ORIGINAL ARTICLE

Frequency of Chikungunya virus infection in samples tested concomitantly for dengue and malaria: experience from clinical laboratory, Karachi Pakistan.

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Abstract

Background
Chikungunya virus (CHIKV) has recently emerged as cause of acute febrile illness. Exact burden of this viral infection in Pakistan, is not known. A cross-sectional study was performed to assess the frequency of CHIKV infection tested in a clinical laboratory as compared to that of dengue and malaria.

Methods
A descriptive cross-sectional study was conducted over a period of 6 months, at the section of microbiology, Aga Khan University Hospital Clinical Laboratories (AKU), in Karachi. Samples for detection of dengue Non-structural protein 1 antigen (NS1Ag) and/or Immunoglobulin M (IgM), Chikungunya IgM and Malaria Immuno-Chromatographic Testing (ICT) from patients of all ages and both genders were selected and enrolled.

Result
A total of 13,271 patient samples with acute febrile illness were received during the study period. Of which (N= 10160) were tested for malaria, (N=1484) were tested for dengue and (N= 595) for Chikungunya infection. Chikungunya IgM was detected in 8.2% (49 cases) samples. Most cases were diagnosed with malaria (256 cases, 2.5% of those tested) and dengue (251 cases, 16.9% of those tested).

Conclusion
We found CHIKV to be a common cause of febrile illness, its proportion of positive results second only to dengue. However, the burden of dengue and malaria experienced at AKU was much higher than CHIKV. Chikungunya is active in the region and should be considered as an important differential for febrile illnesses.

Key words
Chikungunya, Dengue, Malaria, frequency, Pakistan

Background
Communicable disease is a significant cause of morbidity and mortality in Pakistan.[1] Amongst these, vector borne disease like Dengue (DENV), Chikungunya (CHIKV) and malaria, are endemic in the South Asian region[2,3] and contribute to a significant burden of disease. Malaria is caused by a blood parasite of the genus *Plasmodium transmitted by the bite of* female anopheles' mosquito*, four species of which are a cause of human disease, namely: P. falciparum, P. vivax, P. ovale* and *P. malariae. In Pakistan P. vivax* (approximately 64%) and *P. falciparum* (approximately 36%) are responsible for the disease.[4] 500,000 malaria infections and 50,000 malaria-attributable deaths occur each year in Pakistan . *DENV and CHIKV are arboviruses transmitted by the bite of Aedes aegypti* and *Aedes albopictus* mosquitoes*. Dengue belongs to the family* whereas Chikungunya virus is a member of the Togaviridae family. *Co-infections have been reported with grave consequences if left undiagnosed.*[5]

Dengue is endemic since 2003[6] with almost 71,649 cases reported in 2016 from Pakistan.[7] Chikungunya, on the other hand, has recently been reported with an outbreak in Karachi during 2017.[8] Little is known of the epidemiology of CHIKV in our population because of the lack of diagnostics in commercial laboratories before recent outbreak in 2016-17. Although several serological and molecular based diagnostic tools are available for both viruses, serological investigations are routinely used not only due to ease in performance but also for rapid turn-around time allowing prompt diagnosis and disease surveillance. Although CHIKV and DENV are both arboviruses however they belong to a diverse family of viruses. CHIKV is an RNA virus from the *Togaviridae* family, sequence diversity between this and family *flavivivridiae* make them antigenically unrelated[9,10] Thus, diagnosis based on IgM antibody detection for either virus remains one of the common and rapid method as there is minimal chance of cross reactivity. A study on seroprevalence and cross-reactivity of Chikungunya Virus in arbovirus-infected patients showed a low level of cross-reactivity amongst serum samples from flaviviruses-infected patients, 6% particularly of DENV.[11]

These pathogens cause acute febrile illness that may be difficult to differentiate in the early phase with high fever, myalgia, and rash. Diagnosis is essential as treatment with antimicrobial agent is important in cases with malaria whereas DENV and CHIKV require supportive therapy. CHIKV especially mimics

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dengue syndrome in its clinical presentation and is often wrongly diagnosed as dengue even in the absence of positive dengue investigations. One reason can be due to the unavailability of tests identifying Chikungunya virus in commercial laboratories especially in Pakistan. Differentiation between the two viral illnesses is imperative due to different disease complications and outbreak investigations. Late debilitating sequelae of chronic joint disease is associated with the CHIKV disease where as DENV can result in a severe hemorrhagic fever. CDC recommends to refrain from using aspirin and other non-steroidal anti-inflammatory in treating CHIKV cases unless dengue has been ruled out to prevent risk of bleed.[12]

The aim of the study was to determine frequency of IgM antibody positive to Chikungunya virus in serum samples of patients suffering with acute febrile illness and compared to that for dengue and malaria. We determined the frequency of Chikungunya, malaria, and dengue amongst patients with acute febrile illness who submitted samples to AKUH for serodiagnosis of all three diseases.

Materials & Methods

A descriptive cross-sectional study was conducted from January-June 2018 at the clinical microbiology laboratory, Aga Khan University Hospital (AKUH). The study was approved from the Ethical Review Committee of the institution. Non-probability consecutive sampling was used. Samples received from patients for detection of malaria, dengue non-structural protein 1 antigen (NS1Ag) / Immunoglobulin M (IgM) and Chikungunya IgM were selected. Patient samples of all age groups both male and female presenting with acute febrile illness, as identified by the physicians, were included. These were tested for serological evidence for the 3 diseases. Repeat sample from same patient, hemolyzed samples on visual inspection were excluded from the study. Blood samples were collected according to the standard phlebotomy techniques and stored at -80°C after separation of serum for testing. Results were documented as detected/ positive and not detected / negative respectively in the study proforma and entered in Microsoft Excel 2010. Furthermore, samples investigating all three pathogens simultaneously in a patient with acute febrile illness were evaluated to see the possibility of co-infections.

Chikungunya Immunoglobulin M Detection

CHIKV immunoglobulin M (IgM) antibodies were tested using enzyme-linked immunosorbent assay (ELISA) (Inbios USA) according to standard manufacturer instructions. Immune status ratio (ISR) = 1.10 were considered "Reactive" and <0.90 were considered "Non-reactive". Any sample with range of 0.90 < ISR value <1.10 were retested and evaluated in duplicate to verify the sample status. According to manufacturer, the assay has a >90% sensitivity and specificity.

Dengue Immunoglobulin M Detection

DENV immunoglobulin M (IgM) antibodies were tested using enzyme-linked immunosorbent assay (ELISA) (Panbio Diagnostics) according to standard manufacturer instructions. Results were interpreted according to Panbio units that were calculated by multiplying the index value by 10.>11 Panbio units were considered "Positive" and <9 Panbio units were considered "Negative". Any sample with range of 9-11 Panbio units was found equivocal and retested. The assay has a sensitivity of 94.7% and specificity of 100%, as claimed by manufacturer.

Dengue NS1 Antigen

This immunochromatographic assay was used for the qualitative detection of NS1 antigen in serum that can be detected for first day of fever onset up to day nine. Presence of two-colored bands (test and control) within the result window were considered as positive. The assay has >90% sensitivity and specificity.

Malaria Antigen Test

Presence of malaria infection was detected using immunochromatographic assay (SD bioline) for qualitative and differential detection of histidine-rich protein II (HRP-II) antigen of *Plasmodium falciparum (P.f)* and common *Plasmodium* lactate dehydrogenase (pLDH) of *Plasmodium* species (Pan) in human whole blood. Presence of two colored bands (test P.f. and control) or three colored bands (test P.f., test Pan and control) within the result window were considered as positive for falciparum malaria, and two bands (test and Pan) were positive for other *Plasmodium* species. Only one band (test) was considered negative for malaria. According to manufacturer, sensitivity with >50 parasites /µl is >98% and the test is 99.5% specific.

Data Analysis

SPSS 21 was used for analysis. Identifiers were kept anonymous and all cases given study identification numbers. Mean of the continuous variables i.e. age was calculated; frequency and percentage of the categorical variables i.e. gender, CHIKV /DENV/ malaria results were computed.

Result

From January 2018 to June 2018, a total of 12239 patient samples with acute febrile illness were enrolled. The number of samples tested for each disease type included; malaria antigen (N= 10160), dengue (N=1484: NS1Antigen n=773, IgM n= 696) and Chikungunya (N= 595) infection as requested by the physicians (table 1). The geographic distribution of sample received for each test type namely Malaria, Dengue and Chikungunya according to provinces is shown in table 1. Majority of samples were from Sindh. The mean age for malaria, dengue and Chikungunya positive cases was 29.7, 30.8 and 44.7 years respectively. Male: female ratio amongst infected cases was 1.9 (168/88) for malaria, 1.3 (143/108) for dengue and 0.6 (19/30) for Chikungunya infection. Malaria was seen in 2.5% cases (256/10160).

Amongst these, 0.3% were due to *P. falciparum* only and 2.2%

Table 1: Demographics and frequencies for samples tested individually for Chikungunya, Dengue and Malaria infections.

	Chikungunya N= 595 (%)	Dengue N=1484(%)	Malaria N= 10160(%)
Total Positive	49 (8%)	251(17%)	256 (3%)
Province-wise Positivity Rate			
Sindh	49 (8%)	251 (17%)	245 (2%)
Punjab	0 (0%)	0 (0%)	9 (3%)
Khyber Pakhtunkhwa	0 (0%)	0(0%)	0 (0%)
Baluchistan	0 (0%)	0 (0%)	2 (5%)
Mean Age of Positive Cases (Years)	44.7	30.8	29.7
Male (Positive Cases)	19 (39%)	143 (57%)	168 (66%)

N= total number of cases tested for the disease, (%) = percentage, IgM = Immunoglobulin M, NS1Ag= Non-structural protein 1 antigen

were positive for *Plasmodium* species other than *P. falciparum*, most likely *P. vivax*, as *P. malariae* or *P. ovale* are not seen in Pakistan.[4] Of the 1484 patients tested for dengue, 16.9% (251) were positive by either NS1 antigen or IgM. Dengue NS1 antigen was positive in 4.8% cases and 11.9 % were reactive for dengue IgM. Chikungunya IgM was detected in 8.2% samples (49/595), indicating Chikungunya infection in these cases. Forty-seven samples were tested simultaneously for all three pathogens (table 2). Amongst these 27 were males and 20 females. Malaria was seen in 2.1%, dengue was identified as most common infection among patients tested 19.1% of samples concomitantly tested for three pathogens were positive for dengue (both NS1Ag and IgM). None of the concomitantly testes samples showed IgM antibodies to Chikungunya virus. No case of co-infection was observed. The overall positivity rate amongst the samples tested simultaneously for all three diseases, was 21.2%.

Discussion

Chikungunya is known for its epidemic potential worldwide.[5] Recent reports from Pakistan confirm its presence in the country.[8] This study was aimed to determine the frequency of CHIKV infection in patients with acute febrile illness which was found to be 8.2%. This is lower than the frequencies reported from India (25.37%)[13] but slightly higher than those reported from Sri Lanka (3.5%).[14] One of the limitations of this study was that PCR was not performed for patients suspected of CHKV infection, there is a possibility that early cases of CHIK might have been missed. Moreover, 95% of the total samples received at clinical laboratory from suspected acute febrile patients did not request for CHIKV testing, perhaps be due to lack of awareness by the physicians about CHIKV as emerging cause of febrile illness in Pakistan, therefore further studies are needed to investigate the exact burden of CHIKV.

Table 2: Frequencies of infections for samples tested simultaneously for Chikungunya, Dengue and Malaria infections (N=47)

	Chikungunya	Dengue	Malaria
Positive result	0 (0%)	9 (19%)	1 (2%)
Negative results	47 (100%)	38 (81%)	46 (98%)
Co infections	None identified		

Detection of dengue IgM in 21.1% cases and malaria in 2.5% cases highlight the fact that dengue is superseding endemicity of this infection over malaria in our tested population.

Patients with dengue infection had more cases detected by IgM (21.1%) than by NS1Ag (10%). This is most likely due to the fact that dengue IgM is detectable in 80% cases on serological testing around day ten of illness and remains elevated for up to 6 months or longer.[15] We reported 2.2% cases for *Plasmodium* species and 0.3% *P. falciparum*. This finding is strengthened by the observations from other studies in countries reporting *P. species*, (most likely *P. vivax*) as the predominant cause of malaria.[4]

The three infections share similar presentations of acute fever but may be differentiated on basis of other disease characteristics. Malaria has a classic fever pattern of recurring at 12 hours(quotidian – *P. falciparum*), 48 h (tertian – *P. ovale* and *P. vivax*), or 72 h (quartan – *P. malariae*).[16] Dengue can be suspected in presence of decreasing platelets indicating its hemorrhagic potential and disease severity whereas Chikungunya presents predominantly with arthralgia. Thus, clinical presentation can be useful in differentiating the disease but may be absent in the early phase of symptoms. Co-infection is rare[17] and potentially fatal[5], however, no co-infections were identified in this study. This may be due to the short study duration and

limited number of samples tested for all three diseases, and thus needs further evaluation.

One may consider review of this data as passive surveillance however the main objective was to analyze the spread of disease indifferent part of country as patient samples were received from all over country. The study results are generalizable to the population of Sindh only as most samples were received from there, but not the whole of Pakistan. Only forty-seven samples were tested simultaneously for the three diseases and were too few to provide an opportunity to detect co-infection, a rare possibility. Furthermore, clinical details were not recorded as analysis of patients' clinical presentation was out of the scope of this study.

Second limitation was inability to use additional confirmatory tests such as PCR for DENV and CHIKV and peripheral film for Malaria. However, since the intent of study was to see the burden of CHIK virus in the acute febrile patients presenting to clinical lab in comparison to dengue and malaria our study still provides useful information to the readers about the burden of this infection in clinical setup, using regular diagnostic testing methods. Since PCR was not used, early cases of CHIKV might have been missed; therefore, further studies using both diagnostic modalities are required to assess true burden of the disease.

In conclusion, CHIKV is emerging as a cause of acute febrile illness alongside dengue and malaria, and it should be considered in the differential diagnosis of all patients presenting with acute febrile illness in Pakistan. Factors like poor vector control and urbanization increased breeding sites for mosquitos play an important role in disease endemicity. Vector control is integral to limit the spread of these illnesses.

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