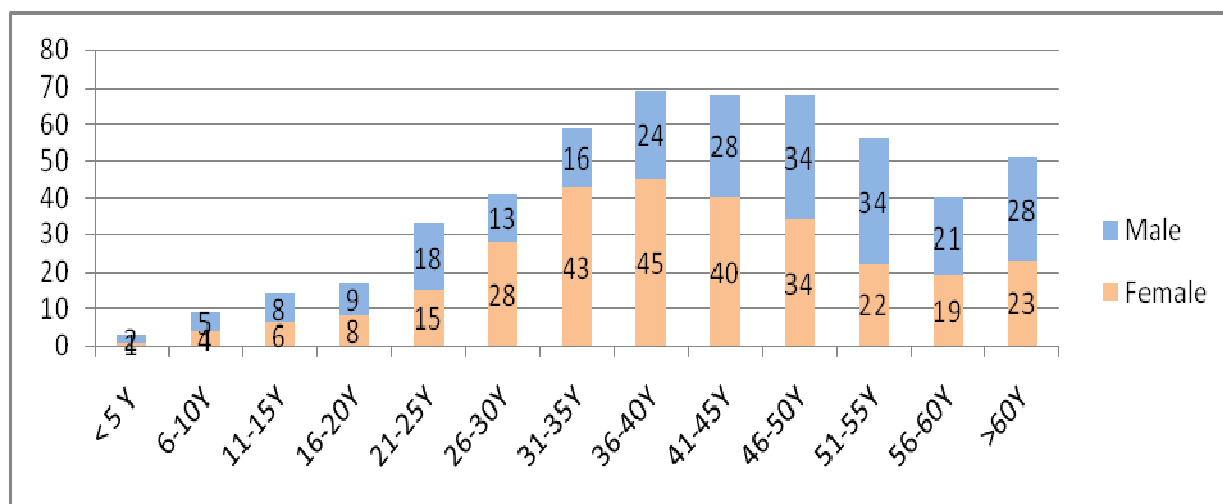
Figure IIB and C: Proportions of IgM positive males and females in different 'stages of life cycle'
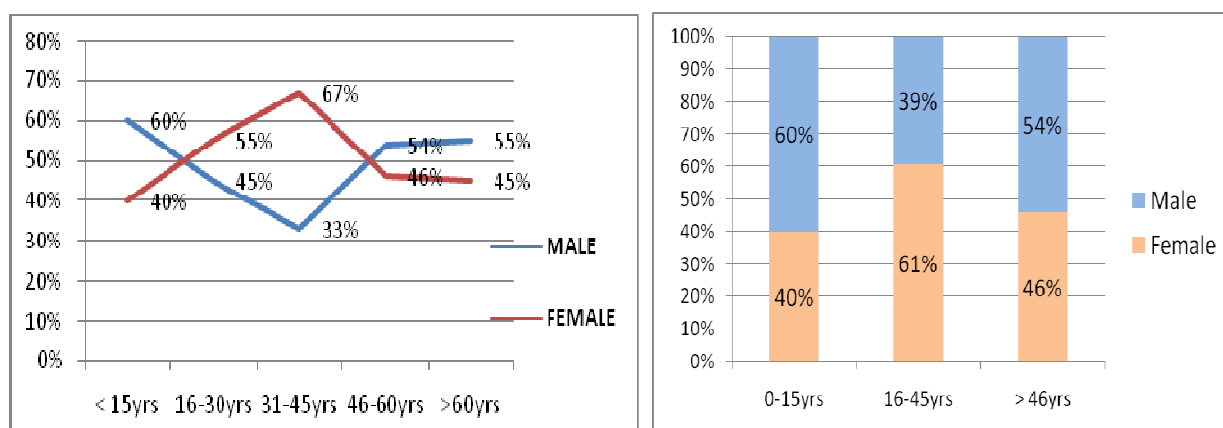
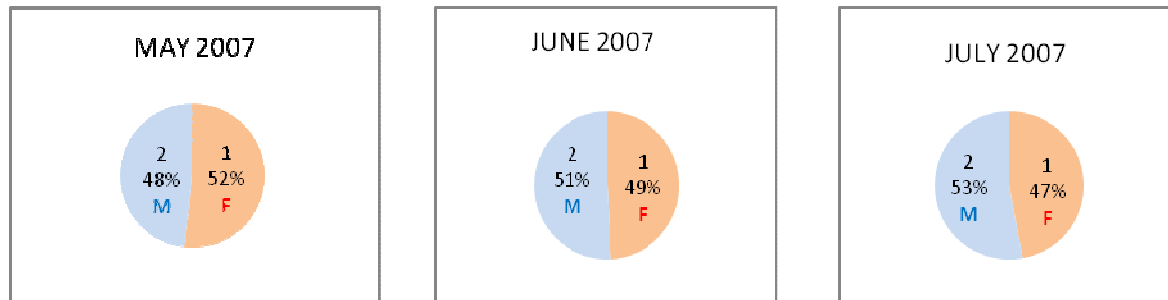Pre-puberty><Menstruating>< Menopause   Pre-puberty   Menstruating   Menopause

The difference between male and female proportions is significant, the Chi square value being 15.6244 and 'p' value, 0.0004.
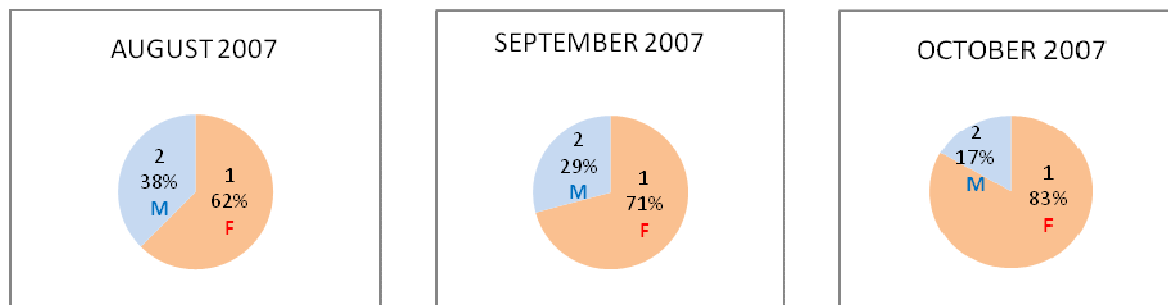
2. Dimorphism during "stages of epidemic":

Analysis of seropositive male:female proportions month-wise, revealed an interesting trend:
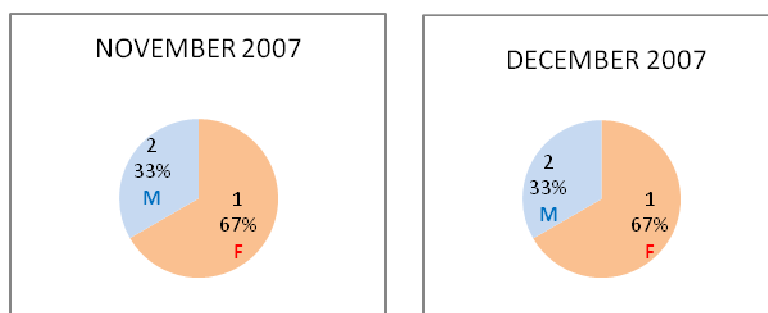
Figure III:
A. Early epidemic

B. Mid Epidemic

C. Late Epidemic

From a situation where IgM positive males and females about equally represented, during the early epidemic, the proportion of positive females increased through mid-epidemic period, almost 'eclipsing' the male segment and then waned during post-epidemic period, the ratio tending to revert to original proportions (**Figure IIIA, B and C**). There is a significant difference between male and female distribution in early, mid and late epidemic periods, the

Chi square value being 11.7705 and p = 0.002.

We have used data covering the year 2007, when most of the cases had occurred, involving all districts of the state (sparing small circumscribed regions in north Kerala). Importantly, it was while A226V mutation was reported in the virus[7,10] with emergence of the IOL strain that caused the devastating 'second wave' of the

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epidemic in Kerala. (The smaller first wave was caused by ECSA strain). Besides, test kits were made available to KSIVID only in 2007.

Testing a sample pool of 1524, near equally represented by the sexes M:F=1:1.02, we found distribution of males and females giving positive results in ratio, 1:1.2. This was in agreement with common consensus, that Chikungunya 'affected' both sexes equally.

A sero survey conducted in plantation areas of middle Kerala in 2007[11] however, reported sero positivity in male / female in ratio 1.2: 1. Cumulative distribution ratio of IgM positive males and females in Kerala, we found at the same time, was 1: 1.2. This apparent disparity in figures is because, said survey was carried out in a cohort population living in and around rubber estates where they worked, heavily infested by *Ae. albopictus*, with a view to assess occupational risk to plantation workers. Seventy four percent of the workers were males who were engaged in ('tapping') work during peak biting hours of the vector.[11]

In other geographical regions, especially in urbanized townships, women who carry out household chores are more exposed to bite of an infected vector.[11,14,15]

Women in Kerala are possibly more exposed to bite of the vector during both its peak biting times, while carrying out household chores .Many of them are housewives who stay at home, with increased chance of acquiring infection in '(wo)man – mosquito –(wo)man' spread of chikungunya – which is a vicious cycle, with more than one infected mosquito feeding on increasing number of viremic members of the household more women than men, they get to bite.[13]

The slight disparity in findings may be explained on basis of 'gender roles', acting as proxy factor for specific behavior (occupational/life style)that causes higher exposure to bite of the vector.[14] Analyzing distribution of IgM positives in males and females separately, in different

age groups they belonged and also in the different phases of the epidemic, revealed two interesting trends:

1. Dimorphism in 'stages of life cycle' :

This dimorphic behavior of sexes seen in **Figure II**, may be attributed to the effect of sex hormone levels fluctuations in different stages of lifecycles of females, influencing immune response, as described above. The difference begins with onset of menarche, increases and continues through reproductive age with high levels of estrogen, and reverts by menopause when hormone levels drop.

2.Dimorphism during 'stages of epidemic':

The latter part of study throws light on virus-vector–host interactions during different stages of the epidemic as it progressed.

Maximum number of IgM positives had clustered between months June to July. Post monsoon increase in vector densities may account for this. Repeated passaging though hosts increasing viral 'virulence' and the vector becoming more competent are other reasons. Notably, A226V mutation in CHIKV was reported from Kerala during this time. 'Herd immunity' building up in host population and falling 'vector densities', may account for the decline in numbers, during late epidemic period.[11]

We have used a single parameter namely 'production of IgM antibody' to CHIKV measured by ELISA, to assess difference between male and female responses to infection. Levels of IgM antibody in serum above 'detectable threshold' of the test, therefore was the key to join the 'positive pool' that was being analysed. Though NIV kit claims a sensitivity to the tune of 95%, it may take a few days before antibody levels in host rise sufficiently high to give that order of 'senitivity.[33,34,35]

Males in particular, who mount a Th type1 response to infection, are slow in producing antibodies may well go

undetected, during early days (4-5days post onset of disease)[34] of illness.

Females on the other hand are robust producers of antibody (Th type 2 response) that may be detected earlier during infection than men. This may account for the higher proportion of sero positive females during early epidemic.

However, multiple infected mosquitoes biting the same (male) host may serve to 'boost' his antibody response(memory cells, and all) easily above threshold that may be detected by ELISA– 'cumulative' result of which over time, is perhaps reflected in the graphs, with the male component increasing in proportion, through late epidemic period, catching up with females.

An ominous thought that crosses our mind, is the possibility of high levels of antibody in females in combination with high viremia, overloading Fc receptors of M2 macrophages with antibody bound virions, leading to 'anergy', allowing 'persistence' of virus in such cells.[7,8,13,24] With no extra human reservoirs (at least in the urban cycle) nor transovarian transmission in mosquitoes, where and how the virus overwinters long periods between epidemics is a pertinent question. The virus persisting in tissue sanctuaries of such chronic joints well be an answer.

Limitations of our study:

Different parameters have been employed in different studies to assess frequency of CHIKV infection in population. Most have included clinical definition of disease to support laboratory based evidence. The latter varied from serological tests for IgM or IgG to virus isolation or PCR.[34] These tests don't tell the same thing,[34,35,36] each being relevant at different stages of the infection, vitiating comparisons.

No data was available on 'pregnancy status' of the subjects.

The study is limited to demonstrating difference between males and females in 'susceptibility' to CHIKV infection. It tells nothing beyond example how they fare

during disease or sequelae[16,17,18,19] that ensued.

Conclusion:

We seek to highlight the importance of collecting and analyzing data separately for males and females categorized in different stages of life cycle - as per W.H.O. guidelines[37], in epidemiological surveys, to obtain unbiased information that may be useful in disease control.

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Authors details:

1. **Corresponding author:** Associate Professor, Department of Microbiology, Pariyaram Medical College, Pariyaram, Kerala- 670503, India; E-mail: rajanjpayyappilly@gmail.com
2. Associate Professor, Department of Community Medicine, Pariyaram Medical College, Pariyaram, Kerala- 670 503
3. Scientist F, Officer-in-Charge Vector Control Research Centre Field Station (ICMR), Department of Health Research, Govt. of India, Kottayam- 686002, Kerala