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RESEARCH ARTICLE

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Serological and molecular evidence of chikungunya virus infection among febrile outpatients seeking healthcare in Northern Malawi

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ABSTRACT

Introduction: Despite global evidence of chikungunya fever (CHIKF) in humans that is caused by chikungunya virus (CHIKV), little is known about the occurrence of CHIKF in Malawi. This study was conducted to determine the seroprevalence of CHIKF and to molecularly confirm the presence of CHIKV ribonucleic acid (RNA) among febrile outpatients seeking health care at Mzuzu Central Hospital in the Northern Region of Malawi.

Methods: Enzyme-immunosorbent assay (ELISA) was used to detect the presence or absence of specific antibodies against CHIKV. Reversetranscription polymerase chain reaction (RT-PCR) was conducted on randomly selected anti-CHIKV IgM-positive samples to detect CHIKV RNA.

Results: Out of 119 CHIKF suspected samples analyzed, 73 tested positive for anti-CHIKV IgM antibodies, with an overall seroprevalence of 61.3%. Most of the CHIKV infected individuals presented with joint pain, abdominal pain, vomiting and nose bleeding with seroprevalence of 45.2%, 41.1%, 16.4% and 12.3%, respectively. All the randomly selected samples that were positive for CHIKV anti-IgM by ELISAhad detectable CHIKV RNA by RT-PCR.

Conclusion: The presence of anti-CHIKV IgM antibodies suggests the presence of recent CHIKV infection. We therefore recommend for the inclusion of CHIKF as the differential diagnosis in febrile ill patients in Mzuzu city, Malawi.

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Chikungunya virus; seroprevalence; molecular; febrile illnesses; Malawi; Arboviruses

Background

Chikungunya fever (CHIKF) is an *Aedes*-transmitted arboviral disease caused by chikungunya virus (CHIKV). CHIKV belongs to alphaviruses of the family *Togaviridae* [1]. The name ''chikungunya'' is derived from the Makonde language in Tanzania, meaning 'to walk bent over' indicating the painful arthralgia experienced by people infected with CHIKV [2].

In the African continent, CHIKF is one of the neglected diseases of great public health concern especially in immunologically naïve human populations and cause epidemics. In poor resource settings such as in Africa, where laboratory diagnostic tools in febrile patients are limited, most acute fevers of unknown origin are not identified, thus they are regarded and treated as malaria [3]. Thus *Aedes*-transmitted arboviruses such as CHIKV go unrecognised in African countries and continued negligence lead to high morbidity of the population at risk. CHIKV has an estimated population at risk (PAR) of 271 million of which of 23% is from the African population [4].

A study done in North-eastern Tanzania found that environmental factors such as living in a house with uncovered containers, keeping hoofed animals and vegetation (<100 m) were associated with high CHIKV IgM seropositivity [5]. The other risk factors for transmission and occurrence of CHIKV include previous CHIKF history from the household neighborhoods, socio-economically disadvantaged populations, high maximal temperatures before the infection, high rainfall in the month before the introduction of CHIKV in the region, poor knowledge on CHIKV transmission, obesity/overweight and occupational inactivity [6].

The incubation period of CHIKF after primary exposure ranges from 2 to 12 days (average, 3–7 days), and the infection is mostly self-limiting [7]. The main symptom of CHIKF is polyarthralgia/polyarthritis and sometimes patients present with acute fever (≥38.9°C) lasting from several days to two weeks [8]. CHIKF presents similar signs and symptoms to other diseases such as Dengue, Malaria, Leptospirosis and Brucellosis [9]. The highly specific laboratory assays are of paramount importance in

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differentiating viral infections which commonly present with fever [10].

Aedes mosquitoes are known to transmit CHIKV and pose a high risk of CHIKF outbreak [11]. *Aedes aegypti* and *Aedes albopictus* are the main vectors of CHIKV transmission to humans [12]). CHIKV is transmitted through urban and sylvatic cycles. The urban cycle is the transmission of CHIKV from human to mosquito to human, while sylvatic transmission refers to the transmission of CHIKV from animal to mosquito to human [13]. The sylvatic cycle is the primary form of CHIKV transmission in Africa [12]. The disease has spread across the entire world causing numerous epidemics that have infected millions of people in Americas, Asia, Europe and Pacific Islands [14].

The emergence and probably the reemergence of CHIKV in the Americas was recorded in 2013, when first local CHIKF cases were diagnosed in Saint Martin [15–17]. Bangkok, Thailand and India were the first countries to record imported CHIKV cases that caused outbreaks in the regions [12]. China's first outbreak of CHIKF occurred in September, 2010 with 51% prevalence of confirmed cases and registering an attack rate of 1% [18]. CHIKF outbreaks in Europe have been reported in Italy, France, Croatia, Madeira and Spain where its emergence was ascertained to globalization including international trade [19].

CHIKV originated in Africa, Tanzania in 1952 [2]. Since then, a number of epidemics had been reported in Angola, Burundi, Central African Republic, Democratic Republic of Congo, Guinea, Malawi, Nigeria, South Africa and Uganda [20]. In June 2004, an epidemic occurred on Lamu Island, Kenya and spread to the Comoros, La Reunion and Indian Ocean islands [21]. Between 2017– 2018, another outbreak occurred in the city of Mombasa whereby 32 laboratory-confirmed cases were reported. CHIKV remains endemic in Kenya [22]. In 2005 and 2007, Madagascar experienced persistent CHIKV circulation [14]. Reported positive confirmed CHIKF cases were also reported in Congo in 2011 [23].

Hospitals and healthcare providers in Malawi do not usually include *Aedes*-transmitted arboviral diseases as a differential diagnosis among patients presenting with fevers and probably misdiagnoses occur [9]. There is probably an underestimation of the burden for mosquito-borne viral diseases in Malawi such as CHIKF that present the same with malaria, which is endemic in the country [24]. Thus, there is absence of published information regarding current prevalence of CHIKF in Malawi. This study was carried out to determine the seroprevalence of CHIKF through detection of antibodies against CHIKV by enzyme-linked immunosorbent assay (ELISA) among outpatients seeking healthcare in Mzuzu city, Malawi. Molecular assays were performed as the confirmatory test to detect CHIKV RNA.

Materials and Methods

Study area

The laboratory analysis for this study were conducted at the Vector- borne Diseases Laboratory, The University of Malawi. The study participants were recruited from Mzuzu city at Mzuzu Central Hospital (MCH) in the year 2019. This is the only and largest referral hospital in northern region of Malawi. Mzuzu city is characterized by a wooded and hilly terrain land. The local people practise trade and agricultural activities such as tea, rubber and coffee farming.

Study participants, sample and data collection

The samples were collected from individuals presenting with febrile illnesses. Participants with fevers ≥ 38°C, for not more than 5 days with symptoms such as chills, headache, joint pains, dizziness, nausea/vomiting, arthralgia and rash were recruited in the study. No restriction to age and gender were considered. Those seriously ill such as in coma and those requiring hospitalisation were excluded from the study.

A structured questionnaire was administered by the clinicians to capture demographic information such as age, sex and clinical manifestations presented by the patients. The outpatients were examined by a qualified clinician at the health facility and enrolled as participants according to the inclusion criteria. The patients were at first screened for malaria using malaria rapid test, SD Bioline Malaria Ag- Pf/Pan kit (Standard Diagnostics, Suwon city, Republic of Korea). Only patients with a malaria negative test result were enrolled into the study. Samples of blood (5 mL) were drawn from all consenting and assenting malaria negative patients using venipuncture and put in red top plain tubes. The blood samples were separated by centrifugation and aliquoted into cryo vials. The sera samples were then sent to The University of Malawi, Vector-borne Diseases Laboratory for permanent storage in −80°C ultralow freezers until analyzed by serological and molecular assays.

Serological assays

Serum samples were tested for anti- CHIKV IgM antibodies using Abcam's ELISA Kit (Abcam, Cambridge, UK) by strictly following manufacturer's

instructions. The diagnostic specificity and sensitivity of the assay was more than 90%. Absorbance of tested samples was read at 450 nm using an ELISA microwell plate reader (Bio-Rad, California, USA). The results were regarded as positive if the absorbance value was greater than 10% over the cut off value. The intensity of the product of IgM anti-CHIKV antibodies and precoated CHIKV antigen in microwell was proportional to the amount of CHIKV specific IgM antibodies in the patient sample. The results were calculated and interpreted in Abcam's unit (NTU) according to manufacturer's instructions. The cut off value in Abcam's unit was 10 NTU, negative result was < 9 NTU and positive was more than 11>NTU as per manufactures instructions. Both positive and negative controls contained in the kit were included in each assay run to ensure the reliability of the test procedure.

Molecular assays

RNA was extracted from fourteen randomly selected anti- CHIKV IgM seropositive samples using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Viral RNA was then stored at −20°C until used for one step RT-PCR. The extracted RNA was used in one step RT-PCR using titan one tube RT-PCR Kit (Roche) in GeneAmp PCR System 9700. NSP1 was amplified using primers Chik NSP1F: 5'-TAGAGCAGGAAATTGATCCC-3' and Chik NSP1R: 5'- CTTTAATCGCCTGGTGGTAT-3'as forward and reverse primers respectively to amplify a 354 bp gene product as previously published.

Preparation of a 25 µL reaction mixture containing 12.5 µL of 2× reaction mix, 1 µL of enzyme (RT/Taq), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 0.5 µL Magnesium salt, 8 µL of nuclease-free water and 2 µL of RNA template was done. PCR reactions were as follows; reverse-transcription reaction at 42°C for 60 minutes, incubation of reverse transcription at 94°C for 3 minutes, denaturing at 94°C for 1 minute, annealing at 54°C and extension at 68°C for 2 minutes for 35 cycles and final extension at 68°C for 7 minutes. PCR products were visualized on agarose gel electrophoresis. Briefly, 2% agarose gel was prepared and run in Tris-acetate ethylenediaminetetraacetic acid buffer. The ladder was loaded into first well and samples (DNA PCR products) on position 1–14. The negative and positive control were loaded on separate wells at the end of the gel. Positive control was the last to be loaded into the gel to prevent contamination. An agarose gel electrophoresis was performed by connecting the electrophoretic tank to the electric current for 40 minutes. The agarose gel was stained with gel red (Bio-Rad, California-USA), visualized on an ultraviolet transilluminator and photos were taken by a digital camera.

Data analysis

Demographic data, the clinical manifestations and ELISA results of the participants were entered and analyzed using Microsoft Office-Excel 2007 (Microsoft, California, USA) and Epi Info version 7.0.8.0 (CDC, Atlanta, USA). Proportions generated were compared using Chi-square independence test at $P \leq 0.005$.

Results

Demographic characteristics of the study participants

A total 119 participants who met the inclusion criteria were recruited into the study. Of these, 79 (66.39%) were females and 40 (33.61%) were males. The participant's' mean age was 31 years (2–83 years).

Seroprevalence and clinical presentation

Of the total 119 sera analyzed for anti-CHIKV IgM antibodies using ELISA, 73 (61.3%) tested positive. Seroprevalence among those who presented with joint pains was 45.21% ($n = 33$). A lower seroprevalence of 41.1% ($n = 30$) was observed among patients who presented with abdominal pains compared to those who did not manifest abdominal pains. A seroprevalence of 12.3% was observed among patients who had nose bleeding history. Those who presented with vomiting had 16.4% ($n = 12$) seroprevalence. Patients with clinical manifestations such as backache, body weakness, chest pain or fast breathing were observed to have the least frequencies when compared with chikungunya seropositivity. Summary of results is presented in Table 1 below.

Seroprevalence by age group

The highest proportion (19.2%; $n = 14$) of seropositivity among anti-CHIKV IgM positive individuals were observed among individual aged 30–39 years. Age groups of 1–9, 40–49 and ≥50 years were the second to be detected to have high proportions of anti-CHIKV IgM antibodies. Age of 20–29 years had 11 (15.1%) cases that were anti-CHIKV IgM antibodies positive. Individuals aged group of 10–19 years had the lowest seroprevalence (12.3%, $n = 9$). The results did not show no any statistical significance in prevalence among age groups in association with seropositivity.

Presence of CHIKV RNA in sera

Fourteen serum samples that were anti-CHIKV IgM positive by ELISA were randomly selected for one step RT-PCR. All the fourteen serum samples had CHIKV RNA detected for RT-PCR upon running

Table 1. Comparison of clinical presentations of participants with CHIKV seropositivity.

Clinical presentations	Number of anti-CHIKV IgM positive 73/119 (61.3%)	Chi square X^2	P-value
Bleeding from nose	9 (12.3%)	1.049	.306
Vomiting	12 (16.4%)	0.018	.892
Joint pain	33 (45.2%)	0.078	.78
Abdominal pain	30 (41.1%)	0.202	.653

on gel electrophoresis. The results of agarose gel electrophoresis were captured by a digital camera as shown in Figure 1 below.

Discussion

As a result of malaria endemicity in Sub-Saharan African countries including Malawi, other aetiologies of febrile illnesses other than malaria are neglected and there is paucity of epidemiological knowledge which lead to improper diagnosis and mismanagement of patients [25]. Although CHIKF has been reported in Malawi's closest neighboring countries such as Tanzania, Mozambique and Zambia [26–28], Malawi remains the only country in Central Africa to lack such published data. In addition, a study conducted in Malawi reported that malaria is often over diagnosed in febrile patients [29]. This may be due to lack of epidemiological data and knowledge gap of other aetiologies of febrile illnesses other than malaria in Malawi. This literature background provided us with a hypothesis that CHIKV is prevalent in Malawi and we sought to conduct the present study to elucidate CHIKV as the aetiology of febrile illnesses other than malaria parasites in Northern Malawi. This is the first study to report the occurrence and the burden of CHIKF in Malawi.

The present study recruited 119 malaria negative participants of which 73 participants had anti-CHIKV IgM antibodies in their sera detected by ELISA. Out of 73 anti-CHIKV IgM positive samples, fourteen samples were randomly selected for molecular assays (RT-PCR) as confirmatory tests. All the fourteen samples had CHIKV RNA amplified and detected by conventional RT-PCR and gel electrophoresis respectively. Most of the CHIKV infected individuals presented with joint pain (45.2%,), abdominal pain (41.1%,), vomiting (16.4%) and nose bleeding (12.3%).

The results indicate a high seroprevalence of 61.3% of CHIKF as detected by presence of anti-CHIKV IgM antibodies in patients' sera. This is despite not testing for anti-CHIKV IgG antibodies and therefore the overall seroprevalence of CHIKF in this population may be underestimated. IgG antibodies against CHIKV indicate if a population is naïve or the patients have a pre-exposure to the pathogen before [30]. Studies elsewhere have reported seroprevalence rates of 0.4 to 75%. The variation has been associated with the use of different sampling techniques such as random and convenience sampling, the magnitude of epidemics, time of virus circulation before survey, demographics and environmental characteristics of different study sites [31].

It should also be noted that serological diagnostic tests are challenged by the patient-specific infection histories and it is important to be conscious of the potential regional limitations of serological tests [32]. Furthermore, specific antibody detection of arboviruses is challenging especially when antigenically conforming viruses co-circulate in the same region of which pre-existing antibodies against other closely conforming arboviral pathogens can cause false-positive results [32,33]. Serological test results for CHIKV must be interpreted cautiously by utmost encompassing interpretation of regionally co-circulating antigenically conforming arboviruses. For instance, CHIKV serological tests are often susceptible to cross-reactivity with antibodies against Mayaro virus (MAYV) or O'nyong-nyong virus (ONNV) [32,33]. However, to the best of our knowledge, there is no documented occurrence of MAYV and

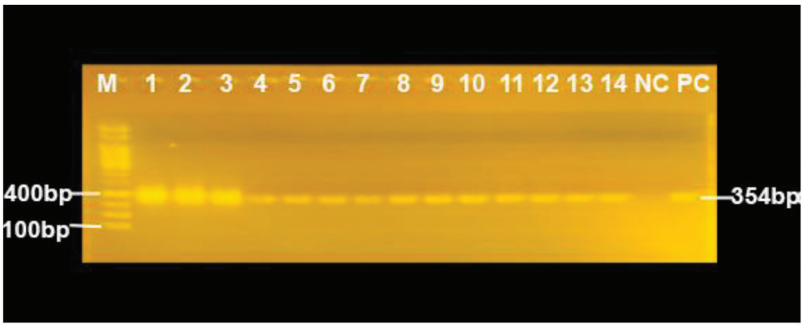

Figure 1. CHIKV RNA detection on agarose gel electrophoresis.
M= 100 bp DNA ladder; PC-positive control; NC-negative control

ONNV in Malawi. By employing molecular assays in this study to detect specific genes for CHIKV, we confidently report the occurrence and prevalence of CHIKF in Malawi with certainty.

The seroprevalence of CHIKF in this study was slightly lower than those reported from Sudan (73.1%) [34], but slightly higher than the seroprevalence reported among febrile patients in Cameroon (51.4%) and Zambia (36.9%) [26,35]. The high seroprevalence in this study can be attributed to the fact that CHIKF occurs through outbreaks in naïve populations [36]. The high seroprevalence in this study is also likely to be attributed to the strict inclusion criteria of suspected patients of CHIKF whereby patients with malaria were excluded from enrolling into the study. This may have probably narrowed down the diagnosis to CHIKF other than malaria which probably increased the chances of detecting antibodies against CHIKV. On the other hand, the effect cross-reactivity of other antibodies need not to be underrated of which might have increased the seropositivity in the present study [37].

The present study further reports that diagnosis of CHIKF based only on clinical presentation of patients such as joint pains is unreliable. Our results are consistent with a study in Brazil where a seroprevalence of 18.3% of anti-CHIKV IgM antibodies was reported among rural community households with no acute clinical manifestations of CHIKF [38]. Although joint pain is the critical clinical manifestation for CHIKF [39], this study has established that even without joint pain, CHIKF is likely to be prevalent in infected patients. This study showed that some patients did not present with joint pains but had CHIKF and therefore accurate diagnosis of CHIKF based on clinical grounds alone may be unreliable. We recommend that for a reliable diagnosis of CHIKF, a combination of clinical manifestations, epidemiological and specific laboratory-based tests should be conducted.

However, in a study done in Cambodia [40], the authors recommended that the significant and commonly reported symptoms of CHIKF should be acknowledged in the classification of clinical criteria for associating potential positive cases when using a syndromic approach in limited resource settings to make a differential diagnosis of CHIKF. This criteria may be particularly useful during the earliest stages of CHIKF outbreak to help health workers to identify potential suspected CHIKF patients and refer them for further laboratory testing for confirmation as lack of access to accurate rapid diagnostics creates a challenge [40,41]. Furthermore, serological diagnosis requires assessment of titers in convalescent samples hence creates minimal value for acute treatment decisions particularly during outbreaks. These challenges highlight the requirement for CHIKV RNA or antigen based rapid diagnostic testing to drive clinical decision making [41].

The present study showed that cases of CHIKF reported here, were recent ones through detection of IgM anti- CHIKV antibodies. By detecting anti-CHIKV IgM specific antibodies, the results suggest a possibility of acute infections. IgM antibodies appear first and can be detected during the first week of the disease [42]. Detection of anti-CHIKV IgM antibodies indicates that CHIKV is prevalent and contributes to the burden of febrile illnesses in Mzuzu city, Malawi. The results from this study suggest that for a long time, CHIKV as the aetiology of febrile illnesses in northern Malawi was not recognized and was incorrectly diagnosed since CHIKF were not differentiated at the health facility the participants were seeking healthcare. This might have led to incorrect management of the patient that resulted in high morbidity of unknown origin.

Our study supports the results published elsewhere that malaria is no longer a huge public threat but other aetiologies of fevers have emerged that are responsible for high morbidity in Sub-Saharan countries [43]. The absence of the sero-epidemiological data of other aetiologies of fevers hampers the proper management of patients and is the main reason why many febrile illnesses are over diagnosed as malaria in Malawi [29]. The detection of CHIKV in this study as an aetiology of febrile illness supports the latter and the need for a continued surveillance of other aetiologies of febrile illnesses such as CHIKV in Malawi.

Limitations of the study

The study encountered some limitations. First, we recruited a small sample size which may have underestimated the true prevalence of CHIKF. Secondly, only febrile patients were targeted which probably may have underestimated the true seroprevalence of CHIKF. Lastly, Malaria positive samples were not tested for anti-CHIKV IgM antibodies and we propose that future research should include malaria positive samples to elucidate possible co-infections which may give overall seropositivity rate in Malawi.

Conclusion

Overall, this study has proved the hypothesis that CHIKV is prevalent in Northern Malawi, and that people have been exposed to the virus. Therefore, clinicians, should consider CHIKF as a differential diagnosis in febrile patients especially when other common causes such as malaria have been excluded.

Abbreviations

SACIDS: Southern African Centre for Infectious Disease Surveillance; CHIKF: chikungunya fever; CHIKV: Chikungunya virus; SUA: Sokoine University of Agriculture; ELISA: Enzyme-linked

immunosorbent assay; IgM: Immunoglobulin M; MCH: Mzuzu Central Hospital; RT-PCR: Reverse transcription- polymerase chain reaction; UNIMA: University of Malawi.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Availability of data and materials

The dataset analyzed during the current study are available from the corresponding author on a reasonable request.

Ethical considerations

Ethical approval to conduct the study was obtained from the College of Medicine Research and Ethics Committee (COMREC) (P.02/20/2956). All study participants were consented and assented by the parents and guardians to participate in the study. The records of all participants were documented through unique identification numbers for anonymity.

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