ORIGINAL ARTICLE

Frequency of Chikungunya, Dengue and Zika Viruses in Acute Non-localizing Febrile Illness via RT-PCR

Tehreem Ansari, Junaid Iqbal, Samreen Sarfaraz, Nida Ghouri, Fouzia Naseer, Syed Ghazanfar Saleem, Shamaila Misbah, Naseem Salahuddin

Department of Infectious Diseases, The Indus Hospital, Korangi Crossing, Karachi.

Abstract

Introduction
Chikungunya and zika are two new emerging threats across the globe. Like dengue, these two viruses also present with acute non-localizing fever and rash, and their route of transmission is the Aedes aegypti mosquito. The hallmark of chikungunya is arthralgia/arthritis which can become chronic, while zika has *in utero* transmission, resulting in congenital anomalies (microcephaly), low birth weight or still births. It is difficult to differentiate these infections on clinical grounds and must be confirmed by PCR or serology. Due to cross reactivity of ELISA serology among flaviviruses, PCR is the gold standard. Pakistan is endemic for dengue, and due to presence of the same vector, chikungunya outbreak was suspected after India's epidemic in 2016. The epidemic in Pakistan was declared in November 2016 in the metropolitan city of Karachi. We conducted this study to know the prevalence of chikungunya, presence and prevalence of zika, and to assess the associated clinical and laboratory parameters.

Methodology
A total number of 183 participants fulfilling the case definition of dengue (Dengue Expert Advisory Group), chikungunya and zika (World Health Organization) were included. Patients with other viral exanthematous illness like chicken pox, measles or an obvious source of infection were excluded. The patients were tested for dengue, chikungunya and zika via QiaAmp Viral RNA Mini kit (Qiagen Inc.), dengue IgM serology via Elisa.

Results
51% were male. Overall frequency of arboviruses was 100 (54.6%), 95 (52%) were PCR positive for one or two arboviruses; serology for DENV was positive in 5 (2.7%). The over all frequency of CHIKV and DENV were 91(49.7%) and 13(7%) respectively. CHIKV/DENV co-infection was found in 4 (2.2%). Zika virus was not isolated in any sample. In CHIKV patients, triad of fever, arthralgia/arthritis and rash was found in 45.1%, joint involvement was predominantly symmetrical and polyarticular. Hemoglobin, hematocrit, TLC, platelets and ALT were normal in majority. No mortality related to CHIKV was noted.

Conclusion
Chikungunya is a newly emergent arboviral illness in this territory. The disease is self limiting with negligible mortality and high morbidity due to joint involvement. Pakistan is a high risk area for zika virus as well. Vector control and improvement in sanitation is vital for avoiding future epidemics.

Keywords
Chikungunya, Dengue, Zika, RT- PCR, Fever, Rash, Triad, Arthritis, Co-infection, mosquito –borne infections

Corresponding Author: Tehreem Ansari
B-67, Tipu Sultan Cooperative Housing Society, Opposite Check Post #6, Malir Cantt, Karachi, Pakistan.
Email: tahreemansari@hotmail.com

Introduction

Globalization has led to rapid travel and with it, a parallel increase in spread of diseases in areas which once were considered "disease free". Rapidly emerging arboviral infection, particularly chikungunya (CHIKV) and zika (ZIKV), are examples of mosquito –borne infections resulting in outbreaks across the globe. Dengue (DENV), CHIKV, and ZIKV are arthropod transmitted viruses, classified as flaviviruses (DENV and ZIKV) and alphavirus (CHIKV), sharing certain common features such as vector and clinical presentation.

DENV is a worldwide growing problem. Bhatt *et al* estimate 390 million DENV infections per year, of which 96 million (average 67–136 million) manifesting clinically with varying degree of disease severity[1], and the number of cases doubling every decade, from 8·3 million in 1990, to 58·4 million in 2013[2]. Pakistan is hyperendemic for DENV, Lahore being the epicenter. The country experienced its first outbreak in 1994 and the largest epidemic in 2011[3] resulted in 20,000 cases and 300 deaths. Out of four DENV serotypes, 2 and 3 are most prevalent in Pakistan.[4]

CHIKV is another rapidly spreading arboviral infection. The causative virus is a positive sense, single stranded RNA member of genus Alphaviridae, family Togaviridae[5], first described during an outbreak in a Swahili village in the Newala district of Tanzania, Africa in 1953. Phylogenetic analysis on partial sequences of NS4 and E1 genes reveals 3 distinct groups: the West African, the East-Central-South African (ECSA), and the Asian.[6,7] Asia experienced its first epidemic in 1954 in Bangkok,

Thailand, and continued till 1964. The presence of the virus in Pakistan dates back to 1980s when Darwish *et al* confirmed the presence of antibodies to CHIKV and ZIKV along with other arboviruses in rodents, domestic animals and humans[8] and in 2011, antibodies were detected by Afzal *et al* in a pediatric population.[9] Outbreaks continued across the globe: in 2016 India experienced a large outbreak that was followed by the current outbreak in Pakistan, starting from mid of September 2016, and confirmed by NIH-Islamabad in December 2016. According to WHO, Italy and France are facing the epidemic of 2017.[10,11]

ZIKV, another emerging threat for Pakistan, is the enveloped, single stranded RNA virus, described first in rhesus monkeys in Zika Forest, Uganda. Only a few outbreaks have been noted due to asymptomatic clinical course and close resemblances with other flaviviruses. WHO reported active circulation of zika virus in 38 countries in 2016.[12] The presence of ZIKV virus antibodies were confirmed in sera of 2.4% of all samples from rodents, domestic animals and humans.[8] Pakistan is at increased risk for ZIKV after confirmation of 3 cases in Ahmedabad, India in 2017.[13]

The common route of transmission in all three viral infections is the mosquito vector - Aedes aegypti and Albopictus. Some features of these infections are in common; however, there are specific features for each infection: arthritis and debilitating arthralgia are the hall mark for CHIKV; bone pain and hemorrhage in DENV fever; and neonatal microcephaly and other pregnancy -related complications with ZIKV. Signs and symptoms come in close differential of fever presenting with rash arthralgia/myalgia and thrombocytopenia, making it difficult to distinguish on clinical grounds alone. Antibodies against these viruses have shown cross reactivity with each other and the only gold standard for diagnosis of each is RT-PCR during acute febrile phase. Malaria is another close clinical differential, but is easily diagnosed on smear or rapid ICT malaria test.

We have observed that a significant number of patients presenting with acute febrile illness are negative for malaria or DENV. This observation of serologically negative cases for DENV/malaria, absence of serological evidence of ZIKV,[8] and confirmed existence of vector and recent outbreak of CHIKV and ZIKV in neighboring country India[13] led us to conduct the study to unmask the frequency of other two viruses. The purposes of this study were confirmation of the CHIKV and subsequent analysis of its clinical and laboratory parameters and early detection of ZIKV. Also, the study will be helpful in identifying and approaching the areas of major epidemics for effective vector control measures and public awareness sessions on prevention in future.

Materials and Methods

This was a descriptive, cross sectional study. Total 183 participants were included after non-probability, consecutive sampling. The study was conducted at The Indus Hospital, Karachi. Indus Hospital is 150 bed, charity based tertiary care hospital providing state of art facilities. Samples were collected from December 27, 2016 till June 12, 2017 from cohort fulfilling inclusion criteria. Patients were recruited from The Indus Hospital (Korangi), Star General Hospital and Al-Tibri Hospital (Malir), Patel Hospital (Gulshan-e-Iqbal), Ziauddin Hospital (North Nazimabad), and directly from laboratories in Malir. Majority of our participants belonged to Malir town, located in the eastern part of Karachi, Sindh, Pakistan, with a multi-ethnic population, bordered by the Jinnah International Airport and the Malir River. Patients of either gender, presenting to the Emergency Department or Out-patient clinics and referred from different hospitals with non-localizing acute febrile illness of less than 14 days duration were included. Patients with obvious source of fever, such as upper respiratory tract, urinary tract etc. or with rash suggestive of any other cause of viral fever e.g. varicella, measles, etc. were excluded.

Sample collection

After hospital institutional review board (IRB) approval and informed consent, information was noted on a performa. Pediatric population was evaluated by a pediatrician to exclude other causes of fever. Blood samples were collected and sent for complete blood picture, ALT, malarial parasite (thick and thin films with Giemsa stain), MP-ICT, dengue serology IgM (fever >5 days) and RT-PCR for dengue, chikungunya and zika (fever =5 days), and blood cultures where indicated. Viral RNA from serum samples was extracted through QiaAmp Viral RNA Mini kit (Qiagen Inc.) according to manufacturer's instructions. Extracted RNA was employed in real-time multiplex PCR (RT-PCR) mix containing probes and primers to detect dengue, chikungunya and zika viral RNA (RT-PCR kit that was kindly provided by Center for Disease Control, USA).

Operational Case Definition

Standard definitions for suspected, probable and confirmed cases have been used for DENV[14], CHIKV[15] and ZIKV[16] fever.

DENV[14]:

Confirmed Case: Detection of viral ribonucleic acid (RNA) by PCR or IgM antibodies in serum.

CHIKV[15]:

Confirmed case: Presence of viral ribonucleic acid (RNA) in acute-phase sera as determined with RT-PCR.

ZIKV[16]:

Confirmed case: Presence of viral ribonucleic acid (RNA) in serum determined with RT-PCR

Data Analysis

Data was entered and analyzed using SPSS version 24. Mean ± SD or median (IQR) was computed as appropriate for all the quantitative variables. Frequencies and percentages were calculated for all the categorical data. Pearson chi square and Fisher exact test were applied to assess significant association of various categorical variables with chikungunya status.

Furthermore, Independent sample T-test and Mann Whitney-U test were applied as appropriate to assess significant differences in quantitative variables between chikungunya status. P-value<0.05 was considered statistically significant.

Results

Out of 183 samples collected, 140 (76.5 %) were from Indus hospital and 43 (23.5%) from different hospitals in Karachi and laboratories from Malir. The overall frequency of arboviruses was 100 (54.6%). 95 (52%) were PCR positive for one or two arboviruses; serology for DENV was positive in 5 (2.7%). The frequency of CHIKV and DENV were 91 (49.7%) and 13 (7%) respectively (Figures 1& 2).

Male to female ratio was almost 1:1, while age ranged between 2-81 years with a mean of 31.7 ± 14.6 years with most of the patients falling between 14-44 years. 40 patients had associated comorbids, while hypertension and diabetes mellitus were most frequent, as it is in the general population. One patient was hemophiliac with HIV/HCV, co infection. 10 (13%) females were pregnant. None of the participants was on immunosuppressive agents.

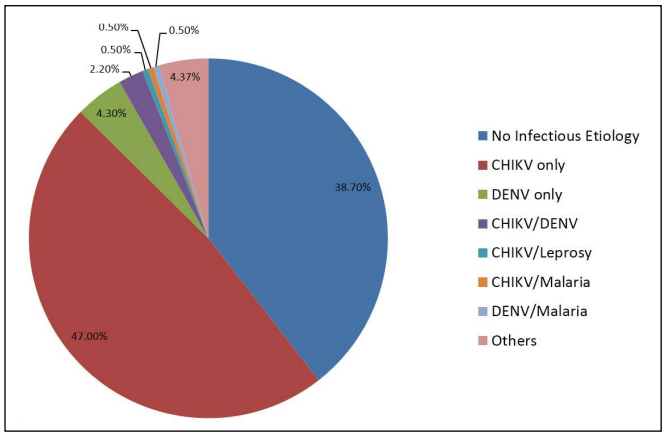

Figure 1: Infectious etiology among participants

CHIKV= Chkiungunya virus, DENV= Dengue virus, ZIKV= Zika Virus, PCR= Polymerase Chain Reaction, /=Coinfection

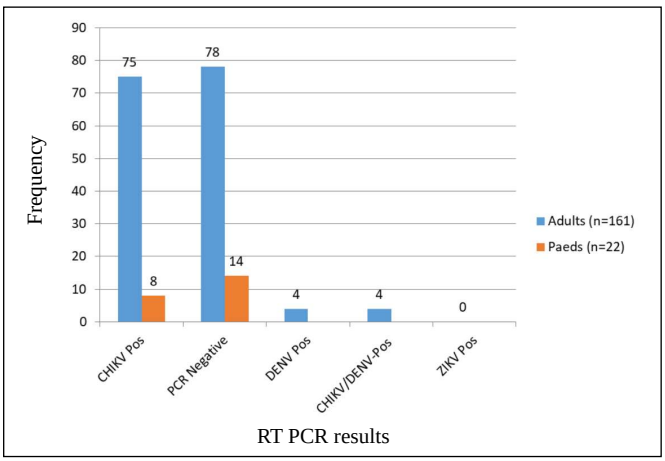

Figure 2: Age Group Based Frequency of Arboviruses via RT-PCR Only.

CHIKV= Chkiungunya virus, DENV= Dengue virus, ZIKV= Zika Virus, PCR= Polymerase Chain Reaction, Pos=Positive

CHIKV was found to be the predominant virus, showing two peaks (figure 3). The degree of maximum temperature noted was higher in PCR positive patients (p=0.028) than in PCR negative. The over all duration of fever ranged 1-90 days (6.4 ± 11.6). Percentages for symptoms were calculated (See Table 1). Only 4 patients were hypotensive, 7 had relative bradycardia but this was not a consistent feature.

Joints involved were predominantly symmetrical and multiple (Table 2). Though not statistically significant, joint pain lasted longer (12 months v/s 6 months) in PCR positive patients.

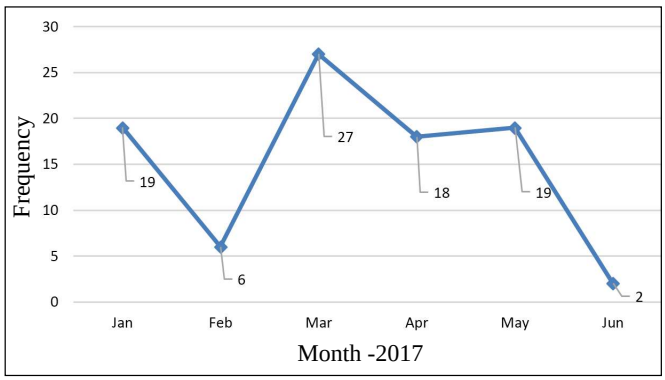

Figure 3: Month wise distribution of CHIKV cases

Table 1: Descriptive Analysis and Comparison of Frequency of Symptoms in CHIKV Positive and Negative Cases (n=160)

Symptom	Total	Positive	Negative	P- Value
Vomiting	48(30)	25(30.1)	23(29.9)	
Rash	78(48.8)	43(51.8)	35(45.5)	
Joint pain	138(86.25)	78(94)[b]	60(77.9)	
Minor bleed (e,g gum bleed, epistaxis)	5(3.1)	1(1.2)	4(5.2)	
Major bleeding (e.g hemetemsis, malena)	2(1.3)	1(1.2)	1(1.3)	
Rigors	38(23.8)	26(31.3)[b]	12(15.6)	
Shivering	50(31.3)	22(26.5)	28(36.4)	0.003[*t I]
Chills	76(47.5)	47(56.6)[b]	29(37.7)	
Muscle pain	70(43.8)	38(45.8)	32(41.6)	
Headache	75(46.9)	39(47)	36(46.8)	
Nausea	46(28.8)	31(37.3)[b]	15(19.5)	
Diarrhea	14(8.8)	6(7.2)	8(10.4)	
Itching	11(6.9)	7(8.4)	4(5.2)	
Abdominal pain	6(3.8)	3(3.6)	3(3.9)	
Redness of eye	5(3.1)	2(2.4)	4(5.2)	
Cough	10(6.3)	2(2.4)	8(10.4)[a]	
Other	42(26.3)	21(25.3)	21(27.3)	

*P-value<0.05, I Pearson Chi Square test, -For significant pair, the key of the category (a=positive, b=negative) appears in the superscript

Table 2: Comparison of Joint Manifestation in CHIKV Positive and Negative Patients

Characteristic	Positive n(%)	Negative n(%)	Total n(%)	P-Value
Arthralgia (n=160)	**78(94)[b]**	**60(77.9)**	**138(86.25)**	**0.003*[I]**
Arthritis (n=139)	**50(60.9)**	**32(39)**	**82 (58.99)**	
Restricted movements	27(54)	16(50)	43(42.4)	0.449 I
Joints involved				
Ankle	14(29.8)	15(50)	29(37.7)	
Wrist	22(46.8)	12(40)	34(44.2)	
Elbow	13(27.7)	6(20)	19(24.7)	
Knee	39(83)[b]	18(60)	57(74.0)	
Small joints of Feet (IP and MTl)	6(12.8)	3(10)	9(11.7)	0.209 [I]
Back	3(6.4)	2(6.7)	5(6.5)	
Small joints of Hand (IP and MCP)	17(36.2)	10(33.3)	27(35.1)	
Only Toes (MTP or IP only)	1(2.1)	2(6.7)	3(3.9)	
Hip	1(2.1)	1(3.3)	2(2.6)	
Shoulder	4(8.5)	0(0)	4(5.2)	
Fingers(IP only)	2(4.3)	0(0)	2(2.6)	
Type				
Large joint pain	27(60)	18(40)	45(100)	
Small joint pain	3(50)	3(50)	6(100)	0.771
Both large and small joints	18(64.3)	10(35.7)	28(100)	
Total	48(60.8)	31(39.2)	79(100)	
Symmetry				
All symmetrical	39(73.6)	19(63.3)	58(69.9)	
All asymmetrical	11(20.8)	8(26.7)	19(22.9)	0.518
Both symmetrical and asymmetrical	3(5.7)	3(10)	6(7.2)	
Total	53(100)	30(100)	83(100)	
Number of Joints Involved (arthralgia/arthirits)				
Mono (single joint)	5(9.4)	2(6.5)	7(8.3)	
Oligo (2-4)	16(30.2)	16(51.6)	32(38.1)	0.177
Poly (=5)	32(60.4)	13(41.9)	45(53.6)	
Total	53(100)	31(100)	84(100)	
Duration of joint pain lasted				
<1month	6(14.3)	2(6.7)	8(11.1)	
1-3months	26(61.9)	23(76.7)	49(68.1)	0.437
>3months	10(23.8)	5(16.7)	15(20.8)	
Total	42(100)	30(100)	72(100)	
Presence of Fever, joint pain and rash (n=183)				
Fever only	11(12.1)	29(31.5)	40(21.9)	
Rash + Fever	2(2.2)	3(3.3)	5(2.7)	0.010*
Joint pain + Fever	37(40.7)	28(30.4)	65(35.5)	
All three	41(45.1)	32(34.8)	73(39.9)	
Total	91(100)	92(100)	183(100)	

*P-value<0.05, **P-value<0.0001, I Pearson Chi Square test, Fisher's Exact test. IP Interphalangeal, MCP metacarpophalyngeal, MTP metatarsophalyngeal

35% cases with CHIKV had hematocrit >40. Mean hemoglobin, hematocrit, TLC, platelet count values were found to be 12.4 (± 2.3) v/s 12.6 (±2.4), 38.2 (±6.1) v/s 39.5 (±7.8), 6.9(±3.3) v/s 7.4(±4.2), 222.7(±86) v/s 233.6(±138.7) in PCR positive v/s negative patients (statistically insignificant). Only 8 patients manifested thrombocytopenia and anemia. SGPT was within normal range in CHIKV positive cases in all, except 4 patients. There was no statistically significant difference in terms of age, gender, visit to outbreak area, family history of similar illness and employment status between the two groups.

Two mortalities recorded were not due to CHIKV or related complications.

Discussion

Acute febrile illness is one of the most frequent reasons for patients seeking healthcare. The entity comprises of various infectious and noninfectious causes. Due to lack of diagnostic facilities, most cases are treated clinically, sometimes resulting in unnecessary antimicrobial administration despite high suspicion of viral illness.

The study confirms the presence of CHIKV in Pakistan. Frequency of chikungunya in our study (49.7%) is higher than that found by Kaur[17] (24.1%) and Marlen[18] (30%) who found higher rates of DENV and co-infections.

In the six months of sample collection, CHIKV outnumbered other viruses with two peaks (figure 3), in January (20.9%) and then in March-May 2017 (29.7%). The reason for fall in cases in February could be a climatic change affecting vector breeding. Frequency of DENV Virus (n=8) and DENV/CHIKV co-infections (n=4) were too low to show any seasonality. A study of longer duration is required to establish behavior of the virus. Our observation is entirely different from Marlen[18], who noted an even distribution of CHIKV throughout the period under review and two peaks of DENV. Comparing with DENV and malaria in Pakistan, malaria peaked significantly from May to October, while dengue cases occurred more frequently between September to December and declined afterwards.[19]

The demographic analysis between RNA PCR positive v/s negative groups was statistically insignificant in terms of gender (p=0.713), age (p=0.168), employment status (p=0.108) and history of mobilization outside or within Karachi in areas of ongoing epidemic (p=0.193). The frequency of involvement of other family members with CHIKV like illness was similar in both groups (78.6 v/s 76%) but number of household members involved in CHIKV +ve was higher (range 1-20 v/s 1-4). Anish[20] also found 377 households in which 71.4% (67.5-74.3%) had at least one member affected by chikungunya. Since we could not do CHIKV serology we may have missed this diagnosis in some patients.

An interesting observation was that out of the 72 CHIKV PCR +ve cases 18 had become afebrile at the time of sample collection, indicating that viremia may persist even after fever resolution. In contrast to this, afebrile patients were sero positive in one study.[21] We found positive CHIKV PCR after 14 days of fever (n=1), suggesting viremia may persist up to 2 weeks (biphasic nature of disease). In a study done in travellers returning from India, RT-PCR remained positive till day 10.[22] Temperature more than >40ºC was noted in one patient with positive PCR. Waggoner[23] and Lee[24] noted mean temperature of 37.4ºC (±0.9) and 39ºC (T -max) with CHIKV respectively.

CHIKV is known for its debilitating course due to joint involvement which may persist for>3 months, few may require steroids or disease modifying therapy. There is disagreement between Chang *et al*[25] and Brad *et al*[26] regarding persistence of virus in synovial membrane as the cause of chronic joint involvement, but both have consensus on autoimmunity and exacerbation of preexisting joint disease. In our study, the shortest duration of joint involvement was 3 days, and maximum up to 12 months in CHIKV positive patients. The finding is likely due to earlier (within 1 week of fever onset) presentation in CHIKV positive cohort, increasing the chances of PCR positivity. Symptoms persisted for > 3 months in only 10 PCR positive patients. Stratification done on the basis of symmetry, number of joints, presence of synovitis and type of joints involvement (Table 2) is comparable with international data.[25,27] Kaur *et al*[17] found more frequent joint restriction (97%). The presence of inflammatory arthritis in CHIKV chronic arthritis can be confused with other inflammatory arthritides. Further studies are required to establish differentiating points.

Like Cunha[28], we also found blanchable erythema/macular or morbiliform rash as a common presentation, generalized in most cases and pruritic in a few cases. The rash was found in 43 out of 78 (51%) CHIKV positive cases which is higher as compared to Kaur *et al*[17] (30%). One patient developed rash after 6 days of fever. Petechiae were seen in CHIKV PCR negative group (11 v/s 125). Unlike Cunha[28], we did not notice other skin manifestations (e.g. photosensitivity, erythema nodosum, vesicles, blisters hyperpigmentation etc.).

Altered level of consciousness was present in 3 patients: only one had CHIKV PCR positive in blood. Unlike Kaur *et al*[17] who confirmed CHIKV encephalitis via positive CHIKV PCR in cerebrospinal fluid (CSF), we did not check CHIKV PCR in CSF of these patients. None of our patients developed neurological sequelae. We did not find visceromegaly or lymphadenopathy specifically associated with CHIKV.

In contrast to Kaur[17] and Torres[29] who correlate hypotension with complicated cases of CHIKV, hypotension (Systolic BP<90, diastolic BP<60) was seen in only four patients complicated by other severe infections or co morbidities: an HIV/HCV +ve, hemophilia with XDR Klebsiella bacteremia, a pregnant female in her third trimester and CHIKV with complicated malaria.

46(73%) PCR positive cases had total leukocyte count within normal limits with leftward shift (neutrophilia>monocytosis) but leukopenia (n=9) and leukocytosis (n=8) were also found. The finding has also been noted in other studies.[24,29] Leukocytosis or leukopenia in the face of high fever and tachycardia mimicked sepsis, and despite negative blood cultures, led to injudicious use of antibiotics.

There were only 12 cases of DENV, too small a number to compare with CHIKV positive patients. DENV presenting as abdominal pain, ascites, pleural effusion, visceromegaly, neurological involvement, but no joint manifestations in our as well as international studies [30,31]. Another study found similar hematological and biochemical parameters.[24]

8 out of 10 pregnant females (80%) were PCR positive for CHIKV only, 1 had CHIKV/DENV co-infection and 1 was negative for any arbovirus. 50% were in their third trimester. In contrast to Kaur,[17] no intrauterine death or adverse pregnancy outcomes were subsequently noted. Follow up of neonates for manifestation was out of scope of this study.

Majority of our patients responded well to initial non-steroidal anti-inflammatory drugs (NSAIDs) and hydration in case of CHIKV and hydration and acetaminophen in DENV infections.

There were no cases of ZIKV but we are at high alert for this emerging disease after the 2018 outbreak of the virus in India. CDC categories Pakistan as a risk area for ZIKV. Serology has also been reported positive in Pakistan by Darwesh *et al* in rodents.[8] In 71(38.7%) cases, we did not find any alternate diagnosis. This could be explained either by lack of availability of serological test for CHIKV; or infections with other viruses known for causing fever with exanthems (adenovirus, rubella, rubeola, coxsackie, parvovirus B19) leading a new path for research.

The transmission of viruses can be halted with adequate vector control, improved sanitation and personal protective measures e.g. mosquito repellants, nets etc. Public awareness sessions should be arranged in areas of epidemic. CHIKV related debilitating arthritis has a financial impact on daily wage earners due to absenteeism.

To the author's best knowledge, no other study has been published so far from Pakistan with descriptive analysis of multiple variables and spectrum of disease. Previously published studies were either done with serology[9] or did not consider comparative analysis.[32] This study can be a stepping stone for future studies from the region.

Study Limitations

The duration of study was short to assess seasonality. The sample size was small and thus head to head comparison of DENV and CHIKV manifestations was not possible. Samples collected directly from labs lacked uniform clinical data. We were unable to find cross reactivity among viruses due to unavailability of serology kits for CHIKV and ZIKV, and were unable to identify serology positive cases in late cases.

Conclusion

Chikungunya is a newly emergent problem in Pakistan, while we are at high risk for zika virus as well. CHIKV can be co-infected with other viruses or malaria, and may mimic more severe illness such as sepsis and rheumatoid arthritis. Laboratory tests are usually nonspecific in CHIKV. Non-infectious diseases can co-exist with CHIKV. The hall mark triad of fever, joint pains and skin rash in CHIKV was significantly higher, and if present, can help in clinically differentiating from other illnesses. CHIKV is a self-limiting, though temporarily disabling disease, and does not require specific therapy, while supportive care for fever and pain control are sufficient. There is a great need to improve diagnostic tests, and most importantly, conditions for prevention of mosquito breeding need urgent attention.

Future Directions

In future, studies can be done on arthritis and its correlation with other parameters.

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Abbreviations

CHIKV Chikungunya Virus
DENV Dengue Virus
RA Rheumatoid Arthritis
RT-PCR Reverse Transcriptase Polymerase Chain Reaction
TLC Total Leucocyte count
ZIKV Zika Virus

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