Epidemiology of *Plasmodium* spp. infections among school-age children living in rural and urban areas of Kinshasa in Democratic Republic of Congo

By:

NUNDU SABITI SABIN

"Dissertation submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biomedical Sciences"

Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases
Department of International Health and Medical Anthropology
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2018 April – 2022 March

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Supervisor: **Professor Taro Yamamoto**
Department of International Health and Medical Anthropology
Institute of Tropical Medicine
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Dedication

To my wife, BAHATI MUTINGWA Parfaite and my daughter ZABIBU SABITI Gift who have endured my absence for a long period of this training. I feel proud of you!!! This is the crowning achievement of your sacrifice and patience. I finally finished this training, but it is also the beginning of a long scientific and research career. To my elder brother and tutor, YENGULA MAKTUMY Hassan, please find here the result of your investments. I also dedicate this thesis to my mother TATU MWASHITI for her continuous prayers for the success of my training abroad. I can never forget my late father NUNDU SUMAILI for giving me a good education and guidance when he was alive, this is the greatest achievement of your long sacrifice. To my siblings, friends, and colleagues, please find here the results of your encouragements.

Declaration

I hereby declare that the content of this dissertation is my own work and has not been submitted to another University or institution for any award or degree.

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Summary

Background and rationale

Malaria is a threatening tropical disease that still causes morbidity and mortality in middle- and low-income countries despite remarkable efforts done for its control. The recent World Health Organization (WHO) report showed an estimated 229 million cases and 409 000 deaths globally in 2019 of which the Democratic Republic of Congo (DRC) accounted for 12% of cases and 11% of deaths ranged in second place in the world after Nigeria. However, Malaria is preventable and treatable as many patients easily recovered from the infection when correctly treated with effective antimalarial drug or individuals are protected when correctly applied preventive measures. Thus, the promotion of malaria control and surveillance strategy requires political will, inter collaboration of local governments, stakeholders, civil organizations, and research communities. For malaria control and prevention, WHO recommends prompt diagnosis using microscopic examination and/or malaria rapid diagnostic tests (mRDTs), and effective treatment for suspected malaria case management, use of insecticide-treated nets and indoor residual spraying, preventive chemotherapy including chemoprophylaxis for travelers, intermittent preventive treatment for infants (children under 12 months living in high-transmission areas of Africa) and pregnant women living in moderate-to-high transmission areas, seasonal malaria chemoprevention for preschool children living in areas of the Sahel sub-region of Africa and mass drug administration. However, most of these recommendations mostly target children under five and pregnant women.

School-age children are a neglected group, not usually covered by household-based cluster surveys and/or malaria interventions and so represent an untreated demographic

that may harbour a significant parasite reservoir thus posing a major challenge for malaria control, surveillance, and elimination strategies. Even tough school-age children have low risk to develop complicated forms of malaria, they are subjects of chronic malaria with major health consequences including anemia, cognitive disorders absenteeism, poor performance and dropouts. Therefore, the understanding of the burden of malaria among school-aged children using school-based malaria survey is essential to justify the impact of capturing schools and school-age children in national malaria control program on malaria control and elimination strategies in the country. Also, the evaluation of PfHRP2-based RDTs, the mostly diagnostic method used, and continuous molecular evaluation of the current antimalaria drugs as well as the evaluation of the current national malaria control strategy will significantly contribute to the improvement of malaria control policy decision-making in the country.

Objectives

The **main objective** of this thesis was to collect and analyse data that may contribute to an improved malaria control strategy in the DRC. The **specific objectives** were a) to determine malaria parasite species composition of *Plasmodium* infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, DRC; b) to identify the *Plasmodium falciparum* parasites lacking *P. falciparum* histidine-rich protein 2 and 3 (*pfhrp2/3*) genes in isolates collected from them and evaluate the performance PfHRP2-based RDTs; c) to identify polymorphisms in *P. falciparum Kelch* 13 (*pfK13*), multidrug resistance 1 (*pfmdr1*), dihydrofolate reductase (*pfdhfr*), dihydropteroate synthase (*pfdhps*) and chloroquine resistance transporter (*pfcrt*) gene mutations in isolates and d) to identify unmet needs of the current national malaria control

strategy in DRC by showing the importance of capturing schools and school-age children onto national malaria survey useful for malaria control and elimination.

Methods

A cross-sectional study was undertaken between October and November 2019 among school-age children aged 6 to 14 years at primary schools and health facilities in the rural area of Mont-Ngafula 2 Health Zone (HZ) and the urban area of Selembao HZ in Kinshasa, DRC. A total of 634 samples were collected from 427 asymptomatic children in selected schools and 217 symptomatic children in selected health facilities. Microscopy, mRDTs, and filter papers spotted blood were performed in Kinshasa. The DNA samples were extracted in laboratory of Institute of Tropical Medicine at Nagasaki University in Nagasaki city and PCRs were performed for genotyping of *Plasmodium* species, *pfhrp*2/3 and genotyping and sequencing of *P. ovale* spp., *pfk13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt* using specific primers.

Major findings

The overall prevalence of *Plasmodium* spp. was 33%, 42% and 62% among asymptomatic children and 59%, 64% and 94% in symptomatic children by microscopy, RDT and PCR, respectively. In asymptomatic carriages, *P. falciparum*, *P. malariae* and *P. ovale* spp. accounted for 77%, 31% and 17% in rural area and 40%, 9% and 5% in urban settings, respectively. Among symptomatic carriers *P. falciparum*, *P. malariae* and *P. ovale* spp. accounted for 96%, 8% and 18% in rural area and 90%, 18% and 14% in urban settings, respectively. Residence in the rural area was an approximately five times greater risk of asymptomatic carriage of malaria parasites as opposed to the urban area.

The prevalence of the *pfhrp2* gene deletion was 2% while it was 1% for the *pfhrp3* gene among RDT positive results. None of RDT negative result was *pfhrp*2/3 gene deleted. The prevalence of *pfcrt* K76T, *pfdhps* K540E and *pfmdr*1 N86Y was low with 27%, 20% and 9%, respectively. None of the isolates showed artemisinin derivates gene resistance and two new Kelch 13 mutations C532S and Q613E were discovered in the country.

The evaluation of the malaria control policy in the DRC showed that the WHO recommendations are still partially implemented by the national malaria control program in DRC and that could be the root of the delay in reducing the burden of malaria in the country.

Résumé

Contexte et justification de la thèse

Le paludisme est une maladie tropicale qui continue de menacer les pays à revenu intermédiaire et faible, provoquant une morbidité et une mortalité élevées malgré les efforts remarquables déployés pour le contrôler au fil des décennies. Selon le récent rapport de l'organisation mondiale de la santé (OMS), le paludisme était responsable d'environ 229 million de cas et 409 000 décès dans le monde en 2019, dont la République Démocratique du Congo (RDC) représentait 12% des cas et 11% des décès se classant au deuxième rang mondial après le Nigéria.

Cependant, le paludisme est évitable et traitable car de nombreux patients guérissent facilement de l'infection lorsqu'ils sont correctement traités avec un médicament antipaludique efficace, mais aussi les individus sont également protégés lorsqu'ils appliquent correctement les mesures préventives recommandées par l'OMS. Ainsi, la

promotion d'une stratégie de contrôle et de surveillance du paludisme requiert une volonté politique, une inter-collaboration des entités locales, des parties prenantes ainsi qu'un engagement des organisations civiles et des communautés dans la lutte et la prévention de la maladie. Pour contrôler et prévenir le paludisme, l'OMS recommande un diagnostic rapide à l'aide d'examens microscopiques et/ou de tests de diagnostic rapide du paludisme (TDR), un traitement efficace pour la prise en charge des cas suspects de paludisme, l'utilisation de moustiquaires imprégnées d'insecticide et de pulvérisations intradomiciliaires d'insecticide avec effet rémanent ainsi que le traitement préventif pour les personnes vulnérables (enfants de moins de 5 ans, femmes enceintes et voyageurs vivant dans des zones non endémiques de paludisme). Il se fait que dans les zones endémiques du paludisme, la plupart de recommandations de l'OMS ciblent principalement les enfants de moins de cinq ans et les femmes enceintes, et les enfants d'âge scolaire qui sont un groupe négligé par les enquêtes épidémiologiques posant un défi majeur pour les stratégies de contrôle, de surveillance et d'élimination du paludisme car ces enfants d'âge scolaire représentent le réservoir des microbes du paludisme facilitant et maintenant la transmission. Aussi même si ces enfants ne développent pas souvent des formes symptomatiques du paludisme, ils développent des formes asymptomatiques et chroniques de la maladie qui peuvent entraîner des conséquences majeures sur leur santé et les exposer à l'anémie et aux troubles cognitifs avec diminution de la concentration, absentéisme avec comme conséquences un mauvais rendement scolaire et parfois abandons. Par conséquent, comprendre le fardeau du paludisme chez les enfants d'âge scolaire à travers des enquêtes en milieu scolaire est essentiel pour justifier l'impact de leur intégration ainsi que l'intégration des écoles dans le programme national de lutte contre le paludisme, en particulier dans les enquêtes épidémiologiques

nationales qui pourraient renforcer la lutte contre le paludisme et les stratégies d'élimination de la maladie dans le pays. En outre, l'évaluation des TDR basés sur la PfHRP2, la méthode de diagnostic principalement utilisée dans le pays, l'évaluation moléculaire continue des médicaments antipaludiques actuels ainsi que l'évaluation de la stratégie nationale actuelle de lutte contre le paludisme pourraient contribuer significativement à l'amélioration de prise de décision dans la lutte contre le paludisme ainsi que son élimination dans le pays.

Objectifs

L'objectif principal de cette thèse était de collecter et d'analyser des données pouvant contribuer à une meilleure stratégie de lutte contre le paludisme en RDC. Les objectifs spécifiques étaient : a) Déterminer la composition des espèces du parasite responsable de l'infection et la transmission du paludisme auprès des enfants d'âge scolaire asymptomatiques et symptomatiques vivant dans les zones rurales et urbaines de Kinshasa, RDC; b) Identifier les parasites *Plasmodium falciparum* dépourvus de gènes du *P. falciparum* riches en protéines histidine 2 et 3 (pfhrp2/3) dans les isolats collectés auprès des enfants d'âge scolaire et d'évaluer les performances des tests de diagnostic rapide (TDR) basés sur PfHRP2; c) Identifier les mutations génétiques de *P. falciparum* Kelch 13 (pfK13), du gène 1 de *P. falciparum* avec résistance multiple aux antipaludiques (*pfmdr*1), de gène du *P. falciparum* responsable de l'enzyme dihydrofolate reductase (pfdhfr), de gène du *P. falciparum* responsable de l'enzyme dihydroptéroate synthase (pfdhps) et ainsi que les mutations du gène de *P. falciparum* contenant le transporteur de résistance à la chloroquine (*pfcrt*) dans les isolats et d) Identifier les besoins non satisfaits de la stratégie nationale actuelle de lutte contre le paludisme en RDC et montrer

l'importance d'inclure les écoles et les enfants d'âge scolaire dans l'enquête nationale sur le paludisme utile pour le contrôle et l'élimination du paludisme**.**

Méthodes

Une étude transversale a été menée entre octobre et novembre 2019 auprès d'enfants d'âge scolaire âgés de 6 à 14 ans dans les écoles primaires et les formations sanitaires de la zone de santé rurale de Mont-Ngafula 2 et de la zone de santé urbaine de Selembao à Kinshasa, RDC. Au total, 634 échantillons ont été collectés auprès de 427 enfants asymptomatiques dans des écoles sélectionnées et 217 enfants symptomatiques dans des établissements de santé. La microscopie, les TDR et les papiers filtres tachetés de sang ont été réalisés à Kinshasa. Les échantillons d'ADN ont été extraits au laboratoire de l'Institut de médecine tropicale de l'Université de Nagasaki dans la ville de Nagasaki et des PCR ont été effectuées pour le génotypage des espèces de *Plasmodium*, *pfhrp*2/3 et le génotypage et le séquençage de *P. ovale* spp., *pfk*13, *pfmdr*1, *pfdhfr*, *pfdhps* et *pfcrt* en utilisant des amorces spécifiques.

Importants résultats

La prévalence globale de *Plasmodium* spp. était de 33%, 42% et 62% chez les enfants asymptomatiques et de 59%, 64% et 94% chez les enfants symptomatiques par microscopie, TDR et PCR, respectivement. Parmi les porteurs asymptomatiques, *P. falciparum*, *P. malariae* et *P. ovale* spp. représentaient 77%, 31% et 17% en milieu rural et 40%, 9% et 5% en milieu urbain, respectivement. Parmi les porteurs symptomatiques

P. falciparum, *P. malariae* et *P. ovale* spp. représentaient respectivement 96%, 8% et 18% en milieu rural et 90%, 18% et 14% en milieu urbain. La résidence dans la zone rurale présentait un risque environ cinq fois plus élevé de portage asymptomatique des parasites du paludisme par rapport à la zone urbaine.

La prévalence de la délétion du gène *pfhrp*2 était de 2% alors qu'elle était de 1% pour le gène *pfhrp*3 parmi les résultats positifs au TDR. Aucun des résultats négatifs du TDR n'a montré la délétion du gène *pfhrp*2/3.

La prévalence de *pfcrt* K76T, *pfdhps* K540E et *pfmdr*1 N86Y était faible avec respectivement 27%, 20% et 9%. Aucun des isolats n'a montré des mutations validées associées à la résistance génétique aux dérivés de l'artémisinine. Néanmoins deux nouvelles mutations Kelch 13 C532S et Q613E ont été découvertes dans le pays.

L'évaluation de la politique de lutte contre le paludisme en RDC a montré que les recommandations de l'OMS sont encore partiellement mises en œuvre par le programme national de lutte contre le paludisme en RDC et que cela pourrait être à l'origine du retard dans la réduction du fardeau du paludisme dans le pays.

要旨

背景と理論的根拠

マラリアは、その蔓延コントロールのために多くの時間と資源が注がれてきた熱帯病である。一方で、中低所得国では依然として罹患率や死亡率が高く、人類の脅威となっている。World Health Organization（WHO）の最新の報告では、2019 年の 1 年間に 2 億 2900 万人が感染し、40 万 9000 人が死亡したとされている。また、コンゴ民主共和国の感染者数は全世界の 12%を占め、また死亡者数は 11%を占めており、ナイジェリアに次ぐ蔓延国となっている。しかしながら、マラリアは効果的な抗マラリア薬の投与や個人レベルでの予防によって、適切な治療や予防が可能な疾患でもある。そのため、マラリア制圧や監視戦略を進めていくためには、政治家や自治体、利害関係者、市民団体、研究者が相互に協力し合う必要がある。マラリアの予防管理のため、WHO は顕微鏡検査もしくはマラリア迅速診断キット（mRDTs）による速やかな診断やマラリア疑いの症例に対する効果的な治療、殺虫剤処理をしたネットや屋内用スプレーの使用、そして旅行者の化学的防除、乳幼児やアフリカの高蔓延地域で生活をする 12 歳以下の小児または妊婦を対象とした継続的な予防的治療、サヘル地域の就学前小児の季節性マラリアに対する化学的予防と集団投与などの化学療法を推奨している。しかしこれらの推奨事項の多くは 5 歳未満児と妊婦を対象としてきた。

学童児は通常、マラリアの世帯調査や介入調査の対象にならないため、多くの学童児が未治療のままマラリア原虫の保因者となっている場合、マラリアのコントロール、監視、撲滅戦略に課題をもたらしている可能性がある。学童児はマラリアの複雑な合併症を発症しにくいものの、貧血や認知機能の低下、無気力などを引き起こすことも知られている。したがって学童児におけるマラリア感染状況を調査して全体像を把握することは、国レベルでのマラリア管理・撲滅戦略を講じる上で、学童児マラリア対策の正当性を保証することにつながる。また、現在最も用いられているPfHRP2をターゲットとした迅速診断キットによる評価や分子生物学的な抗マラリア薬の評価は、マラリア政策の推進に貢献する可能性がある。

目的

本研究では、コンゴ民主共和国のマラリア対策戦略の改善に貢献する可能性のあるデータを収集して分析することを目的とした。

方法

2019 年 10 月-11 月に、コンゴ民主共和国のモンガフラ（農村部）とセレンボ（都市部）の両地区において、それぞれ小学校と保健施設で横断研究を行った。選定した小学校から427名の無症候性学童児が調査に参加し、217名の症候性学童児が保健施設から採用され、計 634 名が研究対象となった。顕微鏡検査、mRDTs、濾紙への血液採取を行い、血液から長崎大学熱帯医学研究所で DNA を抽出した。特異的プライマーを用いてマラリア種のジェノタイピングと *pfhrp2/3* 遺伝子の検出、さらに *P. ovale* spp.、*pfk13*、*pfmdr1*、*pfdhfr*、*pfdhps*、*pfcrt* のジェノタイピングと シークエンス解析を行った。

主要な結果

顕微鏡検査、RDT、そしてPCRによるそれぞれのマラリア陽性率は、無症候性学童児で33%、42%、62%であり、症候性学童児では、59％、64％、94％であった。無症候性感染者のうち、*P. falciparum*、*P. malariae*、*P. ovale* spp.の陽性率は農村部で77%、31%、17%であり、都市部では40%、9%、5%であった。また、症候性感染者では、農村部で96%、8%、18%であり、都市部で90%、18%、14%であった。農村部で生活をする学童児は都市部で生活する学童児に比べて約5倍の無症候性感染リスクがあった。
RDT陽性者のうち、*pfhrp2*欠損は2%で*pfhrp3*欠損は1%（1/173）であった。RDT陰性者では*pfhrp*2/3の遺伝子欠損は検出されなかった。
pfcrt K76T、*pfdhps* K540E、そして*pfmdr*1 N86Y の変異保有率はそれぞれ27%、20%、9%と低かった。アルテミシンに対する変異遺伝子は検出されなかった一方で、コンゴ民主共和国内では初となるKelch 13 変異（C532SおよびQ613E）が検出された。
WHO が推奨しているマラリア対策は、コンゴ民主共和国における全国的なマラリア制圧プログラム内で部分的にのみ実施されており、それがマラリアによる負荷を減らすことの遅れを生み出している可能性がある。

Abbreviations

ACT	Artemisinin combination therapy
AL	Artemether plus Lumefantrine
pfk13	*P. falciparum kelch 13*
A	Artemether
AMA-1	Apical membrane antigen 1
AMD	Antimalarial drug
AP2-G	Apetala2 gametocyte
AQ	Amodiaquine
ART	Artemisinin
AS	Artesunate
ASAQ	Artesunate plus Amodiaquine
ASMQ	Artesunate plus mefloquine
ASSP	Artesunate plus sulfadoxine-pyrimethamine
CAM	Cell adhesion molecule
CCMm	Community case management of malaria
CD	Cluster of differentiation
CDPK6	Calcium dependent protein kinase 6
CelTOS	Cell-traversal protein for ookinetes and sporozoites
CIDR	Cysteine-rich interdomain region
CMPS	Community-based malaria prevalence surveys

cox3	Mitochondrial cytochrome c oxidase III
CPR	Cytochrome P450 reductase
CQ	Chloroquine
CR1	Complement receptor 1
CRP	C-reactive protein
CRT	Chloroquine resistance transporter
CSP	Circumsporozoite protein
CYP2D6	Cytochrome P450 2D6
CyRPA	Cysteine-rich protective antigen
DHA	Dihydroartemisin
DHAPQ	Dihydroartemisin plus piperaquine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EBA-175	Erythrocyte binding antigen 175
EBL	Erythrocyte binding-like protein
ECM	Extracellular matrix
EEF	Exo-erythrocytic form
EPCR	Endothelial protein C receptor
EphA2	ephrin receptor A2
FPPIX	Ferriprotoporphyrin IX
G6PD	Glucose-6-phosphate-dehydrogenase

	Gamete egress and sporozoite traversal
GEST	protein
GpA	Glycophorin A
GPI	Glycophosphatidylinositol
HSPG	Heparan sulfate proteoglycan
HZ	Health Zone
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon γ
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
IPTi	Intermittent preventive treatment for infants
	intermittent preventive treatment for
IPTp	pregnant women
IPTp3+	Three doses of IPTp
IRS	Indoor residual spraying
ITN	Insecticide-treated net
Ldh	lactate dehydrogenase
L	Lumefantrine
LFA	Leukocyte function antigen
LS	Liver stage
	Membrane attack / perforin complex type
MAC/PF	domain

M-CSF	Macrophage Colony-Stimulating Factor
MDA	Mass drug administration
MQ	Mefloquine
mRDT	Malaria rapid diagnostic test
MSP	Merozoite surface protein
NCBI	National center for biotechnology information
NJ	Neighbor-Joining
NMCP	National malaria control program
OH-PQm	Hydroxyl-metabolites namely hydroxylated-PQ metabolites
PCR	Polymerase chain reaction
PECAM	Platelet/endothelial cell adhesion molecule
Pf	*Plasmodium falcparum*
PFATP6	*P. falciparum* adenosine triphosphate 6
PfCDPK5	*P. falciparum* calcium-dependent protein kinase 5
PfCK2	*P. falciparum* casein kinase 2
pfdhfr	*P. falciparum* dihydrofolate reductase
pfdhps	*P. falciparum* dihydropteroate synthase
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfHRP2	*P. falciparum* histidine-rich protein-2

PfHRP3	*P. falciparum* histidine-rich protein 3
	Plasmodium falciparum multidrug resistance
pfmdr1	1 gene
	P. falciparum reticulocyte-binding protein
PfRhs	homologs
PfRipr	Rh5-interacting protein
Pfs	*P. falciparum* surface protein
pH	Potential of Hydrogen
PLP1	Perforin-like protein 1
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
PQ	Primaquine
PV	Parasitophorous vacuole
RBC	Red blood cell
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RON	Rhoptry neck protein
SBC	Social and behavioral change
SERCA	Sarcoendoplasmic reticulum Ca2+ ATPase
SMC	Seasonal malaria chemoprevention
SMPS	School-based malaria prevalence surveys
SP	Sulfadoxine plus pyrimethamine

SPECT	Sporozoite microneme protein essential for cell traversal
SR-B1	Scavenger receptor B1
SSA	sub-Saharan Africa
TLP	Trap-like protein
TM	Thrombomodulin
TNF-α	Tumor necrosis factor α
TRAP	Thrombospondin related anonymous protein
TSR	Thrombospondin repeat
VCAM	Vascular cell adhesion molecule
WHO	World Health Organization

CHAPTER I. General introduction

1.1 Malaria Burden

Malaria is the most prevalent and life-threatening human vector borne disease causing more deaths worldwide where there is risk of infection (1). Some countries have achieved malaria elimination long time ego including the United States of America during the 1950s, Japan in 1960s and European countries in the years post 1970s (2, 3). Nowadays, African countries are improving their efforts and financial support for malaria control and intervention strategies for the past two decades in collaboration with their partners (4). Despite remarkable efforts made, the disease still occurs in more than 85 countries and territories worldwide and about half of the world's population is at risk (5). Its distribution mainly depends on the climate factors such as temperature, humidity, and rainfall necessary for mosquito *Anopheles* and *Plasmodium* parasite development (6-8). Malaria is present in regions where the temperature ranged between 20°C and 32°C, the transmission may be absent in temperature below 20°C and above 33°C (2, 8). The map below (**Figure 1**) (9) shows approximately malaria transmission areas worldwide.

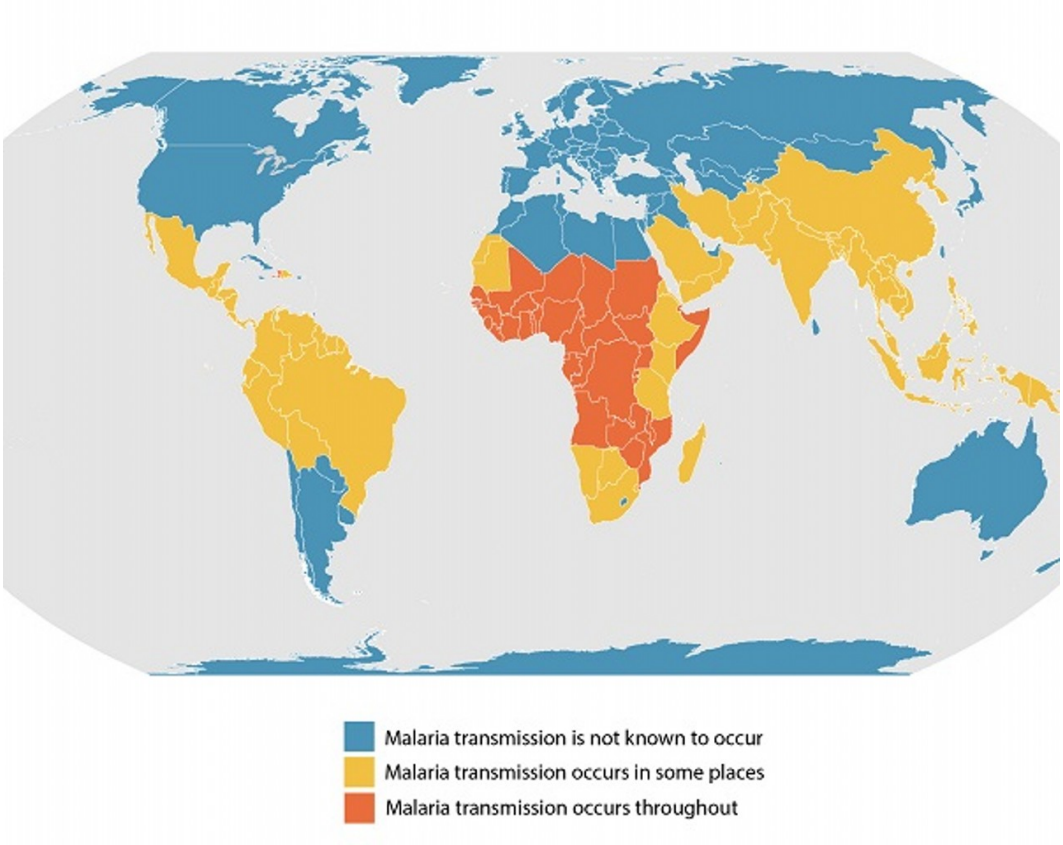

Figure 1. Endemic and non-endemic zones of malaria transmission

Over the last 20 years, global malaria control efforts have resulted in marked decreases in malaria-attributable morbidity and mortality. During the first decade, malaria control efforts focused more on reducing mortality rather than morbidity. Between 2000 and 2010, the estimated cases increased from 238 million to 247 million while that for death decreased from 736000 to 594000. Between 2010 and 2015, malaria control efforts were made in both morbidity and mortality rate from 247 million to 218 million and 549,000 to 453,000, respectively. However, between 2015 and 2019 progress has slowed compared to that achieved in the preceding 15 years with the increase of morbidity from 218 million to 229 million and reduction of mortality from 453,000 to 409,000. Malaria incidence was reduced from 80 per 1000 population at risk in 2000 to 58 per 1000

population at risk in 2010 representing 27% of reduction while it was reduced from 58 per 1000 population at risk in 2015 to 57 per 1000 population at risk in 2019 accounting for 2% of reduction only. Between 2010 and 2019, the estimated cases increased by 21% while the number of deaths decreased by 24% (5).

Considering World Health Organization (WHO) malaria region , WHO African region remains the most affected region and accounted for 94% of both cases and deaths of which six countries captured about half of all global malaria deaths including Nigeria (23%), Democratic Republic of Congo (DRC) (11%), Tanzania (5%), Niger (4%), Burkina Faso (4%) and Mozambique (4%) in 2019 (1, 5). Eleven countries were certified malaria-free by the WHO over the last two decades while 27 countries reported fewer than 100 indigenous cases and two countries (China and El Salvador) achieved at least three consecutive years of zero indigenous cases (**Figure 2**) (5). During this year, DRC accounted for 12% of all estimated malaria cases and 11% of deaths globally (5).

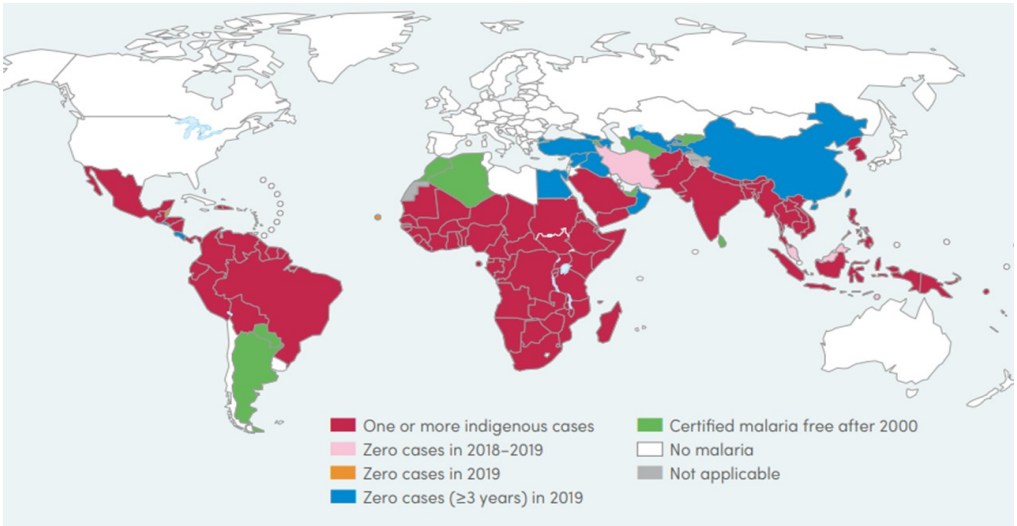

Figure 2. Global malaria burden

Countries with indigenous cases in 2000 and their status by 2019. Source WHO database (5).

1.2 Aetiologic agent of malaria

Malaria in human is a disease caused by the parasites of phylum Apicomplexa, family *Plamodiidae*, genus *Plasmodium* in the individual's blood or tissues (10). Six *Plasmodium* species including *Plasmodium falciparum* (11, 12), *Plasmodium vivax* (11, 12), *Plasmodium malariae* (13), *Plasmodium ovale* spp.(14) counting *P. ovale curtisi* and P. *ovale wallikeri* (15), and *Plasmodium knowlesi* (16, 17), are responsible of the disease in humans, and *P. falciparum* is the most prevalent and threatening in Africa (1, 5).

Plasmodium vivax is mostly prevalent across endemic Asia and South America but also occurs in few countries of Africa and associated with severe morbidity and mortality (5, 18, 19). This parasite is hardly ever frequent in DRC (20, 21).

Plasmodium ovale spp. is responsible of benign tertian malaria and rarely causes severe malaria and deaths in humans (21-24). The parasite is endemic in Africa (15, 25-28), but it is relatively rare in some Asian countries (29, 30). *Plasmodium ovale* spp. and *P. vivax* are responsible for relapses after months or even years without symptoms due to the presence of hypnozoites (31, 32).

Plasmodium malariae is relatively uncommon in Africa including in DRC (20, 28, 33), responsible of quartan malaria (33) and rarely causes severe malaria but may be responsible for chronic nephrotic syndrome (34) which may be fatal (34, 35). *Plasmodium malariae* and *P. ovale* spp. frequently occur as mixed infections with *P. falciparum* or *P. vivax* where vivax malaria is frequent, which can lead to the underestimation of their true prevalence (28, 36-40).

Plasmodium knowlesi is not yet found in sub-Saharan Africa (SSA), but frequent in Asia (16, 17). *Plasmodium falciparum, P. ovale* spp. and *P. vivax* are responsible of

tertian malaria while *P. malariae* is responsible of quartan malaria and *P. knowlesi* responsible of daily malaria paroxysms (41, 42).

1.3 Malaria transmission agent

Malaria is transmitted from a person to another through infected female mosquito bites (43) of the genus *Anopheles* (43, 44). Out of existing more than 400 different *Anopheles* species, around 30 are major malaria vectors (1). *Anopheles gambiae*, *An. funestus*, *An. arabiensis*, *An. melas*, *An. meris, An. moucheti* and *An. nili* are dominant *Anopheles* vector species for human malaria in Africa (45) and *Anopheles gambiae*, the major vector (46-52) including in DRC (53, 54).

1.4 Clinical manifestations and Treatment of malaria

Clinical manifestations of malaria may depend on several factors including host / parasite genetics, age of the patient and transmission intensity (55, 56). These regulate three presentation including asymptomatic, symptomatic uncomplicated and symptomatic severe malaria infections.

1.4.1 Asymptomatic malaria:

This happens when individuals harbor malarial parasites without showing any symptoms (57). This may be as the result of being continuously exposed to malaria parasites, in high transmission settings offering a partial immunity or premunition (58), which generally occurs in school-age children and adults due to repeated exposure (59-63).

Asymptomatic carriers usually represent an untreated group, serve as potential reservoir of gametocyte parasites (64-66) responsible for malaria transmission and its persistence in the communities (66). Moreover, they frequently harbor malaria parasites at low parasitemia levels, cannot clear them (57) but facilitate their transmission to others. Malaria premunition is influenced by factors such as age, genetic background of the host, pregnancy, nutritional status, and co-infection may also influence the development of anti-malarial immunity (67).

1.4.2 Symptomatic uncomplicated (mild form) malaria

When symptoms are present, especially fever but there is no evidence of clinical or laboratory signs of severity or vital organ dysfunction (68). It is the most common and widespread manifestation of the disease depending on the physiological diversity of malaria parasite biology (69).

The symptomatology of malaria is not common as symptoms of malaria can also be found in other tropical diseases. Although malaria symptoms are resembling to other tropical diseases, such as flu (70-72) and vary by age and immunologic status (73), most of patients show fever (1, 72, 74). Other nonspecific symptoms found in uncomplicated malaria include headache, chills, fatigue, body aches, sweating, myalgia, cough, nausea, vomiting, and others (1, 70-73, 75, 76).

Artemisinin (ART) combination therapy (ACT) is the current treatment to deal with this form in SSA (77). In DRC, Artesunate (AS) plus Amodiaquine (AQ) (ASAQ) since 2005 (78) or Artemether (A) plus Lumefantrine (AL) since 2010 are the two ACTs adopted by DRC malaria policy as first-line treatment for uncomplicated malaria (79) and Quinine plus Clindamycin in case of first-line treatment failure (77).

1.4.3. Symptomatic complicated (severe form) malaria:

This happens when there are vital organ disturbances complicated by serious organ failures or abnormalities in the patient's blood or metabolism generally in case of *P. falcipatum* infection in Africa and *P. falciparum*, *P. vivax* and *P. knowlesi* out of Africa.

Its clinical manifestations depend on the virulence of the parasite, genetic factors of the host and age in addition to the intensity of transmission and physiological changes such as during pregnancy or the host's defense against infection is altered (80). It has been shown that this malaria form is more common in areas with low endemicity with peak incidence in children aged 3-4 years (81).

Severe malaria includes severe anemia (due to massive destruction of erythrocytes); cerebral malaria associated with altered consciousness, seizures, coma or other neurological abnormalities; hemoglobinuria (due to massive hemolysis); acute respiratory distress syndrome (due to deep breathing resulting from metabolic acidosis); low blood pressure (due to cardiovascular collapse); acute renal failure; hyperparasitaemia (when more than 5% of Red blood cells (RBCs) are infected); Metabolic acidosis (excessive acidity in blood and tissue fluids); hypoglycemia and other signs of severity (82).

To deal with severe malaria, WHO recommends injectable artesunate/artemether or intravenous quinine (77). In DRC, severe malaria is dealing with injectable artesunate as the first treatment option, followed by injectable artemether or intravenous quinine as second line (79).

1.5 Biology and life cycle of malaria

Human Plasmodium undergoes its live cycle onto two hosts: an exogenous sexual phase (named sporogony) in female *Anopheles* mosquitos, and an endogenous asexual phase (named schizogony) in human hosts (83). In human hosts, the parasites undergo a two-stage development namely pre-erythrocytic schizogony (liver stage or a tissue phase) and erythrocytic schizogony (RBC stage or blood phase). **Figure 3** summarizes the life cycle of *P. falciparum* (84)

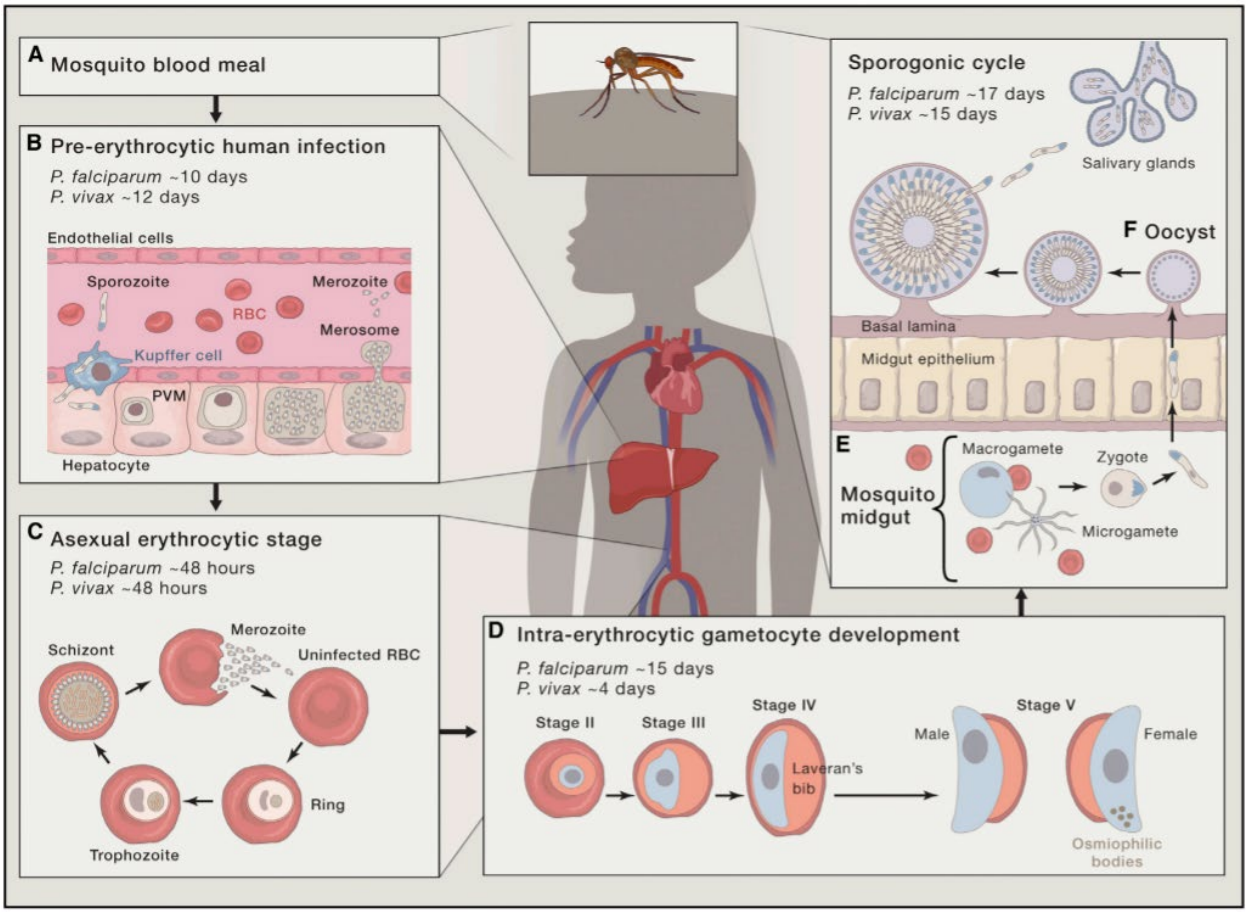

Figure 3. Life Cycle of *P. falciparum*

(Step A) Initially, female anopheline mosquito injects sporozoites into the dermis during a blood meal. (Step B) The sporozoites enter the bloodstream and reach liver, exit the sinusoids through Kupffer or endothelial cells and enter a hepatocyte. Cellular traversal is prior to active invasion until an appropriate hepatocyte is found. After entering

hepatocyte, sporozoites form a parasitophorous vacuolar membrane and undergo exo-erythrocytic schizogony and release tens of thousands of daughter merozoites in packets of merosomes into the bloodstream. (Step C) Released merozoites enter and colonize RBCs and begin a continuous asexual erythrocytic schizogony. (Step D) Some asexual parasites undergo gametocytogenesis and differentiate into sexual erythrocytic (Gametocytes). (Step E) Within a 15-day period, produced gametocytes, for their maturation, sequester and develop within the bone marrow (Stage I-IV) and enter the peripheral circulation once mature waiting to be ingested by a female mosquito where they emerge as extracellular male (microgametes) and female (macrogametes) gametes in the midgut. (Step F) Microgamete and macrogamete mate and produce a zygote which transforms over 24 hours into a ookinete that encysts to become an oocyst after migrating through the mosquito midgut epithelium where produced oocysts undergo asexual sporogonic replication, rupture and release motile sporozoites into the hemocoel and pass into salivary glands waiting to be injected into the next human host (84).

Malaria parasites, known as Apicomplexa, are made up of a set of apical organelles that help the parasite enter its host cells called rhoptries, dense granules and micronemes (85). The micronemes are secretory organelles located at the apical end of the parasite which secretes binding proteins to host cell receptors during invasion facilitating by the action of the actinomyosin motor for its motility and contains the proteases in the plasma membrane at the basal end which result in the excretion of parasitic ligands from the surface (86), while rhoptries are secretory organelles larger than microneme attached to the very apical end of the parasite, pear-shaped or club-shaped with one end, resembling secretory lysosomal organelles (87). On the other hand dense granules are secretory organelles not located at the apical end but rather are found throughout the cell and are released directly after invasion and throughout intracellular replication and involve in the modification of RBCs (88).

Plasmodium spp. is composed of three invasive forms including the sporozoite, the merozoite and the ookinete which involve the apical organelles located at one end of the parasite during their entry into the host cells (89).

1.5.1 Schizogony phase

1.5.1.1 Pre-erythrocytic schizogony:

This phase starts with tissue phase during a blood feed of an infected female *Anopheles* mosquito which bites and injects about 10-100 sporozoites from its salivary glands onto the human's dermal tissue and take about one to three hours for some of them to enter the bloodstream. At the same time, mosquito bites with its piercing proboscis that probes the dermis for a blood vessel and ejects saliva, which, via its anticoagulant activity, facilitates blood ingestion (90).

The remaining sporozoites into host's dermal tissue are may be destroyed and drained by the lymphatics by the first human immune response activation or injected sporozoites take hours in human dermis with a slow release into capillaries and lymphatic system as well (91, 92) or they take 15 minutes to a few hours into of BALB/C mice' skin to migrate into the liver and invade hepatocytes (93).

It seems that the protein Trap-like protein (TLP) may play a role of facilitating the exit of sporozoites from host's dermal tissue to enter bloodstream but cannot enter the circulation. Those motile sporozoites that enter the bloodstream quickly invade the host's hepatocytes by crossing the sinusoidal barrier including macrophage- like Kupffer cells and fenestrated endothelial cells (94). Sporozoites invade hepatocytes through a moving junction to form a replicative parasitophorous vacuole (PV) facilitated by various proteins. Proteins that facilitate the traversal of hepatocyte barriers include phospholipase (PL) (95), cell traversal protein for ookinetes and sporozoites (CelTOS) (96), gamete egress and sporozoite traversal protein (GEST), sporozoite microneme protein essential for cell traversal (SPECT) (97) and perforin-like protein 1 (PLP1, also called SPECT2) (98, 99). The function of these proteins in cell crossing is not well understood.

It appears that PLP1 possesses a membrane attack / perforin complex type domain (MAC / PF) to poke holes in membranes (99) and help sporozoites pass through cells forming a transient vacuole and combined activities of PLP1 and pH sensing are involved in the exit vacuoles avoiding their degradation by host lysosomes (98). A signal for this change is the recognition of hepatocytes by binding of higher sulfated forms of heparin sulfate proteoglycans (HSPGs) activating calcium dependent protein kinase 6 (CDPK6) (100).

The invasion of the sporozoites of *P. falciparum* and its formation into a PV are facilitated by human hepatocyte surface proteins, namely Tetraspanin CD81 (101) and the scavenger receptor B1 (SR-B1) (102) while the hepatocyte ephrin receptor A2 (EphA2) only facilitates the intra-hepatocytic development by formation of the PV by interaction with parasitic proteins p52 and p36 (103). For instance, the circumsporozoite protein (CSP), a major protein which covers the surface of the sporozoite synthesized by motile sporozoites in the salivary gland that plays important role in sporozoite infectivity to the host (104, 105), is a key protein for invasion parasites into hepatocytes which contains a highly repetitive region and a type I thrombospondin repeat (TSR). CSP binds to HSPG and becomes active leading to removal of the N-terminus exposing the TSR domain (106).

The following steps involve other proteins such as the thrombospondin related anonymous protein (TRAP), a micronemal protein responsible of the gliding motility and invasion in both mammalian host and salivary gland of the mosquito vector. It participates in a capping process that drives both sporozoite gliding and cell invasion (107), and the apical membrane antigen-1 (AMA-1), another microneme protein that is involved and expressed in sporozoites and could play a role during invasion of hepatocytes (108, 109) with adhesive domains released by the apical organelles (micronemes and rhoptries) for

the establishment of hepatocyte infection. It has been established that AMA-1 disappears after sporozoite invasion and is only re-expressed in liver merozoites, underlining that it only plays a role in sporozoite invasion of hepatocytes (108, 109) and in merozoite invasion of erythrocytes (110). Once the infection of the hepatocytes is established, sporozoites undergo their replication, each sporozoite transforms over the next 2-10 days into a liver stage (LS) or exo-erythrocytic form (EEF) and becomes a tissue schizont that contains thousands of merozoites at development peaks and releases up to 30 000 - 40 000 merozoites per hepatocyte (up to 30 000 in *P. falciparum* and about 10 000 in *P.vivax / P.ovale*) in the bloodstream by budding of vesicles filled with parasites called merosomes (111).

Hepatic infection usually lasts 8 to 12 days and no clinical symptoms occur at this stage depending on the *Plasmodium* species. The liver stage of *P. vivax* and *P. ovale* can persist for years, named hypnozoites, which can release merozoites into the bloodstream months and even years after the infective mosquito bite, responsible of the phenomenon of relapse without a new infection.

1.5.1.2 Erythrocytic schizogony

Free merozoites released from hepatocytes invade host RBCs, initiating the erythrocytic schizogony stage. The invasion of RBC takes around two minutes following three steps including pre-invasion, active invasion, and echinocytosis (112).

Pre-invasion requires a strong and tight interaction between merozoites and erythrocytes, resulting in motor deformation of the actomyosin of the host cell (112). This step is facilitated by merozoite surface protein (MSP), especially MSP1 which is the

major glycophosphatidylinositol (GPI)-associated protein on the merozoite surface (113) and acts as a platform on the merozoite surface comprising at least three large complexes with different external proteins that bind to erythrocytes (114).

It has been shown that merozoites do not absolutely require MSP1 to invade RBCs, as those lacking MSP1 can also invade erythrocytes (115). Thus, MSP1 appears to play a role of ligand-presenting proteins involved in evading host responses rather than directly playing a role in merozoite invasion. Host cell binding mechanisms are specific and differ between parasite lineages and particular developmental stages (116, 117), and are therefore primary determinants of host cell tropism.

For falciparum malaria, two ligand families of *P. falciparum* MSP1, the erythrocyte binding-like proteins (EBLs) and *P. falciparum* reticulocyte-binding protein homologs (PfRhs) are essential for *P. falciparum* merozoite invasion and involved by binding specific receptors including glycophorin A, B, C and complement receptor 1 (CR1) (118). They also play a role in signaling activation of subsequent steps in invasion. After their release from RBCs, exposure of *P. falciparum* merozoites to low concentrations of potassium ions in blood plasma resulting in increased levels of cytosolic calcium through a phospholipase C-mediated pathway, activating the release of a member of the EBL family, namely EBA-175 (119).

Thus, the binding of EBA-175 to its receptor, glycophorin A (GpA), triggers release of proteins from the rhoptries, as it has been shown that some merozoite proteins are maintained in rhoptries and micronemes during schizont development, then confine to the surface of the merozoite before or shortly after the merozoite exits the schizont through several mechanisms (120).

Likewise, a member of PfRh, named PfRh1, binds to Ca^{2+} signaling in the merozoite (121), and phosphorylation of the cytoplasmic tail of PfRh4, another member of PfRh, by *P. falciparum* casein kinase 2 (PfCK2) is essential for invasion via the parasite-host interaction PfRh4-CR1 (122). Calcineurin which is a calcium-regulated phosphatase in *Plasmodium* spp. (123, 124) also involves in attachment of merozoites, possibly by stabilizing dimerization of EBL and PfRh proteins, as this is essential for host-receptor ligation and signal transduction for subsequent invasion events (125). Following erythrocyte deformation, PfRh5, another member of PfRh but not part of MSP1, forms a complex with PfRipr (Rh5-interacting protein) (126) and cysteine-rich protective antigen (CyRPA) (127) and are involved in reorienting the merozoite so that the apical end abuts the erythrocyte membrane (118). PfRh5 binds to the host basigin receptor (BR), which is also required for merozoite invasion (128). The interaction between the complex PfRh5 and BR is associated with an influx of Ca^{2+} into the host cell (112, 129).

Once the merozoites are attached to RBCs, the process is irreversible leading to the formation of a tight junction formed between parasite-derived proteins, which commits the merozoite for invasion. This step is facilitated by the interaction between AMA-1 (one of several proteins released from the parasite micronemes) and the rhoptry neck protein (RON) complex (appears to be released from the merozoite's rhoptry organelles prior to penetration) called RON2 (130, 131). AMA-1, after its release onto the merozoite surface, facilitates the reorientation of a merozoite-internal actomyosin complex before attachment (132).

Once the merozoite is driven into the erythrocyte due to force generated by the parasite actomyosin motor, rhoptry contents form the PV membrane (133).

After the active invasion phase, fusion of membranes at the posterior end of the merozoite occurs and seal the parasite into the PV membrane surrounding the merozoite and then echinocytosis process starts and causes the erythrocyte to shrink and form spiky protrusions. This process is facilitated by Ca^{2+} influx into the RBC during interaction of the PfRh5 complex with BR (112).

Once erythrocyte infection is established, over the subsequent 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, or 72 hours for *P. malariae* and 24 hours for *P. knowlesi*, cell division (schizogony) results in 16–32 merozoites that egress and invade new host cells. The coordinated process of merozoite egress is tightly regulated and involves a number of protein kinases, including the plant-like calcium-dependent protein kinase PfCDPK5 (134) and cGMP-dependent protein kinase (PfPKG) (135). MSP1 has a role in egress of merozoites from *P. falciparum*-infected cells through subtilisin 1 processing on the merozoite surface that activates its ability to bind the erythrocyte membrane protein spectrin (115). **Figure 4** summarizes the steps of merozoite invasion of RBCs (84).

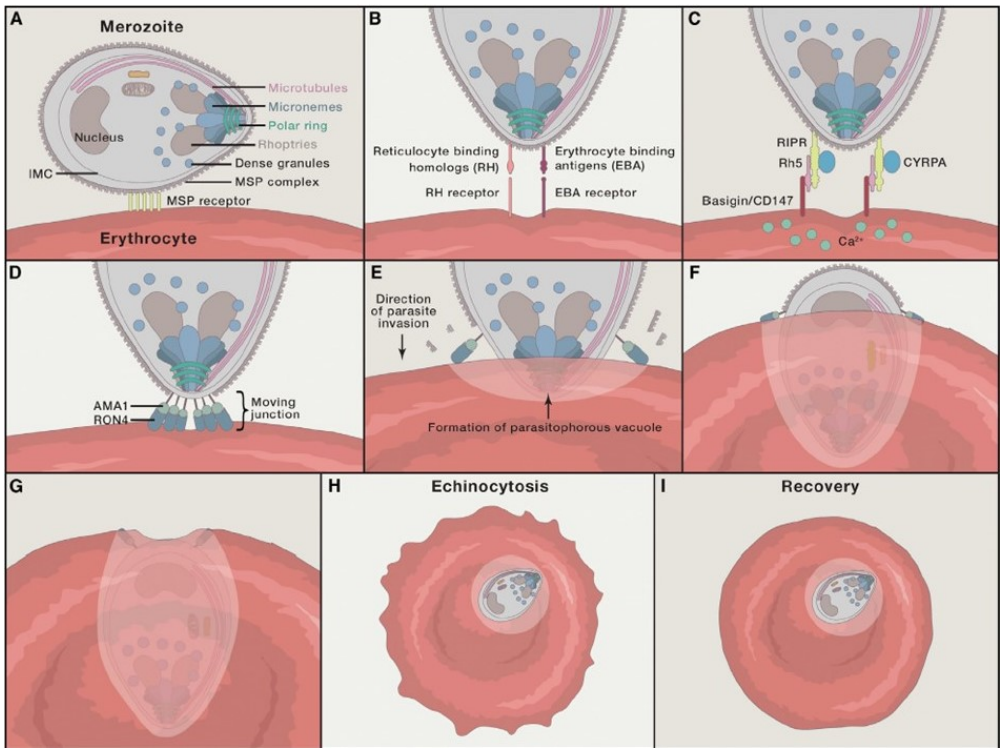

Figure 4. Merozoite invasion of RBCs

(Step A) Contact between merozoite and RBC facilitated by low-affinity interactions between RBC and merozoite surface coat proteins; (Step B) reorientation of the apical end of the merozoite into direct contact with the erythrocyte membrane following by interaction and biding to specific ligand-receptor; (Step C) mediated by proteins of the EBA and PfRh family members facilitating by the binding of the PfRh5 complex to the host BR ; (Step D) interaction between the complex PfRh5 and BR is associated with an influx of Ca^{2+} into the erythrocyte and microneme secretion, permitting deposition of the RON complex into the RBC membrane and its interaction with AMA-1. (Step E-G) Reorientation of a merozoite-internal actomyosin complex, pushing the merozoite into the RBC membrane following by the discharge of rhoptry contents leading to the formation of a PV membrane surrounding the merozoite. (Step H) Due to the loss of water from the cytosol of erythrocytes during the sealing of the PV membrane and the plasma membrane of erythrocytes, a period of echinocytosis occurs (Step I) followed by recovery of erythrocyte homeostasis (84).

The prepatent period (time from the infection by mosquito bite and the first appearance of the trophozoites in the erythrocytes) lasts 9 days in *P. falciparum*, 11-13 days in *P.vivax*, 10-14 days in *P.ovale*, 15 days in *P.malariae* and 9-12 days in *P.knowlesi*.

The last invade again RBC and the process goes on repeatedly every 24 hours for and *P. knowlesi*, 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* or 72 hours for *P. malariae*.

During rounds of schizogony in the bloodstream, generally 48 hours, a proportion of parasites undergo a sexual development to form male (microgametocytes) and female (macrogametocytes) gametocytes necessary for the transmission of malaria from humans to mosquitoes. Some factors such as high parasitemia and exposure to drugs are associated with increased conversion to gametocyte production. It has been found in vitro that extracellular vesicles containing protein, RNA and DNA trafficking between parasites provide a means of cell-cell communication which increases the production of gametocytes (136, 137). Additionally, epigenetic regulation (the process by which the activity of a particular gene is controlled by the structure of nearby chromatin) is essential for the control of sexual differentiation, and the transcription factor AP2-G is a primary regulator of gametocytogenesis (138).

For *P. falciparum* it takes 11 days for mature gametocytes, the infectious form to develop into mosquitoes which found in the peripheral circulation. The young forms (I-IV) remain sequestered within bone marrow (139), avoiding splenic clearance until emerging into the peripheral circulation when they become mature. It has been shown that gametocytes take few hours longer than their asexual cycle to develop to the mature forms then remain for only a short period of about 5 to 12 hours before degenerating and disappearing from the blood.

1.5.2 Sporogonic cycle (sexual development) of malaria parasite

During a blood meal, mature female and male gametocytes are ingested by female *Anopheles* mosquito. The location of the host by the mosquito is influenced by physical

(heat, humidity, visual) and chemical signals that play a role during orientation and landing (140, 141). It has been shown that skin bacteria play an important role in the production of human body odors and convert non-volatile compounds to volatile compounds with characteristic odors. Thus, individuals with greater skin microbial diversity and greater abundance of *Variovorax* spp. and *Pseudomonas* were less attractive to mosquitoes highlighting the genes of major histocompatibility complex may control an innate defense system (142-144).

In the mosquito midgut the macrogametocyte released by the erythrocytes to becomes a macrogamete, generally, within 5 minutes while microgametocyte divides its nucleus more slowly, generally, about 20 minutes into eight flagellated microgametes responsible of the fertilization of macrogamete, that takes about one hour (145). The produced zygote evolved into a slowly motile ookinete that actively penetrate the peritrophic membrane and the midgut epithelium and develops into oocyst generally twenty-four to forty-eight hours after blood meal. CelTOS is essential for traversal of ookinete into mosquito midgut (96). It appears that CelTOS is unique among traversal proteins as it is essential for traversal of malaria parasites in both human host and the mosquito vector and thus plays a critical role for malaria transmission and disease pathogenesis (96, 146).

In case of *P. falciparum*, some gamete surface proteins such as Pfs48/45 and Pfs230 are expressed and involved in the fertilization of macrogametes by microgametes (147) while ookinete surface Pfs25 constitutes a postfertilization antigen expressed (148) and plays a role in the traversal of the mosquito midgut epithelium (149). Additionally, Pfs48/45 and Pfs230 are also expressed in gametocytes circulating in the human blood resulting to their easily antibody detections among exposed individuals (150, 151).

Subsequently, oocyst undergoes its division and maturation and release thousands of sporozoites for about 7 to 30 days depending on environmental factors and type of *Plasmodium* species. Then, the only 25 % of sporozoites produced by oocyst actively and irreversibly migrate through the hemocoelomic fluid to the acinal cells of salivary glands and become highly infective a day after (152), and wait to be injected into a host during a new blood meal.

Malaria life cycle is summarized in **Figure 5** (153).

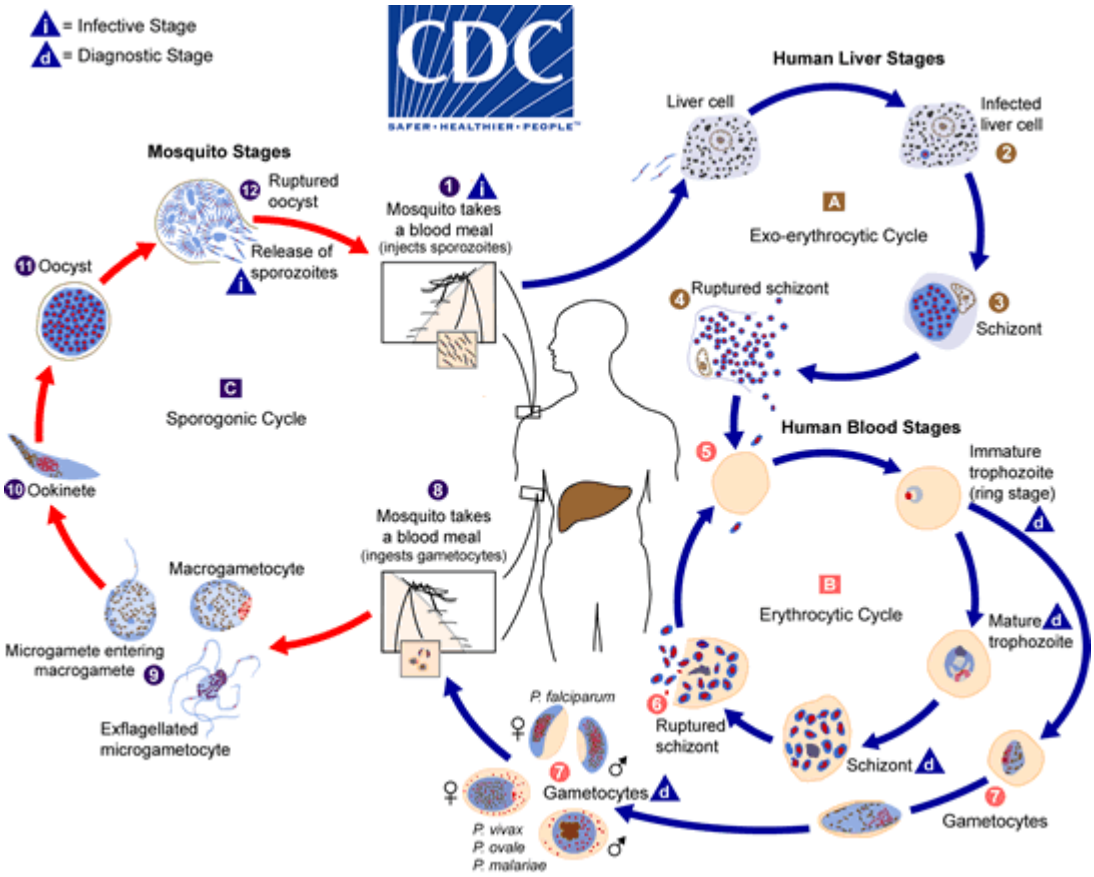

Figure 5. Malaria life cycle

The malaria parasite life cycle involves two hosts (invertebrate and vertebrate hosts). Schizogony stage (asexual multiplication): During a blood meal, a malaria-infected female *Anopheles* mosquito injects sporozoites into the human's dermal tissue from its salivary gland and enter bloodstream (Step 1). After their entrance into bloodstream, sporozoites enter liver and colonize hepatocytes (Step 2) undergo exo-erythrocytic

schizogony (Step A) leading to mature schizonts (Step 3), which rupture and release merozoites (Step 4). (In case of *P. vivax* and *P. ovale* not all merozoites are released, a dormant stage [hypnozoites] can persist in the liver which are responsible of relapses by invading the bloodstream weeks, or even years later without new infection). Once free merozoites are released into bloodstream, the parasites undergo erythrocytic schizogony (Step B). Merozoites enter and colonize RBCs and multiply (Step 5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites into bloodstream which infect other RBCs and so on (Step 6). However, later, some parasites differentiate into sexual erythrocytic stages (gametocytes) (Step 7). Blood stage parasites are responsible for the clinical manifestations of the disease. Sporogonic stage (Step C) starts when a female *Anopheles* mosquito ingests the gametocytes, male (microgametocytes) and female (macrogametocytes) during a blood meal (Step 8) and enter the mosquito's stomach and become microgametes and macrogametes. The microgametes penetrate the macrogametes producing zygote (Step 9). Which become motile and elongated (ookinetes) (Step 10) and invade the midgut wall of the mosquito where they develop into oocysts (Step 11). The oocysts grow, rupture, and release sporozoites (Step 12) which invade the mosquito's salivary glands waiting to be integrated into a new host and the cycle continues (153).

1.6 Malaria pathogenicity

1.6.1. Uncomplicated malaria

Continuous merozoite reinvasion followed by trophozoite development, and schizont rupture over 24 to 72 hours implies the level of parasitemia and the level of human response leading to clinical manifestation (such as fever) and activation of proteins of inflammatory response such as C-reactive protein (CRP), and tumor necrosis factor α (TNF-α) until the patient crosses a threshold of awareness and "feels ill" (154). TNF-α is released in response to an initial infection, as result of parallel reaction between parasite development and host response activation including macrophage ingestion of merozoites, ruptured schizonts, or antigen-presenting trophozoites in the circulation or spleen (155, 156), responsible for fever, that interreacts with other molecules such as interleukin 10 (IL-10) and interferon γ (IFN-γ) among others (157-160).

During infection, macrophage–T-cell–B-cell axis of the immune system is responsible of antibody production prior confers additional macrophage activity which increases clearance of parasites and production of new antibodies (160-163). Once the human host immune system works perfectly, the process will continue to ensure efficient protection.

Plasmodium falciparum infects any age of RBCs and is responsible for frequent multiple infections and multiple ring forms can be found in infected RBCs with large cytoplasm counting 1 to 2 small chromatin and trophozoites with compact cytoplasm counting dark pigment are in peripheral blood, schizonts mature counting 8 to 24 small merozoites and gametocytes in crescent or sausage shape (chromatin in a single mass in macrogametocyte or diffuse in microgametocyte) (153). Mature schizonts and young gametocytes (I-IV) are generally absent in peripheral blood. *Plasmodium falciparum* is able to modify the surface of the infected RBC at any age and creates an adhesive phenotype, responsible for cytoadherence for about half of the asexual life cycle time (164) which help the parasite to avoids splenic clearance. Thus, infected RBC binding can occur with endothelium, platelets, or uninfected RBCs (165-167) which is facilitated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (165).

Plasmodium vivax, *P. ovale* spp., *P. malariae*, *P. knowlesi*, a prolonged period of sequestration is not present (168). The parasites are commonly seen on a peripheral blood smear, frequently exposed to clearance by the spleen; *Plasmodium vivax*, *P. ovale* and *P. knowlesi* prefer to infect younger RBCs (reticulocytes), but *P. knowlesi* can, over time, adapt to infect older RBCs (169).

As compared to other malaria parasites, *P. vivax* mostly infects RBCs that possess the Duffy antigen, but it can also infect RBCs lacking Duffy antigen (170, 171). RBCs

(reticulocytes) infected by *P. vivax* and *P. ovale* spp. are larger than mature RBCs. They appear larger than mature RBCs around them on peripheral blood smear possessing characteristic Schuffner's dots, which are caveola–vesicle structures (153, 172). Trophozoite and gametocyte forms of *P. vivax* are very similar to *P. ovale* spp. Trophozoites are in peripheral blood with large amoeboid cytoplasm counting large chromatin while gametocytes are round or oval containing fine Schüffner's dots; compact, eccentric in macrogametocyte or diffuse in microgametocyte. Mature schizonts of *P. vivax* count 12 to 24 large merozoites with coalesced pigment while those for *P. ovale* spp. count large 6 to 14 merozoites with dark-brown pigment (153).

Inversely, *P. malariae* prefers to infect oldest RBCs and cause the most benign form of malaria infection with several distinct clinical features (33, 173, 174). Ring forms can be found in infected RBCs with sturdy cytoplasm counting large chromatin, trophozoites are in peripheral blood with compact cytoplasm with large chromatin, the number of merozoites produced with each schizont rupture is lower, generally 6 to 12 merozoites with large nuclei (153). Thus, the parasitemias are lower overall compared with others human malaria parasites (33).

Plasmodium knowlesi may provoke fatal complications with higher frequency as seen in *P. falciparum* and *P. vivax* (175). Within the infected RBCs, early trophozoites of *P. knowlesi* appeared as ring forms resembling to those of *P. falciparum* with double chromatin dots, multiply-infected erythrocytes (17, 176) and can be easily mistaken in falciparum malaria endemic regions, while its late and mature trophozoites, schizonts and gametocytes were generally indistinguishable from those of *P. malariae* (17, 177), but with some schizonts lacking symmetrical arranged merozoites surrounding clumps of malaria pigments or "rosette pattern (178). Additionally, its mature schizonts count 12 to

24 large merozoites with coalesced pigment and it produces round to oval gametocytes with fine Schüffner's dots; compact, eccentric in macrogametocyte or diffuse in microgametocyte (153).

1.6.2. Severe malaria

The pathogenesis of malaria is complex and most likely involves nonimmunologic and immunologic mechanisms (179).

Immunologic mechanisms or cytokine-based theory which consists of the release of inflammatory mediators such as cytokines and nitric oxide released by host cells subsequent exposure to malaria "toxins", trigger for the onset of the pathology of severe malaria. Severe malaria is the result of alterations in many tissues and organs frequently leading to metabolic acidosis and localized ischemia. It has been gathered that hyper parasitemia (180) or parasite factors inducing production of cytokines contribute to the severity of disease. Of note concerning the latter aspect, it has been firmly established that glycosylphosphatidylinositols from *Plasmodium* is an important pathogenic factors through their ability to induce TNF-α and Interleukin 1 (IL-1) (181). Thus, cytokines can contribute either directly or indirectly to many pathological outcomes (182) such as cerebral malaria or severe anemia.

Nonimmunologic mechanisms or mechanical theory which involves sequestration of infected RBCs in microcirculation of vital host organs, resulting to mechanical obstruction of blood flow, hypoxia, tissue damage and, ultimately, organ failure, as responsible of severe malaria (179). Parasite sequestration, which help the parasite to avoid the host's normal splenic clearance mechanisms (183), is facilitated by the adhesion to host receptors by mature forms of RBCs infected with *P. falciparum* which are expressed on the endothelium lining the host's capillaries (184) and on uninfected

erythrocytes producing "Rosettes" (185) and on platelets making platelet-mediated clusters (186), leading to microvascular obstruction (187, 188), metabolic disturbances including acidosis (189), and release of damaging inflammatory mediators (190, 191), which when combined is responsible of the severity of the disease and even death of the patient.

In Africa, most malaria deaths occur in children and are dominated by three syndromes that can occur separately or in combination: severe anemia, cerebral malaria, and respiratory distress (192), generally among vulnerable untreated or partially treated individuals, mostly children under five of age, for uncomplicated malaria. During sequestration, the activation of endothelial plays a major role in the microvascular pathology of *P. falciparum* resulting to reduction of nitric oxide bioavailability (193), and increase levels of angiopoietin 2 (194), responsible of the severity of the disease, but also increases the expression of several receptors to which the infected erythrocytes can bind, which also increase endothelial changes.

Endothelial protein C receptor (EPCR) as a ligand for PfEMP1-mediated infected erythrocyte binding plays a fundamental role in endothelial stabilization by promoting protein C activation. Binding of infected erythrocytes to EPCR through the cysteine-rich interdomain region **(**CIDR) domain of PfEMP1, that confer binding properties to different host receptors (195), blocks protein C activation and leads to highly localized coagulopathy (196). It appears that although EPCR-mediated cytoadherence occurs in many vascular beds, localized coagulopathy is particularly likely to take place in the brain due to the low constitutive expression of EPCR and thrombomodulin (TM) protective receptors (197). The PfEMP1 variants capable of binding to EPCR have been associated

with severe malaria (196) delivers a potentially important joining link between parasite and host factors mediating severity.

1.6.2.1 Cerebral malaria

Cerebral malaria (CM) happens when extensive sequestration is located in the cerebral micro-vasculature (198), characterized by a coma, situation in patients with *P. falciparum* infection that is often accompanied by metabolic acidosis, seizures, and hypoglycemia (179). Not all proinflammatory cytokines are equally relevant for the development of cerebral malaria, IFN-γ, TNF-α, and IL-12 are mostly involved, not IL-6 (199) nor IL-4 and IL-1O (200) nor Nitric oxide (NO) (201). Of note concerning proinflammatory cytokines, it has been shown that CM in humans are associated with local production of TNF-α, IFN-γ, and IL-1β but also of IL-10 (202).

Cell adhesion is facilitated by cell adhesion molecules (CAMs) located on the cell surface involved in binding with other cells or with the extracellular matrix (ECM). Cell adhesion has been responsible of mediating cerebral and non-cerebral cytoadherence of *P. falciparum*-infected RBCs to the host endothelium (203), mediating cerebral and non-cerebral cytoadherence of *P. falciparum*-infected RBCs to the host endothelium (203). Immunoglobulin superfamily CAMs (IgSF CAMs) are type of CAMs that bind integrins or different immunoglobulin (Ig) superfamily cell adhesion molecule. Most of them such as CD36, intercellular adhesion molecule 1 (ICAM-1, CD54), platelet/endothelial cell adhesion molecule 1 (PECAM-1, CD31), vascular cell adhesion molecule (VCAM-1), thrombospondin, E-selectin, P-selectin and chondroitin sulphate A, are receptors of PfEMP1 for cytoadherence (204).

CD36 is responsible of thrombocytopenia of malaria infection. ICAM-1 plays a role in inflammation and immunity processes and constitutes a ligand for leukocyte

function antigen-1 (LFA-1) by directing them to areas of inflammation, generally expressed on several tissues including endothelial cells, monocytes, and lymphocytes. During malaria infection, activation of macrophages leads to TNF-α production, the increase of which stimulates activation of ICAM-1. ICAM-1 was associated with CM (205, 206) and more expressed in brain tissue from patients died from CM lining to parasitized RBCs, platelets, and leukocytes in brain endothelium (205, 207-210).

However, it has been firmly established that ICAM-1 is not correlated with the severity of malaria when found outside of cerebral microvasculature (211). Inversely, VCAM-1 (CD106) is member of immunoglobulin superfamily and encrypts a cell-surface sialoglycoprotein expressed by cytokine-activated endothelial, dendritic cells, macrophages, and epithelium. VCAM-1 is lower expressed on unstimulated cells and not common in severe malaria among African individuals (209). In some cases, elevated plasma or serum levels of VCAM-1 have been found in the febrile phase of falciparum malaria (212).

1.6.2.2 Severe anemia

It has been hypothesized that host-related factors including hyperhaemolysis are the main responsible for malarial anemia (213). However, low level of IL-12, a cytokine that boosts erythropoiesis, appears to be associated with anemia (214, 215). Additionally, macrophage inhibitory factor, that inhibits erythropoiesis in vitro, has been associated with the development of anemia (216). Furthermore, alteration in platelets could be linked to the overproduction of cytokines, hence, elevated levels of M-CSF is responsible of platelet disorder (thrombocytopenia) by increasing macrophage phagocytic activity (217).

The highest levels of TNF-α coupled with low level of IL-10 (215, 218, 219) and Neopterin accumulation in serum (220, 221) have been correlated to severe anemia in human malaria.

1.7 Antimalarial drugs and antimalarial drug resistance and its mechanisms of action

The vast majority of antimalarials act to eliminate asexual blood stage parasites. Aryl-amino alcohols, 4-aminoquinolines, Endoperoxides and Antibiotics (Doxycycline, Clindamycin) target blood-stage parasites while Antifolates and Naphthoquinones (Atovaquone) target both liver and blood-stage parasites (222). The vast majority of current antimalarial drugs target the proliferative trophozoites and schizonts (223). Furthermore, Artemisinin derivatives also block the ring stages (224-226) and sexual stages (227, 228). **Figure 6** (222) shows the life cycle of Plasmodium and target sites of antimalarial drugs and drug resistance.

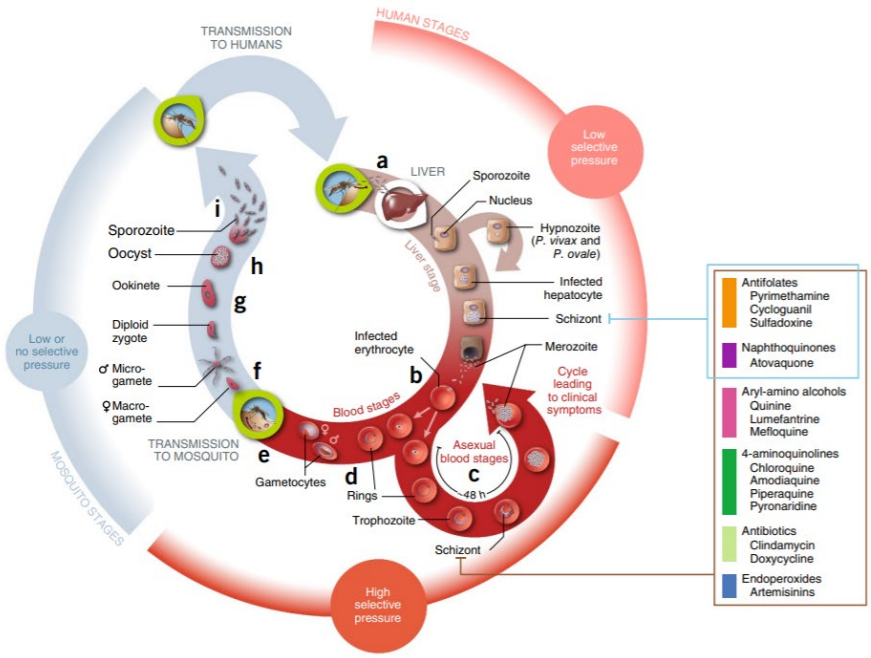

Figure 6. Life cycle of Plasmodium showing target sites of antimalarial drugs and drug resistance

Antimalarial drugs (AMDs) have a power to disrupt metabolic processes or pathways in different subcellular organelles and most of them target the Plasmodium erythrocyte stages, resulting in inhibition or death of malaria parasites.

To be promising, antimalarial drugs must be fast-acting, highly effective against blood-stage parasites, with minimal toxicity, and must be eagerly offered and inexpensive to residents, especially in malaria endemic settings (229).

AMDs target different stages of parasite development including asexual erythrocyte stages, liver hypnozoites (dormant stage) and the gametocyte stage (human sexual stage) (230).

AMDs are classified by the chemical group to which they belong to and following their mode of action (82, 231):

- The 4-aminoquinolines, including chloroquine, hydroxychloroquine, and amodiaquine.
- The Arylaminoalcohols
    - The 4-methanolquinoline derivatives, such as quinine, quinidine and mefloquine
    - The dichlorobenzylidine comprising lumefantrine
    - The 9-phenanthrenemethanols, comprising halofantrine
- The 8-aminoquinoline, counting primaquine and tafenoquine
- The antifolates
    - The diaminopyrimidines, comprising pyrimethamine
    - The sulfonamides and sulfones, such as sulfadoxine and sulfametopyrazine, sulfalene and dapsone
- The sesquiterpene lactones, such as artemisinin and its derivatives
- The biguanides, such as proguanil and chlorproguanil
- The hydroxynaphthoquinones, comprising atovaquone
- The antibiotics
    - The tetracyclines, such as doxycycline and tetracycline
    - The lincosamide including clindamycin

1.7.1 4-aminoquinolines

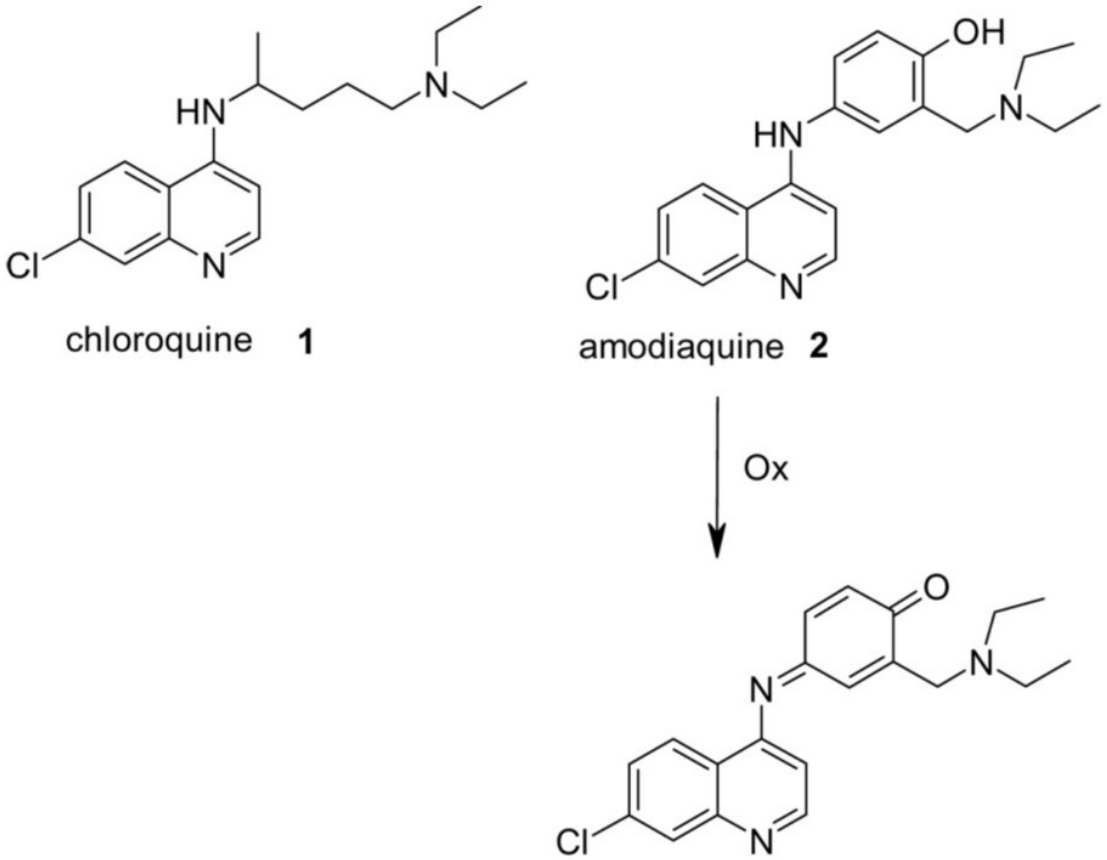

Figure 7. Chemical structure of some 4-aminoquinolines

There are rapid-acting blood schizonticides with some gametocytocidal activity. It has been firmly established that malaria parasite digests the hemoglobin of the hosts cells to obtain essential amino acids resulting to the release of large amount of heme which is toxic to the parasite. Hence, the parasite polymerizes the heme to non-toxic hemozoin which is sequestered in the parasite's food vacuole to protect itself. Thus, 4-Aminoquinolines form complexes with ferriprotoporphyrin IX (FPPIX), inhibiting polymerization into non-toxic hemozoin released from the heme during parasite's digestion (232-234). It has been shown, naturally, CQ is able to infuse and enter the

membrane of the food vacuole but once inside it becomes protonated and cannot pass the membrane to exit it (235, 236).

Resistance to 4-aminoquinolines is the result of a K76T mutation in the chloroquine resistance transporter (CRT) gene located in the membrane of the digestive vacuole which allows the removal of 4-aminoquinolines from the digestive vacuole (237-242). Of note concerning the latter aspect about 4-aminoquinolines, currently, chloroquine (CQ) and amodiaquine (AQ) are no longer used in monotherapy treatment for uncomplicated malaria in SSA countries; however, AQ is used in combination with AS as treatment for uncomplicated malaria (243).

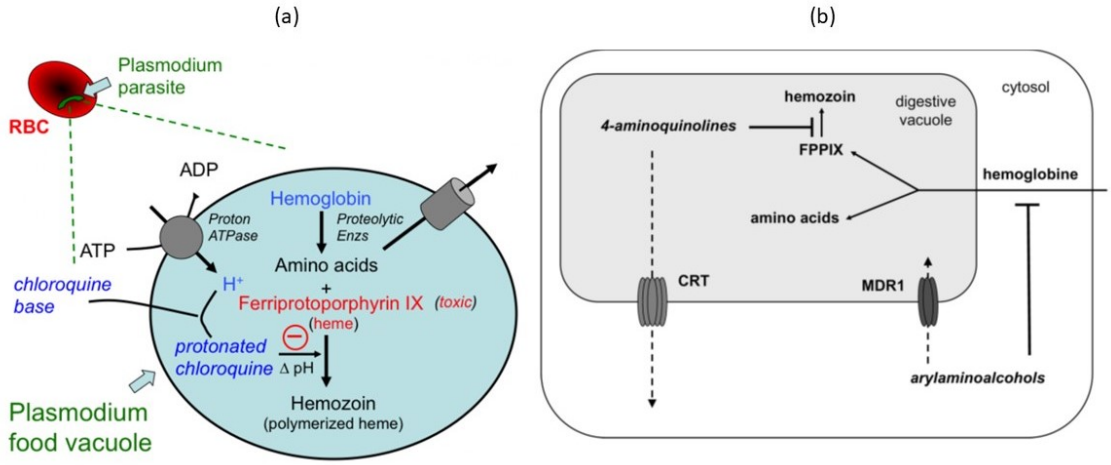

Figure 8. Mechanisms of action of 4-aminoquinolines and CQ resistance

In the parasite's food vacuole, the parasite digests the hemoglobin of the hosts cells to obtain essential amino acids, free radicals and FPPIX (toxic). CQ base diffuses into the food vacuole, interacts with FPPIX, and blocks the polymerization into hemozoin (non-toxic). Also, accumulation of CQ raises the pH within the vacuole, which reduces the rate of non-enzymatic polymerization FPPIX (a) (244). Mutation in the CRT gene located in the membrane of the digestive vacuole allows the removal of CQ from the digestive vacuole (b).

1.7.2 4-methanolquinoline derivatives (Arylaminoalcohols)

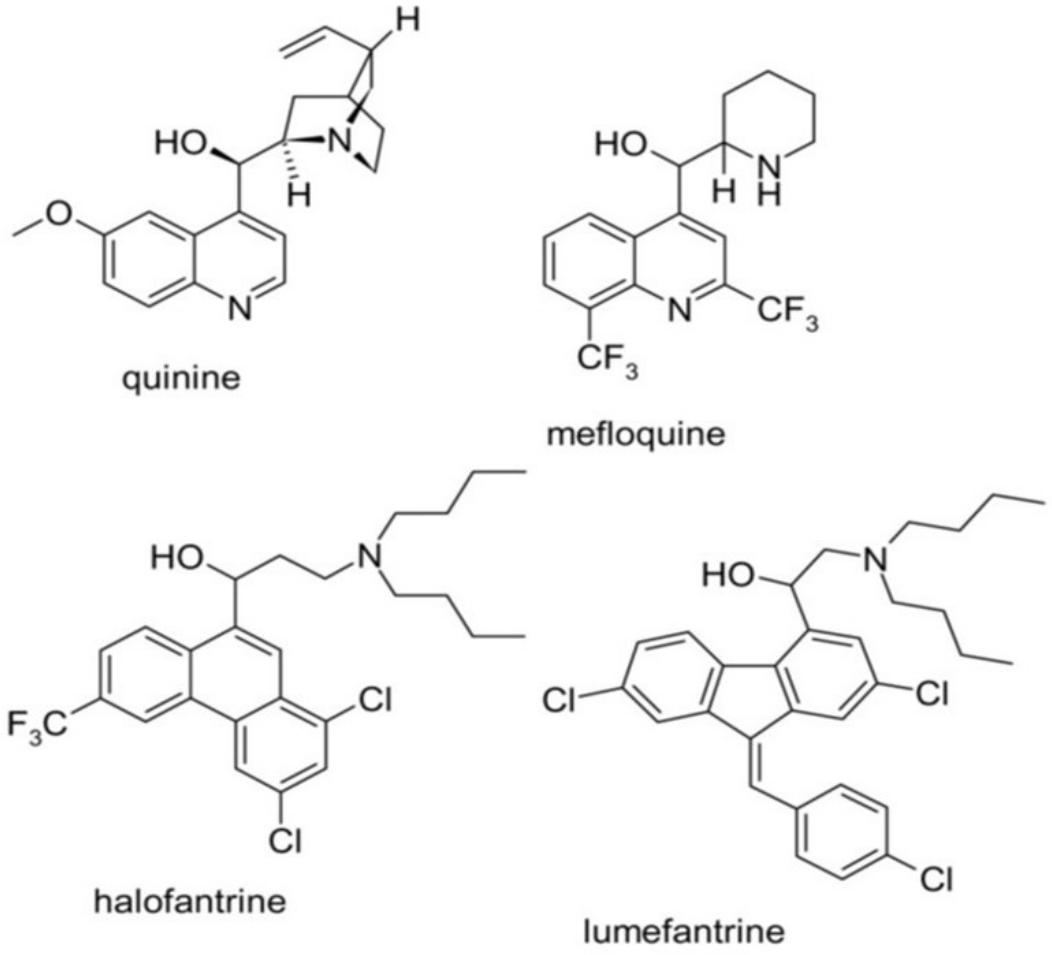

Figure 9. Chemical structure of Arylaminoalcohols

Arylaminoalcohols play like in manner as 4-aminoquinolines with some differences, they appear to interfere with the heme digestion (245, 246). As examples, chloroquine is responsible of clumping of the malaria pigment, while quinine antagonizes this process (247). Moreover, as compared to CQ, quinine is a weaker base, interacts weakly with heme (248) and has less affinity for heme, implying that mechanisms other than ion transport into the food vacuole and heme-drug interactions are essential for the action of these drugs (249). Also, it has been gathered that quinine inhibits heme

polymerization (250, 251) and heme catalase activity (252). Thus, of note concerning the latter role, it has been firmly established that in the absence of a specific transporter, quinine is likely to be accumulated less efficiently in the food vacuole than CQ.

It has been established that resistance to arylaminoalcohols is associated to the amplification of the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr*1) gene (253-257), which is located in the membrane of the digestive vacuole which serves for the transport of arylaminoalcohols into the digestive vacuole (258). *pfmdr*1 gene has been shown to be a structure expressed in a *pfmdr*1 transporter located in the food vacuole of *P. falciparum*. It plays a role similar to that of the mammalian multidrug resistance gene involving the expulsion of the drug from the cell and it encodes a P-glycoprotein (drug transporter that determines the uptake and efflux of a range of drugs) homologue-1 multi-drug resistant transporter (259). Thus, *pfmdr*1 facilitates the production of P-glycoprotein homolog 1, a protein located to the food vacuole membrane (260, 261) supporting the hydrolysis of ATP to the translocation of solutes across cell membranes (260). *pfmdr1* has been shown located along the membrane of the food vacuole and participates in the transport of xenobiotics, including CQ, to the food vacuole and repels CQ from the cytosol (262, 263). Mutations in *pfmdr*1 are associated with decreased influx of various antimalarial drugs reducing their intracellular absorption (264, 265). Thus, *pfmdr1* gene mutations stop the movement of antimalarials from the cytosol into the food vacuole, reducing their potency, but not for drugs that inhibit targets outside the food vacuole (266).

Quinine is still one of the recommended drugs for the treatment of uncomplicated malaria (267, 268), generally in case of ACT failure in combination with clindamycin or doxycycline or tetracycline is generally recommended (243, 267, 268). For severe malaria,

it is usually used after injectable artesunate failure or lack of injectable artesunate because preparations for intravenous applications are available, artesunate is used as first-line treatment for severe malaria caused by all *Plasmodium* species (269, 270).

Mefloquine (MQ) has been proving of having a very long half-life (271, 272). Hence, it is recommended for prophylaxis despite its possible neuropsychiatric side-effects which evidence is not yet conclusive (273, 274), while lumefantrine has been never used in monotherapy but showed synergism with artemether in vitro (275). Both lumefantrine (276) and mefloquine are currently used in combination with artemisinin derivatives in African countries as treatment for uncomplicated malaria (243).

1.7.3. 8-aminoquinolines

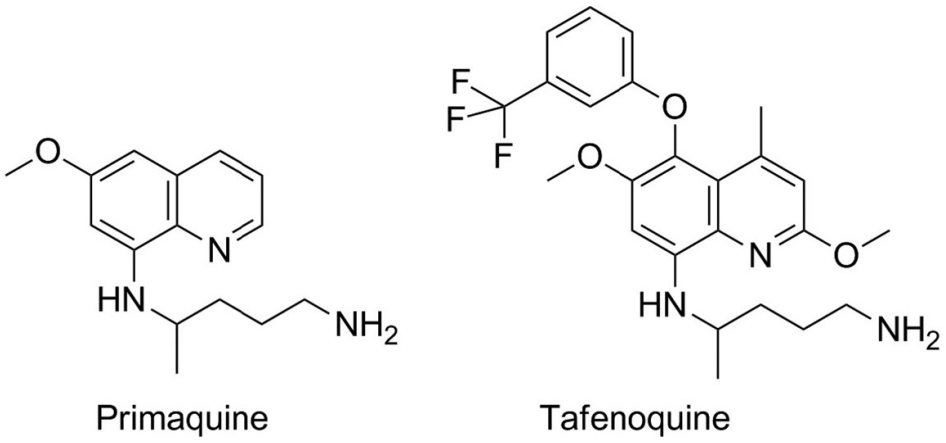

Figure 10. Chemical structure of 8-aminoquinolines

As compared to antimalarials described above, primaquine (PQ) is active against the liver and the sexual blood stages of different plasmodia useful as antirelapse therapy of *P. vivax* (277, 278) or *P. ovale* spp (279, 280). infections. It has been also shown to be effective in the chemoprophylaxis of *P. falciparum* infections (278, 281-283). However,

it is likely responsible of serious side effects such as effect life-threatening hemolysis in humans with glucose-6-phosphate-dehydrogenase (G6PD) deficiency, mostly in Africa and Asia (284). Its mode of action is not well understood. It appears the efficacy of PQ depends on cytochrome P450 2D6 (CYP2D6) gene and its redox partner cytochrome P450 reductase (CPR) which are essential for oxidation of PQ resulting to hydroxyl-metabolites namely hydroxylated-PQ metabolites (OH-PQm).

The improvement in the efficacy of OH-PQm is due to the direct reduction of quinoneimine metabolites by CPR with the concomitant and excessive generation of Hydrogen peroxide (H_2O_2), resulting in the death of the parasite. In addition, clinical and laboratory evidence suggested that the efficacy of PQ may depend on genetic variation in CYP2D6 drug-metabolizing enzyme activity (285-289) Resistance to PQ appears to be attributed to failure of the CYP2D6-mediated conversion of PQ to its active redox metabolite (s) (290).

1.7.4. Antifolates

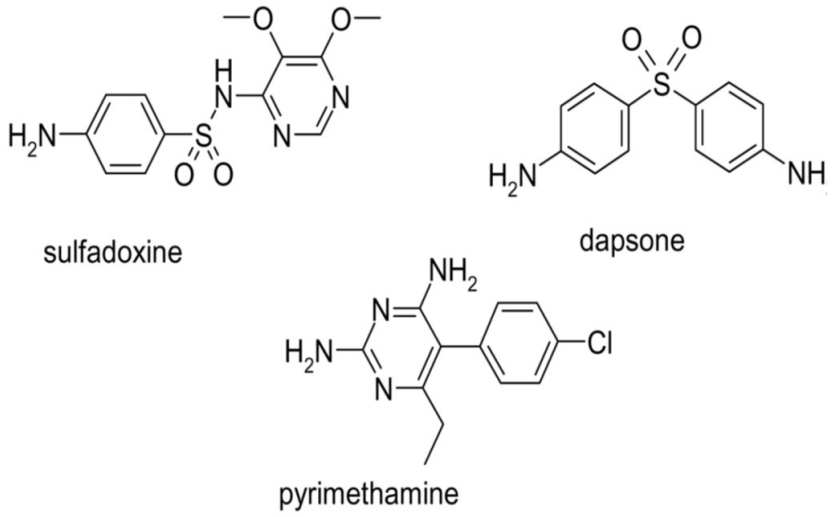

Figure 11. Chemical structure of sulfones

Two enzymes of the biosynthesis of tetrahydrofolate, the dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) are targeted by the antifolates (291-294).

Both two enzymes, DHFR and DHPS, are central in parasite metabolism essential in *de novo* folate biosynthesis, resulting in the biosynthesis of pyrimidines, purines, and some amino acids, a folate metabolism pathway vital for malaria parasite survival. Thus, these two enzymes are involved in the production of reduced folate cofactors vital for parasite DNA synthesis and the metabolism of amino acids. The antifolate drugs inhibit either DHFR (pyrimethamine, cycloguanil) (295), preventing the biosynthesis of purines and pyrimidines and therefore halting the processes of DNA synthesis or DHPS (sulfadoxine) (296), inhibiting the use of para-aminobenzoic acid during the synthesis of dihydropteroic acid.

Sulfadoxine and pyrimethamine act synergistically when used in combination (297), resulting to the blocking of both *dhps* and *dhfr*. The combination sulfadoxine plus pyrimethamine (SP) is currently used for malaria prevention in SSA.

Resistance to antifolates is due to point mutations in both *dhps* and *dhfr*, which mostly occur at codons 51, 59, 108, 164 of the *dhfr* and codons 436, 437, 540, 581, 613 of the *dhps* gene (296, 298-302). It has been shown a correlation between increased frequency of such mutations and resistance to SP drugs across the world (303-305).

1.7.5. Sesquiterpene lactones (Artemisinin and its derivatives)

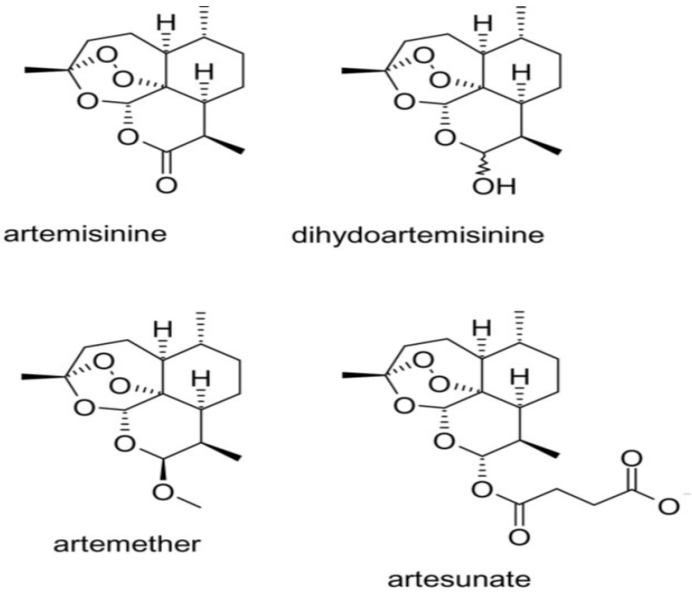

Figure 12. Chemical structure of artemisinin and its derivatives

Artemisinin has a unique trioxane structure with an endoperoxide bond that is required for antimalarial activity (306). Due to the low solubility of artemisinin, its semisynthetic derivatives AS (artemisinin's acylation to the succinic acid hemiester), artemether (artemisinin's methylation to the acetal) and dihydroartemisinin (DHA) (artemisinin's lactone substructure to a hemiacetal) are used clinically (307). When used, both AS and artemether composites are rapidly transformed into DHA (308), which has a rather short elimination half-life of 40–60 minutes (309, 310).

The mode of action of artemisinin and its derivatives is not well understood. It appears depending on hemoglobin degradation, whether they non-specifically modify several targets such as proteins and heme in the digestive vacuole (307, 309, 311, 312) or they specifically inhibit the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) ortholog of *P. falciparum* (PfATP6) (313). Hemoglobin degradation free radical production which can irreversibly bind and inhibit SERCA (313) and *P. falciparum*

phosphatidylinositol-3-kinase (314) of the parasite thereby inhibiting parasite growth but appears also to inhibit formation of transport vesicles like the food vacuole of the parasite. Artemisinin and its derivatives target mainly against the late ring stages, but unlike other antimalarials also against the small ring stages present in erythrocytes a few hours after infection (315).

The artemisinin derivatives are generally not used alone because of their short half-lives and to prevent the selection of resistant parasites as well (316). Of note concerning the latter aspect, some combination artemisinin derivatives and other molecules are currently used such as artesunate ASAQ, AS plus MQ, AS plus SP (ASSP), AL, DHA plus piperaquine (PQ) (DHAPQ) and AS plus MQ (ASMQ). Resistance to artemisinin has been found to depend on Kelch 13 (K13) propeller gene has been identified as a molecular marker of ART resistance (317). K13 is known as an amino acid protein located at the cytostome (known as the cell mouth) that helps the parasite to take up hemoglobin (318).

Artemisinin clinical failure has been found in Southeast Asian countries including Thailand, Cambodia and other Asian countries which clinically manifested as delayed clearance of parasitemia following treatment with artemisinin derivatives (319).

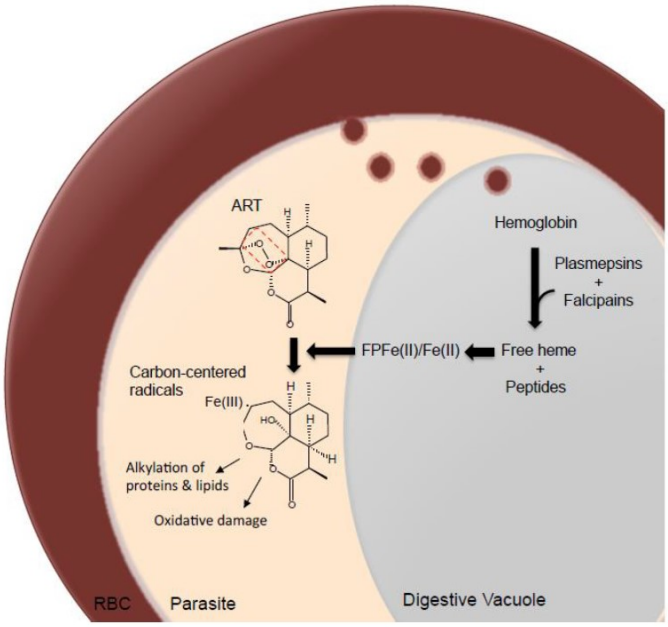

Figure 13. Artemisinin resistance mechanism of action
Adapted from Krungkrai *et al*., 1987 (320).

Among antimalarial drugs that target asexual blood stage, the majority is involved in the degradation and detoxification of host hemoglobin into PV where the parasite import hemoglobin for its survival (222). **Figure 14** summarizes the mechanism of action of antimalarial drugs and drug resistance.

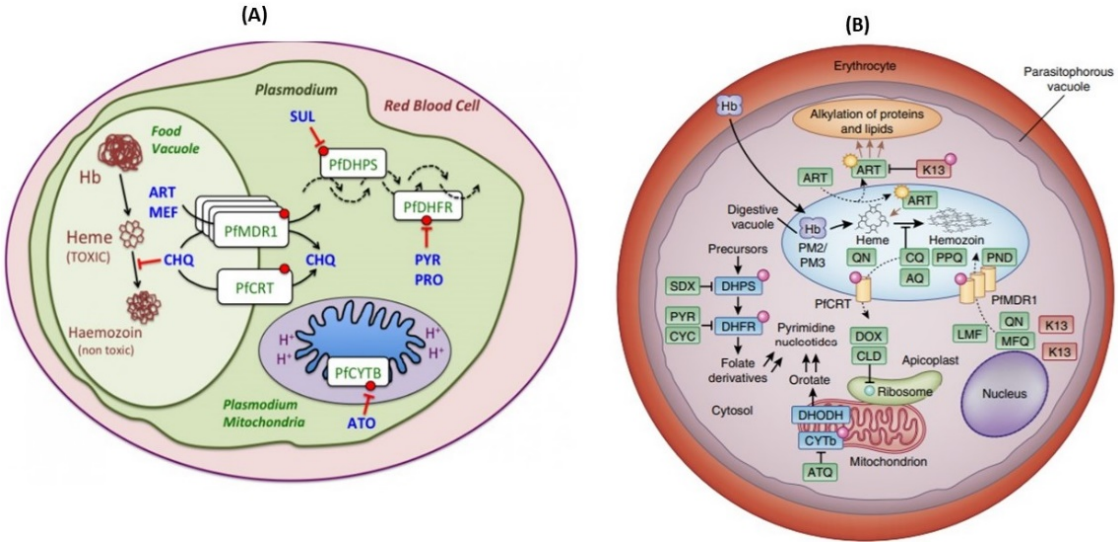

Figure 14. Mechanisms of resistance to antimalarial drugs

In the parasite's food vacuole, CQ is involved in the detoxification of heme useful for the parasite survival. Mutations of the *pfcrt* as well as increased copies of *pfmdr1* are associated with CQ resistance as they reduce its concentration in the digestive food vacuole. Also, *pfmdr*1 gene mutations has also been established to be implicated in MQ and ART resistance. Additionally, mutations in *pfdhfr* or/and *pfdhps* have been shown to be associated with Pyrimethamine or/and Sulfadoxine resistance (Adapted from Ding *et al*., 2012) (**Figure 14a**) (321).

Targeted biological pathways often include heme detoxification in the digestive vacuole, electron transport in the mitochondrion and folate and pyrimidine biosynthesis in the cytosol. CQ, AQ, PPQ and quinine are all thought to concentrate in the digestive vacuole blocking the detoxification of heme (toxic) into hemozoin (non-toxic). Point mutations (pink circles) in *pfcrt* and *pfmdr1* mediate the resistance to 4-aminoquinolines while PPQ resistance is associated with increased expression of the hemoglobinases plasmepsin 2 and 3 and possibly some *pfcrt* gene mutants. Activation of the endoperoxide bridge of ART derivatives (star symbol) through Ferrous (Fe2+) iron-heme produced after parasite protease-mediated degradation of imported host hemoglobin. Different forms of K13, located in endoplasmic reticulum and vesicular structures, constitute a mediator of ring-stage parasite resistance to ART. Also increase copy-number in *pfmdr*1, as well as different point mutations in *pfcrt* and *pfmdr*1 reduce the parasite's susceptibility to quinine, lumefantrine and MQ and can modulate ART potency. In addition, mutations in *pfdhps* and/or *pfdhfr* mediate the resistance to antifolates sulfadoxine or/and pyrimethamine / cycloguanil (adapted from Blasco et al., 2017) (**Figure 14b**) (222).

1.8 Challenges of malaria control strategies

In the absence of effective vaccines, malaria control strategies still depend upon integrated effective case management, preventive chemotherapy, and vector control. To

control malaria, WHO recommends early prompt diagnosis and effective treatment for suspected malaria case management to prevent severe cases leading to deaths; vector control (use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) and others) to deal with mosquito aggressivity and transmission; Preventive chemotherapy (chemoprophylaxis for travelers, intermittent preventive treatment for infants (IPTi), children under 12 months living in high-transmission areas of Africa and pregnant women (IPTp) living in moderate-to-high transmission areas, seasonal malaria chemoprevention (SMC) for preschool children living in areas of the Sahel sub-region of Africa and mass drug administration (MDA) for epidemic control, to prevent malaria occurrence et clear asymptomatic carriages, as components of malaria control and elimination efforts (5, 322). Early prompt diagnostic is the root of the success of malaria case management and surveillance Rapid diagnostic tests (RDTs) and microscopy are routinely used prior to the treatment (323), and Polymerase chain reaction (PCR), which is more sensitive (324) but requires expensive equipment and reagents, is usually used for malaria surveillance for specific issues. In remote areas, where there are problems of electricity and inexperieced microscopists, RDTs are more used as they are cost-effective and provide an easy-to-use alternative to microscopy (325), which requires skilled microscopists to be optimally effective (326, 327). In regions where *P. falciparum* is the most prevalent malaria parasite species such as in SSA, the most frequently used RDTs target *P. falciparum* histidine-rich protein-2 (*Pf*HRP2) and represented 64% of all RDTs distributed by national malaria control programs worldwide in 2018 (72) and continues to increase nowadays. However, *pfhrp*2 gene may be deleted in some parasites rendering them undetectable by *Pf*HRRP2-based RDTs (5) leading to false-negative results resulting to exclusion of some true cases from getting treatment. HRP2 is localized in the cytoplasm of *P. falciparum* and in the

infected erythrocyte membrane (328). It is involved in heme detoxification (329-331). It has been also shown that HRP2 and its homolog HRP3 are present in the food vacuole and play a role in heme polymerization (331). Synthetic peptides corresponding to the HRP2 repetitive sequence have been shown to bind heme and inhibit hemozoin formation in vitro (332).

Early effective antimalarial drug is crucial for the malaria case management and preventive chemotherapy strategies. The emergence and spread of *Plasmodium falciparum* resistance to antimalarial drugs may be a major obstacle to malaria control and elimination strategies. CQ, known in the past as an inexpensive, safe, and effective anti-malarial, was one of the first drugs to be used on a wide scale for malaria treatment and control for decades (333) and a pillar of 20th century malaria eradication and control efforts. However, some years after its use, chloroquine resistance emerged firstly in Southeast Asia and in Latin America (247, 334-336) before spreading in Africa (337, 338). It has been shown that the presence of threonine (T) in place of lysine (K) at position 76 of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene, constitutes the molecular marker for chloroquine resistance (339, 340). Clinically, the K76T substitution raises the of CQ treatment failure sevenfold (341). It has been also shown that CQ resistance was associated to *pfcrt* 76T and *pfmdr*1 allele 86Y (342) and pf*mdr*1 alleles 86Y, 184Y, and 1246Y (343). However, *pfmdr*1 mutations are not responsible of CQ resistance but react synergically with *pfcrt* gene mutations by amplifying CQ resistance. It is noteworthy that despite the fact of the resistance of *P. falciparum* to chloroquine in majority of countries worldwide, it is still being used as first-line treatment of *P. vivax* in some countries where it is still effective (231). But, for the management of uncomplicated malaria, in SSA countries, ACT is the antimalarial drug

of choice since 2001 and the combination SP alone or associated with other regiments is used for preventive chemotherapy, as recommended by the WHO (5, 322).

Artemisinin derivatives are the main components of ACT used in combination of other drugs for uncomplicated malaria or alone for severe malaria. Mutations in the propeller domain of the *P. falciparum kelch 13* (*pfk13*) gene have been associated with ART resistance in vivo and in vitro (317, 344) and spread across the Greater Mekong Subregion of Southeast Asia (345) and some SSA countries (346-348). So far, there is no evidence of approved *pfk13* gene markers associated with the resistance of artemisinin derivatives in DRC (349, 350). It is noteworthy that clinical resistance to or delayed clearance of infections ACT with components including AQ, L, PQ, MQ or SP was found in some areas, especially in south-east Asia (351, 352) but hardly ever in Africa (353, 354). Challenges in the future would be to recognize a three-fold ACT combination (355) or two or even three waves of ACT (356) to deal with delayed clearance of malaria infections during treatment of uncomplicated malaria. Some African countries including DRC, have reported negligeable ASAQ or AL treatment failure rate after 28-day follow-up (72, 357).

Despite resistance to SP, it is still using alone or combined to another drug for the malaria prevention. SP resistance is associated to mutations in *P. falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes. Several studies have reported mutations in these two genes (78, 358-363). It has been shown that mutations in *pfdhps* (S436, A436, K540, A581, K613) and *pfdhfr* (N51, C59, S108, I164) especially the double *pfdhps* (A437, K540) with triple *pfdhfr* (N51, C59, S108) combination (quintuple mutant), and *pfdhps* (A581) combination (sextuple mutant) are associated with the level of clinical resistance to SP (364, 365). Thus, WHO recommends

IPTi with SP only where the prevalence of *pfdhps* 540E is under 50% (366), and the discontinuation of IPTp when the prevalence of *pfdhps* 540E is over 90% and *pfdhps* 581G is above 10% (367), but it may continue be used if IPTp-SP remains effective in preventing pregnancy outcomes even in case of quintuple mutation (322). Of note concerning the latter aspect, it is important to keep in mind the development of a new drug for SP replacement for malaria prevention in infants and pregnancy. Vector control is dealing, especially, by use of ITNs and IRS which need insecticide for their design to be more effective. Apart from the problem of their supplies which arise in malaria endemic countries, insecticide resistance to about all available insecticides used in vector control programs is increasing (368) and could be an obstacle for vector control strategies. Dealing with insecticide resistance requires a strong insecticide resistance management that implies the implementation of a plan for insecticide-resistance monitoring and management in each country (72).

Malaria control strategies often target malaria vulnerable individuals including children under five of age , pregnant women and travelers from non-endemic malaria regions (5, 72). In 2019, children under 5 years of age accounted for 67% of all deaths worldwide and 35% of pregnancy were exposed to malaria infection in 2019 (1, 5). However, school-aged children (5–15 years) are also vulnerable sub-population while their malaria burden are usually underestimated (369, 370).

Given the result of a systematic review, it has been shown that the prevalence of malaria infection among school-aged children ranging from 18 to 83% sub-Saharan countries (371). However, in moderate and high malaria transmission settings younger children are mostly exposed on both severe malaria and deaths and the peak of malaria hospital admissions and mortality is generally between 1-2 years (56, 372-374), while the

distribution is relatively even across all ages under-10 years with increasing transmission intensity in younger age groups for uncomplicated clinical malaria (372). Inversely, in low malaria transmission settings where individuals are occasionally exposed to infected mosquito bites, malaria occurs in all age groups, often most frequently in adults who have an occupational risk (375). The majority of SSA countries are exposed to moderate and high malaria transmission intensity and thus, malaria control measures are mostly focused on the protection of vulnerable individuals including young children and pregnant women. School-age children is a sub-group population not usually covered by malaria control and intervention strategies and so represents an untreated sub-group that may harbour a significant parasite reservoir (376-384), thus posing a major challenge for malaria control, surveillance, and elimination strategies (376, 385-387). In high transmission sites, symptomatic malaria is often relating to children under five of years whereas, asymptomatic infections generally concern school-aged children and adults that acquired an immunity against the disease during their exposure time (59-62).

Thus, asymptomatic malaria cases are known to be predominant in stable malaria transmission areas and school-aged children are the most prevalent (385, 388-393) as compared to children under five of age and pregnant women (371, 382, 394). Even though school-age children rarely develop complicated forms of malaria, chronic infection among this group is an important major contributor to pathology, including anaemia, and thus may have profound consequences for neuro-cognitive development and educational achievement including increased absenteeism, poor school performance, and cognitive disorders (395-400). It has been shown that malaria was responsible of about 13–50% of all school absenteeism among school-aged children in endemic settings (401).

Therefore, the understanding of malaria burden in school-age children is essential to justify the impact of school-based malaria control interventions and find delivery mechanisms for the inclusion of this neglected population in the national malaria control program (401). Thus, this thesis aimed to understand the epidemiology of malaria among this underserved group population in Kinshasa in order to inform the design and employment of schools and school-age children into national malaria control and interventions.

1.9 Objectives

1.9.1 Main objective

The main objective of this thesis was to collect and analyse data that may contribute to an improved malaria control strategy in the DRC

1.9.2 Specific objectives

- **i.** To determine malaria parasite species composition of *Plasmodium* infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, DRC
- **ii.** To identify *Plasmodium falciparum* parasites lacking *P. falciparum* histidine-rich protein 2 and 3 (*pfhrp2/3*) genes among asymptomatic and symptomatic school-age children in Kinshasa and evaluate the performance of the current mRDTs used in DRC
- **iii.** to identify the prevalence of polymorphisms in *pfk13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt* genes in isolates from asymptomatic and symptomatic school-age children in Kinshasa

iv. To identify unmet needs of the current national malaria control strategy by showing the importance of capturing schools and school-age children onto national malaria survey useful for malaria control and elimination in the country.

1.10 Body of the thesis

This thesis is subdivided onto six chapters

- Chapter I. General introduction
- Chapter II. Malaria parasite species composition of *Plasmodium* infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, Democratic Republic of Congo (Published in Malaria Journal https://doi.org/10.1186/s12936-021-03919-4)
- Chapter III. Low prevalence of *Plasmodium falciparum* parasites lacking *pfhrp2/3* genes among asymptomatic and symptomatic school-age children in Kinshasa, Democratic Republic of Congo (Manuscript submitted to Malaria Journal)
- Chapter IV. Identification of polymorphisms in genes associated with drug resistance in *Plasmodium falciparum* isolates from school-age children in Kinshasa, Democratic Republic of Congo (Published in Parasitology International) https://doi.org/10.1016/j.parint.2022.102541
- Chapter V. Review: It is time to strengthen the malaria control policy of the Democratic Republic of Congo and include schools and school-age children in malaria control measures (Manuscript submitted to Parasitology International)
- Chapter VI. General discussion and conclusion

CHAPTER II. Malaria parasite species composition of Plasmodium infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, Democratic Republic of Congo

Sabin S. Nundu[1, 2, 3], Richard Culleton[4]*, Shirley V. Simpson[1, 2,], Hiroaki Arima[1], Jean-Jacques Muyembe[3], Toshihiro Mita[5], Steve Ahuka[3] and Taro Yamamoto[1, 2]

[1]Department of International Health and Medical Anthropology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan;

[2] Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;

[3]Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo;

[4]Division of Molecular Parasitology, Proteo-Science Center, Ehime University, Ehime, Japan;

[5]Department of Tropical Medicine and Parasitology, Faculty of Medicine, Juntendo University, Tokyo, Japan.

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2.1 Abstract

Background. Malaria remains a major public health concern in Democratic Republic of Congo (DRC), and school-age children are relatively neglected in malaria prevalence surveys and may constitute a significant reservoir of transmission. This study aimed to understand the burden of malaria infections in school-age children in Kinshasa/DRC.

Methods. 634 (427 asymptomatic and 207 symptomatic) blood samples collected from school-age children aged 6 to 14 years were analysed by microscopy, RDT and Nested-PCR.

Results. The overall prevalence of *Plasmodium* spp. by microscopy, RDT and PCR was 33%, 42% and 62% among asymptomatic children and 59%, 64% and 95% in symptomatic children, respectively. The prevalence of *Plasmodium falciparum, Plasmodium malariae and Plasmodium ovale* spp. by PCR was 58%, 20% and 11% among asymptomatic and 93%, 13%and 16% in symptomatic children, respectively. Living in a rural as opposed to an urban area was associated with a five-fold greater risk of asymptomatic malaria parasite carriers ($p<0.001$). Amongst asymptomatic malaria parasite carriers, 43% and 16% of children harboured mixed non-*Plasmodium* with *P. falciparum* infections in the rural and the urban areas, respectively whereas in symptomatic malaria infections, it was 22% and 26%, respectively. Few children carried single infections of *P. malariae* (2.2%) and *P. ovale* spp. (1.9%).

Conclusion. School-age children are at significant risk from both asymptomatic and symptomatic malaria infections. Continuous systematic screening and treatment of school-age children in high-transmission settings is needed.

2.2 Background

Despite a widespread reduction in malaria-asscociated morbidity and mortality in the past decade, malaria remains a major public health problem in sub-Saharan Africa. In 2018, 228 million estimated cases of malaria resulting in 405,000 deaths occurred worldwide of which 93% of cases and 94% of deaths occurred in Africa (72). In sub-Saharan Africa, the vast majority of malaria cases are caused by *Plasmodium falciparum* , with this parasite responsible for 99.7% of all cases recorded in the region in 2018 (72). *Plasmodium malariae* and *Plasmodium ovale* spp. are thought to be relatively uncommon (15, 28, 33) with a prevalence varying between 1% and 17% (26, 28, 402, 403). *Plasmodium malariae* and *Plasmodium ovale* spp. frequently occur as mixed infections with other parasites, which can lead to underestimation of their true prevalence (28, 36-39, 404). *Plasmodium malariae* infection frequently results in low parasitemia and commonly occurs in mixed infections with the more common *P. falciparum* or *P. vivax* (33, 405). Infections with *P. malariae* may remain asymptomatic for long periods, but it can cause chronic nephrotic syndrome leading to mortality (406-411). *Plasmodium ovale wallikeri* and *P. ovale curtisii* are responsible of benign tertian malaria, rarely causing severe malaria (412). These species may, however, cause jaundice, severe anemia, and pulmonary impairments (413), and even death if there is a delay in management (414, 415). The two species of *P. ovale* spp. display differences in morphology, clinical characteristics, laboratory parameters and genetics (26, 37, 402, 403, 416-419). *Plasmodium vivax* has occasionally been identified among populations in sub-Saharan Africa (420-423), but it is considered uncommon due to the high proportion of Duffy negativity amongst the local populations of west and central Africa (424, 425). The four species commonly found in sub-Saharan Africa, *P. falciparum*, *P. malariae P. ovale*

wallikeri and *P. ovale curtisi* share overlapping ranges and vectors. They are often found infecting the same human populations at the same time, and mixed infections in individual hosts are common. Currently, there is relatively little known about the consequences of their within-host or within-population interactions, and more baseline data regarding their relative prevalence in populations with varying malaria transmission dynamics is required. Furthermore, there is a lack of data regarding the clinical outcomes of mixed species versus single species infections. For malaria case management and control, rapid diagnostic tests (RDTs) and microscopy are the most widely used diagnostic tools that inform treatment (323). PCR, whilst more sensitive, is relatively expensive and technically challenging, and so is rarely used as a point-of care diagnostic in endemic areas (324). Lately, the popularity of RDTs has increased as they are cost-effective and provide an easy-to-use alternative to microscopy (426), which requires skilled microscopists to be optimally effective (326, 327). However, RDTs are only sensitive and specific for parasitemia above 200 parasites per µl blood (427) and the most widely used are those that detect *Plasmodium falciparum* histidine-rich protein 2 (*pf*hrp2) (72). The low sensitivity and specificity of both PfHRP2-based RDTs and microscopy, and a lack of experienced microscopists may result in poor malaria parasite detection (28, 428). Moreover, the prevalence of non-falciparum parasites may also be underestimated, especially when in co-infections with *P. falciparum* (28, 36-39). PCR remains the most sensitive method for detecting *non-falciparum* species, even in cases of a very low parasitemia (428). Regarding malaria burden, in high transmission sites, symptomatic malaria occurs most often in children under five years old, whereas asymptomatic infections generally occur in older people, school-age children and adults that have acquired immunity against the disease due to repeated exposure (59-62). School-age

children are not usually covered by household-based cluster surveys and/or malaria interventions and so represent an untreated demographic that may harbour a significant parasite reservoir (376-381), thus posing a major challenge for malaria control, surveillance, and elimination strategies (376, 385-387). Even though school-age children rarely develop complicated forms of malaria, chronic infection among this group is an important major contributor to pathology, including anaemia, and thus may have profound consequences for neuro-cognitive development and educational achievement including increased absenteeism, poor school performance, and cognitive disorders (395-400).

The Democratic Republic of Congo (DRC) is the second most malaria-affected country in the world after Nigeria, accounted for 12% and 11% of all estimated malaria cases and deaths worldwide, respectively (72). About 97% of its inhabitants live in perennial malaria transmission zones, in which transmission occurs for 8 to 12 months yearly (79). In DRC, malaria is still the major cause of morbidity and mortality, accounting for more than 44% of all outpatient visits, and for 22% of deaths among children under five years (79). *Plasmodium falciparum* is the most prevalent malaria parasite species responsible for most severe cases (72, 79). *Plasmodium malariae* and *P. ovale spp.* are uncommon and are mostly observed in co-infections with *P. falciparum* (33). Of the two *P. ovale* species, only *P. ovale wallikeri* has been identified in the country so far (416). Reports of the presence of *P. vivax* are rare (422, 429).

Here, the burden of malaria among asymptomatic and symptomatic school-age children living in rural and urban areas of Kinshasa/DRC was assessed and the distribution of Plasmodium species in rural areas compared to urban areas was investigated in order to

inform the design and employment of school-based malaria control interventions for malaria control in this underserved population.

2.3 Methods

Study design

A cross-sectional study was undertaken between October and November 2019 among school-age children aged 6 to 14 years in Kinshasa, DRC.

Study area and study population

The study was conducted at primary schools and health facilities in the rural area of Mont-Ngafula 2 Health Zone (HZ) and the urban area of Selembao HZ in Kinshasa city (**Figure 15**). Selembao HZ is classified as an area at moderate risk while Mont-Ngafula 2 is an area at high risk of malaria (430).

In selected schools, we included all children aged 6 to 14 years attending school on the day of survey and who had a body temperature less than 37.5˚C during a physical examination and who did not have malaria-related symptoms (including fever, headache, fatigue, chills, nausea, vomiting, etc.) in the two weeks prior to the survey.

In selected health facilities, we included all outpatient children aged 6 to 14 years seeking for healthcare within the period of survey and who exhibited fever and/or malaria-related symptoms (headache, fatigue, chills, nausea, vomiting, etc.) within the three days prior to medical consultation and who had not taken antimalarial drugs prior to the consultation.

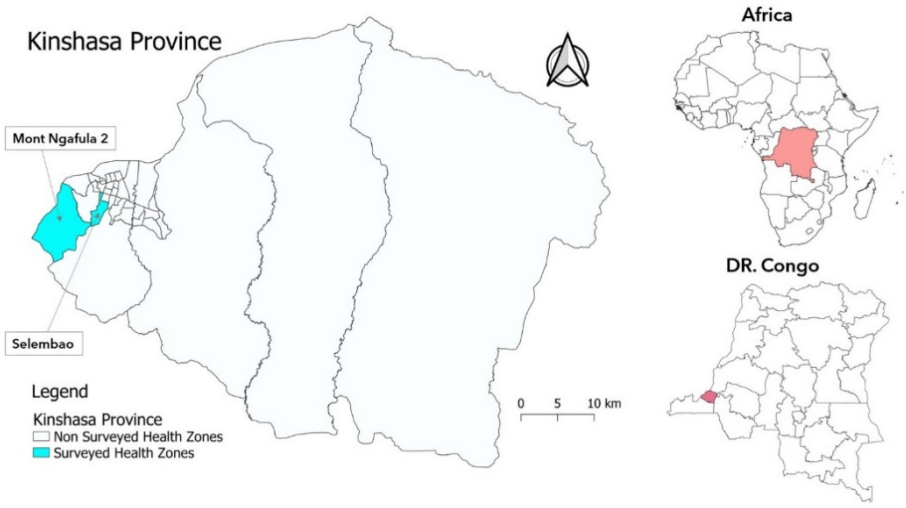

Figure 15. Sample collection sites

Sampling and Sample size determination

Both hospital-based and school-based surveys were conducted. A two-stage stratified cluster sampling protocol was performed to select two health zones (HZ) among 35 within Kinshasa and their constituent health facilities and primary schools. A simple random sampling was performed to selected two HZ (one rural and one urban) among 35 HZ, and among each HZ to select at least one health facility among health facilities and one school among schools. All school children belonging to selected schools or seeking treatment at the selected health facility with parenteral consent were included in the study.

The sample size was determined using the standard statistical formula $n= (Z^2p\ (1-p)/d^2)$ considering 95% confidence interval, 50% estimated prevalence and 5% precision.

Based on this assumption, 634 school-age children (427 asymptomatic and 207 symptomatic) were included in this study.

Data collection

In schools, two visits were necessary for sample collection. The first visit consisted of the selection of primary classrooms of each primary grade followed by the distribution of written consent forms to all children belonging to the selected classroom for their parents/guardians. On the second visit, after obtaining written consent from parents/guardians, information related to sex, age, and body temperature were recorded and a physical examination carried out. An interview was conducted for asymptomatic school-age children from 4^{th} to 6^{th} primary grades using a semi-structured questionnaire to record information related to the history of fever and malaria-related symptoms and treatment; sociodemographic status of parents (parent marital status, number of father's partners, father's education level, mother's education level, family size) and information related to the use of mosquito bed nets and/or indoor insecticide spray within households. The questionnaire was written in English by the research team, and later translated into French (official language). When children were unable to give correct answers to certain questions (questions related to the education level of parent, marital status, etc.), the question(s) were addressed to parents through telephone calls.

In health facilities samples were collected day by day depending on health facility attendance. Malaria-related symptoms and information related to gender, age, and body temperature were recorded followed by physical examination in health facilities after obtaining consent from outpatient parents/guardians.

Malaria Rapid diagnostic test (mRDTs)

An experienced laboratory technician collected approximately 5 µl finger-prick blood from each child to perform RDT. The CareStart™ Malaria *pf (hrp2)* Ag RDT (RMOM-02571) was used for the qualitative detection of malaria histidine-rich protein 2 in whole

blood according to the manufacturer's instructions. The device remained intact for 20 min at room temperature, and the result was registered accordingly.

Microscopy of Giemsa's solution-stained blood films

Thick and thin blood smears were made on the same slide in schools) and health facilities. Slides made in schools were sent to the University Hospital, University of Kinshasa for reading. Slides were stained for 10 minutes with a 10% Giemsa's solution and washed with distilled water and air-dried. Slides were read by two independent experienced laboratory technicians to determine the *Plasmodium* species and parasitemia. The asexual forms were counted with reference to 200 leukocytes. In the case that fewer than 100 parasites were counted against 200 leukocytes, the count continued until 500 leukocytes were observed. Parasite density was calculated based on a total of 8000 leukocytes/µl using the following formula: (Number of Parasites counted × 8000)/Number of counted leukocytes. The parasitemia was obtained by averaging the results of the two independents readers.

In the event of discrepancy results, a third laboratory technician was involved for confirmation. The laboratory technicians counted a minimum of 100 consecutive fields in the thick blood film before classifying a slide as negative. All slides prepared in schools were read by the experienced microscopists of the parasitology unit of the University Hospital, University of Kinshasa.

DNA Extraction

In both schools and health facilities, three capillary blood drops from finger-prick were collected and spotted onto of Whatman 903™ filter paper (Whatman plc, UK) for PCR analysis. Whatman 903™ filter papers containing blood samples were dried and stored

in individual plastic bags containing desiccant and stored at -20˚C before transportation to Nagasaki University for PCR analysis. All DNA templates were extracted from three punched discs (6 mm in diameter) of blood spots using the QIAamp DNA Mini Kit® (Qiagen, USA) according to the manufacturer's instructions. DNA was eluted in 50 µL of the provided buffer.

Nested-PCR for malaria parasite species typing

A nested PCR using primers targeting the *Plasmodium* mitochondrial cytochrome c oxidase III (*cox3*) gene was performed using the protocol described by Isozumi *et al* (431) with minor modifications; in particular, the *P. vivax* specific primers were redesigned due to concerns regarding the non-specific binding of the originally described primers (**Annex: Table 1**). PCR products were visualized under UV light on 1.5% agarose gels run at 100 volts for 30 minutes and stained with Gel Red® solution for 30 minutes. The outer PCR product was diluted 1:100 with sterile water and used as template for inner PCRs. Plasmodium species genotyping was performed using *Plasmodium* spp. outer PCR products to separately amplify specific products of *P. falciparum, P. malariae, P. ovale* spp. *and P. vivax*. PCR products were visualized under UV light on 2% agarose gels run at 100 volts for 30 minutes and stained with Gel Red® solution for 30 minutes. The outer PCR product was diluted 1:100 with sterile water and used as template for inner PCRs.

Seventy one out of 79 *P. ovale* spp. positive PCR products were successfully sequenced using the inner PCR forward (Nst_ovaF) primer. There was insufficient material of the remaining eight samples to make a species-level identification. Sequence data files were analysed using MEGA software. Sequence data from NCBI for reference (*P. ovale curtisi* [GenBank: HQ712052.1] and *P. ovale wallikeri* [GenBank: HQ712053.1]) were used as references. From the sequence data sets, a phylogenetic tree was generated using the NJ

method. According to the phylogenetic tree, the samples were assigned species designations based on clustering with the reference sequences.

Data management and analysis

Data was double-entered and validated in EPI INFO version 3.5.1 and analysed using STATA version 14.2 (College Station. Texas, USA). Descriptive variables were analysed as proportions (categorical variables) or by median and interquartile range (continuous variables). Chi-square tests (or Fisher's exact tests when appropriate) and logistic regression analysis were used to assess associations between independent variables and *Plasmodium* spp. infection prevalence. Odds ratios (ORs) and 95% confidence intervals (CIs) were derived. Significance was set at $p < 0.05$.

Ethical considerations

The study was approved by the ethics committees of the School of Public Health, University of Kinshasa, DRC (Approval number: ESP/CE/042/2019) and the Institute of Tropical Medicine, Nagasaki University (Approval number: 190110208-2). Written informed consent was obtained from children's parents/guardians and assent from children ≥ 7 years old were sought. The written informed consent document was provided either in French (official language) or Lingala (local language) depending on the parent's educational background. All malaria positive cases from mRDTs were treated according to national malaria diagnosis and treatment guidelines.

2.4. Results

Description of study population

A total of 634 (210 asymptomatic and 105 symptomatic in the rural area; 217 asymptomatic and 102 symptomatic in the urban area) children aged 6 to 14 years old were included in this study. Their median (interquartile range) age was 9 (7-11) [asymptomatic: 9 (7-11); symptomatic: 8 (7-9)] in the rural area, and 9 (7-10) [asymptomatic: 8 (7-10); symptomatic: 9 (7-12)] in the urban area. Children aged 6 to 9 years accounted for 59.7% (188/315) [asymptomatic: 50.5% (106/210); symptomatic: 78.1% (82/105)] in the rural area, and 63.3% (202/319) [asymptomatic: 68.2% (148/217); symptomatic: 52.9% (54/102)] in the urban setting; 49.5% (156/315) [asymptomatic: 49.5% (104/210); symptomatic: 49.5% (52/105)] in the rural area, and 51.4% (164/319) [asymptomatic: 54.8% (119/217); symptomatic: 41.1% (45/102)] in the urban setting were females (**Table 1**).

Table 1. Description of study population

	Rural area			Urban area		
Variables	**Asymptomatic (N=210) Number (%)**	**Symptomatic (N=105) Number (%)**	**Total (N=315) Number (%)**	**Asymptomatic (N=217) Number (%)**	**Symptomatic (N=102) Number (%)**	**Total (N=319) Number (%)**
Gender						
Female	104 (49.5)	52 (49.5)	156 (49.5)	119 (54.8)	45 (44.1)	164 (51.4)
Male	106 (50.5)	53 (50.5)	159 (50.5)	98 (45.2)	57 (55.9)	155 (48.6)
Age, Median (IQR)	**9 (7-11)**	**8 (7-9)**	**9 (7-11)**	**8 (7-10)**	**9 (7-12)**	**9 (7-10)**
6-9	106 (50.5)	82 (78.1)	188 (59.7)	148 (68.2)	54 (52.9)	202 (63.3)
10-14	104 (49.5)	23 (21.9)	127 (40.3)	69 (31.8)	48 (47.1)	117 (36.7)

Information relating to sociodemographic characteristics of asymptomatic school-age children and malaria preventive measures

Among 227 (131 in the rural area and 96 in the urban area) asymptomatic school-age children who were interviewed, most of children (>80%) lived together with their parents in both rural and urban areas, 46% (rural: 60%, urban: 28%) had at least one mosquito bed net and 29% (rural: 31%, urban: 25%) slept under a mosquito net the night before the interview (**Table 2**).

Table 2. Sociodemographic characteristics of asymptomatic school-age children

Variables	Overall (N=227) Number	%	Rural area (N=131) Number	%	Urban area (N=96) Number	%
Parent marital status						
Live together	201	88.5	124	94.7	77	80.2
Separated	26	11.5	7	5.3	19	19.8
Father partner size						
One	183	80.6	101	77.1	82	85.4
Two or more	44	19.4	30	22.9	14	14.6
Father education level						
Primary	10	4.4	10	7.6	0	0
Junior school	46	20.3	37	28.2	9	9.4
High school	86	37.9	50	38.2	36	37.5
University	55	24.2	24	18.3	31	32.3
Don’t know	30	13.2	10	7.6	20	20.8
Mother education level						
Primary	28	12.3	23	17.6	5	5.2
Junior school	58	25.6	47	35.9	11	11.5
High school	82	36.1	45	34.3	37	38.5
University	30	13.2	9	6.9	21	21.9
Don’t know	29	12.8	7	5.3	22	22.9
House status						
Own/family house	118	52.0	73	55.7	45	46.9
Rental house	109	48.0	58	44.3	51	53.1
Family size						
≤ 4	71	31.3	43	32.8	57	59.4
> 4	156	68.7	88	67.2	39	40.6
Insecticide spray in the household						
Yes	64	28.2	50	38.2	14	14.6
No	163	71.8	81	61.8	82	85.4
Mosquito net in the household						
Yes	105	46.3	78	59.5	27	28.1
No	122	53.7	53	40.5	69	71.9
Slept last night with Mosquito net						
Yes	65	28.6	41	31.3	24	25.0
No	162	71.4	90	68.7	72	75.0

History of last fever and/or malaria-like symptoms

Among 131 children interviewed in the rural area, 54% reported a malaria episode in the three months and above prior to the survey while 37% reported a malaria episode between two weeks and one month and only in 9% reported a malaria episode between one month and three months. Only 34% went to a health facility, and the majority of these (82%) went to a health centre. Forty-four percent of children reported having self-medicated (of which 33% had used pyrimethamine/sulfadoxine and 11% artemisinin-based combination therapy) given by their parents without a confirmed malaria diagnosis. Fifty-two percent of children missed classes and among these, 39% missed five days or more (**Figure 11**). Among 96 children interviewed in the urban area, 78% reported a malaria episode in the three months and above prior to the survey while 16% reported a malaria episode between two weeks and one month and only in 6% reported a malaria episode between one month and three months. Only 39% went to a health facility, and the majority of these (95%) went to a health centre. Seventeen percent of children reported having self-medicated with antimalarial drugs (of which 25% used Pyrimethamine/sulfadoxine, 25% used artemisinin-based combination therapy and 50% did not remember the drug's name) given by their parents without a confirmed malaria diagnosis. Forty-four percent of children missed classes and among these, 38% missed five days or more (**Figure 16**).

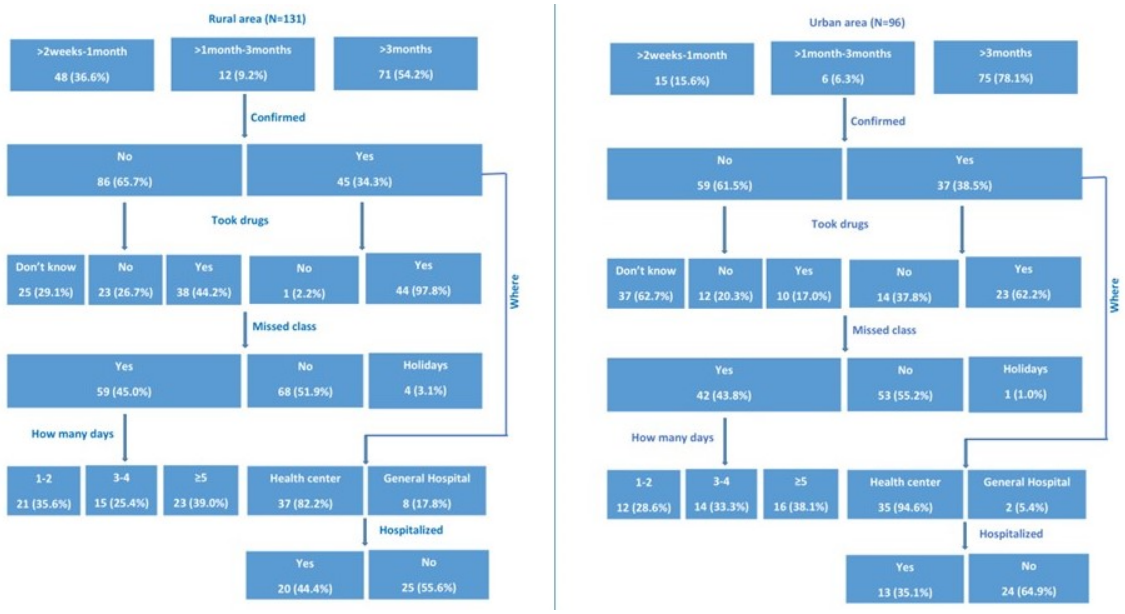

Figure 16. History of last episode of malaria infection among asymptomatic school-age children

Symptoms of outpatient school-age children at admission

All 207 children (105 in the rural area and 102 in the urban area) attending health facilities had fever symptoms (100%), followed by fatigue (39%, rural: 28%, urban: 50%), headache (38%, rural: 6%, urban: 72%), vomiting (36%, rural: 37%, urban: 35%), lack of appetite (25%, rural: 2%, urban: 49%) and abdominal pain (25%, rural: 2%, urban: 48%) as major symptoms (**Table 3**).

Table 3. Symptoms of outpatient school-age children at admission

Variables	Overall (N=207)		Rural area (N= 105)		Urban area (N= 102)	
	Number	%	Number	%	Number	%
Fever						
Yes	207	100.0	105	100.0	102	100.0
No	0	0.0	0	0.0	0	0.0
Headache						
Yes	76	38.2	6	5.7	73	71.6
No	128	61.8	99	94.3	29	28.4
Vertigo						
Yes	21	10.1	4	3.8	17	16.7
No	186	89.9	101	96.2	85	83.3
Abdominal pain						
Yes	51	24.6	2	1.9	49	48.0
No	156	75.4	103	98.1	53	52.0
Nausea						
Yes	40	19.3	9	8.6	31	30.4
No	167	80.7	96	91.4	71	69.6
Lack of appetite						
Yes	52	25.1	2	1.9	50	49.0
No	155	74.9	103	98.1	52	51.0
Vomiting						
Yes	75	36.2	39	37.1	36	35.3
No	132	63.8	66	62.9	66	64.7
Diarrhea						
Yes	41	19.8	24	22.9	17	16.7
No	166	80.2	81	77.1	85	83.3
Body heat						
Yes	15	7.3	2	1.9	13	12.7
No	192	97.7	103	98.1	89	87.3
Chills						
Yes	41	19.8	2	1.9	38	37.3
No	166	80.2	103	98.1	64	62.7
Fatigue						
Yes	80	38.7	29	27.6	51	50.0
No	127	61.3	76	72.4	51	50.0
Lumbago						
Yes	29	14.0	2	1.9	27	26.5
No	178	86.0	103	98.1	75	73.5

Prevalence of Plasmodium spp. *infections by microscopy, RDTs and PCR*

The overall prevalence of *Plasmodium* spp. was 42% (asymptomatic 33%, symptomatic: 59%), 49% (asymptomatic: 42%, symptomatic: 64%) and 73% (asymptomatic: 62%, symptomatic: 95%) by microscopy, RDT and PCR, respectively (**Table 4**).

Table 4. Prevalence of *Plasmodium* spp. infections by microscopy, RDTs and PCR

	Overall (N=634)	**Asymptomatic infection (N=427)**	**Symptomatic infection (N=207)**
Technique	n (%)	n (%)	n (%)
Microscopy	263 (41.5)	140 (32.8)	123 (59.4)
RDT	310 (48.9)	177 (41.5)	133 (64.3)
PCR	462 (72.9)	266 (62.3)	196 (94.7)

Comparison between Microscopy or RDT and PCR

Among 263 *Plasmodium* spp. detected by microscopy, the vast majority (overall: 252/263 (95.8%), asymptomatic: 130/140 (92.9%) and symptomatic: 122/123 (99.2%)) were positive by PCR. Among 310 *Plasmodium* spp. detected by RDT, the vast majority (overall: 305/310 (98.4%), asymptomatic: 174/177 (98.3%) and symptomatic: 131/133 (98.5%)) were positive by PCR. Among 476 *Plasmodium* spp. isolates, 210/476 (44.1%) were positive by PCR while negative by microscopy, 157/476 (33.0%) were positive by PCR while negative by RDT and only 11/476 (0.2%) and 5/476 (0.1%) were positive by microscopy and RDT while negative by PCR, respectively (**Figure 17**).

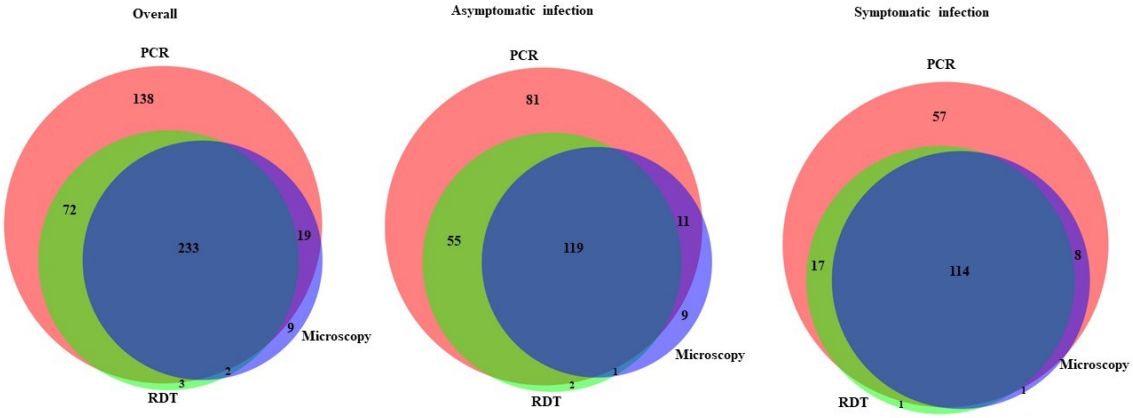

Figure 17. Comparison between microscopy or RDT and PCR

Overall (N=476) and in asymptomatic (N=278) and symptomatic (N=198) infections

Of 12 *P. malariae* and 11 *P. ovale* spp. single infections identified by PCR, six were positive by *Pf*HRP*2*-based RDT; one child harboured *P. malariae* and five children carried *P. ovale* spp. infections (**Table 5**)

Table 5. Number of *P. malariae* (N=12) and *P. ovale spp.* (N=11) while *P. falciparum* is absent by PCR compared to RDT results

	Overall (n=8)			**Rural area Asymptomatic (n=7)**			**Symptomatic (n=1)**			**Overall (n=13)**			**Urban area Asymptomatic (n=11)**			**Symptomatic (n=2)**		
RDTs	***Pm***	***Pm + Po***	***Po***	***Pm***	***Pm + Po***	***Po***	***Pm***	***Pm + Po***	***Po***	***Pm***	***Pm + Po***	***Po***	***Pm***	***Pm + Po***	***Po***	***Pm***	***Pm + Po***	***Po***
Negative	3	0	4	3	0	4	0	0	0	2	2	4	1	2	4	1	0	0
Positive	0	0	1	0	0	0	0	0	1	5	0	0	4	0	0	1	0	0
Total	3	0	5	3	0	4	0	0	1	7	2	4	5	2	4	2	0	0

Pm: P. malariae; Po: P. ovale spp.

Comparison of Plasmodium infection prevalence between rural and urban areas among asymptomatic and symptomatic school-age children by PCR

All malaria parasite species were significantly more prevalent in the rural area compared to the urban setting in asymptomatic infections (p<0.001), whereas in symptomatic infections, *P. malariae* was significantly less prevalent in the rural area compared to the urban setting (7.6 versus 17.7%, p=0.03). No *P. vivax* infections were observed (**Table 6**).

Table 6. Comparison of Plasmodium species including mono- and mixed infections between rural and urban areas in asymptomatic and symptomatic infections

	Asymptomatic infection					**Symptomatic infection**				
	Rural area (N=210)		**Urban area (N=217)**			**Rural area (N= 105)**		**Urban area (N= 102)**		
Malaria infections	**no**	**%)**	**no**	**%**	**p-value**	**no**	**%**	**no**	**%**	**p-value**
Plasmodium species										
Plasmodium spp.	168	80.0	98	45.2	<0.001	102	97.1	94	92.2	0.11
P. falciparum	161	76.7	87	40.0	<0.001	101	96.2	92	90.2	0.09
*P. malariae**	66	31.4	19	8.8	<0.001	8	7.6	18	17.7	0.03
P. avale spp.	35	16.7	11	5.1	<0.001	19	18.1	14	13.7	0.39
Type of Plasmodium infection										
Single infection	96	45.7	82	37.8	<0.001	80	76.2	70	68.3	0.10
Mixed infection	72	34.3	16	7.4	<0.001	22	21	24	23.5	0.22

Out of 71 children infected with *P. ovale* spp., 53 (75%), 17 (24%) and 1 (1%) harboured *P. ovale curtisi*, *P. ovale wallikeri* and mixed *P. ovale curtisi* + *P. ovale wallikeri* infections, respectively. *Plasmodium ovale curtisi* was more frequent than *P. ovale wallikeri* in the rural area (86.0 vs 14.0%) while they were equally distributed in urban area (48% vs 48 %) with one child (5%) harbouring a mixed *P. ovale curtisi* + *P. ovale wallikeri* infection in the urban area (**Table 7**).

Table 7. Distribution of *P. ovale curtisi* and *P. ovale wallikery* by location, health status, age, and gender

	Poc (N=53)	**Pow (N=17)**	**Poc+Pow (N=1)**
	n (%)	**n (%)**	**n (%)**
Location			
Rural	43 (86.0)	7 (14.0)	0 (0.0)
Urban	10 (47.6)	10 (47.6)	1 (4.8)
Health status			
Asymptomatic	33 (76.7)	10 (23.3)	0 (0.0)
Symptomatic	20 (71.4)	7 (25.0)	1 (3.6)
Age (years)			
6-9	28 (70.0)	11 (27.5)	1 (2.5)
10-14	25 (80.7)	6 (19.3)	0 (0.0)
Gender			
Female	22 (73.3)	7 (23.3)	1 (3.3)
Male	31 (75.6)	10 (24.4)	0 (0.0)

Poc : *P. ovale curtisi* ; Pow : *P. ovale wallikeri*

Plasmodium species composition

Of the 462 malaria positive children, 270 (168 asymptomatic and 102 symptomatic) resided in the rural area and 192 (98 asymptomatic and 94 symptomatic) in the urban setting.

In the rural area, 262 (97%) [161 (96%) asymptomatic and 101 (99%) symptomatic] carried *P. falciparum*, 74 (27%) [66 (39%) asymptomatic and 8 (8%) symptomatic] carried *P. malariae*, and 54 (20%) [35 (21%) asymptomatic and 19 (19%) symptomatic) carried *P. ovale spp.* parasites. In the urban area, 179 (93%) [87 (89%) asymptomatic and 92 (98%) symptomatic] carried *P. falciparum*, 37 (19%) [19 (19%) asymptomatic and 18 (15%) symptomatic] carried *P. malariae*, and 25 (13.0%) [11 (11%) asymptomatic and 14 (18%) symptomatic] carried *P. ovale spp.* parasites. There were 89 (53%), 3 (2%) and 4 (2%) single infections of *P. falciparum*, *P. malariae* and *P. ovale spp.*, in asymptomatic participants in the rural area, while there were 79 (77%), 0 and 1 (1%) in symptomatic participants, respectively. There were 73 (75%), 5 (5%) and 4 (4%) single infections of *P. falciparum*, *P. malariae* and *P. ovale*, in asymptomatic participants in the urban area, while there were 68 (72%), 2 (2%) and 0 in symptomatic participants, respectively. There were significantly more infections involving *P. malariae* and *P. ovale spp.* in asymptomatic children compared to symptomatic children in the rural area, whereas there were similar rates of carriage of these species in asymptomatic and symptomatic infections in the urban setting (**Table 8, Figure 13**).

***Plasmodium* mono and mixed infections**

In the rural area, 94 (35%) [72 (43%) asymptomatic and 22 (22%) symptomatic] individuals carried mixed infections, whereas in the urban area 40 (21%) [16 (16%) asymptomatic and 24 (26%) symptomatic] carried mixed infections ((**Table 8, Figure 13**).

Table 8. Proportion of Plasmodium species composition in asymptomatic and symptomatic infections by location

	Rural area			**Urban area**		
	Overall (N=270) n (%)	**Asymptomatic (N= 168)** n (%)	**Symptomatic (N= 102)** n (%)	**Overall (N=192)** n (%)	**Asymptomatic (N= 98)** n (%)	**Symptomatic (N= 94)** n (%)
Type infection						
Single infection	176 (65.2)	96 (57.1)	80 (78.4)	152 (79.2)	82 (83.7)	70 (74.5)
Mixed infection	94 (34.8)	72 (42.9)	22 (21.6)	40 (20.8)	16 (16.3)	24 (25.5)
***Plasmodium* species composition**						
Pf	168 (62.2)	89 (53.0)	79 (77.4)	141 (73.4)	73 (74.5)	68 (72.3)
Pm	3 (1.1)	3 (1.8)	0 (0.0)	7 (3.7)	5 (5.1)	2 (2.1)
Po	5 (1.9)	4 (2.4)	1 (1.0)	4 (2.1)	4 (4.1)	0 (0.0)
Pf+*Pm*	45 (16.7)	41 (24.4)	4 (3.9)	19 (9.9)	9 (9.2)	10 (10.6)
Pf+ *Po*	23 (8.5)	9 (5.4)	14 (13.7)	10 (5.2)	2 (2.0)	8 (8.5)
Pm+*Po*	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	2 (2.0)	0 (0.0)
Pf+*Pm*+*Po*	26 (9.6)	22 (13.1)	4 (3.9)	9 (4.7)	3 (3.1)	6 (6.4)

Pf: *Plasmodium falciparum*; *Pm*: *Plasmodium malariae*; *Po*: *Plasmodium ovale* spp.

In the rural setting there was a large difference in the proportion of single and mixed species infections between asymptomatic and symptomatic children, with more mixed species infections observed in asymptomatic children compared to symptomatic children, however, in the urban region there was little difference in the proportion of single and mixed species infections between asymptomatic and symptomatic children (**Figure 18**).

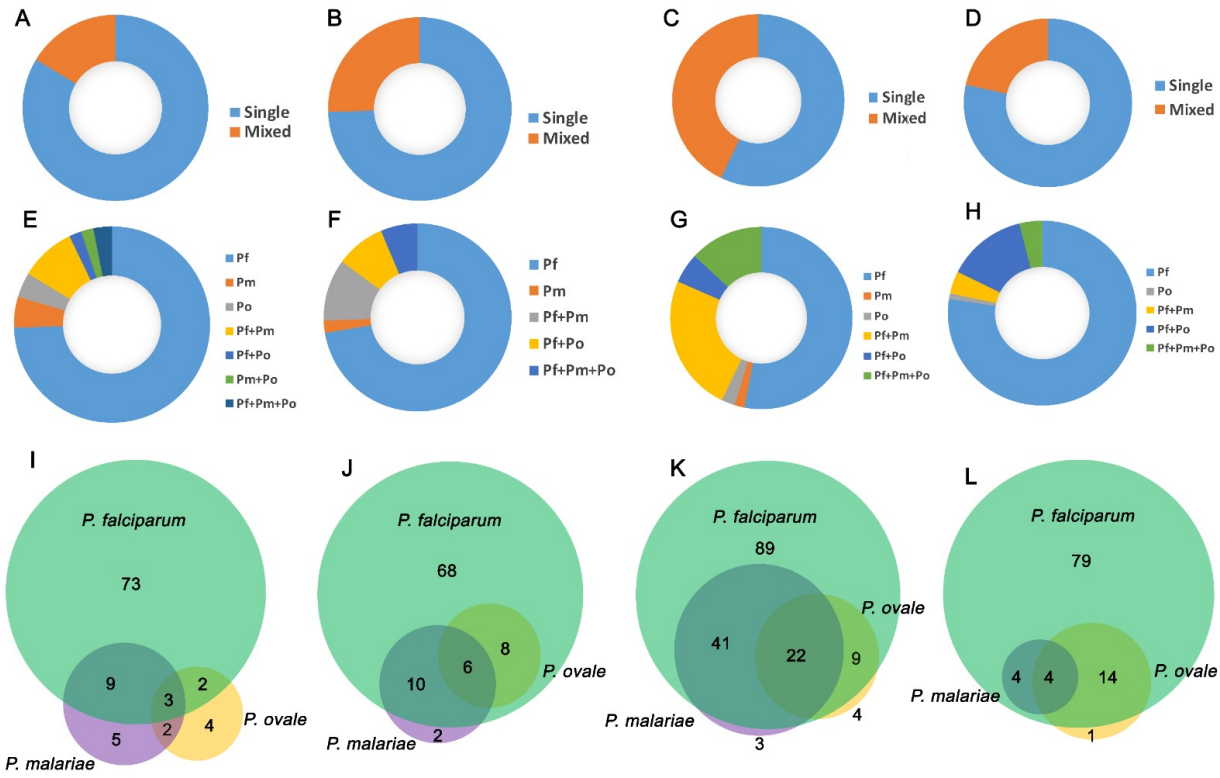

Figure 18. Species composition of Plasmodium infections

(98 asymptomatic (Panels A, E and I) and 94 symptomatic (B, F, and J) children in the urban Selembao health zone (HZ), and 168 asymptomatic (Panels C, G and K) and 102 symptomatic (Panels D, H, and L) children in the rural Mont-Ngafula 2 HZ)

The urban Selembao HZ: Single and mixed infections in asymptomatic (A) and symptomatic (B) children. Malaria parasite species composition in asymptomatic (E & I) and symptomatic (F & J) children. The rural Mont-Ngafula 2 HZ: Single and mixed infections in asymptomatic (C) and symptomatic (D) children. Malaria parasite species composition in asymptomatic (G & K) and symptomatic (H & L). Pf = *Plasmodium falciparum*, Pm = *Plasmodium malariae*, Po = *P. ovale wallikeri* and *P. ovale curtisi ("P. ovale"* includes both *P. ovale* species*").*

Distribution of Plasmodium species infections by age stratified by location

Regarding the distribution of the malaria parasite species infecting asymptomatic children by age in the rural and urban areas, the trend of *P. falciparum* did not change with age in the rural setting, while it increased with age in the urban area. *Plasmodium malariae* and *P. ovale* spp. infections were more common in children aged 10 years and above in the rural area (**Figure 19**).

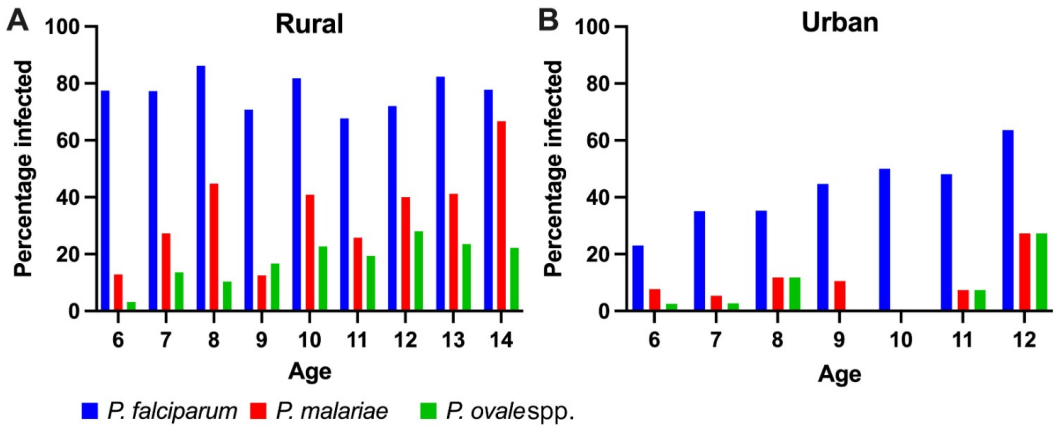

Figure 19. Distribution of Plasmodium species infections by age stratified by location (rural and urban areas)

Association age / gender with *Plasmodium* species infection prevalence

In the rural area, asymptomatic children aged 10 to 14 years were significantly more likely to be infected with *P. malariae* (38.5 versus 24.5%, p=0.03) and *P. ovale* spp. (23.1 versus 10.4%, p=0.014) than those aged 6 to 9 years. In the urban area, however, older children were more likely to carry *P. falciparum* than younger children (50.7 versus 35.1%, p=0.029). There was no association between gender and any particular *Plasmodium* species infection in either the rural or urban areas (**Table 9**).

Table 9. Association Plasmodium species with age and gender by location in asymptomatic infections

		Overall (N=427)							
		Plasmodium spp.		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p**	**n (%)**	**p**	**n (%)**	**p-value**
Gender			0.56		0.77		0.29		0.21
Female	223	136 (61.0)		128 (57.4)		40 (17.9)		20 (9.0)	
Male	204	130 (63.7)		120 (58.8)		45 (22.1)		26 (12.8)	
Age (years)			**0.022**		**0.012**		**0.004**		**0.001**
6-9	254	147 (57.9)		135 (53.2)		39 (15.4)		17 (6.7)	
10-14	173	119 (68.8)		113 (65.3)		46 (26.6)		29 (16.8)	

		Rural area (N=210)							
		Plasmodium spp.		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Gender			0.27		0.22		0.27		0.39
Female	104	80 (76.9)		76 (73.1)		29 (27.9)		15 (14.4)	
Male	106	88 (83.0)		85 (80.2)		37 (34.9)		20 (18.9)	
Age (years)			0.45		0.57		0.03		0.014
6-9	106	87 (82.1)		83 (78.3)		26 (24.5)		11 (10.4)	

10-14	104	81 (77.9)		78 (75.0)		40 (38.5)		24 (23.1)	
		Urban area (N=217)							
		Plasmodium spp.		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Gender			0.54		0.23		0.78		0.52
Female	119	56 (47.1)		52 (43.7)		11 (9.2)		5 (4.2)	
Male	98	42 (42.9)		35 (35.7)		8 (8.2)		6 (6.1)	
Age (years)			0.045		0.029		0.98		0.32
6-9	148	60 (40.5)		52 (35.1)		13 (8.8)		6 (4.1)	
10-14	69	38 (55.1)		35 (50.7)		6 (8.7)		5 (7.3)	

In symptomatic children, there was no association between age or gender with particular malaria parasite infections in either the rural or the urban areas except for *P. ovale* spp. which infected younger children more often in the urban setting (4.2 versus 22.2 %, p=0.009) (**Table 10**).

Table 10. Association Plasmodium species with age and gender by location in symptomatic infections

		Overall (N=207)							
		***Plasmodium* spp.**		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Gender			0.18		0.16		0.08		0.35
Female	97	94 (96.9)		93 (95.9)		8 (8.3)		13 (13.4)	
Male	110	102 (92.7)		100 (90.9)		18 (16.4)		20 (18.2)	
Age (years)			**0.035**		0.20		0.97		**0.011**
6-9	136	132 (97.1)		129 (94.8)		17 (12.5)		28 (20.6)	
10-14	71	64 (90.1)		64 (90.1)		9 (12.7)		5 (7.0)	

		Rural area (N=105)							
		***Plasmodium* spp.**		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Gender			0.57		0.32		0.15		0.48
Female	52	51 (98.1)		51 (98.1)		2 (3.9)		8 (15.4)	
Male	53	51 (96.2)		50 (94.3)		6 (11.3)		11 (20.8)	
Age (years)			0.057		0.17		0.83		0.48
6-9	82	81 (98.8)		80 (97.6)		6 (7.3)		16 (19.5)	

10-14	23	21 (91.3)		21 (91.3)		2 (8.7)		3 (13.0)	

		Urban area (N=102)							
		***Plasmodium* spp.**		***P. falciparum***		***P. malariae***		***P. ovale* spp.**	
Variables	**Number**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Gender			0.26		0.34		0.31		0.49
Female	45	43 (95.6)		42 (93.3)		6 (13.3)		5 (11.1)	
Male	57	51 (89.5)		50 (87.7)		12 (21.1)		9 (15.8)	
Age (years)			0.36		0.84		0.44		**0.009**
6-9	54	51 (94.4)		49 (90.7)		11 (20.4)		12 (22.2)	
10-14	48	43 (89.6)		43 (89.6)		7 (14.6)		2 (4.2)	

Older children were less likely to harbour single species (33.7 versus 57.6%) and more likely to harbour mixed species infections (44.2 versus 24.5 %), (p=0.02) infections in the rural area, whereas in the urban area older children were insignificantly more likely to harbour both single species (46.4 versus 33.8%) and mixed species infections (8.7 versus 6.8 %), (p=0.13) in asymptomatic infections. There is was no association between gender and Plasmodium species mono and mixed infections in both rural and urban areas(**Table 11**).

Table 11. Association of Plasmodium species mono and mixed infections with age and gender by location in asymptomatic infections

	Rural area (N=210)			
		Single infection	**Mixed infection**	
Variables	**Number**	n (%)	n (%)	p-value
Gender				0.32
Female	104	49 (47.1)	31 (29.8)	
Male	106	47 (44.3)	41 (38.7)	
Age (years)				**0.002**
6-9	106	61 (57.6)	26 (24.5)	
10-14	104	35 (33.7)	46 (44.2)	
	Urban area (N=217)			
		Single infection	**Mixed infection**	
Variables	**Number**	n (%)	n (%)	p-value
Gender				0.74
Female	119	46 (38.7)	10 (8.4)	
Male	98	36 (36.7)	6 (6.1)	
Age (years)				0.13
6-9	148	50 (33.8)	10 (6.8)	
10-14	69	32 (46.4)	6 (8.7)	

Both age and gender were not associated with carriage of *Plasmodium* species single and mixed infections in either the rural or the urban areas in symptomatic infections (**Table 12**).

Table 12. Association of Plasmodium species mono and mixed infections with age and gender by location in symptomatic infections

		Rural area (N=105)		
		Single infection	Mixed infection	
Variables	**Number**	n (%)	n (%)	p-value
Gender				0.53
Female	52	42 (80.8)	9 (17.3)	
Male	53	38 (71.7)	13 (24.5)	
Age (years)				0.12
6-9	82	62 (75.6)	19 (23.2)	
10-14	23	18 (78.3)	3 (13.0)	

		Urban area (N=102)		
		Single infection	Mixed infection	
Variables		n (%)	n (%)	p-value
Gender				0.087
Female	45	36 (80.0)	7 (15.6)	
Male	57	34 (59.7)	17 (29.8)	
Age (years)				0.11
6-9	54	34 (63.0)	17 (31.5)	
10-14	48	36 (75.0)	7 (14.6)	

Predictors of asymptomatic malaria infection

Residence in the rural setting was associated with an increased risk of asymptomatic malaria parasite carriage. There was an approximately five times greater risk of asymptomatic carriage of malaria parasites for children living in the rural, as opposed to

urban area ($p<0.001$). Other investigated factors including use of mosquito nets were not associated with increased risk of asymptomatic malaria infection (**Table 13).**

Table 13. Predictors for asymptomatic malaria infections (Univariate versus Multivariate)

Predictors	COR	95% CI	CPR	95%CI	AOR	95% CI	p	APR	95%CI	p
Gender										
Female	1.0		1.0		1.0			1.0		
Male	1.2	0.8-1.7	1.0	0.9-1.2	1.0	0.5-2.0	0.91	1.0	0.9-1.5	0.65
Age (years)										
6-9	1.0		1.0		1.0			1.0		
10-14	1.6	1.1-2.4	1.2	1.0-1.4	2.0	0.9-4.4	0.08	1.2	0.9-1.5	0.21
Marital status										
Live together	1.0		1.0		1.0			1.0		
Separated	1.2	0.5-2.9	1.1	0.8-1.4	3.0	0.8-11.2	0.11	1.1	0.8-1.4	0.88
Family size										
2-4	1.0				1.0					
>4	1.3	0.7-2.3	1.1	0.9-1.4	0.9	0.4-1.8	0.7	1.0	0.9-1.3	0.65
Father education level										
High	1.0		1.0		1.0			1.0		
Low	1.3	0.6-2.5	1.0	0.8-1.2	0.7	0.2-1.9	0.45	0.9	0.7-1.3	0.35
Mother education level										
High	1.0				1.0			1.0		
Low	1.3	0.7-2.3	1.0	0.9-1.3	1.2	0.4-3.4	0.72	1.0	0.8-1.2	0.78
Location of children										

Urban	1.0		1.0		1.0			1.0		
Rural	4.9	3.2-7.5	1.8	1.5-2.1	4.9	2.1-11.5	<0.001	1.5	1.2-1.9	<0.001
Insecticide spray in household										
Yes	1.0		1.0		1.0			1.0		
No	0.7	0.4-1.3	0.9	0.7-1.1	0.7	0.3-1.4	0.23	1.0	0.9-1.2	0.76
Mosquito-net in household										
Yes	1.0				1.0					
No	0.8	0.4-1.4	0.9	0.8-1.1	1.8	0.6-4.8	0.26	1.0	0.8-1.3	0.81
Slept last night under Mosquito-net										
Yes	1.0				1.0			1.0		
No	1.1	0.6-1.9	1.0	0.8-1.3	0.8	0.3-2.1	0.62	1.0	0.8-1.2	0.82

COR: Crude odds ratio; AOR: Adjusted odd ratio; CI: Confidence interval

CPR: Crude Prevalence ratio; APR: Adjusted Prevalence ratio

2.5 Discussion

This study aimed to measure the burden of *Plasmodium* spp. infections including *P. falciparum* and non-*Plasmodium falciparum* single and mixed infections amongst asymptomatic and symptomatic school-age children living in rural and urban areas in Kinshasa, DRC.

Kinshasa, the capital city of DRC, constitutes an urban malaria facies where malaria prevalence is moderate, with an average of 12% of the population infected at any given time in the city, with increase prevalence variations away from the city centre (79, 432). Malaria transmission rates are not homogenous throughout the city and depend on the population density and level of urbanization. The prevalence is highest in the more densely populated and less urbanized zones in the periphery (79, 430).

Additionally, malaria infection usually follows a seasonal pattern regulated by mosquito population fluctuations controlled by climate (53, 430). This study was conducted at the beginning of the rainy season between October and November when conditions of temperature and humidity are favourable for malaria transmission. Temperature, humidity, and rainfall constitute important drivers of mosquito dynamics and malaria risk (53, 433, 434).

The overall prevalence of *Plasmodium* spp. was 32%, 41%and 62% among asymptomatic children and 59%, 64%and 95% in symptomatic children by microscopy, RDT and PCR, respectively. The vast majority (>95%) of microscopic and RDT positive results were positive by PCR. Also, around 44% of microscopy and 33% of RDT negative results were positive by PCR while hardly ever (<1%) negative results by PCR were positive by microscopy or RDT. These findings underline that PCR is more sensitive than

microscopy and RDT (324, 428, 435) in low parasitemia detecting parasitemia below 5 parasites/μL (436) with a range 0.1-10 parasites/μL blood (437-439) while in low parasitaemia, less than 50-100 per μL blood for microscopy (440, 441) and less than 100-200 per μL blood for RDTs (427, 440, 442) there is a risk of false negative results. We found eleven DNA samples were positive by microscopy while negative by PCR. This may be due to misinterpretation of the results. It has been shown that the quality of diagnosis based on microscopy is often inadequate and there is a possibility of inadequate quality control, misdiagnosis due to low parasitaemia or mixed infections when microscopists are not experienced enough (443-447). We also found five DNA samples were positive by RDT while negative by PCR. This may be due to the major constraint of false positives by HRP2-based RDTs as HRP2 may still circulate in the blood for several days after infection clearance (448).

The high prevalence found by PCR in both asymptomatic and symptomatic children highlight the importance of malaria in this underserved population in Kinshasa.

In asymptomatic children, all *Plasmodium* species infections were significantly more prevalent in the rural area compared to the urban setting. There was a significant difference in malaria prevalence in children living in the rural area as opposed to the urban setting, with the former significantly more likely to be infected with malaria parasites. This finding agrees with numerous previous reports, and likely reflects the fact that the ratio of mosquitoes to humans is higher in rural areas than in urban areas (53, 430).

We found that age, generally, was not associated with *Plasmodium* spp. infections. However, there was a significant association between age and asymptomatic *P. malariae* and *P. ovale* spp. infections in the rural area, and *P. falciparum* infections in the urban setting, with children aged 10 to 14 years more infected than those aged 6 to 9 years.

Older children were also the group most likely to harbour more single and mixed infections than younger ones. The proportion of children infected with *P. falciparum* remained constant for all ages in the rural area, while it increased with age in the urban setting. *Plasmodium malariae* and *P. ovale* spp. infections increased with age in the rural area while they did not do so in the urban setting. That difference may be due to age-related acquisition of parasite-tolerating immunity (59-62, 449-451). It has been shown that in malaria tropical facies, malaria pre-immunity starts building up around 10 years (452). It may also reflect the relative force of infection of the species, with that of *P. falciparum* being higher than the other two species in the rural setting.

We found a low prevalence of single infections of *P. malariae* and *P. ovale* spp., and a high prevalence of single infection of *P. falciparum* and mixed species infections in both rural and urban areas in agreement with previous reports from Africa (26, 28, 36-39, 404). Among children infected with *P. ovale* spp., we found 75%, 24% and 1% children harboured *P. ovale curtisi*, *P. ovale wallikeri* and mixed *P. ovale curtisi* + *P. ovale wallikeri*. This finding highlights the fact that *P. ovale curtisi* appears to be more prevalent than *P. ovale wallikeri* (453). This is the first report of *P. ovale curtisi* in DRC; prior to this study only *P. ovale wallikeri* has been reported (414-416). Additionally, this study confirms that *P. ovale curtisi* and *P. ovale wallikeri* can be found in co-infections of the same host (26, 37, 454).

There is a need, perhaps, to focus more attention on the non-*falciparum* malaria parasites of Africa. The ability of *P. ovale curtisi* and *P. ovale wallikeri* to produce hypnozoites, and the quartan intra-erythrocytic cycle time of *P. malariae* may provide these species with a mechanism for evading the current artemisinin-based combination therapies for uncomplicated malaria (37, 455).

The presence of *P. ovale* spp. and *P. malariae*, co-infected with *P. falciparum*, highlights the impact of those two parasites in asymptomatic and chronic malaria infection. *Plasmodium malariae* and *P. ovale* spp. are not usually associated with severe malaria but *P. malariae* may be responsible for chronic nephrotic syndrome (406-409, 456, 457) which can be fatal (406-408, 456), and chronic infections that can last for years (408, 457), even after leaving endemic regions (408, 457). *Plasmodium ovale* spp. is responsible for relapses after months or even years without symptoms due to the presence of hypnozoites (412, 458-463) and it has been shown to cause severe disease and even death on occasion (413-415, 464, 465).

Our data are in agreement with previous reports that showed that *P. malariae* is much less likely to be observed in mixed species infections with *P. falciparum* in symptomatic malaria infections when the transmission rate of malaria is high (466, 467). The reason for this is currently unclear. It is possible that there is a protective effect of mixed infection with *P. malariae* on the severity of the disease caused by *P. falciparum*, perhaps mediated through cross-immunity. It is also possible that in symptomatic *P. falciparum* infections, this species competitively excludes co-infecting species due to increased parasitaemia. This exclusion could result from within-host competition for resources (468), or through host-immune mediated mechanisms in which the innate immune response triggered by the high parasitaemia *P. falciparum* disproportionately affects the less dominant of the species in the co-infection. A further possibility is that the nested PCR methodology used here to determine parasite species may miss the less common of co-infecting species when the disparity between them is large as is likely in symptomatic *P. falciparum* infected patients.

2.6 Limitations

The sample size is not that big to give a real situation of the burden of malaria among school-age children in the country. A large-scale survey targeting school-age children is needed.

2.7 Conclusion

There is a need to include school-age children in malaria control, surveillance, and elimination strategies. Therefore, a continuous systematic school-based prevention, screening, and treatment of children in high-transmission settings may strengthen malaria intervention measures.

2.8 Acknowledgements

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CHAPTER III. Low prevalence of *Plasmodium falciparum* parasites lacking *pfhrp2/3* genes among asymptomatic and symptomatic school-age children in Kinshasa, Democratic Republic of Congo

Sabin S. Nundu[1, 2, 3]*, Hiroaki Arima[2], Shirley V. Simpson[1, 2], Ben-Yeddy Abel Chitama[6], Yannick Bazitama Munyeku[7], Jean-Jacques Muyembe[3], Toshihiro Mita[5], Steve Ahuka[3], Richard Culleton[4]* and Taro Yamamoto[1, 2]

[1] *Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;*

[2]*Department of International Health and Medical Anthropology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan;*

[3]*Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo;*

[4]*Division of Molecular Parasitology, Proteo-Science Center, Ehime University, Ehime, Japan;*

[5]*Department of Tropical Medicine and Parasitology, Faculty of Medicine, Juntendo University, Tokyo, Japan.*

[6]*Department of Parasitology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan;*

[8]*Division of Global Epidemiology, International Institute for Zoonosis Control, Hokkaido University, Sapporo, Japan*

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3.1 Abstract

Background. Loss of efficacy of malaria diagnostic tests may lead to untreated or mistreated cases, compromising malaria case management and control. There is an increasing reliance on RDTs, with the most widely used of these targeting the *Plasmodium falciparum* histidine-rich protein 2 (*pfhrp*2) gene. There are numerous reports of the deletion of this gene in *P. falciparum* parasites in some populations, rendering them undetectable by *Pf*HRP2 RDTs. We aimed to identify *P. falciparum* parasites lacking the *P. falciparum* histidine rich protein 2 and 3 (*pfhrp2/3*) genes isolated from asymptomatic and symptomatic school-age children in Kinshasa, Democratic Republic of Congo.

Methods. We assessed the performance of *Pf*HRP2-based RDTs in comparison to microscopy and PCR. PCR was then used to identify parasite isolates lacking *pfhrp2/3* genes.

Results. Of 462 DNA samples analysed, deletions of the *pfhrp2* and *pfhrp3* genes were found in only three (2%) samples and one (1%) sample in the RDT positive subgroup, respectively. No parasites lacking the *pfhrp2/3* genes were found in the RDT negative subgroup.

Conclusion. *Plasmodium falciparum* histidine-rich protein 2/3 gene deletions are uncommon in the surveyed population, and do not result in diagnostic failure. We encourage the use of rigorous PCR methods to identify *pfhrp*2/3 gene deletions in order to minimize the overestimation of their prevalence.

Key words: Malaria, Rapid diagnostic tests, School-age children, Democratic Republic of Congo.

3.2 Introduction

Despite concerted malaria control efforts, malaria remains a serious public health problem in DRC. The country accounted for 12% of all estimated malaria cases and 11% of deaths globally in 2019 (5). Malaria case management is based on rapid and accurate diagnosis and prompt treatment with effective antimalarial drugs (469).

WHO recommends malaria diagnosis is performed by microscopy or RDTs for all individuals presenting with malaria-like symptoms prior to the commencement of treatment (470). However, although microscopy is the gold standard for diagnosis (471), its use is challenging and subject to both false positive and negative results when performed by inexperienced microscopists, especially in the case of poor blood film preparation and when parasitemia is low (443, 444, 472-475). RDTs are frequently used as an alternative, especially in remote areas (442, 476-478). In regions where *P. falciparum* is the most prevalent malaria parasite species, the most frequently used RDTs target *Pf*HRP2. Sixty-four percent of all RDTs distributed by national malaria control programs worldwide in 2018 were of this type (72). Moreover, *Pf*HRP2-based RDTs have better sensitivity (479, 480) and greater thermal stability (481) than other RDTs (480). Furthermore, numerous antibodies used to detect *Pf*HRP2 also detect *P. falciparum* histidine-rich protein 3 (*Pf*HRP3) as they have a high degree of similarity in their amino acid sequences (482, 483). However, the sensitivity of RDTs is dependent on the level of parasitaemia in the patient. Parasitemias lower than 200 per µL of blood may be associated with false negative results (427). Moreover, *pfhrp*2 and *pfhrp*3 (*pfhrp*2/3) may be deleted in some parasites rendering them undetectable by *Pf*HRRP2-based RDTs (5). This loss of efficacy can lead to untreated or mistreated malaria cases, thus compromising malaria case management and control (480). Thus, the WHO recommends continuous

nationwide surveillance of parasites harbouring *pfhrp*2/3 deletions. It is recommended that if their prevalence exceeds 5%, alternative RDTs should be used (5). In the DRC, the 2013-2014 nationwide demographic and health survey revealed a *pfhrp*2 gene deletion prevalence of 6.4% overall and 21.9% in Kinshasa among asymptomatic children under five (484). Interestingly, no *pfhrp*2/3 gene deletions were detected among symptomatic individuals (485). Munyeku *et al* (486), found an overall prevalence of 9.2% of parasites isolated from symptomatic malaria patients living Kwilu province, (near Kinshasa) carried *pfhrp2* gene deletions. However, only 9.9% of isolates that gave false negative *Pf*HRP2-based RDTs results in that study carried *pfhrp*2 gene deletions, suggesting that the vast majority of RDT failures are not due to *pfhrp2* gene deletions in that region.

This study aimed to assess the prevalence of *P. falciparum* parasites lacking the *pfhrp*2/3 genes in isolates from asymptomatic and symptomatic school-age children in Kinshasa.

3.3 Methods

Study design, Study area and Selection of participants

Samples used in this study were collected from a previous cross-sectional survey carried out in Mont-Ngafula-2 rural health zone (HZ) and Selembao urban HZ of Kinshasa, Democratic Republic of Congo (**Figure 15**) (487).

Initially, 634 school-age children were enrolled in the study (427 asymptomatic and 207 symptomatic). DNA extraction, *Pf*HRP2-based RDTs, microscopic examination and nested-PCR targeting the *Plasmodium* mitochondrial cytochrome c oxidase III (Cox3) were described in our previous study (487). Four hundred and sixty-two (266

asymptomatic and 196 symptomatic) *pfcox3* PCR positive DNA samples were used in this study.

Detection of P. falciparum infection & selection of samples for pfhrp2/3 PCR

We performed real-time PCR (qPCR) targeting the *P. falciparum* lactate dehydrogenase gene (*pfldh*) to quantify the number of parasite genomes per µL of extracted DNA solution from each of the samples using a serial dilution of laboratory cultured *P. falciparum* 3D7 strain DNA for calibration. The limit of detection of the *pfhrp2* and *pfhrp3* PCR assays used in this study was 1 x 10^{-3} ng/µL. In order to ensure that only samples with sufficient DNA for the amplification of *pfhrp2* and *pfhrp3* was used, only those samples with greater than 3 x 10^{-3} ng/µL of DNA as determined by *pfldh* qPCR were considered for further analysis (**Annex: Table 2**) (485, 488).

A calibration curve was prepared using the results of qPCR with control samples (0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL and 0.0001/µL). Duplicated samples were loaded in 96-wells plates along with serially diluted positive controls (using gDNA extracted from cultured *P. falciparum* 3D7) as well as negative controls consisting of DNA samples from known malaria negative individuals (RDT-, microscopy- and PCR-) and distilled water for checking contamination. We repeated the assay for all discordant duplicates and counted three consistent results for confirmation. The DNA concentration of samples were quantified from each Ct values and the calibration curve.

Detection of pfhrp2/3 gene deletions

Pfhrp2 and *pfhrp3* PCR genotyping was performed as previously described (488), with minor modifications using conventional single step PCR with primers targeting exon 2 of

the genes. Genomic DNA from 3D7 (*pfhrp2/3* positive), Dd2 (*pfhrp2* negative) and HB3 (*pfhrp3* negative) were used as controls. PCR products were visualized under UV light on 1.5% agarose gels run at 100 volts for 30 minutes and stained with Gel Red® solution (Biotium. California, USA) for 30 minutes.

Statistical analyses

Data was analysed using STATA version 14.2 (College Station. Texas, USA). Descriptive variables are presented as proportions (categorical variables) or median and interquartile range (continuous variables). Chi-square tests (or Fisher's exact tests when appropriate) were used to assess associations between categorical variables and *pfhrp2/3* gene deletion prevalence. Sensitivity (=true positive / (true positive + false negative) and specificity (=true negative / (true negative + false positive) of RDTs were calculated using PCR and microscopy as gold standard. Agreement between diagnostic techniques was assessed using Cohen's kappa coefficient. Using parasite densities, we assessed the sensitivity and the specificity of RDTs and microscopy at densities between 4 and 120 genomes/µL of extracted DNA, and those greater than 120 genomes/µL (assuming that a DNA concentration of 1 x 10^{-4} ng/µL corresponds to four parasite genomes per µL (488)). P-values of below 0.05 were considered significant.

3.4 Results

Socio-demographic characteristics of the participants and malaria diagnosis

We enrolled 462 school-age children, of which 266 were asymptomatic, and 196 were symptomatic. Of the 266 asymptomatic children, 136/266 (51%) were female, 147/266 (55%) were between the ages of 6 and 9 and 168/266 (63%) lived in rural areas. Of the

196 symptomatic children, 94/196 (48%) were female, 132/196 (67%) were between the ages of 6 and 9 and 102/196 (52%) lived in rural areas **(Table 14)**.

Table 14. Socio-demographic characteristics of asymptomatic and symptomatic children
Socio-demographic characteristics of asymptomatic and symptomatic children

Variables		number (%)
Asymptomatic infection (N=266)		
Sex		
	Female	136 (51)
	Male	130 (49)
Age med. (IQR)		9 (7-11)
	6-9	147 (55)
	10-14	119 (48)
Location		
	Rural	168 (63)
	Urban	98 (37)
Symptomatic infection (N=196)		
Sex		
	Female	94 (48)
	Male	102 (52)
Age med. (IQR)		8 (7-11)
	6-9	132 (67)
	10-14	64 (33)
Location		
	Rural	102 (52)
	Urban	94 (48)

Comparison of RDTs with PCR and Microscopy

Among 266 DNA samples from asymptomatic children, 174/266 (65%), 187/266 (70%) and 130/266 (49%) were *Pf*HRP2_RDT, *pfldh*-qPCR and microscopy positive, respectively. The sensitivity and specificity of RDTs compared to PCR were 150/187 (80%) and 55/79 (70%) while the sensitivity and specificity of RDTs compared to microscopy were 119/130 (92%) and 81/136 (60%), respectively. Agreement between

*Pf*HRP2-based RDTs and PCR was moderate (Cohen's kappa = 0.48) as was the agreement between *pfhrp*2-based RDTs and microscopy (Cohen's kappa = 0.51) (**Table 16**).

Among 196 DNA samples from symptomatic infections, 131/196 (67%), 171/196 (87%) and 122/196 (62%) were *Pf*HRP2-based RDTs, *pfldh*-qPCR and microscopy positive, respectively. The sensitivity and specificity of RDTs compared to PCR were 128/171 (75%) and 22/25 (88%) while sensitivity and specificity of RDTs compared to microscopy were 114/ 122 (93%) and 57/74 (77%), respectively. Findings showed satisfactory agreement between *Pf*HRP2-based RDTs and microscopy (Cohen's kappa = 0.72) and fair agreement between *Pf*HRP2-based RDTs and PCR (Cohen's kappa = 0.37) (**Table 15**).

Table 15. RDT performance compared to PCR and microscopy examination in asymptomatic and symptomatic infections

	Asymptomatic infections (N=266)					
	***Pfldh*_qPCR**			**Microscopy**		
RDTs	Positive	Negative	Total	Positive	Negative	Total
Positive	150	24	174	119	55	174
Negative	37	55	92	11	81	92
Total	187	79	266	130	136	266
Se (%) (CI 95%)	80 (74, 86)			92 (85, 96)		
Sp (%) (CI 95%)	70 (58, 80)			60 (51, 68)		
PPV (%) (CI 95%)	86 (81, 90)			84 (81, 87)		
NPV (%) (CI 95%)	60 (52, 68)			75 (63, 84)		
Kappa*	0.48; p<0.001			0.51; p<0.001		
	Symptomatic infections (N=196)					
	***Pfldh*_qPCR**			**Microscopy**		
RDTs	Positive	Negative	Total	Positive	Negative	Total
Positive	128	3	131	114	17	131
Negative	43	22	65	8	57	65
Total	171	25	196	122	74	196
Se (%) (CI 95%)	75 (68, 81)			93 (88, 97)		
Sp (%) (CI 95%)	88 (69, 98)			77 (66, 86)		
PPV (%) (CI 95%)	98 (94, 99)			97 (95, 98)		
NPV (%) (CI 95%)	34 (28, 41)			64 (47, 78)		
Kappa*	0.37; p<0.001			0.72; p<0.001		

Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence interval. *Statistical analysis using Cohen's kappa coefficient test, significance at p<0.05.

Performance of RDTS and microscopy examinations based on parasite densities

We compared the sensitivity of RDTs and microscopy at lower limits of parasite density (<120 genomes/μL) and above (≥120 genomes/μL). The sensitivity and specificity of RDTs were 96% (symptomatic: 93%; asymptomatic: 100%) and 37% (symptomatic: 55%; asymptomatic: 31%) while the sensitivity and specificity of microscopy were 91%

(symptomatic: 90%; asymptomatic: 94%) and 59% (symptomatic: 65%; asymptomatic: 56%) (**Table 16**).

Table 16. Sensitivity and specificity of RDTs and microscopy based on parasite densities by qPCR overall, in Asymptomatic and Symptomatic infections

	Overall (N=358)					
	RDTs			**Microscopy**		
Parasite densities	Pos.	Neg.	Total	Pos.	Neg.	Total
4-120 genomes/µL	112	73	185	75	110	185
≥ 120 genomes/µL	166	7	173	158	15	173
Total	278	80	358	233	125	358
Se (%) (CI 95%)	96 (92, 98)			91 (86, 95)		
Sp (%) (CI 95%)	37 (31, 45)			59 (52, 67)		
PPV (%) (CI 95%)	84 (83, 86)			89 (87, 90)		
NPV (%) (CI 95%)	73 (56, 85)			67 (55, 77)		

	Asymptomatic infections (N=187)					
	RDTs			**Microscopy**		
Parasite densities	Pos.	Neg.	Total	Pos.	Neg.	Total
4-120 genomes/µL	82	37	119	52	67	119
≥ 120 genomes/µL	68	0	68	64	4	68
Total	150	37	187	116	71	187
Se (%) (CI 95%)	100 (95, 100)			94 (86, 98)		
Sp (%) (CI 95%)	31 (23, 40)			56 (47, 65)		

PPV (%) (CI 95%)	78 (75, 80)	84 (81, 86)
NPV (%) (CI 95%)	100	80 (61, 91)

	Symptomatic infections (N=171)					
	RDTs			**Microscopy**		
Parasite densities	Pos.	Neg.	Total	Pos.	Neg.	Total
4-120 genomes/µL	30	36	66	23	43	66
≥ 120 genomes/µL	98	7	105	94	11	105
Total	128	43	171	117	54	171
Se (%) (CI 95%)	93 (87, 97)			90 (82, 95)		
Sp (%) (CI 95%)	55 (42, 67)			65 (54, 77)		
PPV (%) (CI 95%)	93 (91, 95)			95 (93, 96)		
NPV (%) (CI 95%)	55 (36, 72)			48 (34, 62)		

Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; Acc: Accuracy

NPV: Negative predictive value; CI: Confidence interval. Pos: Positive; Neg: Negative

Detection of Pfhrp2/3 gene deletions

We used a conservative criterion for the detection of *pfhrp*2/3 gene deletions through the selection of samples with DNA concentrations three times higher than the limit of detection of the *pfhrp2/3* PCR assays. Of 462 DNA samples, 173 were selected for *pfhrp2/3* PCR analysis following *pfldh* qPCR. Of the 173 isolates used for *pfhrp*2/3 PCR, three were *pfhrp*2 negative and one was *pfhrp*3 negative (**Figure 20**).

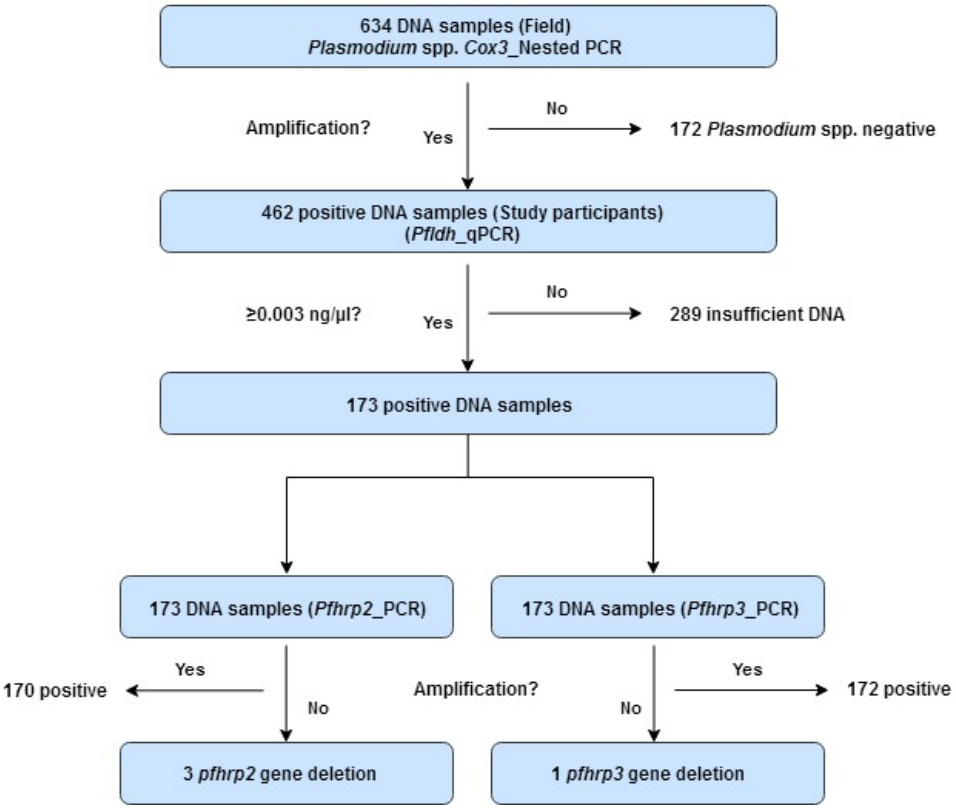

Figure 20. Assessment of *pfhrp*2/3 gene deletion

The overall prevalence of the *pfhrp2* gene deletion was 2% (3/173) while it was 1% (1/173) for the *pfhrp3* gene. All four samples that contained these mutant parasites had returned positive RDT results. Only 7 RDT negative samples had sufficient parasite densities for *pfhrp2/3* deletion, and none of these had *pfhrp*2/3 gene deletions. (**Table 17)**

Table 17. Prevalence of *pfhrp2/3* gene deletion based on PfHRP2_RDT results

	***Pfhrp*2_PCR**			***Pfhrp*3_PCR**		
	Positive	Negative	Total	Positive	Negative	Total
RDTs	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Positive	163 (98)	3 (2)	166 (100)	165 (99)	1 (1)	166 (100)
Negative	7 (100)	0 (0)	7 (100)	7 (100)	0 (0)	7 (100)
Total	170 (98)	3 (2)	173 (100)	172 (99)	1 (1)	173 (100)

Prevalence of P. falciparum hrp2/3 gene deletion by age, sex, health status and location

Among the three samples that harbored *pfhrp*2 gene deletions, two were from children aged six to nine years, and all three were from female children, asymptomatic individuals and children living in the urban area. Age, sex, children health status and location were not associated to phhrp2/3 gene deletion (**Table 18**).

Table 18. Prevalence of *P. falciparum pfhrp2/3* gene deletion by age, sex, health status and location

		Pfhrp2			***Pfhrp3***		
Variables	n	Neg (%)	Pos (%)	p	Neg (%)	Pos (%)	p
Age (years)				1			1
6-9	121	2 (2)	119 (98)		1 (1)	120 (99)	
10-14	52	1 (2)	51 (98)		0 (0)	52 (100)	
Sex				0.09			0.45
Female	78	3 (4)	75 (96)		1 (1)	77 (99)	
Male	95	0 (0)	95 (100)		0 (0)	95 (100)	
Health status				0.06			1
Asymptomatic	68	3 (4)	65 (96)		0 (0)	68 (100)	
Symptomatic	105	0 (0)	105 (100)		1 (1)	104 (99)	
Location				0.09			0.45
Rural	96	0 (0)	96 (100)		0 (0)	96 (100)	
Urban	77	3 (4)	74 (96)		1 (1)	76 (99)	

n: number; Pos: Positive number; Neg: Negative number; p: P-value

3.5 Discussion

Malaria rapid diagnostic tests play an important role in malaria case management and surveillance. Based on several reports that assess the prevalence of *pfhrp*2/3 gene

deletions, the WHO has recently recommended continuous surveillance of *Pfhrp2/3*-deleted *P. falciparum* (480, 489, 490). This study used a rigorous method of DNA sample selection for evaluation of *Pfhrp2/3*-deleted *P. falciparum* (485, 488) which minimizes the overestimation of *pfhrp2/3*-deleted *P. falciparum* results by conventional approaches (484, 491, 492). It is important to consider DNA quantity in samples subjected to PCR to identify *pfhrp2/3* deletions, as low DNA levels may lead to false *pfhrp2*-negative results and overestimate the prevalence of *pfhrp2/3* gene deletions.

We found three isolates harboring a *pfhrp2* gene deletion and one isolate harboring a *pfhrp*3 gene deletion among *pfhrp*2-based RDT positive results. The sample harboring a *pfhrp*3 gene deletion was from a symptomatic child while the three samples harboring *pfhrp*2 gene deletions were from asymptomatic children. It has been shown that *pfhrp*2/3-deleted parasites do not differ from wild-type parasites in their ability to cause malaria symptoms (493). Previous studies conducted in the DRC have found a *pfhrp2* gene deletion prevalence of 6.4% across the country and 21.9% in Kinshasa in a nationwide demographic and health survey among asymptomatic children (484) and 9.2% amongst symptomatic individuals in a neighboring province of Kinshasa (486). This difference may be explained by different methods used for the detection of *Pfhrp2/3* deletions. Prior to our study, another study conducted in the DRC using a similar method of selection of samples with sufficient parasite isolates useful for the detection of *Pfhrp*2/3 gene deletions, did not find any isolates harboring pfhrp*2/3*-deletions among symptomatic children (485) highlighting the fact that the method used in the previous large survey of asymptomatic parasite carriers (484) may have overestimated the prevalence of the *pfhrp2* gene deletion.

Seven isolates were negative by RDT, but positive by qPCR with over 3 $x10^{-3}$ ng of parasite DNA per µL of extracted DNA solution. Five of these samples were negative by microscopy, suggesting relatively low parasitemia. RDT failure in these cases may be explained by data recording errors, operator-dependent and manufacturing quality (494-496) or by the presence of anti-*pfhrp*2 antibodies binding to the circulating antigens (497) or possibly due to the presence of mixed infection *pfhrp*2-negative and *pfhrp*2-positive parasites in the same isolates (498).

Among 196 isolates from symptomatic children, the sensitivity of *Pf*HRP2-based RDTs compared to *pfldh*-qPCR was 75%. Of 43 *pfhrp2* RDT negative isolates when PCR was positive, 36 (84%) had lower than 120 parasites genomes per µL of extracted DNA, and highlight RDTs are less sensitive at low parasitemia as compared to PCR (427). This may exclude some symptomatic children from treatment (487).

Among 266 isolates from asymptomatic children, the sensitivity of *Pf*HRP2-based RDTs compared to *pfldh*-qPCR was 82%. All 37 RDT negative isolates when PCR was positive had below 120 parasite genomes per µL solution, and so highlight the importance of the use of PCR for the diagnosis of asymptomatic malaria parasite carriers (28, 428, 487, 499-501).

Although the samples used in this study did not represent a countrywide survey, the method used permitted the minimization of the overestimation of the prevalence of *P. falciparum* parasites carrying *pfhrp2/3*-deletions which may occur with conventional methods.

3.6 Limitations

The sample size is not that large to give a real overview of *pfhrp2/3*-deletions in the country. A large-scale study targeting the entire population is needed.

3.7 Conclusion

The prevalence of *P. falciparum* parasites carrying deletions of the *pfhrp2/3* gene is low in the population surveyed here, suggesting the use of *Pf*HRP2-based RDTs remains appropriate for the detection of malaria in this region. We encourage the continuous use of rigorous PCR methods for the survey of *pfhrp*2/3 gene deletion prevalence.

3.8 Acknowledgments

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CHAPTER IV. Identification of polymorphisms in genes associated with drug resistance in *Plasmodium falciparum* isolates from school-age children in Kinshasa, Democratic Republic of Congo

Sabin S. Nundu[1, 2, 3], Richard Culleton*[4, 5], Shirley V. Simpson[1, 2], Hiroaki Arima[2], Ben-Yeddy Abel Chitama[5], Jean-Jacques Muyembe[3], Steve Ahuka[3], Dieudonné Mumba[3], Osamu Kaneko[1,5], Toshihiro Mita[6] and Taro Yamamoto[1, 2]

[1] *Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;*

[2] *Department of International Health and Medical Anthropology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan;*

[3] *Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo;*

[4] *Department of Molecular Parasitology, Ehime University, Ehime, Japan;*

[5] *Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan;*

Department of Tropical Medicine and Parasitology, Faculty of Medicine, Juntendo University, Tokyo, Japan

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4.1 Abstract

Background. The emergence and spread of *Plasmodium falciparum* parasites resistant to antimalarial drugs constitutes an obstacle to malaria control and elimination. This study aimed to identify the prevalence of polymorphisms in *pfk13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt* genes in isolates from asymptomatic and symptomatic school-age children in Kinshasa.

Methods. Nested-PCR followed by sequencing was performed for the detection of *pfk13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt* polymorphisms.

Results. Two mutations in *pfk13*, C532S and Q613E were identified in the Democratic Republic of Congo for the first time. The prevalence of the drug-resistance associated mutations *pfcrt* K76T, *pfdhps* K540E and *pfmdr*1 N86Y was low, being 27%, 20% and 9%, respectively.

Conclusion. We found a low prevalence of genetic markers associated with chloroquine and sulfadoxine-pyrimethamine resistance in Kinshasa. Furthermore, no mutations previously associated with resistance against artemisinin and is derivatives were observed in the *pfK13* gene. These findings support the continued use of ACTs and IPTp-SP. Continuous molecular monitoring of antimalarial resistance markers is recommended.

Keywords: *Plasmodium falciparum*, malaria, drug resistance, school-age children, Democratic Republic of Congo.

4.2 Introduction

Malaria remains a major public health concern in the Democratic Republic of Congo (DRC). Effective antimalarial drugs are crucial for malaria case management. The emergence and spread of *Plasmodium falciparum* parasite strains with resistance against antimalarial drugs is a major obstacle for malaria control and elimination in the country. In DRC, chloroquine (CQ) treatment failure was reported in 46% of patients treated in 2001 leading to its replacement by sulfadoxine-pyrimethamine (SP) as the first-line treatment for uncomplicated malaria in 2002 despite the fact that resistance to that drug had already emerged (502). A few years later, resistance to SP spread across the country (502, 503), but it was still used for the management of uncomplicated malaria due to the limited availability of artemisinin-based combination therapy (ACT) (504, 505). Subsequently, despite the widespread occurrence of resistance to SP, it remains the drug of choice for intermittent preventive treatment of malaria in pregnancy (IPTp) (79), as it continues to protect against adverse pregnancy outcomes (506, 507). Meanwhile, ACTs including Artesunate (AS) plus-amodiaquine (AQ) (ASAQ) (since 2005) and artemether plus lumefantrine (AL) (since 2012) are currently used as the first-line treatments for uncomplicated malaria in DRC (503). When failure of ASAQ and/or AL is proven by microscopy, quinine plus clindamycin or doxycycline is used to treat uncomplicated malaria (79).

The mutation K76T in the *P. falciparum* CQ resistance transporter (*pfcrt*) gene constitutes the main molecular marker for chloroquine resistance worldwide (339, 508). Clinically, the K76T substitution raises the probability of treatment failure sevenfold (341). In DRC, from 2000 to 2019, various reports gave the prevalence of *pfcrt* K76T as being 100% in

2000 (509), 93% in 2002 (510), 55.4% in 2007 (511), 83.8% in 2008 (361), 73.2% in 2010 (512), 63.9% in 2014 (349), 28.5% in 2017 (513) and 41.5% in 2018-2019 (350), depending on the study area and population sampled. Reports from Malawi showed a return of CQ susceptibility (514-517), following the cessation of its use which raised the possibility of reintroducing CQ for the treatment of uncomplicated malaria in combination with another anti-malarial drug. The *pfcrt* haplotype SVMNT (Ser-Val-Met-Asn-Thr at positions 72-76) is associated with resistance to AQ (340, 518-520), as it renders parasites resistant to the AQ metabolite monodesethylamodiaquine (340) *in vitro* (519) and *in vivo* (518). This haplotype has not been reported in DRC (350, 512, 513) in spite of the use of AQ in combination with AS as a first-line treatment (79). Certain alleles of *pfmdr1* are also associated with CQ resistance; N86Y *in vivo* (342) and *in vitro* (521, 522), Y184F and D1246Y *in vivo* (343).

Lumefantrine is used in combination with artemether (AL) to treat uncomplicated malaria. Amplification of *pfmdr1* is associated with lumefantrine susceptibility (523). However, there is no strong evidence of an association between increased copy number of *pfmdr*1 and lumefantrine resistance (524), but it may raise the risks of treatment failure with AL and with artesunate–mefloquine (523, 525). Furthermore, cross-resistance between lumefantrine, mefloquine, and/or halofantrine has been shown *in vitro*, suggesting a common mechanism of resistance (526, 527). However, resistance to lumefantrine is still limited in DRC (349, 350, 361, 528). Amplification of particular alleles of *pfmdr1* is linked to resistance to mefloquine, halofantrine, quinine, CQ and/or lumefantrine (259, 529-535).

Monotherapy with artemisinin (ART) derivatives (artesunate or artemether) is used for the treatment of severe malaria (270). Mutations in the propeller domain of the *P. falciparum kelch 13* (*pfk13*) gene are associated with *in vivo* and *in vitro* ART resistance (317, 344) and are most commonly found in the Greater Mekong Subregion of Southeast Asia (345) but also in some sub-Saharan African countries (346-348, 536). Many single nucleotide polymorphisms in this gene have been recorded in the DRC (F495L, S522C, V520A, N498I, N554K, A557S, M476K, N523T, E509D, P506L, E507V, D516E, and G538S), although none are associated with ART resistance (349, 350).

Several studies have reported mutations in *P. falciparum* dihydrofolate reductase (*pfdhfr*) (N51I, C59R, S108N, I164L) and dihydropteroate synthase (*pfdhps*) genes (S436A, A437G, K540E, A581G, K613T), which confer resistance to pyrimethamine and sulfadoxine, respectively, in DRC (78, 358-363). Mutations at *pfdhfr* N51I, C59R, S108N and *pfdhps* A581G are associated with clinical resistance to SP (304, 364). The World Health Organization (WHO) recommends IPTi with SP only where the prevalence of *pfdhps* K540E is under 50% (366), and the discontinuation of IPTp when the prevalence of *pfdhps* K540E is over 90% and *pfdhps* A581G is above 10% (367), but it may continuously be used if IPTp-SP remains effective in preventing pregnancy outcomes even in case of quintuple mutation (322).

Previous reports from Africa have revealed differences in the prevalence of alleles associated with drug resistance between symptomatic and asymptomatic individuals (537-541), and there is a known association between *pfcrt* K76T and severe malaria (541).

The *pfmdr*1 wild type allele N86N was shown to be more frequent in symptomatic individuals and the mixed N86N/Y allele in asymptomatic individuals (540). Furthermore, the *pfmdr*1 single mutation N86Y and double mutation of *pfcrt* K76T + *pfmdr*1 N86Y were more frequent in asymptomatic individuals than symptomatic patients(537). Thus, differences between the prevalence of alleles associated with drug resistance in parasites that cause symptomatic disease compared to those in asymptomatic infections requires further investigation.

This study aimed to assess the profile of *pfk13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt* gene markers in isolates from asymptomatic and symptomatic school-age children in Kinshasa.

4.3 Methods

Study design, study area and study population

Samples used in this study were collected from a cross-sectional survey carried out in Mont-Ngafula-2 rural health zone (HZ) and Selembao urban HZ of Kinshasa, DRC (**Figure 15**) (487).

Initially, a cross-sectional study was conducted in these two HZ (Mont-Ngafula-2 HZ and Selembao urban HZ) and DNA samples from 634 (427 asymptomatic and 207 symptomatic) school-age children were analysed. Asymptomatic children were children aged 6 to 14 years attending school on the day of survey and who had a body temperature less than 37.5˚C during a physical examination and who did not have malaria-related symptoms (including fever, headache, fatigue, chills, nausea, vomiting, etc.) in the two weeks prior to the survey. Symptomatic children were outpatient children aged 6 to 14

years seeking for healthcare within the period of survey and who exhibited fever and/or malaria-related symptoms (headache, fatigue, chills, nausea, vomiting, etc.) within the three days prior to medical consultation and who had not taken antimalarial drugs prior to the consultation.

DNA samples from 634 (427 asymptomatic and 207 symptomatic) school-age children were collected. DNA extraction, *pfhrp*2-based RDTs, microscopic examination and nested polymerase chain reaction (PCR) targeting *Plasmodium* mitochondrial cytochrome c oxidase III (Cox3) were carried out as previously described (487).

Two hundred and twenty-nine (117 asymptomatic and 112 symptomatic) positive DNA samples for both *pfcox3* PCR and microscopy results were analysed for polymorphisms in genes associated with resistance to various antimalarial drugs.

Analysis of *Pfcrt* 72-76, *pfmdr*1, *pfk*13, *pfdhfr* and *pfdhps* single nucleotide polymorphisms (SNPs)

For the analysis of mutations in *pfcrt*, nested PCR was carried out as described by Zhou *et al*. (542). The outer and nested PCR were performed with a final volume of 20 µL, 500 nM primers, 1X One Taq® 2X Master Mix with standard buffer (New England Biolabs, MA, USA) and 2 µL of isolated genomic DNA (1 µL of 100x diluted outer PCR product for nested PCR) (**Annex: Table 3**).

For the detection of *pfmdr1* gene mutations, nested PCR was performed by amplifying a portion of *pfmdr1* containing codons 86 and 184 as described by Humphreys *et al*. (343) with minor modifications (**Annex: Table 3**). The outer and nested PCR were performed for the 'long segment' S1 (for SNPs at codons 86 and 184) with a 24 µL final volume,

200 nM primers, and 1X One Taq® 2X Master Mix with standard buffer and 4 µL of gDNA (4 µL of outer PCR product for nested PCR).

For the detection of polymorphisms in *pfk*13, nested PCR was performed as described by Ariey *et al*. (317, 543) with minor modifications (**Annex: Table 3**). The outer and nested PCR were performed with a 25 µL final volume, 250 nM primers, and 1X One Taq® 2X Master Mix with standard buffer and 5 µL of isolated gDNA (5 µL outer PCR product for nested PCR).

For the detection of *pfdhfr* and *pfdhps* polymorphisms, nested PCR was performed as described by Ruizendaal *et al*.(544) with minor modifications (**Annex: Table 3**). Outer and nested PCR were performed for the amplification of both *pfdhfr* and *pfdhps* with a 25 µL final volume, 400 nM primers, and 1X One Taq® 2X Master Mix with standard buffer and 2.5 µL of gDNA (1 µL (*pfdhfr*) and 2.5 µL (*pfdhps*) of outer PCR product for nested PCR.

Parasite free gDNA (LOT#17765621 (Roche Diagnostics GmbH, Penzberg, Germany)) (negative) and 3D7 clone *P. falciparum* gDNA (positive) were used as controls in all PCR assays. PCR products were subjected to electrophoresis on 2% agarose gels at 100 volts for 30 minutes, stained with Gel Red® solution (Biotium, CA, USA) for 30 minutes and visualized under UV light.

Amplicons were directly sequenced on an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific, MA, USA), with Applied Biosystems Big Dye Terminator V3.1 (Thermo Fisher Scientific, MA, USA). Sequence alignment was performed with MEGA7 software and analysis was performed via BLAST search (www.ncbi.nlm.nih.gov/gene/?term) using the reference *P. falciparum* 3D7 gene

sequence for *pfmdr1* (PF3D7_0523000), *crt* (PF3D7_0709000), *pfdhfr* (PF3D7_0417200), *pfdhps* (PF3D7_0810800) and *pfk13* (www.ncbi.nlm.nih.gov/gene/814205). In the case of mixed infections, we considered secondary peaks as true SNPs if the peak height was above 50% of the major peak at a polymorphic locus.

Haplotype nomenclature

In the results, wild-type amino acids are given in standard type, whilst the mutations are given in bold type and underlined. In the case of mixed infections, where two sequencing peaks were detected at a single position on the electropherogram, the position is denoted with the alternate amino-acids separated by a '/' and placed in brackets. For example, for the *pfcrt* locus, 'CV**IET**' indicates that the isolate was wild type at the first two positions considered, and mutant and the last three. The haplotype 'CV(M/**I**)**ET'** contains a mixed infection in which the third amino-acid considered is polymorphic for the wild-type 'M' and the mutant 'I'.

Statistical analysis

Data were analysed using STATA/BE 17.0 software (College Station, TX, USA). Tests for associations and differences were carried out by chi-square test (or Fisher's exact test when appropriate). Statistical significance was set at P-value less than 0.05.

Ethical considerations

The study was approved by the ethics committees of the School of Public Health, University of Kinshasa, DRC (Approval number: ESP/CE/042/2019) and the Institute of

Tropical Medicine, Nagasaki University (Approval number: 190110208-2). Written informed consent was obtained from children's parents/guardians.

4.4 Results

Socio-demographic characteristics of the study population

We enrolled 229 school-age children, of which 117 were asymptomatic, and 112 were symptomatic. Of the 117 asymptomatic children, 60/117 (51%) were females, 78/117 (67%) lived in rural areas, aged 6-14 years with a median and interquartile range (IQR) of 9 (7-11); children between the ages of 6 and 9 years were 65/117 (56%), while of the 112 symptomatic children, 49/112 (44%) were females, 49/112 (44%) lived in rural areas, aged 6-14 years with median and interquartile range (IQR) 8 (7-10); 73/112 (65%) of children were between the ages of 6 and 9 years **(Table 19)**.

+Table 19. Socio-demographic characteristics of participants

Variables	Overall (N=229) n (%)	Asymptomatic infection (N=117) n (%)	Symptomatic infection (N=112) n (%)
Age median (IQR)	**9 (7-11)**	**9 (7-11)**	**8 (7-10)**
6-9	138 (60)	65 (56)	73 (65)
10-14	91 (40)	52 (44)	39 (35)
Sex			
Female	109 (48)	60 (51)	49 (44)
Male	120 (52)	57 (49)	63 (56)
Location			
Urban	102 (45)	39 (33)	63 (56)
Rural	127 (55)	78 (67)	49 (44)

Prevalence of mutations in *pfcrt* and *pfmdr*1 genes

The *pfcrt* gene was amplified and sequenced from two hundred and twenty isolates. The K76T mutation was present at a prevalence of 27% (60/220). Isolates from children living in rural areas harbored significantly more *pfcrt* K76T mutants compared to those living in urban settings (33% versus 20%, p=0.035) and there were no differences in health status, sex, and age of children that harbored this mutant (p>0.05) (**Table 20**).

Table 20. Prevalence of *pfcrt* K76T by age, sex, child health status and location

	***pfcrt* K76T (N=220)**		
		Positive	
	number	n (%)	p-value
Age (Years)			0.71
6-9	135	38 (28.2)	
10-14	85	22 (25.9)	
Sex			0.24
Female	104	26 (25.0)	
Male	116	34 (29.3)	
Child health status			0.62
Asymptomatic	115	33 (28.7)	
Symptomatic	105	27 (25.7)	
Location			**0.035**
Rural	125	41 (32.8)	
Urban	95	19 (20.0)	
Overall	**220**	**60 (27.3)**	

The majority of isolates carried the CQ sensitive haplotype (CVMNK) and accounted for 73% (160/220). The single *pfcrt* haplotype CV**IET** accounted for 16% (36/220) followed by the mixed infection CV(M/**I**)(N/**E**)(K/**T**) (7%), CV(M/**I**)**ET** (2%), and CV(M/**I**)(**K**/**E**)**T**, CV(M/**I**)(N/**E**)**T** and CV**I**(N/**E**)(K/**T**) accounted for 0.5% each. (**Table 21**).

Table 21. Haplotypes of *pfcrt* in isolates from asymptomatic and symptomatic children

	Overall (N=220)	**Asymptomatic infection (N=115)**	**Symptomatic infection (N=105)**
	n (%)	n (%)	n (%)
***pfcrt* haplotype**			
CVMNK (Wild)	160 (72.7)	82 (71.3)	78 (74.3)
CV**IET**	36 (16.4)	19 (16.5)	17 (16.2)
CV(M/**I**)(N/**E**)(K/**T**)	16 (7.3)	10 (8.7)	6 (5.7)
CV(M/**I**)**ET**	5 (2.3)	3 (2.6)	2 (1.9)
CV**I**(N/**E**)(K/**T**)	1 (0.5)	1 (0.9)	0 (0.0)
CV(M/**I**)(**K**/**E**)**T**	1 (0.5)	0 (0.0)	1 (1.0)
CV(M/**I**)(N/**E**)**T**	1 (0.5)	0 (0.0)	1 (1.0)

pfmdr1 was amplified, sequenced, and analysed from 151 isolates. Most isolates harbored the wild type allele (64% (97/151)) while the most prevalent mutation, Y184F was present in 46% (69/151) of isolates. N86Y was present in 9% (14/151) of isolates, R133K in 2% (3/151), S113R in 1% (1/151) and D156N in 1% (1/151). There was no difference in the prevalence of the most abundant *pfmdr*1 mutation (Y184F) between asymptomatic and symptomatic children (**Figure 21**)

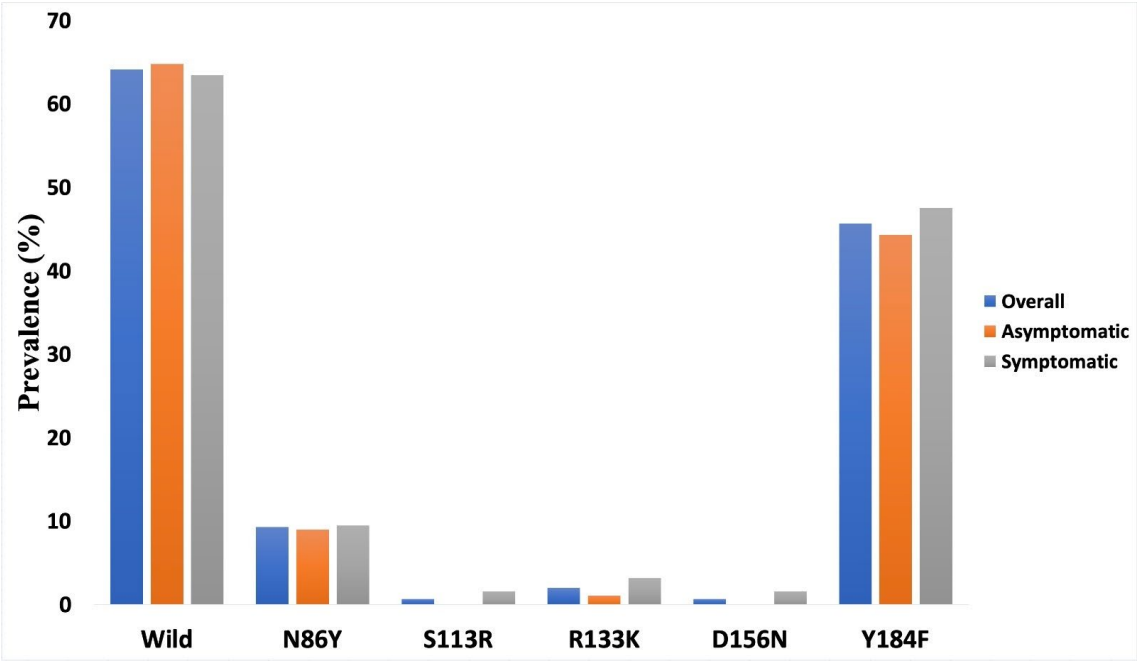

Figure 21. Prevalence of mutations in *pfmdr1* gene in isolates from asymptomatic and symptomatic children

The wild type 86N-113S-133R-156D-184Y (NSRDY) was present in 50% (75/151) of isolates. The most prevalent single *pfmdr*1 haplotype was NSRD**F** and was present in 28% (41/151) of isolates, followed by **Y**SRD**F** (5%,7/151), **Y**SRDY (1%, 2/151) and NSR**NF** (1%, 1/151). The mixed haplotype NSRD**F**/Y was present in 10% (15/151) of isolates followed by NS(R/**K**)DY (2%, 3/151), (N/**Y**)SRD(Y/**F**) (3%, 4/151) and N(S/**R**)RDY (1%, 1/151) (**Table 22**).

Table 22. Haplotypes and mutation of *pfmdr1* in isolates from asymptomatic and symptomatic children

	Overall (N=151)	**Asymptomatic infection (N=88)**	**Symptomatic infection (N=63)**
	n (%)	n (%)	n (%)
***pfmdr1* haplotype**			
NSRDY (Wild)	75 (49.7)	46 (52.3)	29 (46.0)
NSRD**F**	42 (27.8)	25 (28.4)	17 (27.0)
YSRD**F**	7 (4.6)	4 (4.6)	3 (4.8)
YSRDY	2 (1.3)	1 (1.1)	1 (1.6)
NSR**NF**	1 (0.7)	0 (0.0)	1 (1.6)
NSRD**F**/Y	15 (9.9)	8 (9.1)	7 (11.1)
(N/**Y**)SRD(Y/**F**)	4 (2.6)	2 (2.2)	2 (3.2)
NS(R/**K**)DY	3 (2.0)	1 (1.)	2 (3.2)
N(S/**R**)RDY	1 (0.7)	0 (0.0)	1 (1.6)

The mutant *pfcrt* CV**IET** haplotype was found in combination with *pfmdr*1 184Y in 19 isolates and with *pfmdr1* N86Y in three isolates. CV**IET** was found in combination with both *pfmdr*1 N86Yand *pfmdr*1 Y184F in three isolates.

Prevalence of mutations in *pfdhfr* and *pfdhps*

The *pfdhfr* gene was amplified and sequenced from 149 isolates. The majority of isolates harbored three *pfdhfr* gene mutations: N51I (100%, 149/149), C59R (96%, 143/149) and S108N (100%, 149/149) (**Figure 22a**).

Considering positions N51**I** C59**R** and S108**N**, the **IRN** haplotype accounted for 94% (140/149) with no difference in prevalence between symptomatic and asymptomatic children while isolates harboring the **I**C**N** allele accounted for 4% (6/149) and were more prevalent in asymptomatic children than in symptomatic ones (6 versus 2%). The mixed *pfdhfr* haplotype **I**(C/**R**)**N** was recorded in 3/149 (2%) of isolates (**Table 23**).

Table 23. Haplotypes of *pfdhfr* in isolates from asymptomatic and symptomatic children

	Overall (N=149)	**Asymptomatic infection (N=87)**	**Symptomatic infection (N=62)**
	n (%)	n (%)	n (%)
pfdhfr* haplotype			
IC**N**	6 (4.0)	5 (5.7)	1 (1.6)
IRN	140 (94.0)	82 (94.3)	58 (93.6)
I(C/**R**)**N**	3 (2.0)	0 (0.0)	3 (4.8)

*NCS: wild type of *pfdhfr* allele

The *pfdhps* gene was amplified and sequenced from 153 isolates. The prevalence of mutations was low: I431**V** (2%, 3/153), S436**A** (7%, 10/153) and K540**E** (19%, 29/153). Most isolates harbored wild type *pfdhps* (78%, 126/153) (**Figure. 22b**).

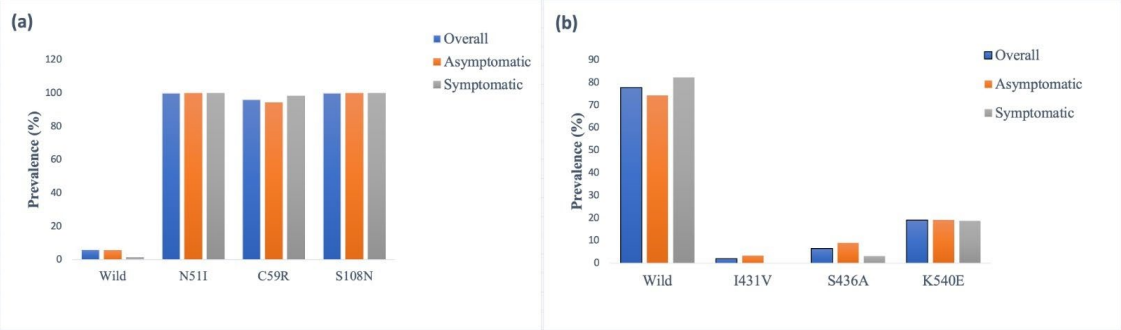

Figure 22. Prevalence of mutations in *pfdhfr* (a) and *pfdhps* (b) genes in isolates from asymptomatic and symptomatic children

Considering mutations at positions I431**V** S436**A** and K540**E**, the majority 75% (114/153) of isolates harbored the wild type haplotype (ISK). The *pfdhps* IS**E** haplotype accounted for 18% (27/153) followed by I**A**K (3%, 4/153) and **VA**K (2%, 3/153). The haplotypes I**A**K and **VA**C were found only in asymptomatic children (**Table 24**).

Table 24. Haplotypes of *pfdhps* in isolates from asymptomatic and symptomatic children

	Overall (153)	**Asymptomatic infection (N=89)**	**Symptomatic infection (N=64)**
	n (%)	n (%)	n (%)
***pfdhps* haplotype**			
ISK (Wild)	114 (74.5)	64 (71.9)	50 (78.1)
IS**E**	27 (17.7)	16 (18.0)	11 (17.2)
I**A**K	4 (2.6)	4 (4.5)	0 (0.0)
VAK	3 (2.0)	3 (3.4)	0 (0.0)
IS(K/**E**)	2 (1.3)	1 (1.1)	1 (1.6)
I(S/**A**)K	3 (2.0)	1 (1.1)	2 (3.1)

The prevalence of *pfdhps* K540E was 29/153 (20%) and this did not differ between asymptomatic and symptomatic children, females and males, young children and older children and children living in urban and rural areas ($p>0.05$ for all comparisons)

Considering both *pfdhfr* and *pfdhps,* the triple mutations **INA** (*pfdhfr* N51I + S108N + *pfdhps* S436A and **INE** (*pfdhfr* N51I + S108N + *pfdhps* K540E) accounted for 1% (1/153) of isolates; the quadruple mutant haplotype **IRNE** (*pfdhfr* N51I + C59R + S108N + *pfdhps* K540E) accounted for 16% (24/153) and **IRNA** (*pfdhfr* N51I + C59R + S108N + *pfdhps* S436A) for 4% (6/153), while the quintuple mutant haplotype **IRNVA** (*pfdhfr* N51I + C59R + S108N + *pfdhps* I431V + S436A) accounted for 2% (3/153) (**Table 25**).

Table 25. Combined *pfdhfr*/*pfdhfr* haplotypes in isolates from asymptomatic and symptomatic children

	Overall (N=153)	**Asymptomatic infection (N=89)**	**Symptomatic infection (N=64)**
	n (%)	n (%)	n (%)
***pfdhfr* + *pfdhps* haplotypes**			
pfdhps K540K	1 (0.7)	1 (1.1)	0 (0.0)
pfdhps K540E	2 (1.3)	1 (1.1)	1 (1.6)
pfdhps K540E/K	1 (0.7)	0 (0.0)	1 (1.6)
pfdhps S436A	1 (0.7)	1 (1.1)	0 (0.0)
pfdhfr N51I + S108N	4 (2.6)	3 (3.4)	1 (1.6)
pfdhfr N51I + S108N + *pfdhps* S436A	1 (0.7)	1 (1.1)	0 (0.0)
pfdhfr N51I + S108N + *pfdhps* K540E	1 (0.7)	1 (1.1)	0 (0.0)
pfdhfr N51I + C59R + S108N	108 (70.6)	59 (66.3)	49 (76.6)
pfdhfr N51I + C59R + S108N + *pfdhps* S436A	6 (3.9)	4 (4.5)	2 (3.1)
pfdhfr N51I + C59R + S108N + *pfdhps* K450E	24 (15.7)	14 (15.7)	10 (15.6)
pfdhfr N51I + C59R + S108N + *pfdhps* K450E/K	1 (0.7)	1 (1.1)	0 (0.0)
pfdhfr N51I + C59R + S108N + *pfdhps* I431V + S436A	3 (2.0)	3 (3.4)	0 (0.0)

Prevalence of mutations in *pfk*13

The *pfk*13 gene was successfully amplified and sequenced from 153 isolates. The majority (99%, 151/153) were wild types. Two isolates harbored mutations, one at codon

532 (C532S) and one at codon 613 (Q613E), both found in asymptomatic children (**Table 26**).

Table 26. Mutations in *pfk*13 in isolates from asymptomatic and symptomatic children

	Overall (153)	**Asymptomatic infection (N=90)**	**Symptomatic infection (N=63)**
	n (%)	n (%)	n (%)
***pfk*13 gene mutation**			
Wild	151 (98.7)	88 (97.8)	63 (100)
C532S	1 (0.7)	1 (1.1)	0
Q613E	1 (0.7)	1 (1.1)	0

Combinations of mutations in *P. falciparum* genes associated with drug resistance in isolates from asymptomatic and symptomatic children

Sixteen (10%) isolates harbored *pfcrt-pfdhfr-pfmdr1* mutations, five (3%) isolates had mutations in *pfcrt-pfdhfr-pfdhps*, one (1%) isolate had mutations in *pfcrt-pfk*13-*pfmdr*1, four (3%) isolates harbored mutations in *pfcrt-pfdhfr-pfdhps-pfmdr*1, and one (1%) isolate had mutations in *pfdhfr-pfdhps-pfk*13-*pfmdr*1 (**Table 27**).

Table 27. Combination of *P. falciparum* gene mutations in isolates from asymptomatic and symptomatic children

	Overall (N=163)	**Asymptomatic infection (n=91)**	**Symptomatic infection (N=72)**
	n (%)	n (%)	n (%)
***P. falciparum* gene mutations**			
pfcrt	10 (6.1)	2 (2.2)	8 (11.1)
pfdhfr	48 (29.5)	27 (29.7)	21 (29.2)
pfdhps	1 (0.6)	0 (0.0)	1 (1.4)
pfcrt + *pfdhfr*	16 (9.8)	11 (12.1)	5 (6.9)
pfdhfr + *pfmdr1*	34 (20.9)	17 (18.7)	17 (23.6)
pfdhfr + *pfdhps*	9 (5.5)	5 (5.5)	4 (5.6)
pfdhps + *pfmdr*1	2 (1.2)	1 (1.1)	1 (1.4)
pfcrt + *pfdhfr* + *pfmdr*1	16 (9.8)	7 (7.7)	9 (12.5)
pfcrt + *pfdhfr* + *pfdhps*	5 (3.1)	4 (4.4)	1 (1.4)
pfdhfr + *pfdhps* + *pfmdr*1	16 (9.8)	11 (12.1)	5 (6.9)
pfdhfr + *pfk*13 + *pfmdr*1	1 (0.6)	1 (1.1)	0 (0.0)
pfcrt + *pfdhfr* + pfdhps +*pfmdr*1	4 (2.5)	4 (4.4)	0 (0.0)
pfdhfr + *pfdhps* + *pfk*13 + *pfmdr*1	1 (0.6)	1 (1.1)	0 (0.0)

4.5 Discussion

This study utilized samples collected from Kinshasa city in 2019 (487) to assess the prevalence of polymorphisms associated with resistance to antimalarial drugs in pfk*13*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfcrt*.

The prevalence of genetic markers associated with resistance against the antimalarial drug chloroquine (CQ) has decreased in the 18 years following its withdrawal

as the first-line treatment for uncomplicated malaria in the DRC (502). The prevalence of *pfcrt* 76T has decreased steadily from 100% in 2000 (509), 73% in 2010 (512), 49% in 2017 (513), 33% in 2018-2019 (350) to 27% in this current (2019) survey in Kinshasa city, despite the continuous use of AQ in combination with artesunate as a first-line treatment for uncomplicated malaria since 2005 (79). It has been shown that the use of AQ may facilitate the selection of CQ resistance even after removal of CQ (545). Notably, we did not find the Papua New Guinea/South America **S**VMN**T** haplotype (546-549) associated with AQ resistance and present in Tanzania and Angola (550, 551), underlining the fact that AQ resistance is not present in Kinshasa, in agreement with previous reports (349, 350, 512, 513). The most common mutant haplotype identified here was *pfcrt* CV**IET**, the most common haplotype in Africa (552), including the DRC (350, 512, 513) which first emerged in Asia (553).

We found a low prevalence of the *pfmdr*1 N86Y mutation (9%) and moderate prevalence of the *pfmdr*1 Y184F mutation (46%). The *pfmdr1* N86Y point mutation is associated with CQ and AQ resistance *in vivo* (341) and with CQ alone *in vitro* (521, 522). Additionally, the *pfmdr1* N86Y and Y184F point mutations are also thought to be associated with AQ resistance resulting in ASAQ treatment failure (343). The decline of the prevalence of *pfmdr*1 N86Y found in this study has been also shown in other studies (554, 555), nevertheless, this alone is not sufficient evidence of the return of CQ efficacy as *pfcrt* mutations play a more important role in CQ resistance (515).

In agreement with previous studies conducted throughout the DRC (344, 349, 350), this study also demonstrated a low prevalence of single nucleotide polymorphisms in the Kelch13 gene of the 153 isolates analysed. We did not find any *pfk*13 mutations

associated with resistance against ART (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H or C580Y) (556) . We found two SNPs in the Kelch 13 gene of two isolates at codons 532 (C532S) and 613 (Q613E) not previously reported in DRC (344, 349, 350). The *pfk*13 Q613E point mutation has been reported in Congo, Angola and elsewhere (557) while *pfk*13 C532S has not previously been reported in Africa.

We found low prevalence of the *pfdhps* mutation K540E (20%) and we did not find the quintuple mutant *pfdhfr* (N51I, C59R, S108N) + *pfdhps* (A437, K540E) which is associated with high-level SP resistance (304, 364). We found a low prevalence of the quadruple mutation *pfdhfr* (N51I, C59R, S108N) + *pfdhps* (K540E) and *pfdhfr* (N51I, C59R, S108N) + *pfdhps* (S436A) and the quintuple mutant *pfdhfr* (N51I, C59R, S108N) + *pfdhps* (I431V, S436A). The triple mutant *pfdhfr* (N51I, C59R, S108N) and quintuple mutant *pfdhfr* + *pfdhps* are associated with SP resistance (341). We found a high prevalence of *pfdhfr* (N51I, C59R, S108N) as previously reported in Kinshasa-DRC (358, 361). We found 24 isolates carrying quadruple mutations at *pfdhfr* N51I + C59R + S108N + *pfdhps* K450E and it is known that double mutations of *pfdhfr* C59R and *pfdhps* K540E are strongly predictive of SP treatment failure (301, 558).

It has previously been reported that symptomatic and asymptomatic individuals residing in the same region are infected differentially by *P. falciparum* parasites carrying alternate alleles of genes linked to drug resistance reported (537, 541). We did not find an association between the presence of either *pfcrt* K76T or N86Y and clinical status, as previously reported (537, 541). Furthermore, we did not observe a significant difference in the prevalence of *pfmdr*1 N86Y, *pfmdr*1 Y184F and *pfdhps* K540E between symptomatic and symptomatic individuals

The WHO recommends the discontinuation of IPTp when the prevalence of *pfdhps* 540E is over 90% and *pfdhps* 581G is above 10% (367). Fortunately, with 20% *pfdhps* 540E and no *pfdhps* 581G in the study area, the continued use of IPTp-SP for protection during pregnancy is advised. However, continued molecular surveillance of *pfdhfr* and *pfdhps* (especially the 540E mutation) is recommended.

4.6 Limitations

The sample size is not that large to give a real overview of antimalarial drug resistance in the country. A large-scale study targeting the entire population is needed.

4.7 Conclusion

We report a low prevalence of genetic markers of CQ and SP resistance in Kinshasa, supporting the continued use of IPTp-SP during pregnancy. We did not find mutations in the pfk13 gene associated with reduced susceptibility to ART and/or its derivatives. We encourage continued molecular monitoring of genetic markers associated with antimalarial drug resistance in the DRC.

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CHAPTER V. Review: It is time to strengthen the malaria control policy of the Democratic Republic of Congo and include schools and school-age children in malaria control measures

Sabin S. Nundu[1, 2, 3]*, Richard Culleton4*, Shirley V. Simpson[1, 2], Hiroaki Arima[1], Jean-Jacques Muyembe[3], Toshihiro Mita[5], Steve Ahuka[3] and Taro Yamamoto[1, 2]

[1]*Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;*

[2]*Department of International Health and Medical Anthropology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan;*

[3]*Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo;*
[4]*Division of Molecular Parasitology, Proteo-Science Center, Ehime University, Ehime, Japan;*

[5]*Department of Tropical Medicine and Parasitology, Faculty of Medicine, Juntendo University, Tokyo, Japan.*

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5.1 Abstract

Despite a decade of sustained malaria control, malaria remains a serious public health problem in the Democratic Republic of Congo (DRC). Children under five years of age and school-age children aged 5-15 years remain at high risk of symptomatic and asymptomatic malaria infections. The World Health Organization's malaria control, elimination, and eradication recommendations are still only partially implemented in DRC. For better malaria control and eventual elimination, the integration of all individuals into the national malaria control programme will strengthen malaria control and elimination strategies in the country. Thus, inclusion of schools and school-age children in DRC malaria control interventions is needed.

Key words: Malaria, National malaria control program, School-age children, Democratic Republic Congo

5.2 Introduction

Over the last 20 years, global malaria control efforts have resulted in marked decreases in malaria-attributable morbidity and mortality. However, between 2015 and 2019 progress has slowed compared to that achieved in the preceding 15 years. During the first two decades of the 21st century, malaria case incidence decreased from 363 per 1000 population at risk to 225, and malaria-associated mortality from 680,000 to 386,000 in the African region (5). Despite this reduction in malaria morbidity and mortality, the disease remains a major public health concern in sub-Saharan Africa, where 94% of cases and deaths from malaria occurred in 2019 (1).

WHO recommends: (i) early prompt diagnosis and effective treatment for suspected malaria as case management to prevent severe cases leading to death; (ii) vector control, such as the use of ITNs and IRS; and (iii) preventive chemotherapy. The latter component includes chemoprophylaxis for travelers; IPTi for children under 12 months living in high-transmission areas of Africa, and IPTp for pregnant women living in moderate-to-high transmission areas; SMC for preschool children living in areas of the Sahel region of Africa; and MDA for epidemic control or as a component of malaria elimination efforts (1, 5, 322).

WHO recommendations are followed differently by countries depending on country malaria policy and malaria transmission settings and the progress of their malaria control interventions. Policies must be tailored for countries with high, moderate, or low burdens of the disease. For countries with high disease burdens, WHO recommends a political engagement; specifically, better leadership, policies, and strategies to manage the impact of malaria, and strong national malaria response coordination to deal with the burden of

the disease (1, 322). Although early malaria diagnosis and treatment strategies protect against the development of severe cases leading to death, this policy does not impact on asymptomatic malaria parasite carriers who harbor parasites and serve as reservoirs for transmission.

Routine malaria diagnosis using RDTs and microscopy for confirmation of suspected malaria cases (322, 559) have limits of detection that can lead to false negative evaluations for a significant proportion of low density infections (28, 324, 427, 428). The incorporation of systematic screening and treatment of asymptomatic individuals and/or preventive treatment of malaria among school-age children in high-transmission settings can reinforce malaria control interventions and contribute to malaria elimination (560, 561). It has been shown that in high transmission sites, symptomatic malaria is common in children under five years old, whereas asymptomatic infections generally occur in school-age children and adults who have built immunity against the disease in response to repeated exposure (59, 61, 62).

School-age children are children between the ages of 5-15 (370, 392, 487, 562) who have been shown to harbor more *P. falciparum* than children under five of years and adult individuals in SSA (381, 382, 392, 563). In SSA, *Plasmodium falciparum* is the most frequent *Plasmodium* species, and its prevalence peaks among school-age children (381, 382, 392, 393, 563, 564) and it is over 50% in many areas (371, 487, 565). Thus, malaria control and interventions have to target all members of the community, including school-age children to promote health and social equity benefits (566), in terms of access to malaria case management and prevention. However, as opposed to children under five and pregnant women, school-age children are the group least likely to profit from malaria

interventions in Africa (5, 566-568) including in DRC (79, 430, 452, 569-571), thus the burden of malaria in this group is not well understood as they are not routinely included in household cluster surveys (79, 430, 452, 562, 569). In DRC, school-age children are the least protected with ITNs as the vast majority of ITNs are distributed through expanded program for immunization (children under five of years) and antenatal care (pregnant women) (79, 572) and rarely in schools (572). Thus, they are given lower priority for the use of ITNs compared to children under five and pregnant women when the number of mosquito nets in the household is insufficient (572). Additionally, they are less likely to develop symptomatic malaria infections and so represent a probable untreated age group (573). Their reservoir of parasite sexual stages makes them an important source of human-to-mosquito malaria transmission (64, 65), thus posing a major challenge for malaria control and elimination efforts (574-576).

Despite a decade of sustained malaria control strategies, malaria remains a serious public health problem in DRC. The DRC has the highest malaria burden worldwide after Nigeria; and in 2019 accounted for 12% of all estimated malaria cases, 11% of deaths globally, and 54% of cases in Central Africa (5). It is the principal cause of morbidity and mortality in the country, accounting for 44% of all outpatients and for 22% of deaths in 2018. Roughly 97% of its population lives in stable malaria transmission zones, in which transmission occurs for 8 to 12 months yearly (79). School-age children are at high risk for both asymptomatic and symptomatic malaria infections (432, 577-579); however, they are neglected and are not included in the DRC malaria control interventions such as demographic health surveys (DHS) (79, 569) and less benefit with ITNs than children under five and pregnant women .

Here, we review the DRC malaria control policy and show reports that support the necessity of the policy revision and the inclusion of schools and school-age children in the country malaria control and elimination strategy.

5.3 Methods

A literature review was undertaken of published literature on World Health Organization malaria guidelines, malaria control and elimination strategies worldwide, in Africa and in the DRC targeting school-age children, preschool children and pregnant women cited within the past 20 years in PubMed, Google scholar and Web page. Key terms such as malaria control policy, malaria chemoprevention, malaria vector control, malaria case management, Plasmodium, school-age children, schools, children under five, pregnant women were used to find the information related to the topic.

5.4 Current DRC malaria control policy

The DRC is still exploring the best mechanisms and strategies for malaria control and prevention which will significantly lower the transmission of the disease and have a positive impact on malaria elimination in the country. Currently, WHO recommended intervention measures are partially implemented under the umbrella of the national malaria control program (NMCP). Briefly, (i) malaria case management involves giving antimalarial drugs only after a positive confirmatory RDT test and/or microscopy; (iii) vector control with ITNs for prevention of malaria is mostly targeting higher risk groups, including children under five and pregnant women; and (iii) IPTp for pregnant women. IRS is also included in the DRC malaria control policy, but has not yet been

implemented (5, 79), only in some private sectors (580, 581). Preventative chemotherapy has also not yet been implemented in infants (IPTi) (79), neither SMC in areas with epidemic waves and MDA, which are important to clear asymptomatic carriers and slow the risk of malaria transmission.

Malaria case management

Microscopy and/or RDTs are the current routine diagnostic tests used for the confirmation of malaria suspected cases prior to antimalarial treatment, in part to prevent drug resistance arising due to an overuse of drugs after erroneous presumptive treatments (231). Despite that microscopic examination is the gold standard diagnostic tool, its use is challenging, especially in rural and semi-urban settings where there is a lack of experienced microscopists, equipment, reagents, and electricity. Thus, RDTs are mostly used as an alternative for quick and accurate diagnosis (442, 476-478). However, the sensitivity and specificity of both microscopy and RDTs are dependent on threshold parasitaemia levels; and in the case of low parasitaemia, microscopic examination (less than 50-100 per ul blood) and RDTs (less than 200 per ul blood) may carry risk of false negative results, compromising treatment for true cases (28, 38, 324, 427, 428, 441, 442).

Plasmodium malariae and *Plasmodium ovale* spp. are typically co-infections with *P. falciparum*, in both symptomatic and asymptomatic individuals (20, 26, 28, 36-40, 403, 455). They have usually shown low parasitemia and are therefore challenging to diagnose by RDTs and microscopy, two routine diagnostic techniques (28, 38, 324, 428), or by microscopy due to the difficulty of distinguishing from *P. falciparum* (174). Given the fact that the detection of *P. malariae* and *P. ovale* spp. is usually underestimated, *P. falciparum* is the most diagnosed species and considered to be responsible for

symptomatic malaria. Therefore, for the management of confirmed cases of uncomplicated malaria, MNCP recommends treatment with ACT, including ASAQ or AL (5, 79).

However, although these two non-falciparum parasites seem to be uncommon in symptomatic malaria infections, it should be considered that there is a need to pay attention to them. Specifically, because *P. malariae* is responsible for the quartan intra-erythrocytic cycle time and chronic infections that can last for years (457, 582); while *P. ovale* spp. may produce hypnozoites (460, 465), allowing them with a mechanism for evading the current ACT, especially AL (37, 455). Also, the current ACT (ASAQ and AL) used as first-line treatments for uncomplicated malaria do not have long enough half-lives to deal with quick *P. falciparum* re-infections after treatment (583, 584). Given this matter, the choice of drugs that have a longer half-life prophylactic effect in combination with ART derivatives such as DHAPQ may be useful (585, 586). Additionally, although RDTs and ACT are typically free of charge to patients, there is a problem of patient adherence to diagnosis and treatment regimens in health care facilities; probably due to limited transport facilities(581, 587, 588) or other reasons including low education, poverty, and remote locations. There is a need to promote community case management of malaria (CCMm) as recommended by the WHO to increase the number of patients with access to RDTs and ACT, especially for remote and marginalized populations (589-591).

Reports conducted in other countries (592-598) and in the DRC [58-60] following WHO strategies based on CCMm, have shown a significant positive effect of adherence to case management guidelines by improving access to RDTs and ACT. This follows the

WHO 'test, treat, and track' (T3) strategy which recommends evidence-based recommendations on diagnostic testing, treatment, and surveillance (599). Indeed, every suspected malaria case should be tested, treated, and tracked to minimize presumptive treatment and save ACT from misuse and the risk of the development of resistance. However, case management only targets symptomatic individuals, and has limited ability to reduce the parasite reservoir harbored in asymptomatic parasite carriers (600).

There is a need to include asymptomatic individuals for sustaining malaria control and elimination strategies. It has been shown that systematic screening followed by treatment of asymptomatic individuals in high-transmission settings may sustain malaria control interventions and contribute to malaria elimination (560, 561). Moreover, school-based malaria prevalence surveys (SMPS) are cost-effective and easier to conduct as compared to community-based malaria prevalence surveys (CMPS) (388, 579). Furthermore, the malaria burden within school-age children is a reliable indicator of the prevalence and transmission intensity of the disease in a defined community (388), as they represent representative smaller administrative units to derive malaria infection estimates, relative transmission risk, and impact of interventions (388, 601). An efficient SMPS may provide valuable information on intervention performance; and it is useful for disease surveillance (388, 602-605). Thus, schools may constitute useful sentinels for the detection of epidemics and promotion of drug supplies in health facilities (606) and health education that may benefit the whole community (606, 607). It will additionally protect this underserved age group (603) by helping to integrate and control other tropical diseases that threaten them, such as soil-transmitted helminths, schistosomes, and filaria. Thus, it is important to enhance control measures for asymptomatic malaria parasite

carriage to protect them against chronic infections leading to chronic anaemia, absenteeism, reduced school performance, and other complications (608-610).

Vector control

ITNs are the only vector control widely implemented in DRC for the prevention of malaria, and with emphasis on children under five and pregnant women. Household ITN ownership increased from 30% to 44%. ITN use among children under five and pregnant women increased from 38% and 43% to 51% and 52% respectively, from 2010 to 2018 (79). However, the improvement of its coverage is still challenging due to the scarcity of mass distribution campaigns; which under DRC malaria policy occur every three years (79). In 2019, ITN coverage was estimated to be around 65% (5). It is meaningful to monitor ITN replacement needs (611) every year after distribution. Additionally, ITN ownership is not usually correlated to ITN use; and low bed net use may be associated to low awareness of malaria prevention (612), low education of mothers and other vulnerable individuals (571, 611, 613), inconvenience of net installation (614, 615), and damaged or worn mosquito nets (616). Thus, follow-up and evaluation and behavior change intervention focusing on malaria prevention, education, and promotion of the use of ITNs may increase the utilization of ITNs among underserved and vulnerable groups (571, 612, 613, 617).

Although the distribution of ITNs targets the general population, the proportion of INT use in school-age children is still quite less compared to children under the age of 5 and pregnant women (567, 568, 613, 616, 618). There is an urgent need to promote a campaign of mass distribution of ITNs among school-age children through school-based or community-based interventions, as they are gametocyte carriers that mediate malaria

transmission (567, 568, 612, 613, 616, 618). Country reports have shown that the use of ITNs has sensibly reduced childhood morbidity and mortality (570, 612, 619) and malaria burden among school-age children (581, 611, 620). IRS is not yet officially implemented in DRC; but given the fact that the proportion of bed net ownership and use remains insufficient, the combination of ITNs and IRS may help to address malaria morbidity and mortality among susceptible groups. A study conducted in southern DRC has shown a positive impact of the combination of these two strategies (ITNs, IRS, CCMm and CMPS) on the reduction of malaria prevalence among children including school-age children (581).

Preventive chemotherapy

Among the three preventive chemotherapy strategies recommended by the WHO, only IPTp-SP is implemented in DRC (79) in pregnant women during antenatal care visits to prevent morbidity and mortality in pregnant women and adverse pregnancy outcomes including anemia, low birth weight, and stillbirths. The WHO recommends the minimum of three doses of IPTp (IPTp3+) with universal coverage of at least 80% (5). However, in DRC, IPTp3+ implementation less than the universal coverage and the majority of pregnant women attending at least four antenatal care visits receive less than three doses of IPTp (5, 621). Despite the increase of SP falciparum resistance in DRC (358-361, 503, 506), IPTp is still used for malaria prevention during pregnancy and protection against maternal anemia and low birthweight; especially when it is given in three or more doses (506), as shown elsewhere (622-626).

Thus, the optimization of IPTp coverage will be cost effective for the most beneficial protection of pregnancy outcomes (625). Nevertheless, not covered by

preventive chemotherapy in infants (IPTi) as recommended by WHO. Also, preventive chemotherapy in all children under five (IPTc) who are more at risk of malaria morbidity and mortality, and in school-age children (IPTsc) who are a large reservoir of asymptomatic carriers for disease transmission may help to protect them from malaria infections. For instance, reports have shown that for IPTc when given every four months combined with timely treatment of febrile malaria illness has significantly reduced malaria-related childhood morbidity and mortality (627-631). Similarly, IPTsc when given every four or three months has provided substantial protection against malaria morbidity and anemia, as well as reduction of school absenteeism and increased school performance (608, 632-634).

However, SP resistance has spread globally, probably due to SP overuse, and in part because of self-medication (635). SP self-medication is still widely used for treatment of uncomplicated malaria (636-642), and the interruption of its misuse and overuse will delay the increase of resistance. There is a need for continuous sensitization and education for women about the benefits of malaria prevention including IPTp during the pregnancy; and for the community members, including community healthcare and private health professionals, about the guideline of malaria case management and malaria prevention (636, 643-647). To minimize the risk of SP resistance, SP may be used in combination with other non-artemisinin derivates used as first-line treatment to protect ACT resistance as previously highlighted (608, 631-633, 648, 649).

Adherence to malaria preventive measures requires the promotion of social and behavioral change (SBC) through education and training to increase the awareness and knowledge of populations - such as parents especially mothers, caregivers, teachers, and

healthcare professionals - about malaria transmission mode, case management and prevention at high and moderate risk of transmission. Another malaria chemoprevention strategy is post-discharge administration of ACT to children after recovering from severe anemia (650, 651). This intervention has shown a positive impact in Malawi of reducing the composite endpoint of death, severe anemia, or severe malaria by 31% when given one and two months after discharge of children under five from hospitals (652) and in Kenya and Uganda reducing all-cause readmission or death by 35% when given two, six, and 10 weeks after discharge (653). For integration measures, it has been shown that the combination of vector control and preventive chemotherapy will reinforce malaria prevention strategies (654, 655).

5.5. Limitations

Lack of publications on DRC malaria control policy. A systematic review or meta-analysis may give real situation of malaria control policy in DRC.

5.6 Conclusion

In the DRC, children under the age of five and school-age children are at risk of symptomatic and asymptomatic malaria infections. In addition to the current control measures, there is an urgent need to also integrate school-age children into the national malaria control interventions including DHS. Case management strategies should be sustained through the promotion of CCMm of malaria. Moreover, for malaria prevention measures, integration of preventive chemotherapy and vector control strategies should also target children under five, school-age children, and pregnant

women especially, with a look at the entire population. SMPS and CMPS should serve as tools to monitor and evaluate preventive chemotherapy and vector control strategies.

The political will to strengthen and support research on drug and insecticide resistance can contribute to the choice of effective drugs and insecticides for the national malaria control policy. To increase the coverage of ITNs, ACTs, and RDT kits used, social behavior change should be promoted, and the continuous supply of drugs distributed by international organizations should be ensured to prevent shortages.

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CHAPTER VI. General discussion

It has been a while that malaria endemic countries have developed strategies to fight malaria. However, despite the widespread reduction of malaria morbidity and mortality due to malaria control and intervention measures based on WHO recommendations, malaria remains a major public health problem in SSA including in DRC. Moreover, most of countries have differently followed WHO recommendations to deal with malaria depending on country malaria policy, malaria transmission settings and the progress of their malaria control interventions. The success of WHO recommendations, particularly in high-burden countries, depends on political commitment, including better leadership, policies, and strategies to manage the impact of malaria, and strong national coordination of the malaria response to address the disease burden. Thus, each country must adapt these recommendations taking into account its community commitment and engagement to national malaria policy as the roots of the success of malaria control and prevention interventions.

In the first study, we showed the burden of malaria among asymptomatic and symptomatic school-age children by determining the prevalence of *Plasmodium* species composition. WHO recommendations mainly target children under five and pregnant women while school-age children, adolescent children and adults are the group least likely to profit from malaria interventions (5, 567, 568). School-age children are neglected and not directly included in the national malaria control measures (79, 569). There is a need to pay attention to them and protect them against malaria outcomes. Although early malaria diagnosis and treatment strategies for malaria case management protect against the development of severe cases leading to death, DRC malaria policy does not target

asymptomatic malaria parasite carriers who harbor parasites and serve as reservoirs for transmission. In fact, it has been shown that in high transmission sites, symptomatic malaria is common in children under five years old, whereas asymptomatic infections generally occur in school-age children and adults who have built immunity against the disease in response to repeated exposure (59, 61, 62). Thus, school-age children are less likely to develop symptomatic malaria infections and so represent a probable untreated age group (573). Also, they constitute the reservoir of parasite sexual stages makes them an important source of human-to-mosquito malaria transmission (64, 65), thus, posing a major challenge for malaria control and elimination efforts (574-576). Our findings showed that school-age children were at high risk in both asymptomatic and symptomatic malaria infections. Our findings are similar to other previous reports (432, 577-579). Thus, there is a need to include school-age children and pay attention to asymptomatic malaria carriers useful for the improvement of malaria control and elimination strategies. It has been gathered that systematic screening followed by treatment of asymptomatic individuals in high-transmission settings may sustain malaria control interventions and contribute to malaria elimination (560, 561). Thus, the promotion of malaria screening with PCR which is more sensitive than microscopy and RDTs is needed.

Our study showed that prevalence of *P. falciparum* by PCR was likely the double of that by microscopy or RDTs and it was difficult to identify *P. malariae* or *P. ovale* spp. by microscopy and RDTs used as routine diagnostic tests in DRC. RDTs used in DRC detect only *P. falciparum* that my underestimate the burden of non-*P. falciparum*. Our findings showed co-infection *P. falciparum*/*P. malariae* or *P. falciparum*/*P. ovale* ssp. in both asymptomatic and symptomatic children. Our results are similar to other previous reports

in Africa (20, 26, 28, 36-40, 403, 455). *Plasmodium malariae* and *P. ovale* ssp. have usually shown low parasitemia and are therefore challenging to diagnose by RDTs and microscopy, two routine diagnostic techniques (28, 38, 324, 428), or by microscopy due to the difficulty of distinguishing them from *P. falciparum* (174). Given the fact that the detection of *P. malariae* and *P. ovale* spp. is usually underestimated, *P. falciparum* is the most diagnosed species and considered to be responsible for symptomatic malaria. The success of malaria control measures does not involve only the clearance of *P. falciparum* but all human malaria parasites. There is, therefore, a need to also target underappreciated malaria parasites (other than *P. falciparum*) to support malaria control and elimination strategies.

In the second study, we assessed the impact of *pfhrp2* gene on the performance of the PfHRP2-based RDTs routinely used prior to the treatment for malaria case management. RDTs and/or microscopy are the routine diagnostic tests currently used to confirm suspected cases of malaria prior to antimalarial treatment, in part to prevent drug resistance due to drug overuse of presumptive treatments (231). RDTs are mostly used as an alternative for quick and accurate diagnosis because of compromise microscopic results due to the lack of experienced microscopists, equipment, reagents, and electricity, especially in remote areas (442, 476-478). However, RDTs target *Pf*HRP2 is that mostly used in DRC and *pfhrp2* gene may be deleted in some parasites rendering them undetectable by *Pf*HRRP2-based RDTs (5) leading to untreated or mistreated malaria cases, thus compromising malaria case management and control (480). Our findings showed low prevalence of *pfhrp2* gene deletion using a rigorous method of DNA sample

selection for evaluation of *Pfhrp2/3*-deleted *P. falciparum*. Our result was likely similar to that previously found in symptomatic children under five (485) and inversely to that among asymptomatic malaria parasite carriers (484) highlighting the fact that the method used in the previous large survey among asymptomatic malaria carriages may have overestimated the prevalence of the *pfhrp2* gene deletion. It has been shown that some false negative results may be associated with lower parasitaemia instead of *pfhrp2/3*-deleted *P. falciparum* as most of *Pf*HRRP2-based RDTs have a limit of detection of less than 200 per µL of blood (427). Thus, a true management of *pfhrp2/3* gene deletions may exclude low parasitaemia which can constitute a bias by underestimating the prevalence of *pfhrp2/3* gene deletion. Therefore, it is important to keep in mind that *pfhrp2* gene deletion is confirmed when there is a *PfHRP2*-based RDT negative test result of an isolate combined to positive pan- or pf-pLDH RDT test result or the isolate is confirmed microscopically as positive for *P. falciparum* by two qualified microscopists and the sample is positive by *pfldh* qPCR which selects a sample with higher concentration than the limit of detection of *pfhrp*2/3 PCR minimizing the overestimation of *pfhrp2/3*-deleted *P. falciparum* results by conventional approaches (484, 491, 492). The use of rigorous method for the determination of *pfhrp*2/3 gene deletions will help to better evaluate the performance of PfHRP2-based RDTs.

In the third study we identified the polymorphisms of genes associated with drug resistance in *Plasmodium falciparum* isolates from school-age children in Kinshasa**.** It has been known that effective antimalarial drug is crucial for the malaria case management and preventive chemotherapy strategies. Thus, the emergence and spread of

Plasmodium falciparum resistance to antimalarial drugs may be a major obstacle to malaria control and elimination. Our findings showed high prevalence of *pfdhfr* gene mutations in isolates while the prevalence of *pfcrt* and *pfmdr*1 gene mutations was low and none of isolates harbored validated gene mutations associated with ART resistance. Our findings highlight remarkable reduction of CQ resistance in Kinshasa eighteen years after its withdrawal as malaria treatment in the country (350, 509, 512, 513) and no proof of AQ and ART resistance as previously shown (344, 349, 350). Our findings also showed low prevalence of SP resistance as well (358, 361) based on WHO statement (367). Thus, our findings support a continuous usage of ACT and IPTp-SP as recommended by the DRC national malaria control program.

The fourth and last study aimed to identify unmet needs of malaria control policy in DRC and show the importance improving the DRC malaria control policy by capturing school-age children and schools into malaria control interventions. The country is still exploring the best mechanisms and strategies for malaria control and prevention which will significantly lower the transmission of the disease and may have a positive impact on malaria elimination in the country. However, currently, the WHO recommendations are partially implemented under the umbrella of the NMCP. Regarding vector control, ITNs are the only vector control widely implemented in DRC for the prevention of malaria while IRS is not yet officially implemented in the country; but given the fact that the coverage of bed net ownership and its use remain insufficient, the combination of ITNs and IRS may help to address malaria morbidity and mortality among susceptible groups. A study conducted in southern DRC has shown a positive impact of the combination of these two strategies on the reduction of malaria prevalence among school-

age children (581). The use of ITNs alone is not enough to prevent the transmission of malaria into the communities as ITN ownership and its use coverage are still under the estimated rate (5, 79). Thus, political will for optimizing the bed-net ownership and its follow-up and evaluation combined to behavior change measures focusing on malaria prevention, health education, and promotion of the use of ITNs may increase the utilization of ITNs among underserved and vulnerable groups (571, 612, 613, 617).

Regarding chemoprevention, only ITPp-SP is used to prevent pregnancy outcomes while ITPi is not yet officially implemented. Malaria case management only targets symptomatic individuals. Chemoprevention may help to clear asymptomatic malaria carriers that are not covered by malaria case management strategies. Regarding chemotherapy, reports have shown that for IPTc when given every four months combined with timely treatment of febrile malaria illness has significantly reduced malaria-related childhood morbidity and mortality (627-631). Similarly, IPTsc when given every four or three months has provided substantial protection against malaria morbidity and anemia, as well as reduction of school absenteeism and increased school performance (608, 632-634). Thus, we advocate DRC NMPC to think about integrating IPTi, IPTc and IPTsc in malaria prevention strategies.

Regarding malaria case management, although RDTs and ACT are typically free of charge to patients, there is a problem of patient adherence to diagnosis and treatment regimens in health care facilities; probably due to limited transport facilities locations (581, 587, 588) or other reasons including low education, poverty, and remote locations. Thus, promotion of CCMm as recommended by WHO may increase the number of patients with access to RDTs and ACT, especially for remote and marginalized

populations (589-591). It has been shown elsewhere (592-598) and throughout the country [58-60] that the use of CCMm strategies has been shown to give a significant positive effect of adherence to case management guidelines by improving access to RDTs and ACT. This follows the WHO 'test, treat, and track' (T3) strategy which recommends evidence-based recommendations on diagnostic testing, treatment, and surveillance (599) to minimize presumptive treatment and save ACT from misuse and the risk of the development of resistance. However, CCMm under case management only targets symptomatic individuals, and has limited ability to reduce the parasite reservoir harbored in asymptomatic malaria parasite carriers (600). Regarding the target of asymptomatic individuals useful for the improvement of malaria control and elimination strategies, it has been shown that systematic screening followed by treatment of asymptomatic individuals in high-transmission settings may sustain malaria control interventions and contribute to malaria elimination (560, 561). Moreover, SMPS are cost-effective and easier to conduct as compared to CMPS (388, 579). Thus, the combination of SMPS and CMPS can help to cover the entire community and CMPS can be used as an indicator for monitoring malaria prevalence in low-income countries.

Limitations

While this study provided insight into the burden of malaria in school-age children and the importance of considering this underserved age group in DRC malaria control policy, data from this study come from the same study site comprising two health zones out of 35 ZS of Kinshasa. The results of these data may not be generalized to all areas of

Kinshasa and to the whole country. The results of this study therefore provide valuable information on the burden of malaria in this neglected age group in selected areas.

Conclusion

Malaria control and elimination in the country may be possible if all age groups are involved in the malaria control and intervention strategies. Moreover, the success of malaria control and intervention strategies implies the combination of several determinants including prompt diagnosis, effective treatment and vector control combined with CMPS and SMPS strategies useful for malaria control and elimination in the country.

Perspectives

Our perfectives are to conduct multicentric prospective comparative studies implicating children under five, school-age children, adolescents, and pregnant women. Also, regarding the lack of in vitro and in vivo studies on ART resistance in the country, we would like to focus on it in the near future to establish the relationship between K13 gene resistance and in vivo and in vitro ART resistance.

Recommendations

We advocate the government through NMCP to

Revise country malaria policy by

- Including Schools and School-age children in national malaria control and intervention strategies including countrywide malaria surveys, malaria prevention strategy
- Introducing antimalarial drug with longer half-life prophylactic effect in combination with ART derivatives such as DHAPQ as first-line treatment for uncomplicated malaria for the management of recrudescence, or relapse forms
- Promoting SMPS, CMPS and mCCM for the reinforcement of malaria case management and prevention
- Promoting social behavior change for community engagement and adherence
- Integrating IPTi and IPTsc in the malaria chemoprevention strategy
- Integrating ITN and IRS for vector control strategy
- Allocating sufficient budget for malaria research including continuous malaria countrywide surveys, monitoring, and evaluation of ongoing malaria intervention strategies

Political will to

- Promote and support research on drug and insecticide resistance can contribute to the choice of effective drugs and insecticides which may contribute to the choice of effective drugs and insecticides for the national malaria control policy
- Increase the coverage of ITNs, ACTs, and RDT kits used, social behavior change should be promoted, and the continuous supply of drugs distributed by international organizations should be ensured to prevent shortages

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Annex

Table 28 (Annex: Table 1). Primer sequences and PCR conditions for Plasmodium spp and Plasmodium genotyping

Species	Primer name	Primer sequence (5' to 3')	Reaction components	Cycling conditions
	Outer			
***Plasmodium* spp.**	MtU.F	CTCGCCATTTGATAGCGGTTAACC	One *Taq* 2X Master Mix with	94˚C/30s;
	MtU.R	CCTGTTATCCCCGGCGAACCTTC	standard buffer: 12.5µL	40 cycles of 94˚C/30s,
			10µM forward primer: 0.5µL	63˚C/1min and 68˚C/1min
			10µM reverse primer: 0.5µL	68˚C/5mins
			Nuclease free water: 6.5µL	12˚C -∞
			DNA template: 5µL	
			25uL reaction volume	
	Inner			
P. falciparum	MtNst_falF	GAACACAATTGTCTTATTCGTACAATTATTC		
	MtNst_falR	CTTCTACCGAATGGTTTATAAATTCTTTC	One *Taq* 2X Master Mix with	
			standard buffer: 12.5µL	
P. malariae	MtNst_malF	CTAGCTTTGTACACAAATTAATTCGTCTAC	10µM forward primer: 0.5µL	
	MtNst_malR	CTTTATAAGAATGATAGATATTTATGACATA	10µM reverse primer: 0.5µL	94˚C/30s;
			Nuclease free water: 9.5µL	40 cycles of 94˚C/30s,
			DNA amplicom: 2µL (100x	
***P. ovale* spp.**	MtNst_ov2F	ATTATTGTCAAATATAAGTACTTTAATC	diluted)	63˚C/1min and 68˚C/1min
	MtNst_ov2R	GGTTGAAGTTTATGATACTAATAATC	25uL reaction volume	68˚C/5mins
				12˚C -∞
P. vivax	Pv_For1	TATTATTGTCTATACTAGATACTATAG		
	Pv_Rev	CTATATTTTCATCATTAGTATCAGGA		
***Plasmodium* spp.**	MtUnst.F	GTAAACATGCwGTCATACATGATGCAC		
(Nested)	MtUnst.R	CCCCGGCGAACCTTCTTACCGT		

Table 29 (Annex: Table 2). Primer sequences and PCR conditions for *P. falciparum ldh* and *hrp2/3* PCR amplification

Target gene	Primer sequence (5' to 3')	Reaction components	Cycling conditions	LOD, ng/µL
Pfldh(initial qPCR)	For: ACGATTTGGCTGGAGCAGAT Rev TCTCTATTCCATTCTTTGTCACTCTTC	LightCycler® 480 SYBR Green I Master: 6µL Forward primer (2.4µM): 1µL Reverse primer (2.4µM): 1µL RNase free water: 3µL Template DNA: 1µL 15uL reaction volume (Primer concentration: 200 nM)	50℃: 2min 95℃: 10min 50 cycles of 95℃: 15s 60℃: 1min 95℃:5s 65℃: 1min 97℃: 5s	10^{-4}
Pfhrp2 ***Pfhrp3***	For: CAAAAGGACTTAATTTAAATAAGAG Rev: AATAAATTTAATGGCGTAGGCA For: AATGCAAAAGGACTTAATTC Rev: TGGTGTAAGTGATGCGTAGT	One *Taq* 2X Master Mix with standard buffer: 12.5µL 10µM forward primer: 1µL 10µM reverse primer: 1µL Nuclease free water: 7.5µL DNA template: 3µL 25µL reaction volume (Primer concentration: 400 nM)	94˚C/10 min 45 cycles of 94˚C/50s, 55˚C/50s and 70˚C/1min; 4 ˚C -∞	10^{-3}

LOD; lower limit of detection

Table 30 (Annex: Table 3). PCR primer sequences and reaction conditions for *pfcrt*, *pfmdr1*, *pfk13*, *pfdhfr* and *pfdhps* fragments

Gene Fragment	Primer Name	Primer sequence (5'-3')	PCR product size (bp)	PCR Cycling Conditions
pfcrt (for SNPS at codons 72 to 76	Outer P1	CCGTTAATAATAAATACACGCAG	537	94°C: 3min 35 cycles of 94°C: 30s, 56°C: 30s and 60°C: 1min
	Outer P2	CGGATGTTACAAAACTATAGTTACC		60°C: 5min
	Inner P1	TGTGCTCATGTGTTTAAACTT	154	94°C: 3min 35 cycles of 94°C: 30s, 48°C: 30s and 65°C: 1min
	Inner P2	CAAAACTATAGTTACCAATTTTG		65°C: 5min
*pfmdr*1 (for SNPS at codons 86 and 184)	Outer Forward P1	AGGTTGAAAAAGAGTTGAAC	578	94°C: 10min 30 cycles of 94°C: 30s, 55°C: 1min and 65°C: 1min
	FN1/1			
	Outer revers P2	ATGACACCACAAACATAAAT		65°C: 5min
	REV/C1			
	Nested Forward D3	ACAAAAAGAGTACCGCTGAAT	534	94°C: 10min 30 cycles of 94°C: 30s, 60°C: 1min and 65°C: 1min
	MDR2/1			
	Nested Reverse D2	AAACGCAAGTAATACATAAAGTC		65°C: 5min
	NEWREV/V1			
*pfk*13 (for SNPS at domain of *P. falciparum* (PF3D7_1343700))	K13_PCR_F	CGGAGTGACCAAATCTGGGA	2000	95°C: 15min 30 cycles of 95°C: 30s, 58°C: 2min and 72°C: 2min
	K13_PCR_R	GGGAATCTGGTGGTAACAGC		72°C: 10min

	K13_N1_F	GCCAAGCTGCCATTCATTTG	849	95°C: 15min 40 cycles of 95°C: 30s, 60°C: 2min and 72°C: 2min 72°C: 10min
	K13_N1_R	GCCTTGTTGAAAGAAGCAGA		
pfdhfr (for SNPS at codons N51, C59 and S108)	Nest 1 forward dhfr	TTTATGATGGAACAAGTCTGC		94°C: 10min 30 cycles of 94°C: 1min, 58°C: 1min and 72°C: 1min 72°C: 5min
	Nest 1 reverse dhfr	AGTATATACATCGCTAACAGA		
	Nest 2 forward dhfr	TCTGCGACGTTTTCGATATTT		94°C: 10min 30 cycles of 94°C: 1min, 58°C: 1min and 72°C: 1min 72°C: 5min
	Nest 2 reverse dhfr	CTCATTTTCATTTATTTCTGGA		
pfdhps (for SNPS at codons A437 and K540)	Nest 1 forward dhps	AACCTAAACGTGCTGTTCAA		94°C: 10min 30 cycles of 94°C: 1min, 58°C: 1min and 72°C: 1min 72°C: 5min
	Nest 1 reverse dhps	AATTGTGTGATTTGTCCACAA		
	Nest 2 forward dhps	CCTAAACGTGCTGTTCAAAGAA		94°C: 10min 30 cycles of 94°C: 1min, 62°C: 1min and 74°C: 1min 74°C: 5min
	Nest 2 reverse dhps	TTGTTCATCATGTAATTTTTGTTGTG		
	Nest 2 reverse dhpd Sequencing only	CAATACTTATAATTGGTTTCGCATCA		
	Nest 1 reverse 540 dhps Sequencing	TTCGCAAATCCTAATCCAATATC		