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Diagnosis of Chikungunya Virus in Febrile Patients From a Malaria Holoendemic Area

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ABSTRACT

Introduction: Accurate diagnosis of chikungunya (CHIK) is essential for effective disease management and surveillance. In a cohort of febrile Congolese patients, available diagnostic methods widely used in CHIK diagnosis were evaluated. In addition, plasma cytokines were quantified in CHIK patients and those coinfected with malaria compared with healthy controls.

Methods: Between June and November 2019, a total of 107 febrile patients with suspected CHIK were subjected to differential diagnosis both for CHIK and malaria. Patients were screened for CHIK virus using molecular diagnosis by real-time PCR, serologic testing by IgM-specific and IgG-specific ELISAs, and lateral flow-based method with rapid diagnostic test (RDT), while malaria diagnosis was confirmed by PCR methods. Pro-inflammatory (IL-12, IL-16, IFN-γ, TNF-α) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines were quantified in patients and healthy controls by ELISA assays.

Results: Molecular diagnoses revealed that 57% (61/107) were positive for CHIK by RT-PCR, while serologic testing revealed 31% (33/107) and 9% (10/107) seropositivity for anti- IgM and IgG, respectively. None of the patients were CHIK RDT-positive. Also, 27% (29/107) were PCR-positive for malaria. Among the malaria-positive patients, 14% (15/107) were co-infected with CHIK and 13% (14/107) were monoinfection. Plasma IL-12 and TNF-α levels were increased in patients with malaria and IL-13 levels were increased in patients with co-infection ($p<0.05$).

Conclusion: Co-infection of malaria and CHIK were common in febrile Congolese patients. Real-time PCR was a better tool for detecting actual occurrences of CHIK in a malaria holoendemic area.

Introduction

Chikungunya (CHIK), caused by the Chikungunya virus (CHIKV), is a vector-borne disease primarily transmitted by an infectious bite from *Aedes aegypti* or *Aedes albopictus* mosquitoes. While the disease sporadically occurs in Asia and Africa, outbreaks are also

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reported in Europe and America (Harapan *et al*., 2019), with an estimated 3 million infections each year. The onset of the disease usually occurs 4-8 days later and is characterised by an abrupt onset of fever, often accompanied by joint pain, muscle aches, joint swelling, headache, nausea, fatigue, and rash (Schilte *et al*., 2013; Hoarau *et al*., 2010). The Republic of Congo is a holoendemic area for malaria (Koukouikila-Koussounda and Ntoumi, 2016), where CHIKV infections are often misdiagnosed as malaria due to overlapping symptoms. In Congo, a differential diagnosis of CHIKV is often not considered in the clinical setting. Early diagnosis of CHIKV should support effective case management, especially reducing the frequency of misdiagnosis and mistreatment with antibiotics or malaria drugs (Joshi *et al*., 2008; Sow *et al*., 2016). Early reporting with appropriate testing and treatment can complement community tracking and early vector control, and thus reduce potential regional spread.

The WHO recommends both serological and nucleic acid tests in suspected CHIKV patients within the first week of symptoms (WHO 2017). The reference laboratory in Congo relies on IgM/IgG ELISA-based diagnosis of CHIKV. However, cross reactivity with other arboviruses, failure in discriminating an acute and past infection, with a high rate of false positivity cannot be excluded by ELISA-based diagnostic methods. Low sensitivity is well recognised in ELISA-based diagnosis of acute CHIKV cases compared with nucleic acid testing (Blacksell *et al*., 2011). A study from Singapore has additionally documented the limitation of ELISA, which failed to detect an acute infection caused by a different strain in a second outbreak (Yap *et al*., 2010).

Studies have confirmed that co-infections of CHIKV and malaria occur among African populations (Waggoner *et al*., 2017; Ayorinde *et al*., 2016; Kinimi *et al*., 2018; António *et al*., 2018; Sow *et al*., 2016). The host's ability to control an early infection largely depends on immune mediators, especially pro-inflammatory and anti-inflammatory immune responses (Clark *et al*., 2008). Early infection with *Plasmodium* parasites favours CHIKV clearance and is associated with an increase in Th1 and pro-inflammatory immune responses, in particular elevated interferon gamma (IFN-γ) levels (Theo *et al*., 2018).

Given the limitations in diagnosing CHIKV with ELISA-based methods, this study aimed to compare rapid diagnostic test (RDT), ELISA, and nucleic acid tests based on real-time PCR. In addition, inflammatory cytokines that modulate immune responses, including Interleukins (IL) IL-4, IL-10, IL-12, IL-13, IL-16, tumour necrosis factor-alpha (TNF-α), and IFN-γ were quantified in 102 patients with acute CHIKV infection and with malaria coinfections.

Materials and Methods

Ethical approval

This study was approved by the independent and institutional ethics committee of the Fondation Congolaise Pour la Recherche Médicale (FCRM). The samples from all patients and healthy controls were collected after signed informed consent and assent from the participant and/or guardian, respectively. Confidentiality of data was ensured.

Study population

In February 2019, the Republic of Congo officially reported on a CHIK outbreak (WHO 2019) and 37 suspected CHIK cases were reported by June 2020 (ECDC 2020). The samples were collected as a prospective cross-sectional study in the months June to November 2019, during the outbreak of CHIKV disease. The study also used retrospective samples confirmed with CHIKV infection by the National Laboratory of Public Health (LNSP). The study population consisted of patients with fever and suspected of CHIKV (aged 1-89 years) (n=107) who presented at health centres in the provinces of Pointe-Noire, Dolisie and Kouilou in the Republic of Congo. Clinical data of patients were anonymised. In addition, blood samples (n=23) were collected from age-matched and consenting healthy individuals without symptomatic and chronic disease who were examined during the epidemic survey and enrolled as healthy controls. Venous blood samples (10 mL) were collected from consenting patients and healthy controls were bio banked at Centre de Recherches sur les Maladies Infectieuses-Christophe Mérieux (CERMI-CM) at -80°C until further use.

Screening for malaria parasites

Microscopy: Giemsa-stained thick films were examined under the microscope. Asexual parasites were counted against 200 white blood cells (WBCs) and expressed as the number of asexual parasites/μL of blood, assuming a WBC count of 8,000/μL of blood.

Molecular detection: DNA from the whole blood was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Multiplex PCR targeting the *18S rRNA* gene was performed as described earlier (Padley *et al.,* 2003). In brief: a single reverse primer, conserved for *Plasmodium* spp. PFor: 5′-GTATCTGATCGTTCTCTTCACTCCC-3′ and specific reverse primers *Plasmodium falciparum* (PfR: 5′ AACAGACGGGTAGTCATGATTGAG-3′), *Plasmodium vivax* (PvR: 5′-CGGCTTGGGAAGTCCTTGTGT-3′), *Plasmodium ovale* (PoR:5′-CTGTTCTTTGCATTCCTTTATGC-3′), and *Plasmodium malariae* (PmR:5′-CGTTAAGAATAAACGCCAAGCGCG-3′) were used. PCR was performed on a thermal cycler using HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany). Cycling conditions were initial denaturation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, with a final elongation at 72°C for 5 minutes. Positive and negative controls were included for each run. Amplicons were separated on a 1.2% agarose gel electrophoresis with a 1000 bp DNA ladder (Invitrogen, Karlsruhe, Germany). The plasmodial species was confirmed with representative amplicon size: 276 bp for *P. falciparum*, 300 bp for *P. vivax*, 375 bp for *P. ovale*, and 412 bp for *P. malariae.*

Diagnosis of CHIKV using real-time PCR

Chikungunya viral RNA was isolated using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Subsequently, real-time PCR was performed using RealStar® Chikungunya RT-PCR Kit 2.0 (Altona Diagnostics, Hamburg, Germany) following manufacturer's instructions.

Diagnosis of CHIKV using IgG and IgM assays

CHIKV-specific IgM/IgG antibodies were quantified using the IgM and IgG capture ELISA kit (IBL International, Hamburg, Germany) following manufacturer's instructions. The diagnostic specificity and sensitivity were >90%.

Diagnosis of CHIK using rapid diagnostic test

The rapid diagnosis of CHIKV was performed using STANDARD F Chikungunya IgM/IgG FIA kit (SDBIOSENSOR, Gyeonggi-do, Republic of Korea) that qualitatively analyses CHIKV-specific IgM and IgG antibodies using fluorescent immunoassay (sensitivity 94.3% and specificity 97%).

Cytokine measurement

The interleukins (IL) IL-4, IL-10, IL-12, IL-13, IL-16, tumour necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) were

Table 1
Prevalence of chikungunya infection by rtPCR and ELISA.

Characteristics	Study subjects n=107	Positive by RT-PCR n=61	ELISA Positive IgM (n=33)	ELISA Positive IgG (n=10)
Age in years				
01-20 (%)	31 (29)	18	10	4
21-80 (%)	76 (71)	3	23	6
Mean age (SD)	32.7 (±18.5)			
Gender				
Female	68 (63.5)	40	20	5
Male	39 (36.5)	21	13	5
Groups				
Malaria	29 (27)	15	8	4
No malaria	78 (73)	46	25	6
Province				
Pointe-Noire	47 (44)	22	18	0
Kouilou	60 (55)	39	15	10

quantified with the human IL-4 DuoSet ELISA, Human IL-10 DuoSet ELISA, Human IL-12 p70 DuoSet ELISA, Human IL-13 DuoSet ELISA, Human IL-16 DuoSet ELISA, Human TNF-α DuoSet ELISA, Human IFN-gamma DuoSet ELISA (R&D Systems Inc. Minneapolis, USA) were tested according to the manufacturer's instructions on an Infinite® F50 Absorbance Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). The detection limit for the cytokines tested was 31.2 pg/mL (IL-4, IL-10, IL-12), 93.8 pg/mL (IL-13), 15.6 pg/mL (IL-16 and TNF-α), and 9.4 pg/mL (IFN-γ), respectively.

Statistical analysis

The data were analysed using SPSS statistical software (SPSS ver. 24, Chicago, Illinois, USA). Non-parametric Kruskal Wallis and Mann-Whitney tests were computed for observing significant difference in distributions between two or more groups. The level of statistical significance was set at $p<0.05$.

Baseline characteristics of study participants

A total of 107 samples were analysed in this study. The baseline characteristics of the study participants are summarised in Table 1. All febrile patients were aged between 1-83 years, with a mean age of 32.7 years (±18.5), and 63.5% were female. The febrile patients were predominantly from the provinces of Pointe-Noire (44%) and Kouilou (55%). The healthy controls were also enrolled from these two provinces (n=23).

Diagnosis of CHIKV by RT-PCR, ELISA and RDT

A total of 107 samples were analysed by all three methods: 61/107 (57%) were positive for CHIKV by real-time PCR. ELISA results indicated that 33/107 (31%) were positive for IgM and 10/107 (9.3%) were positive for IgG. None of the samples were RDT positive. A high incidence of positivity by real-time PCR was observed in the 1-20 years age group, with no statistical significance. CHIKV infection was more widespread in the province of Kouilou (36%) than in Pointe-Noire (21%).

Diagnosis of malaria by PCR

All 107 febrile patients were also screened for malaria and 29/107 (27%) were positive (Table 1). Of these 29 malaria-positive patients, 14 (13%) were mono-infected with malaria and 15 (14%) were co-infected with CHIK and malaria (Table 2).

Plasma pro-inflammatory and anti-inflammatory cytokines

A total of 78 plasma samples were quantified for IL-4, IL-10, IL-12, IL-13, IL-16, TNF-α, and IFN-γ by ELISA. The measurements were performed in laboratory-confirmed CHIK mono-infections (n=26), CHIK and malaria co-infections (n=15), malaria mono-infections (n=14), and healthy controls (n=23). The mean plasma concentrations of cytokines of the four groups are shown in Figure 1.

The concentration of IL-4 was significantly higher in both the malaria/CHIK co-infection group and the CHIK mono-infection group than in the asymptomatic malaria group (p=0.03) and (p=0.03), respectively (Figure 1A). In contrast, the concentration of IL-10 was not significantly higher in the asymptomatic malaria group than in the CHIK group (p=0.05) and in the asymptomatic malaria/CHIK group (p=0.07) (Figure 1B). IL-13 was significantly higher in the asymptomatic malaria/CHIK co-infection group than in the asymptomatic malaria group (p=0.04) and slightly higher in the asymptomatic malaria group than in the CHIK group at a near significant level (p=0.05) (Figure 1C). IL-12 was significantly higher in the asymptomatic malaria-infected than in the asymptomatic malaria/CHIK coinfection and CHIK groups (p<0.001) (Figure 1D). The difference in IL-16 plasma cytokine levels in the asymptomatic malaria group did not reach a significant level compared with the asymptomatic malaria/CHIK coinfection group (p=0.09), but was significant compared with the CHIK group (p=0.03) (Figure 1E). IFN-γ levels in the asymptomatic malaria/CHIK and CHIK groups were significantly higher than in the asymptomatic malaria group (p<0.001) (Figure 1F). In contrast, TNF-α levels were higher in the asymptomatic malaria group compared with both asymptomatic malaria/CHIK and CHIK groups (p<0.001 and p<0.001, respectively) (Figure 1G). Compared with the healthy control group, the concentration means of: IL-13 and IL-12 were significantly higher; IL-10 and IL-16 were higher but not statistically significant; IFN-γ and TNF-α were statistically higher than the control group; and IL-4 was less in the malaria group than in the control group (Figure 1).

Discussion

This study compared, by ROC, three diagnostic methodologies (RT-PCR, ELISA, and RDT) for detecting CHIK in patients suspected with CHIKV infections. In addition, plasma cytokines in patients diagnosed with CHIK, malaria, and CHIK co-infection with malaria were evaluated.

The malaria positivity by PCR reflecting submicroscopic infection was only seen in the febrile subjects. As is known for malaria, recently treated patients can be PCR-positive for several weeks. Therefore, the positivity of PCR in febrile subjects can be interpreted as a recent malaria infection (Stoler and Awandare, 2016). Indeed, the history of malaria infection was not recorded in this study. The coinfection rate of 14% was higher than those observed in Cameroon (8.95%) (Borris *et al.*, 2021). This discrepancy could reflect the different geographical and population backgrounds.

Table 2
Prevalence of malaria infection, using PCR, among febrile and afebrile patients.

	Total subjects N=107	MP by PCR, N (%)	MP and chikungunya, N (%)	MP only, N (%)
Febrile patients	84	29 (27.0)	15 (14.0)	14 (13.0)
Non-febrile patients	23	0 (0.0)	0	0

MP = Malaria-positive individuals

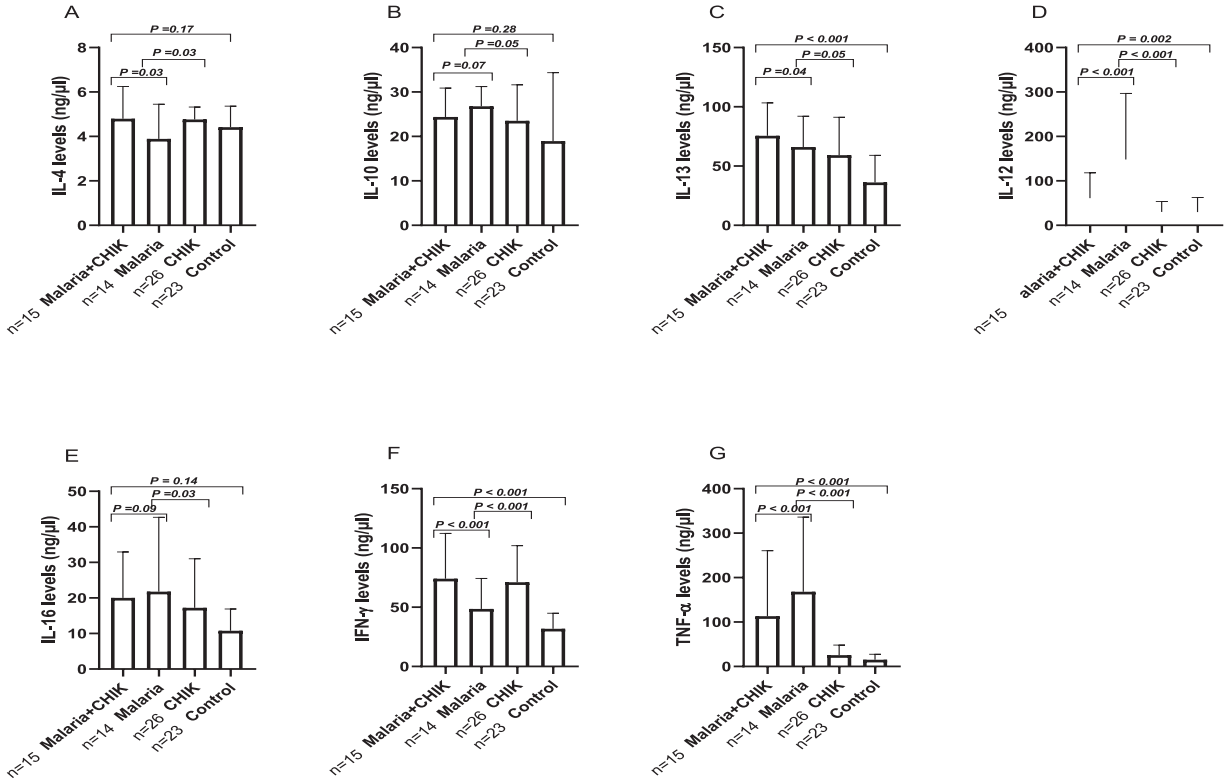

Figure 1. The mean plasma concentrations of cytokines of the four groups: A, concentration of IL-4; B, concentration of IL-10; C, concentration of IL-13; D, concentration of IL-12; E, concentration of IL-16; F, IFN-γ level; G, TNF-α level.
The top of each band shows the average of the interleukin's concentration or the mean of IFN-γ and TNF-α level. The top of the bar is the maximal value of the cytokines.

In the present study, RT-PCR was found to be a sensitive method for early diagnosis of CHIK compared with IgM, IgG ELISA and RDT. The sensitivity and specificity of IgM and IgG ELISA were >90%, according to the manufacturer. Studies have demonstrated high sensitivity of RT-PCR over IgM, IgG ELISA, and RDT methods in diagnosing CHIKV chronic and acute infections (Natrajan *et al.*, 2019; da Cunha and Trinta, 2017; Edward *et al.*, 2017; Panning *et al.*, 2009). None of the serologic tests demonstrated acceptable sensitivity or specificity, confirming previous reports claiming that and their use should be limited to epidemiologic purposes (Vairo *et al.*, 2020). No RDT was found to be positive for CHIK in the current study. Avoiding speculation of the quality of used RDT, false negative RDTs for CHIK have been reported elsewhere (Prat *et al.*, 2014).

The current Congolese participants presented low sensitivity of anti-CHIKV IgM that could be explained by samples probably collected before the fifth day of infection and before the development of anti-CHIKV IgM. It is known that IgM antibodies to CHIKV appear earlier than IgGs, and IgM antibodies to CHIKV are formed 5-7 days after symptoms (Johnson *et al.*, 2016; LoPresti *et al.*, 2014) and anti-CHIKV IgG detection a few days later (LoPresti *et al.*, 2014).

Even though cytokines are produced by the body in response to microbial invasion as a defence strategy, uncontrolled inflammation triggered by cytokines (pro-inflammatory cytokines) such as TNF-α, IFN-γ, IL-16, and IL-12 promotes severity of infection, organ failure and mortality (Shimizu 2020; Schernthaner *et al.*, 2017; Teo *et al.*, 2018; Ragab *et al.*, 2020). In contrast, anti-inflammatory cytokines such as IL-10, IL-4, and IL-13 attenuate pro-inflammatory cytokines, preventing the severity of infection and organ failure (Wojdasiewicz *et al.*, 2014; Venugopalan *et al.*, 2014; Teo *et al.*, 2018).

Significantly elevated levels of the anti-inflammatory IL-13 in the CHIK/malaria co-infection group compared with the other groups were reported in the present study. Furthermore, IL-10 was higher in the asymptomatic malaria group, and IL-4 was higher in the co-infection of CHIK/malaria group, but differences were insignificant. TNF-α and IL-12 were both significantly higher in malaria than other groups, and IFN-γ was significantly elevated in CHIK. Several studies have reported elevated levels of inflammatory cytokines in malaria infection in human and animal models (Ateba-Ngoa et al., 2015; Farrington et al., 2017; Clark and Vissel, 2017; Liehl et al., 2014; Miller et al., 2014; Theo et al., 2018). Anti-inflammatory (IL-10, IL-13) and pro-inflammatory (IL-12, IL-16, TNF-α) cytokine levels were significantly higher in *Plasmodium falciparum*-infected patients than in CHIKV-infected patients; however, cytokines (IL-4 and IFN-γ) were significantly higher in CHIKV-infected patients than in malaria-infected patients. These results suggest that acute malaria infection is characterised by elevated levels of a broad spectrum of cytokines, whether in the Th2 group (IL-10, IL-13) or Th1 group (IL-12, IL-16, TNF-α).

It is well established that Th1 pro-inflammatory cytokines play an important role in eliminating infectious agents and are typically induced during acute asymptomatic malaria infection. Th1 pro-inflammatory cytokines, TNF-α, and IFN-γ inhibit pathogen progression and stimulate the process of phagocytosis. Studies in mice infected with malaria parasites and CHIKV have demonstrated that pro-inflammatory cytokines have an anti-viral role (Teo *et al*., 2013; Teo *et al*. 2018). Concurrent infection of CHIKV with asymptomatic malaria in the current study may have had a positive effect on CHIKV by suppressing viral replication.

This study had some limitations. Microscopic examination was not performed to determine the *Plasmodium* species and the intensity of infection. It was not possible to determine whether the subjects had undergone treatment for malaria prior to specimen collection. The viral copies number of CHIKV and parasite density of *Plasmodium* spp. were not determined, which would have enabled demonstration of the parasite density versus cytokine concentrations in mono and co-infection with CHIK. The parasite density and concentration of viral copies may have influenced the levels of the cytokines. Nevertheless, profiles of cytokines in asymptomatic malaria mono and co-infection with CHIK compared with healthy controls we were able to be presented.

Conclusion

This study reported prevalence of CHIK and malaria in febrile Congolese patients. RT-PCR has been shown to be a useful diagnostic tool for detecting acute and early phase of CHIK infection. It is suggested that the elevated levels of pro-inflammatory cytokines in asymptomatic malaria infection may have inhibited CHIK viral replication. This assertion may be further explored when considering CHIKV intervention programs in endemic areas for malaria and CHIK.

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Conflict of 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.

Authors contributions

LLI, SMP, and NH were involved in the field work. LLI and AA performed lab analyses. FV, AA,AZ, RK EN, SRP, and TPV contributed in the data analysis. FN was responsible for the study. All authors were involved in writing the paper.

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