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Mariangela l'Episcopia, Cécile Doderer-Lang, Edvige Perrotti, Giovanni Battista Priuli, Serena Cavallari, et al.. Polymorphism analysis of drug resistance markers in *Plasmodium falciparum* isolates from Benin. *Acta Tropica*, 2023, 245, pp.106975. 10.1016/j.actatropica.2023.106975 . hal-04224099

HAL Id: hal-04224099

<https://hal.science/hal-04224099>

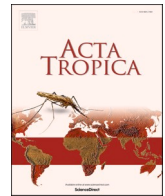
Submitted on 19 Oct 2023

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Polymorphism analysis of drug resistance markers in *Plasmodium falciparum* isolates from Benin

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ARTICLE INFO

Keywords:

Plasmodium falciparum
Malaria
Drug resistance
ACT
kelch 13
Targeted amplicon deep sequencing

ABSTRACT

Like most countries in sub-Saharan African countries, Benin continues to bear a heavy malaria burden. In 2014, the National Malaria Control Programme (NMCP) changed its treatment policy, and recommended the use of artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated *Plasmodium falciparum* cases. The study presented here was conducted to investigate the impact of current antimalarial drug resistance on the country. Molecular surveillance targeting the *Pfcr*, *Pfmdr1*, *Pfkelch13*, *dhfr*, and *dhps* genes was carried out on samples from patients positive for *P. falciparum* malaria by microscopy, LAMP and PCR diagnostic test. Molecular analysis was performed using targeted amplicon deep sequencing (TADS). In addition, the frequency of parasites with dual deletion of the histidine-rich protein 2 and 3 genes (*pfrp2* and *pfrp3*), known to be responsible of the performance of HRP-based malaria rapid diagnostic tests (HRP-RDT), was estimated. Fifty-three *falciparum* samples collected at the Saint Jean de Dieu hospital in Tanguiéta, Benin, were tested. No *Pfkelch13* validated or candidate artemisinin partial resistant variants were identified. A marked prevalence of Asn511Ile (N511I), Cys59Arg (C59R), and Ser108Asn (S108N) mutant alleles was found in the *dhfr* gene, representing the most frequent genotype (64%). Five-point mutations were detected in *dhps*, Ile431Val (I431V), Ser436Ala (S436A), Ala437Gly (A437G), Ala581Gly (A581G), Ala613Ser (A613S) of which the third was the most common (92%). No mutation was identified in *dhps* Lys540Glu (K540E). The quintuple mutant genotype resulting from the combination of the *dhfr* triple mutant (511/59R/108N) with the *dhps* double mutant 436A/437G was detected at a frequency of 30%. Low levels of mutations in *Pfcr* and no mutation at codon 86 in the *Pfmdr1* DNA fragment were observed, whereas a high level of Tyr184Phe (Y184F) polymorphism in the *Pfmdr1* gene was found. These results could be indicative, over a decade after the implementation of ACT therapy, of the return of chloroquine-sensitive but artemether-lumefantrine resistant *falciparum* genotypes in Benin. There was no evidence of HRP2 and HRP3 deletions. Data from the present study support the need for routine monitoring of molecular markers of antimalarial drug resistance as part of surveillance activities aimed to make informed treatment policy decisions at the national level.

1. Introduction

Despite an encouraging reduction in malaria incidence in 2015, the baseline year of the Global Malaria Technical Strategy (GTS) 2016–2030, no reduction in case and death rates was reported in 2021. According to the latest World Malaria Report (WHO, 2022 a), 247

million malaria-related cases and 619,000 related deaths were reported worldwide, mainly in children under five years of age. Although the COVID-19 pandemic was responsible for an estimated increase of more than 13 million cases due to disruptions in prevention, diagnosis, and treatment, new threats, such as the emergence of artemisinin partial resistance (ART-R) in Africa (WHO, 2020), can jeopardized progresses

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<https://doi.org/10.1016/j.actatropica.2023.106975>

Received 5 April 2023; Received in revised form 16 June 2023; Accepted 19 June 2023

Available online 20 June 2023

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made to date in the fight against malaria. ART-R, defined as delayed parasite clearance after treatment with an artemisinin derivative, has been associated with the presence of point mutations in the gene *kelch13* encoding the Kelch protein in *P. falciparum* (PF3D7_1343700).

In Benin, malaria still represent the major cause of mortality in children and morbidity among adults. Benin accounts for about 2% of malaria cases and deaths globally (WHO, 2022a). In 2021, nearly 3 million indigenous *P. falciparum* cases and about 3000 deaths were reported. Compared to 2015, there was no difference in case incidence in 2021 (< 5% increase or decrease) in the country. Although Benin did not achieve the milestones for mortality in the GTS 2021, it did achieve a reduction in the mortality rate of less than 40% (WHO, 2022a). In line with the WHO recommendations, the Beninese health authorities introduced artemether-lumefantrine (AL), an artemisinin combination therapy (ACT), for the treatment of uncomplicated *P. falciparum* and artesunate for severe malaria. An additional ACT, artesunate - pyronaridine, is now included in the list of options for the treatment of uncomplicated malaria as an alternative to AL (WHO, 2022b).

The molecular characterization of the *Pfcr* and *Pfmdr1* markers facilitates the prediction of parasite responses to ACT partner drugs (amodiaquine, lumefantrine and mefloquine).

In high to moderate malaria transmission settings, international recommendations suggest the use of chemoprophylactic drugs for high-risk groups of the population, such as pregnant women. Thus, in Benin, the association of the antifolates sulfadoxine-pyrimethamine (SP) is administered as intermittent prevention therapy during pregnancy, and it has been estimated that its coverage increased between 2020 and 2021 in the country (WHO, 2022a; DHS, 2020). Preserving the efficacy of the currently available drugs, ACTs have become crucial given the serious problem of antimalarial drug resistance and the lack of alternative treatment strategies. SP targets the *P. falciparum* folate metabolism pathway in which dihydrofolate reductase (*PfDHFR*) and dihydropteroate synthase (*DHPS*) enzymes act, used as molecular markers related to the *in vitro* drug susceptibility.

The present study assessed the prevalence of known drug resistance genotypes in the *Pfkelch13*, *Pfcr*, *Pfmdr1*, *dhfr* and *dhps* genes in samples collected in Benin from patients who were tested positive for *P. falciparum* malaria by microscopy, LAMP and PCR diagnosis. In addition, deletion of the histidine-rich protein 2 and 3 genes (*Pfhrp2* and *Pfhrp3*), antigenic markers known to render HRP-based malaria rapid diagnostic tests (HRP-RDTs) ineffective, was assessed.

2. Materials and methods

2.1. Study design and sample collection

Samples were collected from May to June 2019 in the department of Atakora, district of Tanguiéta, located in the northwest of Benin (10°37'N/16°E). This region has a sub-equatorial climate with a dry season from December to May, and a rainy season from June to November. The average annual rainfall is 1300 mm with a temperature range of 22 - 34 °C. All patients presenting with clinical symptoms suggestive of malaria were eligible for blood sampling after obtaining informed consent. The study was approved by the Saint Jean de Dieu Hospital of Tanguiéta-Benin (N° 21/22/HSJDT/DH-SA) and written informed consent was obtained from patients and guardians of eligible children. For all blood samples, identifying information have been removed so that data cannot be linked or re-linked with identifiable human subjects, making anonymous each sample processed in the present study. Samples confirmed as positive for *falciparum* malaria by microscopy (thick and thin films) and LAMP (Illumigene®-Malaria, Meridian Bioscience Europe) were included in the study.

The microscopic examination and the LAMP were performed in Benin, at Saint Jean de Dieu Hospital of Tanguiéta-Benin while the PCR, on dried blood spots collected in Benin, was performed in Rome at the ISS laboratories. All patients were treated with ACT (Artemether /

Lumefantrine) in line with the country's drug policy.

2.2. Nested PCR

Genomic DNA (gDNA) isolation of all samples from two punches of 3 mm dried blood spot was performed using the Purelink Genomic DNA Kit protocol by Invitrogen, eluted in a 100 µl final volume of EB and stored at -20 °C for molecular diagnosis confirmation by nested PCR targeting the 18 s rRNA gene of *Plasmodium* spp. (Snounou and Singh, 2002). PCR amplification was carried out with 10 µl extracted gDNA and 5 µl of primary amplicon for the primary and nested PCR, respectively, using the Gotaq Green Master mix (Promega). Parasite DNA from 3D7 laboratory strain was included in each run as a positive control, and sterile water was used as a negative control.

2.3. Assessing molecular markers associated with antimalarial drug resistance

The eluted DNA was quantified by fluorimetry (Qubit, Thermo Fisher), adjusted to 20 ng/ml and stored at -20 °C. DNA was then analyzed for the presence of point mutations in the *Pfkelch13* propeller domain (codons 430–720) associated with ART-R, in *Pfcr* (at codons 72–76, 93, 97, 145, 218, 343, 350 and 353), *Pfmdr-1* (at codons 86, 184, 1034, 1042 and 1246) genes associated with or suspected of 4-aminoquinoline and amino alcohol resistance, *dhfr* (at codons 16, 51, 59, 108, 164) and *dhps* (at codons 431, 436, 437, 540, 581, 613) genes associated with pyrimethamine and sulfadoxine resistance (WHO, 2020). Primer sequences used for the amplification of the target genes are from Koko et al. (2022).

The target sequences were amplified using multiplexed nested PCR assays with indexed primers, which themselves contain specific tags (barcodes) consisting of individual 8-base indices specific to the sample and adapter sequences (14 or 15 bases) that allow the final PCR product to bind to the sequencing flow cell. A total of 4 µl of PCR reactions from each sample were pooled (96 samples). This was done to increase the sample volume and reduce the amount of sample for downstream steps in the protocol. For each pool, amplicons were then purified using AMPure XP beads (Beckman Coulter) according to the manufacturer's protocol to eliminate dNTP, salts, primers and primer dimers. The eluates containing the purified amplicons were analyzed on a fragment analyzer (Agilent) to assess the quality of the purified PCR products. DNA concentration in the pooled fragments was assessed by fluorometric quantification (Qubit, Thermo Fisher). Pooled libraries were denatured with NaOH to a final concentration 0.1 N and diluted with hybridization buffer before sequencing. Sequencing reactions were carried out with an Illumina MiSeq instrument using paired-end 2 × 150-bp reads (MiSeq Reagent Kit v2 – 300 cycles).

Raw sequences were demultiplexed and quality trimmed with phred score 30 to avoid primer bias. The primer sequences were trimmed from the 5' ends of the sequences to avoid primer bias in the sequenced fragments. Sequences were compared to a custom database comprising the 3D7 reference sequence to call bases. The CLC Genomics Workbench 22 software package (Qiagen) was used for bioinformatics analysis. Laboratory reference parasite strains (Dd2, 7G8, HB3 and Cambodian culture adapted) with known alleles in each gene were used as controls. We considered as interpretable only those samples for which we had an acceptable number of reads (>x10) for each of the targeted genes. Parasites with mixed alleles (in which both wild-type and mutant alleles were present) were considered mutants.

In order to detect a deletion of the *hrp2* or *hrp3* genes, we compared the coverage of each target gene with the coverage of the amplicons generated for *hrp2* and *hrp3*. If the coverage of the *hrp2* or *hrp3* gene was zero or <1% relative to the coverage of the other target genes, we considered the gene to be deleted. In addition, for each plate, we tested control strains for which the presence or absence of *hrp2* and *hrp3* deletions was known (Cambodian strain 3601 and strain 7G8 without

deletions, strain Dd2 with an *hrp2* deletion and strain HB3 with an *hrp3* deletion). We used 2 pairs of primers targeting exon 2 of the *hrp2* and *hrp3* genes.

3. Results

3.1. Data patient analysis

This study included 107 blood samples obtained from patients attending the Saint Jean de Dieu hospital in Tanguiéta, Benin. The study population consisted of 71 female and 36 male patients aged between 5 months to 36 years with a fever of ≥ 37.5 °C. Nested-PCR detected 56 (52.3%) *Plasmodium* positive samples, of which 52 (92.8%) were identified as *P. falciparum*, 1 (1.8%) as *P. ovale*, 1 (1.8%) as *P. vivax* and 1 (1.8%) as *P. malariae*, and one sample (1.8%) was detected as a mixed species infections (*P. falciparum*/*P. ovale*).

3.2. Polymorphisms in antimalarial resistance markers

Among 53 *P. falciparum* isolates confirmed by nested PCR, we identified key mutations in the *Pfcr*, *Pfmdr1*, *Pfkelch13*, *Pfdhfr*, and *Pfdhps* genes (Table 1). Three parasite isolates had the CVIET mutant genotype at codon position 74–76, one of which also harbors the 356T. Two isolates were characterized by the SVMNT mutant genotype, rarely reported from the African continent.

For *Pfmdr1*, the Y184F mutation was highly prevalent at 68% (Table 1).

Sequencing of the antifolate resistance gene *Pfdhfr* showed the N51I, C59R, and S108N mutations close to fixation with a prevalence of 76%, 91%, and 83% respectively (Table 1), with 64% of the triple mutant genotype IRN (Table 2).

In the *Pfdhps* gene, the I431V and A581G were both identified in one sample whereas the A613S mutation was present in 4 different samples. The non-synonymous polymorphisms S436A and A437G had a prevalence of 64% and 92% (Table 1) making IAGAA the most predominant genotype at 49% (Table 2). Combining the two loci *dhfr/dhps*, the frequency of parasites carrying the highly resistant quadruple mutant genotype *dhfr* 51I, 59R, 108N + *dhps* 437G was 21%, followed by the quintuple mutant *dhfr* 51I, 59R, 108N + *dhps* 436A, 437G observed in 32% of samples (Table 2).

None of the successfully obtained sequences of the propeller domain in the *Pfkelch13* gene carried any of the validated or candidate mutations associated with artemisinin partial resistance. Sequence alignment revealed that two isolates carried the non-synonymous mutations N629S and N458D in the propeller domain, respectively (Table 1).

Analysis of the histidine-rich protein 2 and 3 genes (*pfhrp2* and *pfhrp3*), known to cause poor performance in HRP-based malaria rapid diagnostic tests (HRP-RDTs), showed no deletion in the samples collected.

4. Discussion

The emergence and spread of drug-resistant *falciparum* isolates is a major public health concern in many regions of the world, particularly in sub-Saharan Africa. In this context, molecular surveillance of drug

Table 2

Frequency of *dhfr*, *dhps*, and *dhfr/dhps* genes in *P. falciparum* isolates from Benin (N = 53). Bold letter indicate mutant amino acid.

| Genotype/Combined Genotype | Type | N° | Frequency (%) |
|----------------------------|-----------|----|---------------|
| <i>dhfr</i> | | | |
| NCS | Wild | 3 | 5,7 |
| NCN | Single | 2 | 3,8 |
| IRS | Double | 6 | 11,3 |
| NRN | Double | 8 | 15,1 |
| IRN | Triple | 34 | 64,1 |
| <i>dhps</i> | | | |
| ISAAA | Wild | 1 | 1,9 |
| IAAAA | Single | 3 | 5,7 |
| ISGAA | Single | 18 | 33,9 |
| IAGAA | Double | 26 | 49,1 |
| IAGAS | Triple | 4 | 7,5 |
| VAGAG | Quadruple | 1 | 1,9 |
| <i>dhfr/dhps</i> | | | |
| NCS/ISAAA | Wild | 0 | 0 |
| NCN/ISAAA | Single | 1 | 1,9 |
| NCS/IAGAA | Double | 2 | 3,8 |
| NCN/ISGAA | Double | 1 | 1,9 |
| NCS/IAGAS | Triple | 1 | 1,9 |
| NRN/ISGAA | Triple | 4 | 7,5 |
| IRS/ISGAA | Triple | 2 | 3,8 |
| IRS/IAGAA | Quadruple | 4 | 7,5 |
| IRN/ISGAA | Quadruple | 11 | 20,7 |
| NRN/IAGAA | Quadruple | 4 | 7,5 |
| IRN/IAAAA | Quadruple | 3 | 5,7 |
| IRN/IAGAS | Quintuple | 3 | 5,7 |
| IRN/IAGAA | Quintuple | 16 | 30,2 |
| IRN/VAGGA | Septuple | 1 | 1,9 |

resistance is critical for monitoring the effectiveness of antimalarial drugs and for guiding malaria treatment policies. Results from this study revealed a high prevalence (64%) of *dhfr* IRN triple genotype (N51I, C59R, S108N) associated with resistance to pyrimethamine (Lozovsky et al., 2009), and a high prevalence of *dhps* mutations associated with sulphadoxine resistance. The majority of isolates carried the A437G variation in the *dhps* gene with a frequency of 92%, and as quadruple mutant, in combination with the IRN *dhfr* genotype, it was detected at 21%. These findings are similar to results obtained in other studies conducted in many African countries (Fagbemi et al., 2020; Boukoumba et al., 2021; L'Episcopia et al., 2021; Dosoo et al., 2022). As showed before, the continued administration of SP as preventive regimens in pregnant women (Intermittent Preventive Treatment, IPTp), in infants (Intermittent Preventive Treatment, IPTi) and as Seasonal Malaria Chemoprevention (SMC) may contribute to the persistence of genotypes conferring SP resistance. It is worth noting that, although the continued SP drug pressure for IPTp and SMC in the country (Hansen et al., 2021), the frequency at which the *dhfr* and *dhps* mutant genotypes were detected in the present study is lower than the one described by Svigel and colleagues (Svigel et al., 2021) in samples from Djougou (Northern Benin) in 2017 (IRN+437G → 21%vs. 50%; IRN+436A/437G → 30% vs. 34%; IRN → 64%vs. 85%). This reduction is somewhat surprising and may not be explained considering a lack of drug pressure since the introduction of SMC in 2018 in the north of Benin with SP/AQ (Benin Malaria Operational Plan FY, 2020) should have contributed to increasing drug pressure in that area with a following rise instead of a

Table 1

Frequency of non-synonymous mutations in *Pfcr*, *Pfmdr1*, *Pfkelch13*, *Pfdhfr*, and *Pfdhps* genes identified in *P. falciparum* isolates from Benin (N = 53). Bold letter indicates mutant amino acid.

| Pf gene | crt | | | | | mdr1 | | kelch 13 | | dhfr | | dhps | | | | | | | |
|--------------|-----|----|----|----|-----|------|-----|----------|-----|------|----|------|-----|-----|-----|-----|-----|-----|--|
| AA position | 72 | 74 | 75 | 76 | 356 | 86 | 184 | 458 | 629 | 51 | 59 | 108 | 431 | 436 | 437 | 540 | 581 | 613 | |
| Wild-type | C | M | N | K | I | N | Y | N | N | N | C | S | I | S | A | K | A | A | |
| Mutant | S | I | E | T | T | Y | F | D | S | I | R | N | V | A | G | E | G | S | |
| N° of Mutant | 2 | 3 | 3 | 6 | 1 | 0 | 36 | 1 | 1 | 40 | 48 | 44 | 1 | 34 | 49 | 0 | 1 | 4 | |
| Frequency% | 4 | 6 | 6 | 11 | 2 | 0 | 68 | 2 | 2 | 76 | 91 | 83 | 2 | 64 | 92 | 0 | 2 | 8 | |

drop in mutation frequency. Presumably, it may be addressed with a difference in the sampling region, Donga (Svigel et al., 2021) to Atakora. Even if a high level of *dhfr* IRN genotype + 437 G mutation in *dhps* (RII), prevalent in Western and Central Africa (Flegg et al., 2022; L'Episcopia et al., 2021), is a predictor of clinical SP treatment failure, the absence of 540E (RII/RIII high level of resistance) and the presence of a handful isolates having the 581 G mutation (RIII highest level of resistance, Ahmed et al., 2004) demonstrates a low circulation level of SP-RII/RIII genotypes. Moreover, our results illustrate that the absence of 540E mutation does not suggest the need for withdrawal of SP in the country since the WHO thresholds of >95% *Pfdhps* 540E and >10% *pfdhps*581G for IPTp were not observed.

As shown in previous studies conducted in countries of West Africa (Maiga et al., 2021; Sisowath et al., 2009; Otienoburu et al., 2016), where the treatment policy for uncomplicated *P. falciparum* is based on the use of artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ), the *in vivo* selection of both *Pfcr* K76 and *Pfmdr1* N86 genotypes (wild types) occur after artemether-lumefantrine treatment. Those findings emerged in the context of chloroquine withdrawal and increased of lumefantrine (LUM) exposure resulting in the *Pfcr* and *Pfmdr1* wild type alleles-selection. Moreover, the N86, 184F, and D1246 genotype in *mdr1*, which is highly predominant, arises after the extensive use of AL in Africa (Sisowath et al., 2016). Our results regarding *Pfcr* and *Pfmdr1* are consistent with these data. In fact, a high level of *Pfcr* K76 and *Pfmdr1* N86 alleles and no rise in the prevalence of AQ-resistant genotype (*pfm* triple YFY: 86Y, 184F, and 1246Y) was detected in *falciparum* isolates circulating in Benin. Considering the emergence of ART-R in eastern Africa (WHO, 2020), and the slight loss of LUM susceptibility linked to a significant increase (> 99%) of *Pfmdr1* N86 alleles in *ex vivo* parasites (Tumwebaze et al., 2021, 2022), the spread of *Pfcr* and *Pfmdr1* wild genotypes may cause concerns about the efficacy of AL even if, to date, it remains broadly effective across Africa, including Benin (Kpemasse et al., 2021). Regarding the *Pfkelch13* marker gene, two non-synonymous mutations (N629S and N458D) were detected at positions not related to ART-R but previously described in Liberia (Yang et al., 2017) and in one patient returning from Ghana (Zhang et al., 2019). Interestingly, the non-synonymous mutation at codon 458 was observed as N→D amino acid change instead of N→Y, which is the allelic variant validated to be associated with delayed parasite clearance after treatment with ART (WHO, 2020). Based on evidence from the missense 3D tool, this mutation, located on the BC loop connecting blades, is not predicted to cause damage to the *PfKelch13* propeller tertiary structure (Tornyigah et al., 2020). Nevertheless, this amino acid variation could be suggestive of genetic changes linked to possible ART-R emergence in Africa due to selective pressures of ACTs use in the country. The *Pfkelch13* mutations recently observed in East Africa and confirmed to be associated with ART-R (WHO, 2020) were not observed in Benin and in other western African countries. In addition, the present study assessed the status of deletion of the histidine-rich protein 2 and 3 genes. In the absence of microscopy, the gold standard approach for malaria diagnosis, the WHO has recommended the easy-to-use RDTs, able to give quick results in about 15 min (WHO, 2022b). Many of these tests are based on the detection of the histidine-rich protein (HRP2) for *Plasmodium falciparum* infection. In 2010 it has been demonstrated for the first time that the detection of parasites lacking the *pfhrp2* and *pfhrp3* genes (Gamboa et al., 2010) or genetic variations linked to the presence of highly polymorphic repeat domains (Baker et al., 2010; Wurtz et al., 2013) could affect RDT performance increasing false-negative results. Since then, several studies have been conducted across endemic countries showing the spread of parasites with such deletions. Considering the important advantages of HRP2-based RDTs in settings where microscopy may be not available or unreliable, the presence of *Pfhrp2/3* deletions in a high transmission area could substantially affect malaria case management, control, and elimination.

In accordance with results presented in an epidemiological study

conducted in a wide geographical region in Central and West Africa of high malaria endemicity (Krueger et al., 2023) including Benin, this report provides evidence that *P. falciparum* infections from Tanguiéta in Benin show complete levels of HRP2 and HRP3 expression, and no mutated parasites were detected, which should not cause RDT negative results.

In conclusion, our study provides an update on the current state of antimalarial resistance in an area of northern Benin. Moreover, these findings support the need for routine reporting activities as part of malaria control programs aimed at preventing/containing the spread of multidrug-resistant *falciparum* isolates, especially in those endemic African countries where resistance to artemisinin has not yet been identified.

Author statement

All authors, Mariangela L'Episcopia, Cécile Doderer-Lang, Edvige Perrotti, Giovanni Battista Priuli, Serena Cavallari, Carlotta Guidetti, Francesco Bernieri, Didier Menard & Carlo Severini have participated in the preparation, creation and revision of this MS. All authors have read and approved the final version of the MS. We declare that all authors have seen and approved this version of the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

All authors have declared no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

In memoriam of Dr. Joseph Ndasyake, chief technician in charge of the laboratory of the "Saint Jean de Dieu" hospital in Tanguiéta, who sadly passed away in the 2019. These few words are to acknowledge his fundamental contribution to the work carried out at the hospital in the context of this collaboration.

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