Development of molecular methods for screening *Plasmodium* infections: New diagnostics for the era of malaria elimination

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Summary

The causative agents of human malaria include *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri*, and *P. knowlesi*. In addition, zoonotic malaria parasites such as *P. brasilianum*, *P. cynomolgi,* and *P. simium* can also infect humans under experimental and natural conditions. The distributions of these parasite species are overlapping and coexist in many endemic areas leading to mixed-species malaria infections. The morphology-based diagnosis using light microscopy has a low detection threshold and often fails to differentiate cryptic species from the major ones; thereby, prevalence and distribution of co-infection and less virulent species are often vastly underestimated. Unveiling such complex infections requires highly sensitive molecular techniques to obtain accurate epidemiological data to support malaria control and elimination intervention strategies.

As part of this thesis, innovative diagnostic technologies have been developed and utilized to address three challenges that are still major gaps and opportunities in conventional diagnostics for malaria. First, a metagenomics sequencing-based approach was developed to understand the level of *Plasmodium* species co-infections in patients from Gabon, Central Africa. By this approach, a surprisingly high diversity of *Plasmodium* species and genotypes in naturally acquired infections was observed. Cryptic species including recently identified sympatric sub-species—*P. ovale curtisi* and *P. ovale wallikeri*—were found in a large proportion of co-infections in patients with uncomplicated malaria.

Second, a molecular diagnostic technique was developed to show for the first time that transmission of non-human primate species—termed *P. brasilianum*—occurs naturally among Yanomami communities of the Venezuelan Amazon. These findings substantiated that *P. brasilianum* and *P. malariae* are not only the same parasites species but a true anthropozoonosis that infects both human and new world monkey.

Third, a simple molecular test of low-density, submicroscopic *P. falciparum* malaria infections was developed in a format compatible with field application. The test, based on recombinase polymerase amplification (RPA), is highly sensitive, rapid and easy to implement eliminating the need for complex instrumentations. The sensitivity was up to 1000 times higher than conventional malaria diagnostics (microscopy and RDTs)

and had a 95% diagnostic accuracy comparable to ultra-sensitive quantitative PCR (RT-qPCR). Furthermore, the data showed that the assay worked with unprocessed blood for the rapid detection of *P. falciparum*, i.e., without any nucleic acid purification step.

This thesis provides methods that facilitate the accurate diagnosis and case identification of individuals with low-density asymptomatic *Plasmodium* species infections. It enabled the conduct of exemplary studies on current and relevant topics. Such, the first molecular evidence for the coinfection of tertian ovale malaria parasites in Gabon could be delivered. Moreover, it could be proven for the first time that quartan malaria is a true anthropozoonosis. Coupling the newly developed innovative diagnostic method to the current intervention strategies in the field can empower malaria control and elimination efforts of tropical malaria in endemic areas.

Zusammenfassung

Zu den Erregern der Malaria des Menschen gehören *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* und *P. knowlesi*. Darüber hinaus können zoonotische Malariaparasiten wie *P. brasilianum*, *P. cynomolgi* und *P. simium* unter experimentellen und natürlichen Bedingungen auch Menschen infizieren. Die Verbreitungsgebiete dieser Parasitenarten überschneiden sich und koexistieren in vielen endemischen Gebieten, was zu Ko-Infektionen führt. Der morphologische Nachweis mittels Lichtmikroskopie gelingt nur bei vergleichsweise vielen Parasiten im Blut und kryptische Spezies mit niedriger Parasitämie können von den dominierenden Parasiten oft nicht unterschieden werden. Dadurch wird die Prävalenz und Verteilung von Ko-Infektionen vor allem der weniger virulenten *Plasmodium*-Arten häufig stark unterschätzt. Die Aufdeckung solch komplexer Infektionen erfordert hochempfindliche molekulare Techniken, um valide epidemiologische Daten zu erhalten und erfolgreiche Interventionsstrategien zur Malariakontrolle und -beseitigung zu entwickeln.

Im Rahmen dieser Dissertation wurden innovative diagnostische Technologien entwickelt und angewendet, um drei Problembereiche anzugehen, die nach wie vor eine große Herausforderung in der konventionellen Malariadiagnostik darstellen. Zunächst wurde ein auf Metagenomik-Sequenzierung basierender Ansatz entwickelt, um das Ausmaß von Ko-Infektionen verschiedener *Plasmodium*-Arten bei Patienten aus Gabun, Zentralafrika, zu untersuchen. Mit diesem Ansatz wurde eine unerwartet hohe Diversität von *Plasmodium*-Arten und Genotypen bei natürlich erworbenen Infektionen gefunden. Ko-Infektionen mit kryptischen Arten, darunter die kürzlich identifizierten sympatrischen Unterarten *P. ovale curtisi* und *P. ovale wallikeri*, wurden bei einer großen Zahl von Patienten mit unkomplizierter Malaria gefunden.

Zweitens wurde eine molekulardiagnostische Methode entwickelt, mit der die Übertragung von *Plasmodien* nicht-menschlicher Primatenarten Südamerikas– genannt *P. brasilianum*–auf den Menschen in den Yanomami-Gemeinschaften des venezolanischen Amazonasgebietes untersucht werden konnte. Auf der Grundlage dieser Ergebnisse konnte zum ersten Mal systematisch gezeigt werden, dass die quartanen Malariaparasiten *P. brasilianum* und *P. malariae* identisch sind und quartane Malaria damit eine echte Anthropozoonose ist, die sowohl den Menschen als auch die Neuweltaffen infiziert.

Drittens wurde ein einfacher molekularer Test zur Detektion von Infektionen mit *P. falciparum* Parasiten entwickelt. Ein Hauptkriterium bei der Entwicklung des Tests war die verbesserte Sensitivität gegenüber der Mikroskopie. Der Test, der auf der Rekombinase-Polymerase-Amplifikation (RPA) basiert, ist hochempfindlich, schnell und einfach zu implementieren und macht komplexe Instrumente überflüssig, weshalb er auch unter einfachen Bedingungen und mobil angewendet werden kann. Die Sensitivität ist bis zu 1000-mal besser als die konventionelle Malariadiagnostik (Mikroskopie und Schnelltest) und vergleichbar mit der ultra-sensitiven quantitativen PCR (RT-qPCR) bei einer diagnostischen Genauigkeit von 95%. Darüber hinaus zeigen die Daten, dass der Assay mit unverarbeitetem Blut für den schnellen Nachweis von *P. falciparum* funktioniert, d.h. ohne einen Nukleinsäure-Reinigungsschritt.

Diese Arbeit stellt Instrumente bereit, die eine genaue Diagnose und Fallidentifizierung von Individuen mit asymptomatischen *Plasmodium*-Infektionen geringer Dichte erleichtern. Mit Hilfe der entwickelten Methoden konnten expemplarische Studien zu aktuellen und wichtigen Fragestellungen der Malariologie durchgeführt werden. So konnten erstmalig Koinfektionen tertianer Malariaparasiten in Gabon nachgewiesen werden. Des Weiteren konnte zum ersten Mal der molekularbiologische Beweis erbracht werden, dass quartane Malaria eine echte Anthropozoonose ist. Die Anwendung dieser innovativen diagnostischen Methoden kann entscheidend zur Verbesserung aktueller Interventionsstrategien zur Bekämpfung der tropischen Malaria in endemischen Gebieten beitragen.

List of Publications

This cumulative thesis is based on the following publications.

1. **Albert Lalremruata**, The Trong Nguyen, Matthew B.B. McCall, Ghyslain Mombo-Ngoma, Selidji T. Agnandji, Ayôla A. Adegnika, Michael Ramharter, Peter G. Kremsner, Benjamin Mordmüller. *Recombinase Polymerase Amplification and Lateral Flow Assay for Ultrasensitive Detection of Low-Density Plasmodium falciparum Infection from Controlled Human Malaria Infection Studies and Naturally Acquired Infections*. ***J Clin Microbiol* 2020**; 58(5): pii: e01879-19.

2. **Albert Lalremruata**, Sankarganesh Jeyaraj, Thomas Engleitner, Fanny Joanny, Annika Lang, Sabine Bélard, Ghyslain Mombo-Ngoma, Michael Ramharter, Peter G. Kremsner, Benjamin Mordmüller, and Jana Held. *Species and genotype diversity of Plasmodium in malaria patients from Gabon analyzed by Next Generation Sequencing*. ***Malar J*. 2017**; 16(1): 398

3. **Albert Lalremruata**, Magda Magris, Sarai Vivas-Martínez, Maike Koehler, Meral Esen, Prakasha Kempaiah, Sankarganesh Jeyaraj, Douglas Jay Perkins, Benjamin Mordmüller, and Wolfram G Metzger. *Natural infection of Plasmodium brasilianum in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon*. ***EBioMedicine* 2015**; 2(9): 118-1192.

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1. Fernandes JF, Held J, Dorn M, **Lalremruata A**, Schaumburg F, Alabi A, Agbanrin MD, Kokou C, Ben Adande A, Esen M, Eibach D, Adegnika AA, Agnandji ST, Lell B, Eckerle I, Henrichfreise B, Hogan B, May J, Kremsner PG, Grobusch MP, Mordmüller B. *Causes of fever in Gabonese children: a cross-sectional hospital-based study*. *Sci Rep*. 2020; 10(1):2080.

2. Metzger WG, Theurer A, Pfleiderer A, Molnar Z, Maihöfer-Braatting D, Bissinger AL, Sulyok Z, Köhler C, Egger-Adam D, **Lalremruata A**, Esen M, Lee Sim K, Hoffman S, Rabinovich R, Chaccour C, Alonso P, Mordmüller BG, Kremsner PG. *Ivermectin for causal malaria prophylaxis: a randomised controlled human infection trial. Trop Med Int Health.* 2020; 25(3):380-386

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13. **Lalremruata A**, Ball M, Bianucci R, Welte B, Nerlich AG, Kun JF, Pusch CM. *Molecular identification of falciparum malaria and human tuberculosis co-infections in mummies from the Fayum depression* (Lower Egypt). PloS One. 2013; 8(4):e60307.

1 Introduction

Malaria is caused by protozoan parasites of the genus *Plasmodium* (Apicomplexa: Plasmodiidae) which are transmitted by female *Anopheles* mosquitoes (Diptera: Culicidae). There are more than 150 *Plasmodium* species infecting mammals, birds, and reptiles [1]. Of these, six species can naturally infect human beings, namely: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi*; two of these —*P. falciparum* and *P. vivax*—pose the greatest threat. Although *P. vivax* can cause severe illness and death, *P. falciparum* is the malaria parasite with the highest burden of disease. *P. vivax* has a wider distribution than *P. falciparum* and predominates in many countries outside of Africa [2]. The other species—*P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri*—cause less severe morbidity and almost no mortality but are commonly found as co-infections with *P. falciparum*. Whilst, *P. knowlesi* is a zoonotic infection encountered only in Southeast Asia it can cause severe malaria as well [3]. Other zoonotic malaria parasites such as *P. brasilianum*, *P. cynomolgi,* and *P. simium* can also infect humans under experimental and natural condition, but remain often unnoticed by standard malaria diagnostics [4–7].

1.1 Biology

The human malaria parasite life cycle involves two hosts. When an infected female *Anopheles* mosquito probe the human skin for blood meal, a form of parasite known as sporozoites are simultaneously inoculated. Immediately, the sporozoites migrate to the liver via blood circulation and complete their pre-erythrocytic development, which lasts for 1-2 weeks depending on the species. In *P. vivax* and *P. ovale* infections, sporozoites can remain in the liver and become dormant hypnozoites, causing relapses weeks to years after the initial infection [8, 9]. Following the liver stage, merozoites are released into the blood stream to initiate the blood-stage (erythrocytic cycle), where asexual multiplication can produce exponentially increasing parasite numbers causing malaria symptoms. A fraction of merozoites differentiate into male and female sexual forms (gametocytes), which are taken up by the mosquito via a blood meal again. In the mosquito's midgut, male gametocytes exflagellate and fuse with female gametes to form motile zygotes called ookinetes. The ookinetes migrate to the gut wall and form oocysts. Inside oocysts, sporozoites are produced which

migrate to the mosquito salivary glands to start the next life cycle as shown in figure 1.

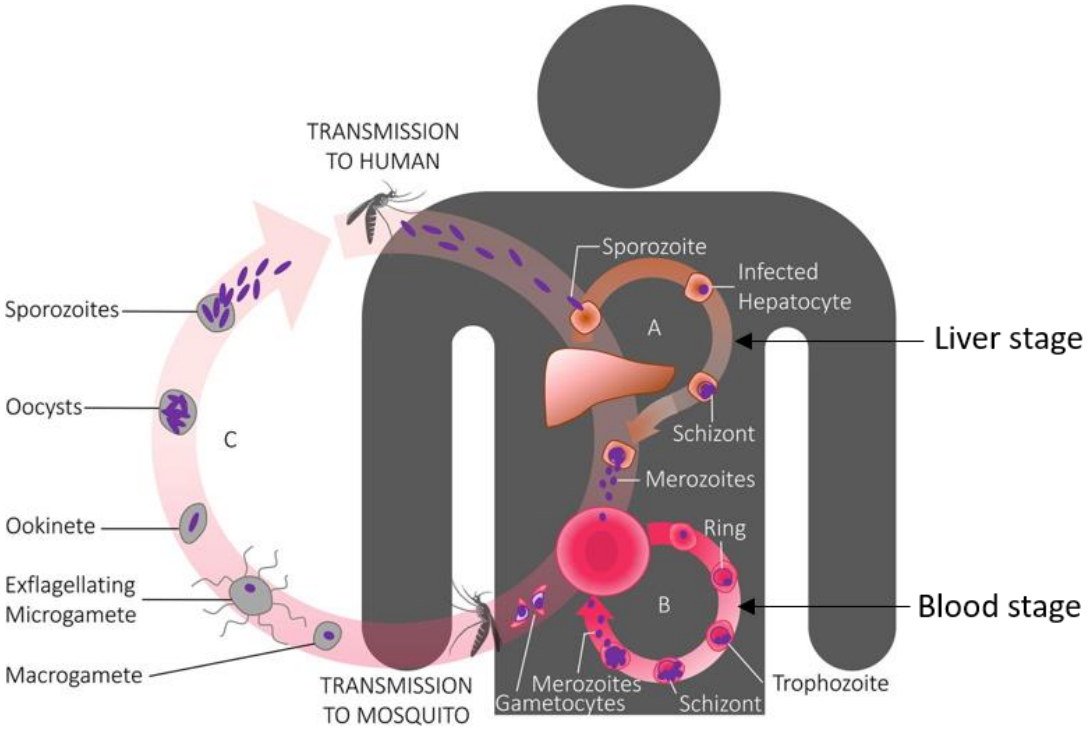

Figure 1. The life cycle of human malaria parasite.

Source: Good *et.al.* Emerging Topics in Life Science 1.6 (2017): 547-552 (with permission provided by Portland Press, Ltd., and Copyright Clearance Center)

1.2 Global malaria situation

Malaria is the most important parasitic disease and a major public health concern worldwide. It causes a heavy economic burden on health systems and economic development to the affected countries. Eradicating malaria, a permanent reduction to zero of the worldwide incidence, has been one of the ultimate public health goals for a century [10].

Despite success in reducing malaria burden between 2000 and 2015, progress in malaria control overall has since stalled, with malaria incidence and mortality remaining unchanged since 2015 [11]. In 2018, there were 228 million cases of malaria with estimated 405,000 deaths, 67% of which were children under 5 years in Africa. *P. falciparum* is the most prevalent malaria parasite species accounting for 99.7% of estimated malaria cases in Africa, 50% in the South-East Asia, 71% in the Eastern

Mediterranean and 65% in the Western Pacific Region. Almost 85% of all malaria cases globally occurred in 19 countries only: India and 18 African countries [12].

2 Malaria elimination and challenges

An early Global Malaria Eradication Program (GMEP) was launched in 1955 with some success in eliminating malaria from Europe, North America, the Caribbean, parts of Asia and South-central America [13]. However, success could not be sustained in large areas, due to the financial constraints in implementing new interventions and the emergence of drug and insecticide resistance, leading to resurgence of cases except in Europe and North America. Thus, GMEP was put on hold in 1969, and the World Health Assembly (WHA) shifted the focus to malaria control, defined as the reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable level as a result of deliberate efforts [14, 15].

Since then, malaria control programs have enormous impact in the fight against malaria. With increased funding and effective control measures taken, the annual global incidence of malaria has fallen by an estimated 41%, and the annual mortality rates by 62% from the year between 2000 and 2015 [11]. The number of countries with endemic malaria have reduced from 106 to 86, and more than half of the world's countries are now malaria free [15].

This global decrease in malaria burden in the last 15 years was mainly attributed to increased funding from endemic countries and the international global partners, from roughly US $1.5 billion in 2000 to $4.3 billion in 2016 [15]. This catalyzed the increased scale-up of malaria control interventions such as: diagnosis with rapid diagnostic tests (RDTs), treatment with artemisinin-based combination therapies (ACTs), distribution of insecticide treated nets (ITNs), and indoor residual spraying (IRS).

Encouraged by the progress made, the World Health Organization (WHO) has launched a new Global Technical Strategy for Malaria 2016-2030 (GTS) in 2015 to help and accelerate progress towards elimination, defined as the reduction to zero incidence of indigenous cases of a specified malaria parasite in defined geographical areas as a result of deliberate activities [16, 14]. The WHO strategy together with the Roll Back Malaria advocacy plan: "Action and investment to defeat Malaria 2016-2030

(AIM)" set a global target of eliminating malaria in at least 21 countries by 2020, and 35 countries by 2030 (figure 2) [16].

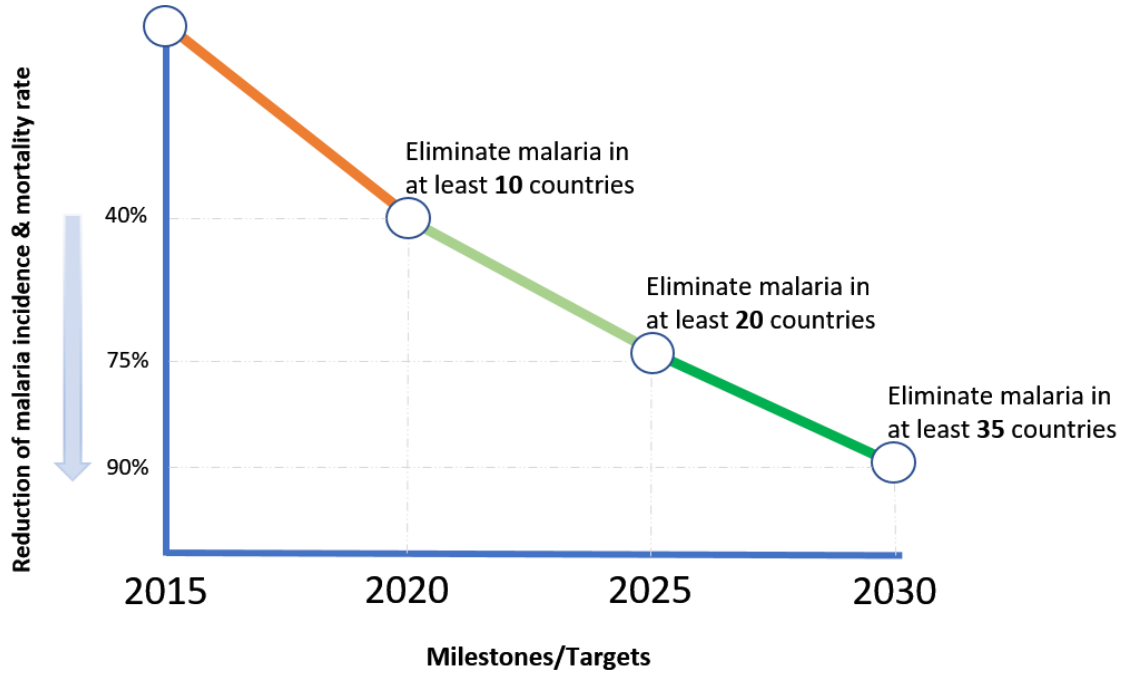

Figure 2. The Global Technical Strategy for malaria 2016-2030.

Source: Adapted from the World Health Organization-GTS report 2015

The challenges that prevent the progress against malaria consist of several interrelated problems. The first and one of the most important challenges is sustainable funding and political commitment of the domestic as well as international partners. The second is the emergence of parasite resistance to antimalarial drugs and of mosquito resistance to insecticides. A change in vector population dynamics and altered behavior has seriously weakened the effectiveness of malaria control strategies in the recent past [17]. The third challenge include poor functionality of the health systems in several endemic regions that lacks adequate tools to diagnose, and human resource capacities to treat efficiently, including high-risk occupational groups, migrant and rural communities who are highly exposed to infections [18].

In regions of low-transmission and pre-elimination settings, an important challenge is the detection of symptom-free individuals with low level parasitemia that is not detected by the current diagnostic tools (healthy carriers). These individuals can serve as an important human parasite reservoir for further malaria transmission. In addition,

the presence of zoonotic *Plasmodium* species present new challenges for malaria control and elimination in some regions [16, 19].

2.1 Asymptomatic malaria

According to the WHO definition, asymptomatic malaria is the presence of asexual parasites in blood without symptoms of illness [14]. Generally, asymptomatic malaria refers to an infection of any density without fever or other symptoms that would lead to seek treatment. This includes early blood-stage parasitemia that has not yet reached fever threshold or chronic infections due to anti-parasitic immunity [20]. Thus, asymptomatic malaria infection can be either microscopic (detectable by light microscopy) or submicroscopic (detectable only by more sensitive molecular tests such as PCR) [21].

The widespread utilization of molecular diagnostics for malaria epidemiological studies have revealed that a large portion of infections in all endemic areas are asymptomatic [22]. The fraction of submicroscopic parasite infections were shown to be highest in low-endemic regions [23]. All forms of *Plasmodium* infections, whether symptomatic or asymptomatic, constitute the infectious reservoir to the mosquito. Even though asymptomatic infections are generally associated with low density parasitemia and low rates of infectiousness to mosquitos as compared to symptomatic infections, the high proportion and chronicity of the infection may pose a greater contribution to the risk of transmission to mosquito than symptomatic malaria [24]. Indeed, studies have shown that submicroscopic parasitemias and/or gametocytemias contribute substantially to malaria transmission [25, 26].

The large size of the asymptomatic parasite reservoir has major consequences for elimination. Without symptoms most people refuse treatment and thus unknowingly contribute to transmission. Even with active surveillance activities, the majority of low-density parasite infections will be missed by the current screening tools employed in the field.

2.2 Zoonotic malaria

A challenge and an obstacle to malaria elimination in certain areas has long been envisaged by the presence of non-human primate malaria parasites [27]. However,

the threat of these zoonotic parasites to public health, and the probability of stable transmission to humans has been thought to be low until the application of advanced molecular techniques for diagnosis.

The genus *Plasmodium* is widespread in primates and there are 27 non-human malaria parasites reported until recently [28]. Of these, 13 species occur in Southeast Asia [28]. Evolutionary history suggests that host-switch events have occurred between non-human primate (NHP) *Plasmodium* species to humans [29]. In fact, two of the most important human malaria parasites, *P. falciparum* and *P. vivax*, have originated from a cross-species transmission event from great apes in Africa [30, 31]. Transmission of primate malaria parasites to human continues till today, such as: *P. knowlesi* and *P. cynomolgi* in Southeast Asia, *P. simium* and *P. brasilianum* in South America, and *P. vivax*-like in the Central African Republic [32]. While, the potential transmission of several other *Plasmodium* species in great apes to humans or humans to apes in certain conditions are yet to be corroborated [33].

Infections with *P. knowlesi* was frequently misdiagnosed as *P. malariae* by routine microscopy until molecular PCR-based diagnosis revealed its occurrence in humans [34]. Since then, the use of PCR techniques has manifest the widespread occurrence of *P. knowlesi* in all Southeast Asian countries and is now accepted as the primary cause of malaria in parts of rural Malaysia [35]. Asymptomatic *P. cynomolgi* infections have also been detected by molecular tests in Cambodia [36] and Malaysia [37]. Recently with the same molecular diagnostic approach, zoonotic *P. simium* outbreaks have been reported in the Atlantic forest region of Brazil; where elimination had been achieved for over 50 years [7].

This accumulating evidence clearly shows that the emerging zoonotic malaria should be critically evaluated for the risk they pose to control and elimination efforts.

2.3 Eliminating all Plasmodium species

The occurrence of *Plasmodium* species overlaps in most malaria endemic regions of the world, often the same mosquito species are involved in their transmission to human hosts [38]. Non-falciparum species are often underestimated by the gold standard test, blood smear analysis by light microscopy [39]. The widespread use of PCR tests in

community surveys clearly showed that non-falciparum species are common in all endemic areas [40].

Historically, the global malaria control and elimination programme has been focused on *P. falciparum*. Obviously, *P. falciparum* receives more attention than the other species because of its paramount contribution to malaria related death cases. However, the contribution of other species to morbidity is not well investigated, mostly due to lack of epidemiological tools. At least *P. vivax* is now included in the framework, but other species, *P. malariae*, *P. ovale*, and *P. knowlesi*, still present a major challenge for elimination [41].

Among human malaria species, *P. vivax* has the most widespread geographical distribution. It has unique features including the ability to preserve dormant hypnozoites in liver hepatocytes, that may reactivate thus leading to blood stage infection and clinical relapses late after the initial transmission [42]. In addition, sexual gametocytes appear much earlier during infections as compared to *P. falciparum* [43]. The biology of *P. vivax* entails that the proportion of the total parasite reservoir, which includes a large proportion of asymptomatic low density parasitemia and the cryptic dormant stage parasites in liver, pose a huge barrier to effective elimination strategies [44].

P. malariae infections are usually asymptomatic and the parasite is known to persist in blood for a long time at very low densities [45]. The parasite is widely distributed throughout sub-Saharan Africa, Southeast Asia, and the Amazon basin of South America [45]. Infection with *P. malariae* is usually considered benign, however it has been implicated with other pathologies such as anemia and nephrotic syndrome [46, 47].

Based on molecular dimorphism observed in the genome, *P. ovale* now consists of two distinct species, *P. ovale curtisi* and *P. ovale wallikeri* [48]. These two are morphologically indistinguishable and coexist in many Asian and African countries [49]. Like *P. malariae*, *P. ovale* counts in blood are usually low compared to *P. falciparum* or *P. vivax* and alone usually cause mild, asymptomatic infections [50, 51]. Similar to *P. vivax*, however, it may cause relapsing infections after months of recovery from the primary infection; especially *P. ovale curtisi* [8, 52].

Increased scale-up of malaria control measures against *P. falciparum* has shown a marked shift in the prevalence of non-falciparum malaria parasite species in many endemic areas [53, 54]. Malaria elimination will require better understanding of the epidemiology of other species. To achieve this, improved surveillance tools for monitoring the transmission potential of each species are required. In particular, identifying asymptomatic carriers of infections is critical for the successful implementation of interventions [55].

3 Tools for malaria elimination

3.1 Antimalarial drugs

Access to safe and effective antimalarial drugs is essential to saving lives and has been the cornerstone of malaria control strategies. Highly effective artemisinin-based combination therapies (ACTs) have played an important role in reducing *P. falciparum* malaria-related mortality and morbidity worldwide. Antimalarial drug treatment that fully clear parasites including the mature sexual stage gametocytes, among symptomatic and asymptomatic carriers, is required in the context of malaria elimination as well as for containment of multidrug resistance parasites. Recently, pharmacologic interventions to interrupt transmission of malaria has been recommended using drugs that actively clear both asexual and gametocytes from individual in low-endemicity and in pre-elimination areas [56]. Such approaches include: mass drug administration (MDA), i.e. mass treatment of the population regardless of symptoms or confirmed infections; mass screening and treatment (MSAT), i.e. testing of an entire population followed by treatment; focal screening and treatment (FSAT), i.e. screening all individual from a defined region or “hot spots” followed by treatment [57]. Antimalarial drug treatment is also used as an effective preventive strategy for the most vulnerable groups, such as pregnant woman and children, called intermittent preventive treatment for pregnant women (IPTp), infants (IPTi) and seasonal malaria chemoprevention (SMC), respectively [18].

3.2 Insecticides

Use of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) are the most important vector-targeting interventions that have significantly contributed

to reduction in malaria cases in sub-Saharan Africa [58]. Between the year 2000 and 2015, LLINs have shown the greatest effect among control interventions in reducing malaria incidence and prevalence across Africa [58]. However, transmission still occurs due to vector populations that escape contact with these indoor interventions, known as residual transmission [59]. Additional tools that target residual transmission using a combination of several approaches known as an integrated vector management (IVM) strategy was established by the WHO to combat vector-borne disease transmission [60]. Such approaches are essential for effective control of malaria control and elimination.

3.3 Vaccines

Vaccines would be the most cost-effective tool for the prevention, control and elimination of the global burden of malaria. Vaccines have enabled previous eradication programs for other infectious diseases such as smallpox, polio, and measles. Out of many vaccine candidates evaluated, RTS,S/AS01 is the first malaria vaccine and the only human anti-parasite vaccine that has completed phase 3 studies. It shows a robust but only partial efficacy against malaria throughout Africa [61, 62]. RTS,S/AS01 is a pre-erythrocytic vaccine that targets an antigen from *P. falciparum* sporozoites (circumsporozoite protein). Because of the positive result of the phase 3 trial, pilot implementation programme for the vaccine was launched in three African countries—Ghana, Kenya and Malawi—in 2019 [63]. Other candidates based on whole sporozoite, blood-stage, or sexual-stage antigens remain in clinical development since efficacy of RTS,S/AS01 does not reach WHO's preferred product characteristics. All vaccine candidates are planned to be used in combination with other interventions to interrupt transmission for malaria elimination [64–66]. The current malaria vaccine technology roadmap targets development of an effective vaccine by 2030, which is at least 75% efficacious against clinical malaria caused by *P. falciparum* and *P. vivax* [67].

3.4 Diagnosis

Malaria diagnosis is one of the most critical tools needed to guide treatment of infected individual and to support surveillance/screening. Early diagnosis and prompt treatment are essential to sustain the success achieved globally in the fight against malaria.

However, not all *Plasmodium* infections lead to clinical symptoms, and vast majority of individuals in endemic areas are healthy carrier. In *P. falciparum* infections this is mainly due to partial immunity (dubbed semi-immunity) that develops after repeated exposure to the parasite. For the other species, virulence and immunity are much less well understood. Thus, mostly symptomatic individuals get tested and treated appropriately. Those individuals with asymptomatic infections are likely not tested nor treated, thereby presenting a huge reservoir of *Plasmodium* parasites for continued transmission. Furthermore, parasite density in asymptomatic individuals are usually lower, often below the threshold detectable by microscopy, termed as submicroscopic parasitemia. All *Plasmodium* species can cause asymptomatic infections [22]. Recent studies have clearly shown that even submicroscopic infections contribute to infectious reservoir [68, 69]; the strategy to detect and treat all asymptomatic infections to achieve elimination would require more sensitive, new diagnostics to support an active case detection system [70].

4 Current diagnostic tools for malaria

Three methods are currently employed for parasitological confirmation of malaria parasites: blood smear microscopy, antigen-detecting rapid diagnostic tests (RDTs), and nucleic acid amplification tests (NAAT), although in many remote places, treatment of malaria still relies on presumptive diagnosis based on clinical signs and symptoms, especially in sub-Saharan Africa [71]. However, even in highly endemic regions (where the *a priori* probability of malaria is high) anti-malarial treatment without parasitological confirmation results in both drug wastage and potentially life-threatening under-treatment of the underlying cause of clinical symptoms. Since 2010, WHO revised their recommended treatment policy, which requires every suspected malaria case be confirmed by microscopy or RDTs prior to treatment, known as the "T3: Test.Treat.Track" strategy [72].

4.1 Light Microscopy (LM)

Microscopic examination of Giemsa-stained thick and thin blood films is the oldest method and is still considered as the gold standard test for malaria [73]. The advantage of LM in addition to the detection of peripheral parasitemia, is the differentiation of species and the stage of malaria parasites. Moreover, parasite density can be

counted, which is vital to the diagnosis (e.g. in hyperparasitemia) and evaluation of antimalarial treatment efficacy. Despite this valuable features, microscopy has several limitations; it is a morphological based diagnosis that rely on parasite characteristics besides the shape and size of infected erythrocytes (iRBCs), which lead to misdiagnosis especially when two or more species occur in the same host. The identification of *P. knowlesi* infections in humans, which were previously misdiagnosed as *P. malariae* by microscopy is one good example [34]. Importantly, the sensitivity of microscopic diagnosis relies on the skill of the operator and reliable equipment. Although expert microscopists can detect up to 5 parasites/microliter (p/μL) in research settings, the threshold of detection ranges from 50–100 p/μL in routine clinical settings. Fluctuation of parasite density during the course of infection, due to sequestration, often lead to parasite level below the limit of microscopy detection, thereby resulting in an under-estimation of infection rates [74].

4.2 Rapid diagnostic tests (RDTs)

Development of malaria diagnosis based abundant blood-stage antigen known as histidine-rich protein 2 (HRP2) began during the early 1990s. The subsequent commercialization of immunochromatographic tests that detect HRP2 in whole blood has made enormous impact and transformed malaria diagnostic landscape [75]. Antigen-detecting rapid diagnostic tests (RDTs) offer several advantages over clinical diagnosis or direct parasite detection by microscopy. RDTs are simple to perform with fast interpretation of results. As a result, they are increasingly used in endemic countries and are included in WHO list of essential *in vitro* diagnostics [76]. Through the widespread roll out of RDTs through donors, the annual malaria diagnoses performed increased from 55 million to more than 233 million, of which more than 75% of the test were performed using RDTs [75]. While HRP2 is an antigen specific to *P. falciparum*, lactate dehydrogenase (LDH) and aldolase are found in all *Plasmodium* species. Thus, there are lactate dehydrogenase-based tests specific for *P. falciparum* (*Pf*-LDH) or *P. vivax* (*Pv*-LDH) as well as for all species detecting tests, based on LDH (pan-LDH) and aldolase (pan-aldolase) [77]. So far, no antigen tests specific for *P. malariae*, *P. ovale*, and *P. knowlesi* are available. In addition, pan-Plasmodium RDTs are less sensitive and more expensive than RDTs that detect *P. falciparum* specific HRP2 [78, 79]. Despite their widespread use, RDTs do have limitations including their

inability to quantify parasite density; false-positive results due to presence of autoantibodies such as rheumatoid factor, or persistence of HRP-2 in blood after clearance of infection; false-negative results due to high levels of HRP2 deletion of the gene encoding HRP-2 protein or prozone effect due to high levels of antigens in hyperparasitemic patients [80]. Like microscopy, accurate diagnosis could be achieved only with parasite density above 100-200 p/µL. With such threshold of detection, RDTs missed many infections that are common in asymptomatic parasite carriers which are important for maintaining transmission cycle.

4.3 Nucleic acid amplification tests (NAATs)

The development of molecular techniques that amplify and detect specific regions of parasite DNA or RNA (nucleic acids) have emerged as alternatives to light microscopy and RDTs. Nucleic acid amplification tests (NAATs) are highly sensitive and specific, enabling accurate diagnosis to species and genotypes level. The NAATs include PCR-based methods such as nested, real-time, multiplex PCRs; isothermal methods such as Loop-mediated nucleic acid amplification (LAMP), nucleic acid sequence-based amplification (NASBA), and recombinase polymerase amplification (RPA). Differential diagnosis of malaria parasites by nested PCR (nPCR) was first developed by Snounou *et. al*. in 1993 [81]. Since then, different methodologies and different PCR chemistries were introduced for diagnosis of malaria parasite: real-time PCR using SYBR or probe-based chemistry, reverse transcription real-time PCR, and parallel detection of several targets in one assay known as multiplex PCR [82]. Most of these commonly used PCR-based assays provide more accurate diagnosis than microscopy or RDT [83]. Given their superior sensitivity to detect low-density parasite infections in asymptomatic carrier and mixed species infections, PCR tests are highly desirable asset in epidemiological research and survey. However, implementation in endemic region poses many challenges due to cost, the need for expert personnel, longer turnaround times compared to light microscopy and certain laboratory settings. To overcome challenges of using PCR-based NAAT in remote areas, several isothermal amplification techniques have emerged. Each isothermal amplification techniques developed has distinctive innovation but with the same principle of quick results, accurate and cost-effective compare to PCR methods. LAMP for testing of *P. falciparum* was first developed by Poon *et. al*. in 2006 [84]. Since then, several

commercial malaria LAMP kits as well as in-house developed assay have been evaluated as for use as field-diagnostics. NASBA which uses RNA template for amplification has been used for quantifying human infectious reservoir by detecting specific messenger RNA (mRNA) from *Plasmodium* gametocytes [85]. However, there is still a lack of sensitive assay that can be deployed as PCR alternative in field screening, importantly LAMP still miss portion of low-density infections that may have implication in malaria elimination efforts [86, 87]. The use of field applicable, highly sensitive NAATs method for low levels of parasites remains elusive. Developing such a tool is still a work in progress that has to be widely tested on the target population in endemic areas [88]

5 Objectives

Development of new diagnostic technologies is one of the research approaches of the WHO's malaria eradication research agenda initiative (malERA) [89]. Sensitive diagnostics are needed for reactive or proactive detection of parasite reservoirs in asymptomatic individuals for interventions aiming malaria elimination. The studies included in this thesis are the steps taken toward the development of molecular diagnostic tools for screening parasites for epidemiologic studies and surveillance.

The objectives of the work presented in this dissertation are as follows:

1. In the first chapter, I aim to investigate the prevalence of *Plasmodium* species and the complexity of parasites infections involved in Gabonese patients with uncomplicated malaria using a metagenomic ultra-deep sequencing approach.

2. In the second chapter, I aim to investigate quartan malaria infections among the forest-dwelling Yanomami communities from Venezuelan Amazon and to determine a possible role of zoonotic parasites for quartan malaria infections in humans.

3. In the last chapter, I aim to develop a simplified molecular method based on recombinase polymerase amplification and lateral-flow test that can be used for field diagnosis of submicroscopic parasitemia in asymptomatic patients.

6 Results

The results of each published paper accumulated for this thesis are summarized into this section.

6.1 Chapter I

Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing.

Lalremruata *et. al.* Malar J. 2017; 16(1): 398

Six *Plasmodium* species are known to naturally infect humans. Infections of the *Plasmodium* species usually occur as monoinfection by single species, most frequently by *Plasmodium falciparum* or *P. vivax*. However, mixed species infections occur regularly but morphological discrimination by microscopy is difficult and multiplicity of infection (MOI) can only be evaluated reliably by molecular DNA amplification methods. Accurate identification of each species involved in a malaria infection is important for appropriate medication; especially in areas where there are two treatment policies: artemisinin-based combination therapy (ACTs) for *P. falciparum,* and chloroquine for non-falciparum species. In addition to the first line treatment, anti-hypnozoite drugs (e.g. primaquine) may be used for *P. vivax* and *P. ovale* infections to prevent relapses.

In this study, the complexity of *Plasmodium* infections in patients treated with ACTs for microscopically detected non-falciparum or mixed species malaria in Gabon were investigated. To understand the dynamics of microscopic and submicroscopic *Plasmodium* species involved in natural infections of 46 Gabonese patients, an ultra-deep sequencing method was developed using nucleus (18S rRNA), mitochondrion (cytochrome b), and apicoplast (caseinolytic protease C) encoded genes.

Compared to the gold standard “nested-PCR with gel-electrophoresis” method, next-generation sequencing (NGS) revealed a large complexity of coinfections in patients with uncomplicated malaria, both on species and genotype levels. Mixed infections involved up to four parasite species (*P. falciparum*, *P. malariae*, *P. ovale curtisi*, and *P. ovale wallikeri*). Multiple genotypes from each species were determined from the asexual 18S rRNA gene. 17 of 46 samples (37%) harbored multiple genotypes of at

least one *Plasmodium* species. The number of genotypes per sample (MOI) was highest in *P. malariae* (n = 4), followed by *P. ovale curtisi* (n = 3), *P. ovale wallikeri* (n = 3), and *P. falciparum* (n = 2). The highest combined genotype complexity in samples that contained mixed-species infections was seven.

In summary, ultra-deep sequencing showed an unexpected breadth of *Plasmodium* species and within species diversity in clinical samples that could not be determined by the standard malaria diagnosis tools including the current gold standard nested PCR method. This method offers a powerful tool for understanding the epidemiology of *Plasmodium* species, especially for providing prevalence data to inform control efforts for each *Plasmodium* species to reduce mortality, morbidity and ultimately eliminate the disease.

6.2 Chapter II

Natural infection of Plasmodium brasilianum in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon

Lalremruata *et. al.* EBioMedicine 2015; 2(9): 118-1192

A majority of pathogens, 61% of them, causing diseases in humans are zoonotic [90]. *Plasmodium knowlesi* is one of those zoonotic parasites. It is a parasite of macaques and it is responsible for about 70% of the malaria cases in humans in Malaysian Borneo [35] and cases have been reported from several other Southeast Asian countries threatening malaria elimination efforts in the region [91]. In South America *P. knowlesi* does not exist but there are other parasites that infect New World monkeys: *P. brasilianum,* a quartan malaria and *P. simium*, a tertian malaria.

P. brasilianum is highly similar and microscopically indistinguishable to the human quartan malaria parasite *P. malariae.* The genomes of the two parasites are also nearly identical, differing only in a range of mutations expected within a species. Therefore, it has long been speculated that the two are the same and that monkeys constitute a potential zoonotic reservoir of malaria transmission in the Amazon region.

We investigated malaria cases from remote Yanomami indigenous communities of the Venezuelan Amazon and analyzed the genes coding for the circumsporozoite protein

(CSP) and the small subunit of ribosomes (18S) by species-specific PCR and capillary based-DNA sequencing.

Based on 18S rRNA gene sequencing, we identified 12 patients harboring malaria parasites which were 100% identical with *P. brasilianum* isolated from the monkey, *Alouatta seniculus*. Translated amino acid sequences of the CS protein gene showed identical immunodominant repeat units between quartan malaria parasites isolated from both humans and monkeys.

This study reports, for the first time, naturally acquired infections in humans with genuine *P. brasilianum*. We conclude that quartan malaria parasites are easily exchanged between humans and monkeys in Latin America. We hypothesize a lack of host specificity in mammalian hosts and consider quartan malaria to be a true anthropozoonosis. The expansive reservoir of mammalian hosts discriminates quartan malaria from other *Plasmodium* spp. and requires particular research efforts for their control and elimination.

6.3 Chapter III

Recombinase Polymerase Amplification and Lateral Flow Assay for Ultrasensitive Detection of Low-Density Plasmodium falciparum Infection from Controlled Human Malaria Infection Studies and Naturally Acquired Infections

Lalremruata *et. al.* J Clin Microbiol 2020; 58(5): pii: e01879-19

In malaria endemic areas, particularly in low-endemic countries, a large portion of individual are asymptomatic with low-density parasitemia that cannot be detected by current field-applicable diagnostic tests. On average, microscopy and rapid diagnostic tests (RDTs) detect only half of PCR-positive *Plasmodium falciparum* infections [92]. These so-called subpatent infections have been associated with sustaining transmission and severe adverse birth outcome in vulnerable groups such as pregnant women [20]. There is a pressing need for new diagnostics, which bridge the usability of RDTs and the sensitivity of PCR-based nucleic acid amplification test (NAAT), in programmes such as: mass screen-and-treat (MSAT) and intermittent screening and treatment of pregnant women (ISTp).

To complement existing diagnostic methods, an isothermal reverse transcription-recombinase polymerase amplification and lateral flow assay (RT-RPA) was developed. We compared the performance with that of ultrasensitive reverse transcription-quantitative PCR (uRT-qPCR) using nucleic acid extracts from blood samples (n=114) obtained during standardized controlled human malaria infection (CHMI) with *P. falciparum* sporozoites. As a preliminary investigation, we also sampled asymptomatic individuals (n=28) in an area of malaria endemicity (Lambaréné, Gabon) to validate RT-RPA and assess its performance with unprocessed blood samples (dbRT-RPA). In 114 samples analyzed from CHMI trials, the positive percent agreement to uRT-qPCR was 90% (95% confidence interval [CI], 80 to 96). The negative percent agreement was 100% (95% CI, 92 to 100). The lower limit of detection was 64 parasites/ml. In Gabon, RT-RPA was 100% accurate with asymptomatic volunteers (n=28), while simplified dbRT-RPA showed 89% accuracy. In a subgroup analysis, RT-RPA detected 9/10 RT-qPCR-positive samples, while loop-mediated isothermal amplification (LAMP) detected 2/10.

In summary, RT-RPA is a reliable diagnostic test for asymptomatic low-density infections. It is particularly useful in settings where highly sensitive PCR is difficult to implement. The technique has the potential to develop further as the new rapid point-of-care molecular testing for malaria diagnosis.

7 General Discussion

Accurate diagnostic tools are needed to differentiate between malaria and other febrile illnesses caused by bacteria and viruses [93]. In sub-Saharan Africa, malaria is the most common diagnosis for febrile patient and misdiagnosis of febrile illness still remains the major problem [94]. From the early 2010, the WHO recommended that every case of suspected malaria must be confirmed by microscopy or RDTs prior to treatment. Treatment based on clinical symptoms are encouraged only in areas where diagnostic tests are not available or as part of pre-referral treatment of severe malaria. Since the launch of a new initiative—the T3 (Test.Treat.Track)—in 2012, malaria diagnosis has become an essential component of malaria control strategies [72]. The importance of malaria diagnostic testing has been further emphasized in the WHO's

Global Technical Strategy 2016–2030, which provides technical guidance to countries and development partners with the aim of reducing global malaria burden by 90% in 2030. Standard diagnostic tests such as microscopy and RDTs play the key role for therapeutic care of febrile patients in endemic areas. Accurate diagnosis and scale-up of testing with new and/or improved diagnostic tools will not only improve rational treatment and therapy, but also help malaria control and elimination efforts to become more efficient and more targeted towards the goal.

With successful malaria control programs, many countries have now shifted the focus to eliminate the disease. In such areas, detecting a parasite has become difficult as a large proportion of infections is of lower parasitemia without symptoms. To eliminate malaria in such areas, detection of parasites require screening for asymptomatic carriers in the community known as active case detection (ACD). Several approaches have been investigated to improve the sensitivity and specificity of malaria diagnostics to uncover asymptomatic reservoir in the context of elimination strategy. One promising approach was the development of a new generation RDT (Alere™ Malaria Ag Pf) in 2017. The new RDT is shown to be ten times higher sensitive than the existing RDTs on the market [95]. In a study conducted to demonstrate the detection of low-density parasite infections, Alere RDT have failed to detect up to 56% of PCR-detectable *P. falciparum* [96]. In addition, Alere RDT is also based on HRP-2 antigen, thus it is still vulnerable to HRP-2 gene deletion, cross-reaction issue with rheumatoid factors, and it does not work with non-falciparum species.

The use of NAATs have several advantages compare to microscopy and RDTs. Therefore, developing field-friendly, simplified molecular methods constitute an active research agenda for malaria elimination. Recent approaches to enhance detection sensitivity are the use of reverse transcriptase to detect total nucleic acids, use of large blood volume, and multicopy genes amplification qPCR [97–99]. Through these approaches, limit of detection below 100 parasites/mL have been achieved enabling ultra-sensitive molecular diagnosis method [24] However, NAATs are prone to carry-over contamination which requires trained technician to perform the assay with a very high standard of laboratory practice in a well-equipped facility. Except the isothermal technique described in the chapter 3, the other methods described in this thesis rely on expensive instruments and are impractical for routine diagnosis outside of the

research laboratory. Non-instrument amplification by a simplified method such as RPA offers a promising point-of-care molecular diagnosis for *Plasmodium* as an alternative in the field.

7.1 Plasmodium species detection by ultra-sensitive sequencing method

The WHO certification of malaria elimination in a country requires a proof that local transmission of all human parasite species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) has been interrupted with zero incidence of indigenous cases for three consecutive years [100]. While the current elimination efforts are focused on malaria caused by *P. falciparum* and *P. vivax*, only relatively little effort has been dedicated to research on the other human malaria species. If elimination of all human species is the goal, non-falciparum species pose additional challenges to achieve elimination due to subtle differences in life cycles, ecology, distribution, and lack of tools to study them [41].

Infections with non-falciparum species usually present with low parasitemia; they can persist for long periods and sometimes remain asymptomatic for years [45]. The interaction between concurrent species within the host may play a role in transmission dynamics and/or infection outcome [101, 102]. Recent studies demonstrated the varying transmission patterns between *P. falciparum* and non-falciparum species in Africa. In Tanzania, despite a reduction in *P. falciparum* transmission, the prevalence of *P. malariae* and *P. ovale* infections was shown to increase from 2010 to 2016 [55]. Similar trends were reported in Burkina Faso and Uganda with increased prevalence of *P. malariae* [103, 104].

Accurate detection of each *Plasmodium* species is important to obtain the incidence and transmission dynamics in distinct areas; so as to provide proper treatment guidelines and to assess the impact of malaria control interventions. Routine diagnosis performed by light microscopy has limited sensitivity and specificity for species identification. Likewise, RDTs lack sensitivity and frequently miss non-falciparum mono-infections [105, 106]. *Plasmodium* and concurrent species infections can be detected more reliably by molecular PCR-based methods. The nested PCR, by

Snounou *et. al*. 1993, has been considered the molecular gold standard for malaria species detection [81].

An alternative approach would be next generation sequencing (NGS) technologies, which allow high-resolution analyses of a heterogeneous mixture of the parasites within the host [107]. Compare to Sanger's method, sequencing by NGS using a targeted approach known as amplicon sequencing enables detection of minor genotypes as well cryptic species and provides a new platform to investigate within-host diversity of *Plasmodium* infections. In recent times, NGS technologies have been used to reconstruct whole genomes of non-falciparum species [108], genotyping of *P. falciparum* for drug and vaccine resistance strains [109, 110], as well as applied for ecological studies for *Plasmodium* origins [111].

In the first chapter of the thesis, a metagenomics approach was applied which allow a snapshot in time; the breadth and intensity of different *Plasmodium* species concurrent infections in a cohort of patients with uncomplicated malaria. The study was the first to apply metagenomics-based approach using three genes—nucleus (18S rRNA), mitochondrion (cytb), and apicoplast (clpC)—in parallel to characterize the different *Plasmodium* species that infect patients with uncomplicated malaria. The advantage of our approach is that by using universal PCR primers (Pan-Plasmodium), that potentially amplify all human and non-human primate Plasmodium species, and deep sequencing, which allows unbiased detection of even minor or rare occurring species, offers robust tool for epidemiological studies to investigate the prevalence, the emergence, and change in composition of *Plasmodium* species.

7.2 Quartan malaria: a zoonotic disease

Since malaria eradication is on the global health agenda again, proper surveillance of parasite reservoir that sustain malaria transmission is highly critical. In this context, non-human primates as source for *Plasmodium* infections in humans have received increased attention [112].

According to the WHO expert committee on zoonosis, zoonoses are diseases transmissible from living animals to humans [113]. *P. brasilianum* is a quartan malaria parasite of New World monkeys. It was first identified by Gonder and von Berenberg-

Gossler in an imported ‘bald-headed uakari’ (Cacajao calvus) in 1908 [114]. *P. brasilianum* is morphologically similar to the human quartan parasite *P. malariae* under the microscope. Experimental infection studies in the 1960s showed that humans could be infected with *P. brasilianum* from monkeys, and likewise, New World monkeys could be experimentally infected with *P. malariae* from humans [114, 115]. Compared to *P. knowlesi*, which have two principal natural monkey hosts in South-east Asian countries, *P. brasilianum* have been identified from more than 35 monkey species in South America [116, 117].

In the second study of the thesis, we investigated quartan malaria cases in Yanomami communities living in remote areas of the Alto Orinoco Casiquiare Biosphere Reserve where humans and non-human primates live in close contact for a possible onward transmission of disease.

The study was designed before the reference draft genome of *P. malariae* was made available in 2017 [118]. At that time, only few gene sequences of quartan malaria parasites were available in the public database, the NCBI Genbank. We found only two gene targets, 18S and CSP, wherein *in silico* analysis of the reference sequences for *P. brasilianum* and *P. malariae* showed differences, which might be useful for differential diagnosis of the two related species. Thus, primers were designed to capture those differences by amplifying the two targets. Amplicons were subjected to conventional Sanger sequencing.

Our study was the first to retrieved in man the 18S gene sequences of *P. brasilianum* similar to those found in Alouatta monkeys from French Guiana [117], implying the evidence of naturally acquired human infections. Indeed, our finding was further corroborated by the new *P. malariae* reference genomes analysis which showed that quartan malaria parasites from New World monkeys are closer and indistinguishable to human *P. malariae* isolates [118]. Thus, confirming our conclusion that in Venezuelan Amazon, especially where contact is high with monkeys in nature, quartan *P. malariae/P. brasilianum* is freely circulating between monkeys and humans without any host’s cross-species restriction. Clearly for *P. malariae,* the issue of zoonotic infections and huge parasite reservoirs in monkeys pose a big challenge for elimination, at least in some South American regions.

7.3 Highly sensitive molecular point-of-care diagnostic for malaria

The WHO defined a set of criteria to determine the suitability of a test for use in endemic countries with limited sources as: Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable (ASSURED) [119]. These criteria are ideals and no current diagnostic test meets all of them. RDTs fulfilled many of the ASSURED criteria, however, the sensitivity especially in the context of detecting low parasitemia hampered their suitability for use in the field, especially for detecting asymptomatic parasite carriers.

Molecular amplification methods such as PCR or real-time PCR are highly sensitive and can detect submicroscopic, low level parasitemia from asymptomatic individuals. However, major challenges for implementing them in endemic countries are the high cost of instrumentation (procurement and maintenance), the need for space and uninterrupted power supply, and well- trained personal for conducting the assay. In recent years, many novel amplification techniques that run on a constant temperature without thermal cyclers (PCR) were developed to translate molecular tests from lab-based to on-site methods for rapid diagnosis of infectious diseases [120]. One of such technology was developed in 2006, known as recombinase polymerase amplification (RPA) [121]. The technique is based on recombinase complex from T4 bacteriophage that initiate the binding of primers to specific sites and amplification mediated by strand displacing DNA polymerase. Thus, RPA can work at a relatively lower and constant temperature (38–42°C). Furthermore, it can be combined with lateral flow detection for a quick read-out of amplification results. This makes the technology particular interesting for a scenario where high sensitivity is essential. Microscopy and RDTs are well suited for clinical decisions in symptomatic patients, whereas they do not perform well in asymptomatic individuals.

Current strategies to interrupt transmission in malaria eliminating countries, such as mass screening and treatment (MSAT) and focal testing and treatment (FSAT), rely on the sensitivity of the diagnostic tests to identify asymptomatic parasite reservoirs for treatment. For such purpose, microscopy and RDTs have been used with limited success due to undetected parasites that still sustained onward transmission.

In the third objective of the thesis, we applied and designed RPA method for accurate diagnosis of the most virulent and most prevalent parasite species in sub-Saharan Africa, *P. falciparum* infection. To enhance the sensitivity, we co-amplified total nucleic acids of both large subunit ribosomal RNA and DNA (28S) in single-step reverse transcription RPA assay (RT-RPA). The test was evaluated using samples from well characterized control human malaria infection trials, and naturally acquired infections. I could demonstrate that RT-RPA has a sensitivity well above the recommended minimum detection threshold needed for a new molecular test – 2000 parasites/mL – , which is considered a huge improvement to the current field diagnostic tools.

The future work will consider the transfer of the knowledge gained from this study to apply and design similar platform for other *Plasmodium* species diagnosis; so that non-falciparum can be accurately detected for all *Plasmodium* species elimination efforts.

8 Conclusions

As malaria control activities have intensified, transmission has declined in many endemic areas. Consequently, malaria incidence has significantly reduced to levels below from what was previously experienced. With the decline in transmission, cases have become more concentrated to a specific populations and communities (“hot pops”) or regions (“hot spots”) [18]. Thus, effective epidemiological surveillance by sensitive diagnostics is essential to sustain and enhance malaria control to elimination.

A particular situation may arrive with potentially very powerful interventions, such as vaccines, being developed against the most important malaria parasite species *Plasmodium falciparum*. In case of successful implementation of such a highly effective intervention the burden of disease due to other, non-falciparum species including zoonotic malaria species may become increasingly important. Interestingly, improved diagnostic techniques have already revealed a greater prevalence of non-falciparum species in areas than previously thought [40]. While little is known about the potential interactions between *Plasmodium* species in humans, recent studies showed non-falciparum infections increased with decreasing *P. falciparum* prevalence, showing the importance of molecular surveys for all *Plasmodium* species [55]. It is clear that transfer of zoonotic malaria parasites to human cannot be prevented entirely, however surveillance using recent advancement in technologies should be applied to high-risk populations or communities living and working near the “hot spots”, where contact with animal reservoirs are high. Diagnostic platforms such as next-generation sequencing (NGS) hold enormous potential for such deeper surveillance of high risk-groups and to better understand the dynamics of transmission of cryptic parasite species to human from animal reservoirs and *vice versa*.

As most countries with a high burden of malaria are low- and middle-income countries, the interest and support for the development of new diagnostics are those technology or platform that can be adopted in low-resource settings and that can impact the health care systems. For a new diagnostic that could be “a game changer”, it is of paramount important that the test is inexpensive, rapid, quantitative, and highly sensitive. Platforms such as recombinase polymerase amplification (RPA) and lateral flow testing, developed in this study, showed the potential for a simple molecular point-of-care test for malaria diagnosis in the future.

9 Personal Contributions

My contributions to the published work compiled for a doctoral thesis are as follows:

9.1 Published paper 1

Species and genotype diversity of *Plasmodium* in malaria patients from Gabon analysed by next generation sequencing**.**

- Contributed to the study design
- Designed PCR primers and established NGS method
- Performed library preparation, sequencing and analysis of data
- Drafted, revised and improved the manuscript for publication

9.2 Published paper 2

Natural infection of *Plasmodium brasilianum* in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon

- Conceived and designed the study
- Designed PCR primers
- Performed PCR, sequencing, data analysis, and interpretation
- Drafted, revised, and improved the manuscript for publication

9.3 Published paper 3

Recombinase Polymerase Amplification and Lateral Flow Assay for Ultrasensitive Detection of Low-Density *Plasmodium falciparum* Infection from Controlled Human Malaria Infection Studies and Naturally Acquired Infections

- Conceived and developed the assay
- Designed primers and probe for the assay
- Performed laboratory optimization and field tests
- Data collection and analysis
- Prepared manuscript

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12 Appendix

a) Published paper 1
b) Published paper 2
c) Published paper 3

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Malaria Journal

RESEARCH

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Species and genotype diversity of *Plasmodium* in malaria patients from Gabon analysed by next generation sequencing

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Abstract

Background: Six *Plasmodium* species are known to naturally infect humans. Mixed species infections occur regularly but morphological discrimination by microscopy is difficult and multiplicity of infection (MOI) can only be evaluated by molecular methods. This study investigated the complexity of *Plasmodium* infections in patients treated for microscopically detected non-falciparum or mixed species malaria in Gabon.

Methods: Ultra-deep sequencing of nucleus (18S rRNA), mitochondrion, and apicoplast encoded genes was used to evaluate *Plasmodium* species diversity and MOI in 46 symptomatic Gabonese patients with microscopically diagnosed non-falciparum or mixed species malaria.

Results: Deep sequencing revealed a large complexity of coinfections in patients with uncomplicated malaria, both on species and genotype levels. Mixed infections involved up to four parasite species (*Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale curtisi,* and *P. ovale wallikeri*). Multiple genotypes from each species were determined from the asexual 18S rRNA gene. 17 of 46 samples (37%) harboured multiple genotypes of at least one *Plasmodium* species. The number of genotypes per sample (MOI) was highest in *P. malariae* (n = 4), followed by *P. ovale curtisi* (n = 3), *P. ovale wallikeri* (n = 3), and *P. falciparum* (n = 2). The highest combined genotype complexity in samples that contained mixed-species infections was seven.

Conclusions: Ultra-deep sequencing showed an unexpected breadth of *Plasmodium* species and within species diversity in clinical samples. MOI of *P. ovale curtisi*, *P. ovale wallikeri* and *P. malariae* infections were higher than anticipated and contribute significantly to the burden of malaria in Gabon.

Keywords: *Plasmodium malariae*, *Plasmodium falciparum*, *Plasmodium ovale wallikeri*, *Plasmodium ovale curtisi*, Next-generation sequencing, Amplicon, Metagenomics

Background

Malaria in humans is caused by six *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi, P. ovale wallikeri* and *Plasmodium knowlesi* which, although zoonotic, is an important pathogen in humans in several regions of South East Asia [1, 2]. Whereas current research is focused on malaria caused by *P. falciparum* and increasingly also *P. vivax,* only relatively little effort has been dedicated to research on the other human malaria species. Infections with these species usually present with low parasitaemia; they can persist for long periods and sometimes remain asymptomatic. Besides mono-infections with one *Plasmodium* species, mixed infections within one individual occur [3, 4] and interaction between concurrent species—although not well

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characterized—may play a role in disease progression and outcome [5, 6]. In clinical care, species determination is commonly performed by light microscopy, which has limited sensitivity and specificity. Double, triple or even quadruple infections can be detected more reliably by molecular methods. In addition, presence of distinct genotypes (strains) of the same species cannot be discerned microscopically. An important proportion of naturally occurring infections consists of multiple genotypes and "multiplicity of infection" (MOI) refers to the number of different genotypes of one species infecting a single host [7]. For *P. falciparum*, MOI has been associated with several conditions, including age of the host, clinical severity, and transmission intensity [8]. Determination of within-host diversity may be a useful marker to assess the impact of interventions [9].

Genotyping of polymorphic genes such as merozoite surface proteins (MSPs) and glutamate-rich protein (*glurp*), by nested-PCR is a gold standard method for assessing MOI in *P. falciparum* infections. An alternative approach uses next generation sequencing (NGS) technologies, which allow high-resolution analyses of a heterogeneous mixture of the parasites within the host [10]. Compared to the standard method, NGS revealed an up to six times higher MOI of *P. falciparum* in a previous study [11]. By generating multiple reads per sample (usually between 100 and 10,000), this technique is very sensitive and able to detect minor alleles. Recent analysis of the conserved cytochrome b gene (*cytb*) using NGS, detected multiple genotypes infections (up to 4) including non-falciparum species in 10% of 437 samples collected in Cameroon [12].

Here, a metagenomics sequencing based approach was applied using three pan-*Plasmodium* primer sets for nucleus (18S rRNA), mitochondrion (*cytb*), and apicoplast (*clpC*) encoded genes to characterize the different *Plasmodium* species that infect patients with microscopically diagnosed non-falciparum malaria in the department of Tsamba-Magotsi, Gabon, a remote area of rural Central Africa [13]. By this approach, parasite diversity in 46 Gabonese symptomatic patients is described, that are sampled over a time period of 3 years and provide a baseline for research on the epidemiology and potential influence of non-falciparum malaria on disease burden in Central Africa.

Methods

Patients

Parasite DNA for this study was extracted from dried blood spots on filter paper of 46 patients with uncomplicated, microscopically diagnosed non-*falciparum* or mixed species malaria, which represents a minority of malaria cases in the region but was found in more than 20% in a cross-sectional survey in the area [13]. Blood samples from two clinical studies were used. From the first study (recruited 2008–2010) samples of 30 patients were included, details of patients' characteristics are published elsewhere [14]. Briefly, patients with uncomplicated malaria, defined as symptoms and presence of *P. ovale* or *P. malariae* in thick blood smear, either as mono or mixed infection were included after informed consent was given. All patients received artemether-lumefantrine as anti-malarial chemotherapy and recovered from the infection. From the second study (recruited 2012–2013) 16 samples of patients that were included for molecular assessment of *Plasmodium* spp. were used. Both studies were approved by the regional ethics committee (Comité d'Ethique Régional Indépendant de Lambaréné) and followed the principles of the Declaration of Helsinki in its 5th revision.

Amplification and 454 sequencing

Capillary blood collected on filter paper from patients prior to anti-malarial treatment was processed for DNA extraction using QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's specifications. For malaria species and genotype identification using NGS, we designed three *Plasmodium* genus-specific primer sets from the conserved region flanking the highly polymorphic nucleotide sequence of the 18S rRNA, the mitochondrial cytochrome b (*cytb)* and the apicoplast caseinolytic protease C gene (*clpC*). All primers were 5′-fused to universal tail sequences. Using 2.5 µl of DNA extract, target specific PCR was carried out by using Phusion High-Fidelity PCR master mix (Finnzymes) for 35 cycles. The 454 MID kit (Multiplicom) was used to perform subsequent PCR addition of a 454-adaptor sequence linked to multiplex identifiers (MID) in order to discriminate the patients in following analysis steps. Here, the 100 times diluted first round PCR product was used as template and amplified for 20 cycles. All reactions were carried out using a Biometra T2 professional Thermocycler. The corresponding primer sequences and annealing temperature are given in Table 1. PCR amplicons were purified using AmPure XP kit (Agencourt) according to standard procedures (Roche Technical Bulletin No. 2011-007). Quality and purity of amplicons were checked using the Agilent DNA 1000 assay kit on a 2100 Bioanalyzer (Agilent Technology) and subsequently quantified using the Quant-iT Picogreen dsDNA reagent (Invitrogen) on a Fluoroskan Ascent microplate Fluorometer (ThermoScientific). Based on individual DNA concentration, each amplicon was diluted to 10^7 molecules/µl stock solutions. Amplicons were pooled in equimolar concentration to generate a single library and further processed following the GS Junior emPCR LibA

Table 1 Primers used in this study

Primer name	5′–3′ sequence[a]	Annealing (°C)
SSU-Fwd	AAGACTCGGCAGCATCTCCAGTGAAATTCTTAGATTTTCTG	58
SSU-Rev	GCGATCGTCACTGTTCTCCACGTGTTGAGTCAAATTAAGC	
Cyt-Fwd	AAGACTCGGCAGCATCTCCAGAGTGGATGGTGTTTTAGAT	58
Cyt-Rev	GCGATCGTCACTGTTCTCCAGTGCTACCATGTAAATGTAA	
Clpc-Fwd	AAGACTCGGCAGCATCTCCAGGTCAATTAACAGAACAA	55
Clpc-Rev	GCGATCGTCACTGTTCTCCATAGTTAATCTATTTAATAATTC	
rPLU6[b]	TTAAAATTGTTGCAGTTAAAACG	
rPLU5[b]	CCTGTTGTTGCCTTAAACTTC	
p-AVL-F	GGAATGACAATGTCGTAAAACAAAGTAT	
P-AVL-R	ATACTTTGTTTTACGACATTGTCATTCC	

[a] Underlined region represents universal tag sequences

[b] Snounou et al. [25]

method (Version April 2011) for emulsion PCR (emPCR) using a low copy per bead ratio (0.25 cpb). 500,000 DNA enriched beads were loaded onto a GS Junior Picotiter plate following the GS Junior sequencing manual (Version April 2011) and sequencing was performed in both, forward and reverse direction using the GS Junior Titanium sequencing kit.

Sequence analysis

Figure 1 summarizes the bioinformatics pipeline used to process *Plasmodium* sequence reads generated by GS Junior. The *sfffile* program (SFF Tools, Roche) was used to split raw sequence data based on multiplex identifier (MID). Low quality and short reads (< 200 bp) were excluded prior to analysis. Sequencing errors (PCR noise) and homopolymer stretches were corrected with Acacia [15]. Chimeric sequences were detected by using the software Uchime [16] and excluded from further analysis. Both programs were run with default parameters. High-quality filtered reads were mapped to a local reference database comprising 18S rRNA, *cytb* and *clpC* gene sequences of *Plasmodium* spp. downloaded from GenBank (Table 2). Unmapped sequences were further analyzed using BLAST searches against the NCBI nucleotide database and the *Plasmodium* database (Plasmodb) [17]. Single nucleotide polymorphisms (SNPs) in the analyzed genes were determined using the probabilistic variant detection method-implemented in the CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark). Haplotypes were determined with DnaSP [18] using the following thresholds: SNP frequency equal or greater than 10% as well as per gene read coverage $\geq$ 10 fold. *Plasmodium* species genotypes were identified by querying consensus sequences against the NCBI GenBank database. All SNP positions were reported as absolute positions in the best matching NCBI reference sequence. Polyclonal infections were identified based on SNPs in the variable region (V5) of the 18S gene, giving the number of genotypes infecting one host (referred to as MOI). The number of genotypes obtained by this marker typically leads to an underestimation of polyclonality and characterization of genotypes based on more polymorphic genes might better estimate the scale of multiple infections. However, this would make comparisons between species more difficult as different genes would be evaluated.

Results

Sequence statistics from patients

The 46 patient samples were analyzed in batches of four sequencing runs on a Roche 454 GS Junior sequencer. The median number of raw reads assigned to each sample was 3829 (range 1563–11,590). However, after removal of chimeric and low-quality reads, the final informative sequences available for each sample was 3165 (range 655–9091). The sequence coverage for each locus per sample is reported in the Additional file 1. Differences in coverage between loci and overall batch to batch variation in the number of raw reads were observed. However, there is no evidence that this substantially influences the results, as the overall coverage per sample is expected to be high enough to detect minor genotypes.

Species discrimination by deep sequencing

Each filtered read was mapped to the target genes of the reference species for identification. Mixed-species malaria infections identified by the metagenomics approach were also detected by conventional nested-PCR in combination with gel electrophoresis [14]. By NGS, it was possible to achieve a more detailed resolution of the species diversity that included identification of nine novel genotypes and polyclonal infections using the same gene in all six investigated *Plasmodium* spp.

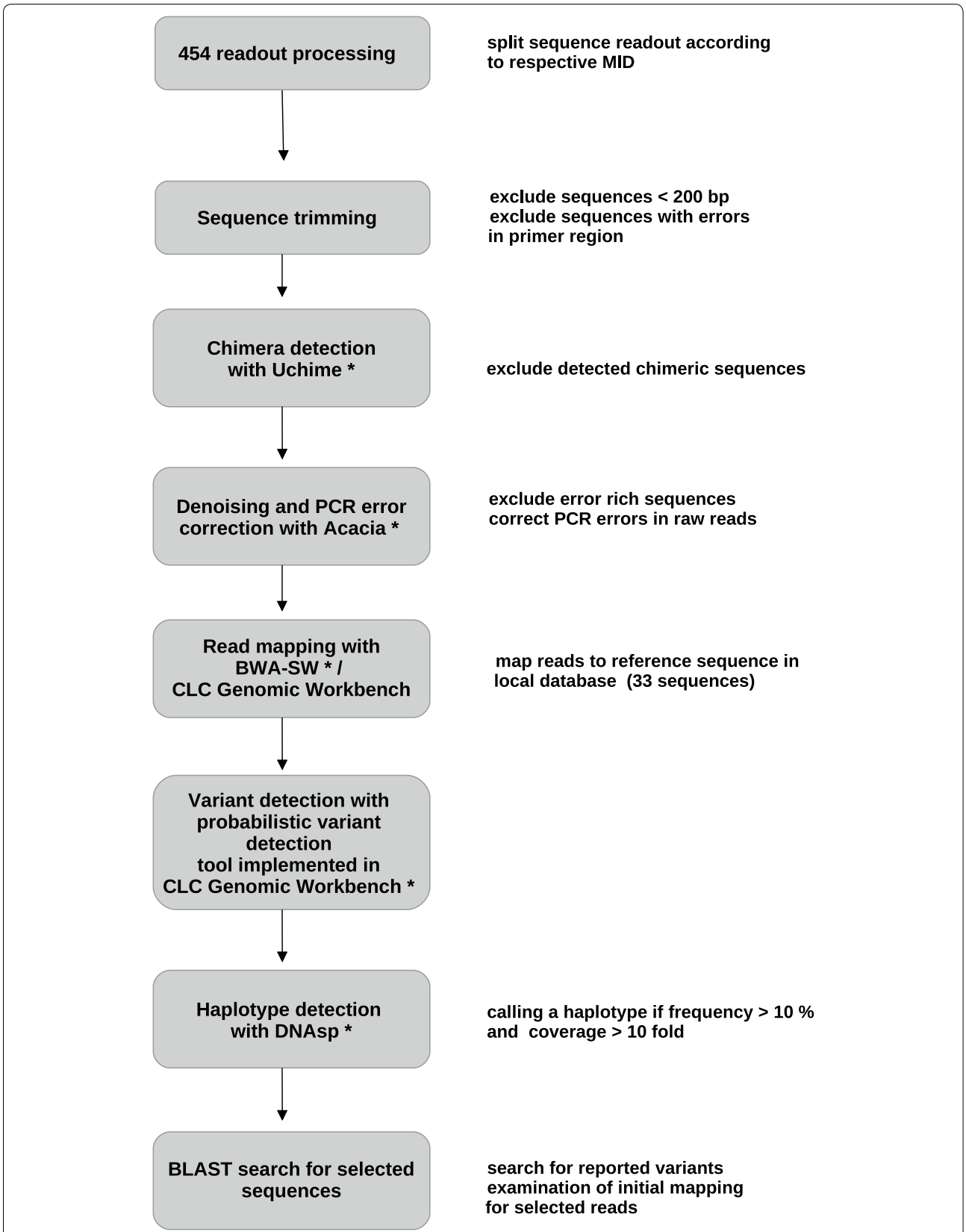

Fig. 1 Bioinformatics pipeline. Flowchart describing bioinformatic pipeline. The pipeline is divided into a quality control part, mapping and variant calling part. * used with default settings

A minimum of three high quality reads had to be assigned to at least one of the target loci to be included in the analysis. There were 44 (96%) patients with *P. falciparum,* 15 (33%) with *P. malariae*, 7 (15%) with *P. ovale curtisi*, and 8 (17%) with *P. ovale wallikeri* infections. Different parasite combinations in mixed infections and up to four species in the same host were detected. Of 46 samples, 24 contained only *P. falciparum*, another two contained only *P. malariae* or *P. ovale wallikeri*. The remaining samples contained double (n = 15), triple (n = 2), and quadruple (n = 3) *Plasmodium* species infections (Table 3).

Table 2 *Plasmodium* spp. reference sequences used for 454-reads mapping

Plasmodium species	GenBank Acc.
Plasmodium falciparum 18S ribosomal RNA gene (S-type)	HQ283222.1
Plasmodium falciparum 18S ribosomal RNA gene (A-type)	JQ627152.1
Plasmodium vivax 18S ribosomal RNA gene (A-Type)	JQ627158.1
Plasmodium vivax 18S ribosomal RNA gene (O-Type)	U93235.1
Plasmodium vivax 18S ribosomal RNA gene (S-Type)	U93234.1
Plasmodium berghei 18S ribosomal RNA gene	M19712.1
Plasmodium berghei 18S ribosomal RNA gene	M14599.1
Plasmodium malariae 18S ribosomal RNA gene	M54897.1
Plasmodium ovale 18S ribosomal RNA gene	AB182493.1
Plasmodium ovale 18S ribosomal RNA gene	AB182489.1
Plasmodium yoelii 18S ribosomal RNA gene	AF180727
Plasmodium knowlesi 18S ribosomal RNA gene	JF714686.1
Plasmodium cf. inui 18S ribosomal RNA gene	FJ619091.1
Plasmodium simiovale 18S ribosomal RNA gene	AB287287.1
Plasmodium ovale wallikeri cytochrome b gene	KJ930413.1
Plasmodium ovale curtisi cytochrome b gene	KP050432.1
Plasmodium malariae cytochrome b gene	LT594637
Plasmodium vivax cytochrome b gene	JN788776
Plasmodium falciparum cytochrome b gene	KC175316.1
Plasmodium berghei cytochrome b gene	DQ414645.1
Plasmodium knowlesi cytochrome b gene	JQ345523.1
Plasmodium spp. from Gabon cytochrome b gene	AF069623.1
Plasmodium yoelii cytochrome b gene	DQ414658.1
Plasmodium falciparum HB3 clpC gene for caseinolytic protease C	DQ642846.1
Plasmodium ovale curtisi clpC gene for caseinolytic protease C	KP050446.1
Plasmodium ovale wallikeri clpC gene for caseinolytic protease C	KP050439
Plasmodium malariae clpC gene for caseinolytic protease C	AB649418.1
Plasmodium vivax clpC gene for caseinolytic protease C	AB471871.1
Plasmodium gaboni clpC gene for caseinolytic protease C	HQ842630.1
Plasmodium knowlesi clpCgene for caseinolytic protease C	AB471880.1
Plasmodium simiovale clpCgene for caseinolytic protease C	AB471881.1
Plasmodium cf. ovale clpC gene for caseinolytic protease C	HQ842632.1
Plasmodium yoelii clpC gene for caseinolytic protease C	DQ417625.1
Plasmodium berghei clpC gene for caseinolytic protease C	AB649421.1

Plasmodium falciparum infections

Plasmodium falciparum specific sequence reads were identified in all but 2 (96%) of the study samples. Based on the 18S A-type gene sequence, four novel genotypes (GenBank KJ170098—KJ170101) were identified, all are a single nucleotide polymorphism when compared to the reference sequence (GenBank JF681166) as shown in Table 4. No other sequences with these SNPs were found when a similarity search against GenBank was performed. Co-infections with other species were found in 20 patients (45%).

Plasmodium malariae infections

A total of 15 infections were observed with the quartan malaria parasite, one as mono and the remaining ones as co-infection with other species. Based on the 18S rRNA gene, the parasites were further characterized as *P. malariae*-Asian type 1 (n = 1) and *P. malariae*-Asian type 2 (n = 14) [19]. Two unique genotypes (GenBank KJ170105, KJ170106) similar to *Plasmodium cf. malariae* type 2 were obtained (GenBank AF488000, 99% identity). Each genotype is defined by either a single nucleotide substitution or insertion compared to *P. malariae*-Asian type 2 (Table 4) (see comments below).

Plasmodium ovale wallikeri and *P. ovale curtisi* infections

Plasmodium ovale wallikeri and *P. ovale curtisi* were identified in the dataset supported by reads matching to specific 18S, *cytb,* or *clpC* sequences of the two *P. ovale* species (Additional file 1). In addition, mixed infections of *P. ovale curtisi* and *P. ovale wallikeri* were observed in four patients (Table 3).

Table 3 Number of *Plasmodium* spp. infections detected by deep sequencing

Species	Infection type	Patients (n)
Pfal	Mono infection	24
Pmal	Mono infection	1
Pow	Mono infection	1
Pfal, *Pmal*	Double infection	10
Pfal, *Poc*	Double infection	3
Pfal, *Pow*	Double infection	2
Pfal, *Pmal*, *Pow*	Triple infection	1
Pfal, *Poc*, *Pow*	Triple infection	1
Pfal, *Pmal*, *Poc*, *Pow*	Quadruple infection	3

Pfal, *P. falciparum*; Pmal, *P. malariae*; Poc, *P. ovale curtisi*; Pow, *P. ovale wallikeri*

Table 4 Polymorphic Nucleotide positions compared to GenBank best-hits reference sequences

Genotypes	Origin	386	407	504	517	529		
P. falciparum (JF681166)	China	G	T	G	A	C		
P. falciparum (KJ170098)	Gabon	A	C	.	–	.		
P. falciparum (KJ170099)	Gabon	.	.	.	.	G		
P. falciparum (KJ170100)	Gabon	A	.	.	.	.		
P. falciparum (KJ170101)	Gabon	.	.	A	.	.		
		1036	**1189**					
P. malariae (AF488000)	Myanmar	G	–					
P. malariae (KJ170105)	Gabon	.	T					
P. malariae (KJ170106)	Gabon	A	T					
		1005	**1026**	**1109**	**1112**	**1128**	**1131**	**1132**
P. ovale wallikeri (AB182493)	Indonesia	A	C	G	G	A	G	A
P. ovale wallikeri (KJ170102)	Gabon	.	.	.	.	G	.	.
P. ovale wallikeri (KJ170103)	Gabon	G	T	A	A		A	G
P. ovale wallikeri (KJ170104)	Gabon	G	T	.	.	.	.	.

Each dot represents nucleotide similarity with the GenBank reference sequences. Dashes represent deletions. Numbers at the column header represent nucleotide positions corresponding to GenBank reference sequences

All of *P. ovale curtisi* genotypes identified from the samples were similar to previously submitted sequences. Three novel genotypes of *P. ovale wallikeri* were identified based on 18S rRNA gene sequence polymorphisms (GenBank KJ170102—KJ170104). One of them possesses a single nucleotide substitution at position 1128. Genotype 2 and Genotype 3 showed six and two nucleotide substitutions when compared to the corresponding reference sequence (GenBank AB182493), respectively (Table 4).

Detection of *P. ovale curtisi* 18S rRNA gene variant

In three of the samples a fraction of reads (range 10–15 reads) could not be mapped to any reference sequence. A sequence identity search against the NCBI database initially did not show significant similarity with respect to query coverage and maximum identity with any of the *Plasmodium* spp. 18S sequence. A 1078 bp length sequence was constructed (GenBank KJ170108), spanning the variable regions 4 and 7 along with the 454 amplicon target (V5) by PCR and Sanger sequencing with pan-*Plasmodium* and specific primers, respectively (Fig. 2). The generated sequence showed 100% similarity to the draft genome of *P. ovale* (*P. ovale* Blast Server, Sanger Institute) and a recently discovered 18S rRNA gene variant (GenBank KF696378) of *P. ovale curtisi* [20].

Multiplicity of infection

An overview of the MOI based on the variable region (V5) of the 18S gene for the different *Plasmodium* species is given in Table 5. The relative frequency of each genotype per sample based on the number of reads is plotted in Fig. 3. Multiple *P. falciparum* genotype (MOI) infections were detected in five patients with a maximum of two genotypes per sample. All *P. falciparum* mono-infections carried a single genotype, except one patient (MID15) who carried two genotypes. Multiple genotype infections were found in two of the seven *P. ovale curtisi* infected samples (MOI of 3). Among eight samples infected with *P. ovale wallikeri*, six harboured multiple *P. ovale wallikeri* genotypes (MOI of 2–3). Analysis of the *cytb* and *clpC* reads showed 100% similarity to the published reference sequences. Nine samples contained multiple *P. malariae* genotypes (MOI of 2–4). The number of different genotypes per sample was greater for *P. malariae* when compared to the other species, showing up to four genotypes within one sample.

Reads mapping to *cytb* and *clpC* showed 100% sequence identity to the reference sequences for all species (KC175316 and DQ642846 for *P. falciparum*; KJ930413 and KP050439 for *P. ovale wallikeri*; KP050432, KP050446 for *P. ovale curtisi*; HQ842634 and AB649418 for *P. malariae*).

Discussion

Gabon is highly endemic for malaria with perennial transmission of *P. falciparum* and co-endemicity of *P. malariae* and *P. ovale* [21, 22]. Gabon has a low population density (6.7 persons per square km in 2015) [23], but population mobility is high, and a large fraction of inhabitants live close to or within non-cultivated areas, that contain diverse and intact wildlife [24]. Originally, the study was designed to assess the efficacy of artemisinin combination therapy (artemether-lumefantrine) in uncomplicated non-*falciparum* and mixed species

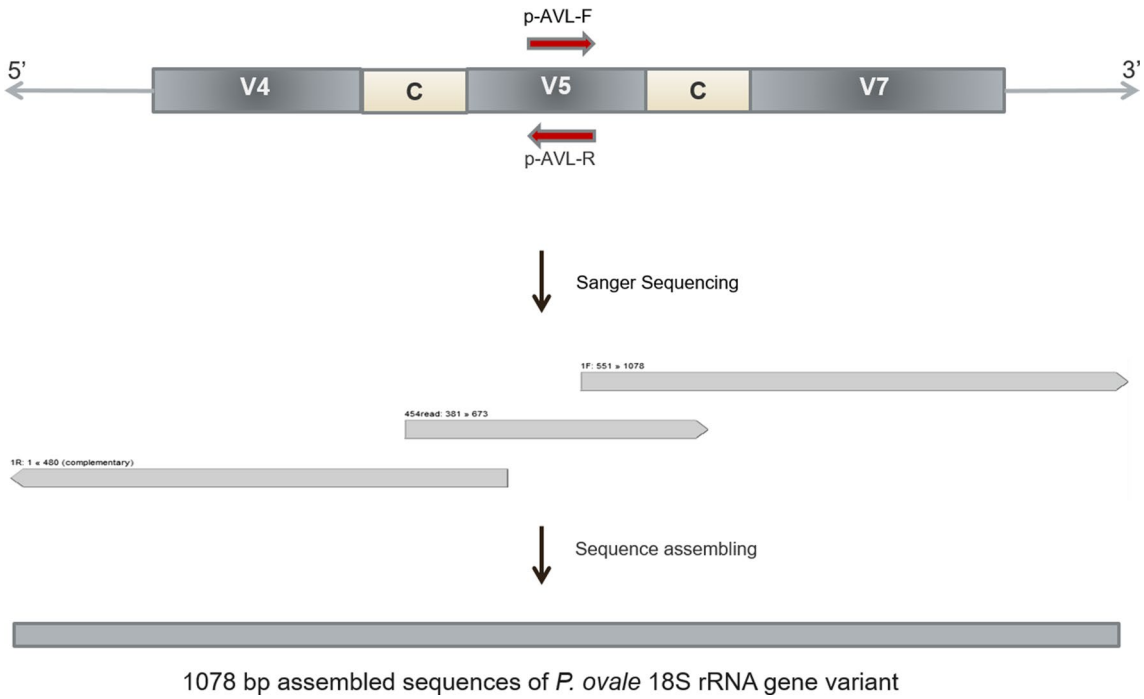

Fig. 2 Schematic representation of the sequencing approach by Sanger method to determine the new 18S rRNA gene type sequence of *P. ovale* spp. Conserved pan-*Plasmodium* 18S primers (rPLU6 and rPLU5) were first used to amplify the gene spanning three variable regions and two conserved blocks. The nucleotide sequences upstream and downstream of the 454 sequencing target region (V5) of the gene were determined by direct sequencing of the initial product using p-AVL-F and p-AVL-R primers. Partial 18S gene (1078 bp length) was obtained by assembling 454 reads with the sequences obtained using p-AVL-F and p-AVL-R primers

Table 5 Multiplicity of *Plasmodium* spp. infections

Sample id	Infecting species	Number of genotypes for each species				
		Pfal	***Pmal***	***Poc***	***Pow***	**Total (MOI)**
MID01	*Pmal*		4			4
MID11	*Pfal, Pmal*	2	2			4
MID15	*Pfal*	2				2
MID16	*Pfal, Pmal, Poc, Pow*	1	4	na	2	7
MID23	*Pfal, Poc, Pow*	2			2	4
MID24	*Pfal, Poc*	1		3		4
MID25	*Pfal, Pmal*	1	3			4
MID27	*Pfal, Pmal*	2	2			4
MID28	*Pfal, Pmal, Poc, Pow*	2	2	1	1	6
MID30	*Pfal, Pmal, Pow*	1	na		2	3
MID31	*Pfal, Poc*	1		3		4
MID32	*Pfal, Pow*	1			3	4
MID34	*Pfal, Pmal*	1	2			3
MID38	*Pfal, Pow*	1			3	4
MID41	*Pfal, Pmal*	1	2			3
MID43	*Pfal, Pmal*	1	2			3
MID46	*Pow*				2	2

Pfal, *P. falciparum*; Pmal, *P. malariae*; Poc, *P. ovale curtisi*; Pow, *P. ovale wallikeri*; na, no 18S sequence obtained

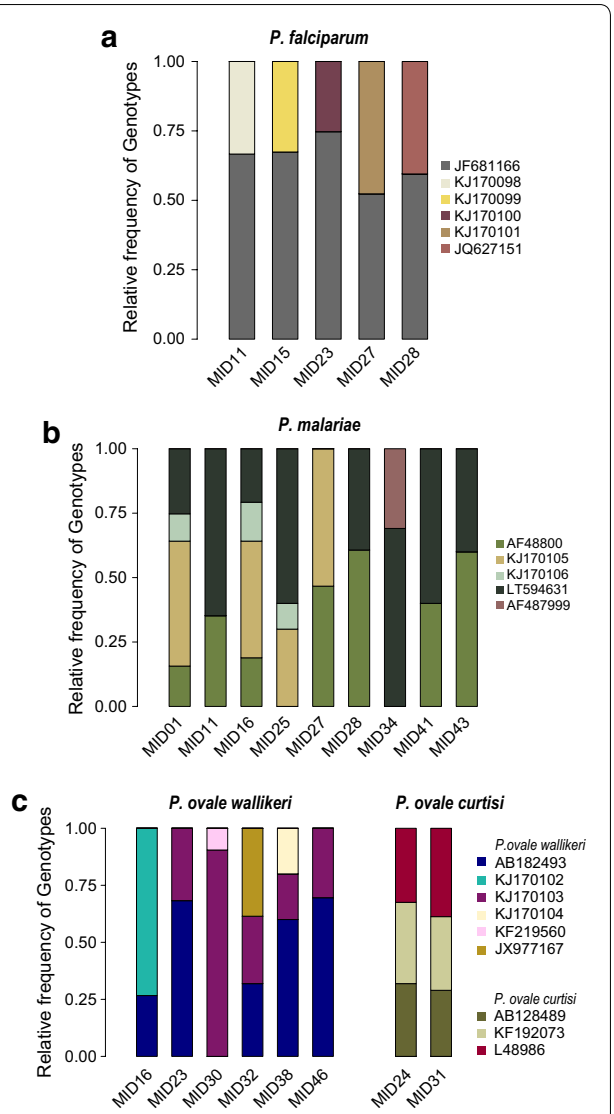

Fig. 3 Relative frequency of each *Plasmodium* spp. genotype within one infected host. **a** Relative frequency of *P. falciparum* genotypes (defined by the 18S A-type gene variation); **b** relative frequency of *P. malariae* genotypes. **c** Relative frequency of *P. ovale curtisi* and *P. ovale wallikeri*

malaria [14]. Species identification was based on microscopy and verified by species-specific nested PCR [25]. Here, a metagenomics approach was designed based on high throughput sequencing to re-analyze the diversity of *Plasmodium* spp. Two studies were conducted recently using NGS to explore the evidence of zoonotic transmission of *Plasmodium* spp. in humans [12, 26]. These studies were designed to differentiate *Laverania* species of apes and humans based on the diagnostic single nucleotide polymorphism found in the mitochondrial DNA (mtDNA). The current study expanded the target genes to three genes including the 18S rRNA and *clpC* gene to improve accuracy and assess the diversity of *Plasmodium* populations in blood samples of malaria patients. The 18S rRNA gene is one of the most commonly used targets in the molecular diagnosis of *Plasmodium* spp. including a widely used nested PCR and a number of real-time PCR assays [25, 27]. Due to the high copy numbers (5–10 copies), and the presence of both highly conserved and variable regions, 18S genes are well-suited and frequently used molecular targets for the detection and discrimination of *Plasmodium* species. Until to date, no study has applied these genes to identify *Plasmodium* spp. using NGS.

As expected, results obtained by conventional species-specific PCR were reproduced upon DNA re-extraction and analysis by deep sequencing. Beyond this, this analysis gives a snapshot in time; showing the breadth of co-infections and an unbiased estimate of infection intensity of the different *Plasmodium* spp. in a cohort of patients with uncomplicated malaria. *P. malariae* is widespread in Africa but rarely characterized by molecular techniques. Based on morphological differences and sequence polymorphisms compared to genuine *P. malariae* (Uganda CDC isolate), two possible subtypes were identified in Asia: *P. cf. malariae* type 1 and *P. cf. malariae* type 2 [19]. It would be interesting to investigate if the Asian and the Ugandan type differ in their sensitivity to artemisinins, since a reduced sensitivity of an infection in Uganda has been observed [28, 29] but all patients in this study responded well to the treatment [14]. Only few studies have looked at MOI of non-falciparum species. It has been shown in one study that *P. malariae* infections in Malawi, often consisted of multiple genotypes per infected individual and showed a surprisingly similar pattern when compared to *P. falciparum* [30]. Up to five genotypes were detected from a single sample by multilocus genotyping based on microsatellite markers in asymptomatic carriers [31]. There are also up to four different genotypes of *P. malariae* in two patients and three different genotypes of *P. ovale wallikeri* and *P. ovale curtisi* each in two patients in the here presented study based on the 18S region, respectively. Many of the *P. falciparum* infections were on the contrary caused only by one genotype when judged by polymorphisms in the 18S rRNA gene. These results are surprising as one would assume a lower MOI in *P. malariae* and both *P. ovale* species as the reported prevalence in this region is low. However, results are also in line with earlier findings from Cambodia for *P. ovale* [32], reflecting the possibility that these parasite species are more prevalent than previously thought. Evidence suggests that *P. malariae* is not completely species-specific and also prevalent in non-human primates [29, 33], having a larger pool of hosts. In addition, infections with these species might be more chronic and long-lived so that multiple genotypes could accumulate in one host. It has to be considered that the marker used

to define a genotype in this study is not as polymorphic as markers commonly used to define genotypes; for example microsatellites, or genes like *msp1*, *msp2* and *glurp* for *P. falciparum* [34]. It is expected that the number of genotypes are larger if more polymorphic markers had been used. The advantage of our approach is that homologous genes between the different species can be compared and one can get an impression on the population structure of the co-infecting species. Particularly, because there is no evidence that selection leads to different mutation rates in this gene region for the different species, this can be one approach to compare diversity between species. These results highlight the potential of amplicon-based high throughput sequencing combined with adequate polymorphic markers to obtain reliable molecular characterization of other non-falciparum species, where only limited data are available. Despite short-read length (< 400 nt) generated by the NGSs technique, significant dimorphism of the targeted genes allowed accurate assignment of reads between *P. ovale curtisi* and *P. ovale wallikeri.* By using a multiple locus approach, co-infections of the two *P. ovale* species in four patients were detected, adding yet further evidence that the two species do not recombine in nature. The primer for the *cytb* gene was selected from a region not including the ape-specific SNPs leading to potential inability to detect non-human species. However, there was no evidence for non-human *Plasmodium* species infections based on the analysis of the other two genes.

Two structurally distinct types of 18S rRNA have been reported in many *Plasmodium* species [35]. In *P. falciparum,* type A and type S has been described with up to 11% difference in the sequences between the two types [36]. The existence of paralogous 18S rRNA genes in *P. malariae*, *P. ovale wallikeri* and *P. ovale curtisi* genome is not well described until now. The new type of 18S rRNA gene sequence from this study, together with the recent report [20], clearly suggests that at least two different forms exist in *P. ovale curtisi* and *P. ovale wallikeri* [20]. A real-time PCR based assay targeting this new sequence showed high sensitivity and specificity and can be used for the differential diagnosis of *P. ovale* species infections (unpublished observation).

The main limitation of techniques that involve PCR is the inherent risk of contamination and the NGS approach is no exception. Thus, an essential aspect of sample preparation for sequencing is the careful adoption of experimental strategies aimed at minimizing cross-contamination. In addition, amplicon-based NGS are prone to errors such as chimeras, a well-known issue in metagenomics analyses of environment bacterial communities. Several measures to minimize and avoid these underlying problems at each step, including a non-template control to check contamination of reagents during each target amplification, a separate work station for DNA extraction, PCR assay set-up, and post-PCR processing are necessary.

The true burden of disease caused by *P. malariae* and the *P. ovale* species is not known as these species are mostly underdiagnosed despite their worldwide distribution [29]. Malaria caused by these parasites is generally more benign when compared to *P. falciparum.* Infections present often with a low level parasitaemia that is difficult to detect by microscopy, but might cause a more chronic illness associated with anaemia [37]. Recent reports reveal a higher than expected prevalence of these species in many African countries when diagnosed by PCR [38], going in line with the deep sequencing result. Currently, deep sequencing methods may not be applicable in field settings but can be helpful in longitudinal epidemiological studies to investigate the emergence and change in composition of plasmodial species. The study population was highly selected and represents only a minority of all malaria cases in the area. Microscopic species differentiation is not reliable, particularly when parasite density is low. Therefore, it is unlikely that the appearance of new plasmodial species is noted without molecular techniques. Diversity of the two *P. ovale* species, and characterization of the *P. malariae* population should be taken into consideration for the design, endpoints and feasibility of malaria control strategies, e.g. longer follow up or separate treatment of hypnozoites. Fortunately, artemisinin combination therapy was efficacious in all patients of the present study but emergence of novel zoonotic species or isolates that may be inherently less responsive to current treatment regimens shall be detected early, to provide a specific treatment regimen, especially when severe malaria can occur, as in the case of *P. knowlesi* infections. The impact on malaria vaccine development shall even be stronger since cross-protection between *Plasmodium* species is limited [39] and mechanisms of high-grade protection are often not known but likely to be different for each parasite species.

Conclusions

Parasite diversity of *Plasmodium* species in naturally acquired malaria is larger than expected. The population structure, especially of non-falciparum species, needs further assessments to better understand the prevalence and biology of these parasites. Metagenomics analysis by deep sequencing provides a tool for this and boosts the understanding of naturally acquired malaria. It will be exciting to expand such investigations to other malaria-endemic regions and larger cohorts.

Additional file

Additional file 1: Table S1. Number of high-quality 454 reads mapped to *Plasmodium* spp. references.

Abbreviations

clpC: caseinolytic protease C; cytb: cytochrome b; MOI: multiplicity of infection; mtDNA: mitochondrial DNA; NGS: next-generation sequencing; PCR: polymerase chain reaction; SNPs: single nucleotide polymorphisms.

Authors' contributions

BM, SJ conceived the idea of the project. SJ, AL, TE, MR, SB, PGK, and BM designed research. AL, SJ performed NGS experiments. AL, SJ, TE, JH and BM analysed data and drafted the manuscript. FJ, AL and GMN contributed to data collection. All authors interpreted the data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. The new 18S genotypes reported in this paper are available in the GenBank under the Accession Number(s): KJ170100.1 to KJ170108.1

Consent for publication

Not applicable.

Ethics approval and consent to participate

Both studies were approved by the responsible regional ethics committee, Comité d'Ethique Régional Indépendant de Lambaréné, Gabon. All study participants consented to participate in the study.

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Research Paper

Natural infection of *Plasmodium brasilianum* in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon

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ABSTRACT

Background: The quartan malaria parasite *Plasmodium malariae* is the widest spread and best adapted human malaria parasite. The simian *Plasmodium brasilianum* causes quartan fever in New World monkeys and resembles *P. malariae* morphologically. Since the genetics of the two parasites are nearly identical, differing only in a range of mutations expected within a species, it has long been speculated that the two are the same. However, no naturally acquired infection with parasites termed as *P. brasilianum* has been found in humans until now.
Methods: We investigated malaria cases from remote Yanomami indigenous communities of the Venezuelan Amazon and analyzed the genes coding for the circumsporozoite protein (CSP) and the small subunit of ribosomes (18S) by species-specific PCR and capillary based-DNA sequencing.
Findings: Based on 18S rRNA gene sequencing, we identified 12 patients harboring malaria parasites which were 100% identical with *P. brasilianum* isolated from the monkey, *Alouatta seniculus*. Translated amino acid sequences of the CS protein gene showed identical immunodominant repeat units between quartan malaria parasites isolated from both humans and monkeys.
Interpretation: This study reports, for the first time, naturally acquired infections in humans with parasites termed as *P. brasilianum*. We conclude that quartan malaria parasites are easily exchanged between humans and monkeys in Latin America. We hypothesize a lack of host specificity in mammalian hosts and consider quartan malaria to be a true anthropozoonosis. Since the name *P. brasilianum* suggests a malaria species distinct from *P. malariae*, we propose that *P. brasilianum* should have a nomenclatorial revision in case further research confirms our findings. The expansive reservoir of mammalian hosts discriminates quartan malaria from other *Plasmodium* spp. and requires particular research efforts.

1. Introduction

Since malaria eradication is on the global health agenda again, non-human primates as source for *Plasmodium* infections in humans have received increased attention (Ramasamy, 2014). In this context, the simian *Plasmodium brasilianum* is particularly interesting. In 1908, a quartan malaria parasite was identified by Gonder and von Berenberg-Gossler in an imported 'bald-headed uakari' (*Cacajao calvus*) and named *P. brasilianum*, the quartan malaria parasite of New World monkeys in Latin America (Gonder and Von Berenberg-Gossler, 1908). *P. brasilianum* resembles the human quartan parasite *Plasmodium malariae* under the microscope, but early cross-species experimental infections by subcutaneous transfer of parasitized blood from black spider monkeys in the 1930s were unsuccessful. Hence, the names of two distinct parasites were maintained (Coatney et al., 2003).

Later investigations in the 1960s demonstrated that humans could very well be experimentally infected with *P. brasilianum* from monkeys, and, vice versa, New World monkeys could be experimentally infected with *P. malariae* from humans (Coatney et al., 2003; Geiman and Siddiqui, 1969). Moreover, studies in the 1980s showed that monoclonal antibodies against the circumsporozoite protein (CSP) of *P. malariae* cross-reacted and even neutralized the infectivity of

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P. brasilianum sporozoites to monkeys and vice versa (Cochrane et al., 1985). Sequencing of the gene coding for CSP confirmed the identity of this otherwise species-specific epitope in the two parasites (Lal et al., 1988).

Another common tool for the molecular species identification of malaria parasites is the gene for the small subunit (18S) of ribosomes (Snounou et al., 1993). In 1999, Fandeur et al. analyzed the 18S gene sequences from quartan malaria parasites (*P. brasilianum*) found in four monkey species from French Guiana with the highest prevalence in *Alouatta* monkeys (Fandeur et al., 2000). The similarity between 18S sequences from *P. brasilianum* and *P. malariae* is more than 99% differing only in single nucleotide polymorphisms (SNPs). SNPs are distributed at random like in the genetic pool of one single species, and no distinctive marker has been identified so far.

Unlike other human *Plasmodium* species, no whole genome sequence is available for quartan malaria parasites. We reviewed other published gene targets (msp-1, dhfr, cytochrome b, microsatellite DNA markers) and found striking homologies in all the markers without any specific identifying SNPs between the two parasite types (Fandeur et al., 2000; Guimarães et al., 2012; Tanomsing et al., 2007). The two parasites are nowadays perceived as variants of the same species, which had specialized on different hosts. Or, in practical terms, when quartan malaria parasites were identified in monkeys, they were designated as *P. brasilianum*. Conversely, when quartan malaria parasites were detected in humans, they were classified as *P. malariae*. Thus, the infected host determined the *Plasmodium* designation.

At the time of writing, altogether thirteen 18S sequences of *P. brasilianum* and thirty-four 18S sequences of *P. malariae* were registered in the NCBI GenBank nucleotide database. Sero-epidemiological studies in Brazil and French Guyana already suggested that non-human primates might constitute a natural reservoir for human malaria, and may contribute to the maintenance of foci for *P. malariae* (Volney et al., 2002). However, as naturally-acquired infections with parasites termed as *P. brasilianum* were never described in humans (Baird, 2009), the idea of host specificity was upheld and the classification of *P. brasilianum* as an independent *Plasmodium* species was retained.

Amazonas, the most southern federal state of Venezuela, is bordering Colombia to the west and Brazil to the east. Half of the population belongs to one of eighteen indigenous ethnic groups with the Yanomami representing one of the largest Amerindian communities. These seminomadic Indians live on both sides of the frontier between Venezuela and Brazil. On the Venezuelan side, about 12,000 Yanomami inhabit the vast forest area where the Orinoco originates and the Casiquiare river bifurcates towards the southern Amazon (Metzger et al., 2008; Humboldt, 1812).

In a traditional Yanomami village, all persons live under one common roof, *shabono*, consisting of a circular open wooden construction that accommodates up to 400 people. Daily life takes place “open air” and the night is spent in hammocks. Many *shabonos* are difficult to reach and are several days walking distance to the nearest health post. It is estimated that around 5000 Yanomami have retreated into the deep jungle with little or no contact to Western culture (Metzger et al., 2008). As forest-dwelling people, Yanomami hunt monkeys as a food source and incorporate them as household's pets.

Overall, *P. vivax* is predominant in Amazonas with roughly 85% of all detected parasites, but the distribution pattern of the malaria species is variable and contingent upon the geographic settlement of the ethnic groups. For example, a pilot study conducted in Yanomami communities from the Upper Orinoco revealed that nearly half of the malaria positive samples were *P. malariae* (Metzger et al., 2008). In contrast, no infections caused by *P. malariae* were detected among indigenous Piaroa from the Middle Orinoco basin (Rodulfo et al., 2007). Interestingly, Yanomami communities have also the highest *P. falciparum* rates (40.3%) compared to other ethnic groups in the region (8.7–22.4%) (Metzger et al., 2009).

The current study was carried out to identify and characterize *Plasmodium* species in the Venezuelan Amazon. Specifically, we investigated quartan malaria cases in Yanomami communities living in remote areas of the Alto Orinoco Casiquiare Biosphere Reserve where humans and non-humans live in such close vicinity that they could be concurrent reservoirs of transmission.

2. Materials and methods

2.1. Samples

Samples for this study originate from surveys in the Yanomami communities of Ocamo, Mavaca, Koyowe, and Platanal situated in the Upper Orinoco area near the Brazilian border, which were carried out as part of governmental malaria and onchocerciasis control activities in the region between 2005 and 2007. Ethical approval was obtained by the Ethical Committees of the *Servicio Autonomo Centro Amazónico de Investigación y Control de Enfermedades Tropicales ‘Simon Bolivar’* (SA-CAICET), Puerto Ayacucho, Venezuela, and the London School of Hygiene & Tropical Medicine, London (LSHTM), UK.

When the team—consisting of medical doctors, scientists and health workers—arrived in a *shabono*, people were invited for a gathering. Malaria control and research activities were explained with the help of translators. Special importance was given to the presence of elders and leaders of the community. Informed consent was obtained orally. All individuals who felt sick were examined, diagnosed and treated for malaria, or the respective disease, according to the guidelines of the Venezuelan Health Ministry (Metzger et al., 2008). The ages of the patients were estimated as the Yanomami have no counting system.

Thick and thin blood smears were taken from individuals who presented with a history of fever and/or headache and/or malaise. Blood samples were collected by finger prick and stored on filter papers. 633 samples were used for the retrospective screening to investigate the molecular genetics of *P. malariae* parasites.

2.2. DNA extraction and PCR diagnosis

Parasite DNA from field samples was extracted from dried blood spots on filter paper using a commercial extraction kit (QIAamp DNA Blood Mini Kit, Qiagen). Screening for *Plasmodium* spp. infection was carried out by conventional nested-PCR assay with genus and species-specific primers based on the small subunit ribosomal RNA genes (18S) described previously by Snounou et al. (1993).

Genomic DNA of the *P. brasilianum* Peruvian III strain (MR4-349) was obtained from the Malaria Research and Reference Resource Center (MR4) to generate reference sequences for analyses at the University of New Mexico School of Medicine, Albuquerque, NM, USA.

2.3. 18S: development of new primers and sequencing

Though a *P. malariae*-specific PCR assay by Snounou et al. (1993) is sensitive and typically employed in the differential diagnosis of species, the 145 bp product of this primer set is too short for extensive sequence analysis. Therefore, we designed new sequencing primers targeting an amplicon spanning the entire variable region 5 (V5), one of the eight highly variable regions in the 18S gene which has considerable sequence variations among *Plasmodium* species. In addition we amplified three variable domains (V4, V5, V7) from the genomic DNA of *P. brasilianum* Peruvian III strain obtained from MR4.

Primer sequences were selected from unique and common regions for *P. malariae* and *P. brasilianum* species. The first primer pair (Pm18S Outer-F and Pm18S Outer-R) amplifies an 808-bp fragment. The second set of nested primers (Pm18S Inner-F and Pm18S Outer-R) amplifies a 763-bp fragment. All amplified samples were purified using Exo-SAP It Kit (USB) and sequenced bi-directionally using forward and reverse primers. Details of primer sequences, PCR amplification, and sequencing methods are shown in the appendix.

2.4. CSP: primers and sequencing

For CSP gene amplification, primers were used from the conserved regions flanking the central repeat region that contains immunodominant epitopes. The amplified CSP gene fragment from each isolate was purified using Exo-Sap It kit (USB), cloned using a TOPO TA cloning kit (Invitrogen), and transformed into TOP10 competent *Escherichia coli* cells (Invitrogen). We sequenced at least 3 clones from each isolate using M13 primers and gene specific internal primers. Details of the methods are given in the appendix.

2.5. Sequence analysis of 18S and CSP gene

For the 18S gene, sequences were aligned and edited using the Vector NTI ContigExpress program version 10 (Invitrogen). Nucleotide sequences generated from each isolate were queried against the NCBI GenBank nucleotide database using BLASTN for similarity search. Out of the total 47 published sequences of *P. malariae* and *P. brasilianum*, 23 sequences overlapped with the amplified target. The remaining 24 sequences were amplified from regions different from Snounou et al. primer site. Detailed information about source, origin, and accession number of all published 18S gene sequences is listed in the supplementary (appendix). A *Plasmodium* phylogenetic tree based on the 18S gene was constructed using the Kimura 3-parameter implemented in MEGA software ver. 6.0 (Tamura et al., 2013). *Theileria* sp. (GenBank Ac. AF162432) was used as outgroup. The reliability of the tree was assessed by the bootstrap significance test with 1000 replications. The final tree was refined using the program FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The nucleotide diversities were calculated using DnaSP (Librado and Rozas, 2009). The maximum likelihood genetic distances based on Kimura 3-parameter, modeled with gamma distribution (shape parameter = 0.21) were calculated with MEGA ver. 6.0. For distance calculation, the best suited maximum likelihood model was obtained by using Modeltest implemented in MEGA ver.6.0. The comparable 18S gene (A-type) sequences of other *Plasmodium* spp. for the analyses were obtained from NCBI GenBank and listed in the appendix. The sequence alignments that were used to infer phylogenetic relationships are available from the authors on request.

For the CSP gene, each plasmid sequence containing the CSP gene fragment was aligned using the Vector NTI ContigExpress program version 10 (Invitrogen). The edited sequences of the CSP gene from each isolate were translated to corresponding amino acid sequences using the ExPASy Translate tool (http://web.expasy.org/translate/). Deduced amino acid sequences were compared to CS protein sequences of *P. malariae* and *P. brasilianum* isolates available in the database.

3. Results

3.1. Diagnosis of P. malariae infection

The determination of malaria by conventional nested-PCR detected the presence of *P. malariae* DNA in 75 of 633 samples collected from different individuals in Yanomami villages, constituting an 11.8% carrier rate in this survey. 25 of 75 samples (33%) were co-infected with *P. vivax* (n = 7), *P. falciparum* (n = 12), or triple infections (n = 6) while the remaining 50 had mono-infections with quartan malaria parasites.

3.2. Differentiating P. brasilianum from P. malariae

Out of the 75 samples PCR-positive for *P. malariae*, the 18S gene from 33 samples (27 mono-infections and 6 mixed infections) was successfully amplified and the resulting 763 bp product was analyzed by sequencing. Upon sequence analysis, 12 of the 33 samples had 18S gene sequences that were 100% identical with a *P. brasilianum* strain (GenBank AF130735) isolated from an infected monkey (*Alouatta seniculus*) in French Guiana (Fandeur et al., 2000). The twelve *P. brasilianum* infected individuals were from five different shabonos. The estimated ages of the patients were from 6 to 60 years.

In addition, isolates from four patients (n = 4) were 100% identical with the *P. malariae* Myanmar strain 1 (GenBank AF487999); six isolates (n = 6) were 100% identical with the *P. malariae* Myanmar strain 2 (GenBank AF488000); and one isolate (n = 1) was 100% identical with the *P. malariae* PNG strain (GenBank AF145336). The remaining ten isolates (n = 10) were 99% identical to either *P. malariae* Myanmar strain 2 or *P. malariae* PNG strain. Variation in the ten samples was represented by nucleotide polymorphisms (i.e., substitutions, insertions and/or deletions). Among these ten isolates, four new variants were identified (appendix).

Alignment of the twenty-three published 18S sequences and newly generated sequences of quartan malaria parasites showed 27 polymorphic sites in the 496-nucleotide sequences spanning V5 region. The overall nucleotide diversity (Pi) was 6.5×10^{-3} (SD 0.001), with an average number of nucleotide differences (k) of 2.935.

Table 1 shows the extent of divergence in the genetic distances between *Plasmodium* species, as calculated from the 18S gene spanning variable domain 5. Although the quartan parasites were genetically distinct from the other *Plasmodium* species, there was no genetic differentiation between the *P. malariae* and *P. brasilianum* isolates (distance, d = 0.005). The average genetic distance between all quartan isolates from human and monkey is comparable to intra-species genetic distance in other *Plasmodium* spp. (Table 1).

The phylogenetic analysis by the Neighbor joining method confirmed the similarity of all quartan malaria parasites (irrespective of the source of isolation, *P. brasilianum* or *P. malariae*) by clustering into a single monophyletic clade with a high bootstrap support of 100% (Fig. 1).

No identifying SNPs specific to either *P. malariae* or *P. brasilianum* was identified in the gene locus conventionally employed for differential diagnosis of malaria species. For example, all published *P. brasilianum* sequences isolated from monkeys and the twelve *P. brasilianum* sequences isolated from humans and sequenced in this study displayed G at position 565. However, the *P. brasilianum* isolate from Peru (MR4) sequenced in this study displayed an A, and three recently sequenced *P. malariae* isolates from humans in Costa Rica displayed a G at position 565 (Table 2).

The new sequences identified in this study were submitted to the NCBI GenBank with accession numbers KJ619941–KJ619947 and KM016331–KM016338.

3.3. Analysis of the CSP gene

The central immunodominant repeat region of the CSP gene was amplified and sequenced from three isolates, each representing *P. brasilianum*, *P. malariae* Myanmar strain 1, and *P. malariae* Myanmar strain 2, identified by 18S gene sequencing. Translated amino acid sequences showed that all three isolates constituted the minor tandem tetrapeptide repeat unit NDAG (N, asparagine; D, aspartic acid; A, alanine; G, glycine) and the major unit NAAG (N, asparagine; A, alanine; G, glycine) varying only by the number of repeat units.

All published isolates from South America, including *P. brasilianum* and *P. malariae*, from this and other studies, started the repeat region with the tetrapeptide NDEG, which is similar to *P. malariae* isolates from Asia. In contrast, African isolates from Uganda, Cameroon, and Cote d'Ivoire started the repeat region with NDAG (Table 3).

4. Discussion

All quartan malaria parasites analyzed in this study would be *P. malariae*, if they had been found fifteen years earlier. However, as Fandeur et al. (2000) detected some 100% identical strains in monkeys in 1999, twelve of the 33 parasites had to be named *P. brasilianum*.

Table 1
Estimates of the average genetic distance between and within *Plasmodium* spp.
The table shows average genetic distances based on variable domain 5 (V5) of the 18S gene sequences between (black) and within (red) *Plasmodium* spp. Standard errors (SE) for interspecies values (black) are shown above the diagonal (blue). The number of sequences (n) in each group of *Plasmodium* spp. is indicated in the second column. All V5 sequences generated in this study (including the MR4 sequence) and all comparable sequences from GenBank were included into the analysis. Sequences for *P. malariae* and *P. brasilianum* are listed in the supplementary appendix. Data show that genetic divergence between *P. brasilianum* and *P. malariae* is not more than within a species (gray boxes). Pmal, *P. malariae*; Pbra, *P. brasilianum*; Pfal, *P. falciparum*; Pviv, *P. vivax*; Pkno, *P. knowlesi*; Pcyn, *P. cynomolgi*; Poc, *P. ovale curtisi*; Pow, *P. ovale wallikeri*.

Species	n	Pbra	Pmal	Pfal	Pviv	Pcyn	Pkno	Poc	Pow
Pbra	14	0.004	0.002	0.011	0.017	0.018	0.020	0.017	0.017
Pmal	18	0.007	0.008	0.011	0.016	0.018	0.020	0.017	0.016
Pfal	8	0.043	0.043	0.001	0.017	0.018	0.019	0.016	0.015
Pviv	11	0.071	0.069	0.073	0.002	0.002	0.005	0.011	0.011
Pcyn	5	0.077	0.076	0.077	0.004	0.002	0.006	0.011	0.011
Pkno	19	0.093	0.091	0.080	0.012	0.017	0.004	0.013	0.013
Poc	10	0.077	0.076	0.068	0.040	0.044	0.054	0.012	0.005
Pow	19	0.071	0.070	0.059	0.039	0.042	0.053	0.017	0.009

Based on phylogenetic analysis standards, *P. malariae* and *P. brasilianum* are one species (Fig. 1), and the punctual differences are not more than differences between strains of other *Plasmodium* species (Tables 1 and 2). So far, the host made the difference, as the infected host has been the classification determinant for *P. malariae* and *P. brasilianum*. Our findings show that this distinction criterion no longer applies.

Malaria history surmises that 500 years ago, Old World Humans introduced *P. malariae* to the New World; some of the parasites crossed the species barrier, adapted to New World monkeys and became *P. brasilianum*, the simian quartan malaria parasite of Latin America (Coatney et al., 2003). This hypothesis was challenged later; based on genetic diversity assumptions, it was reasoned that quartan parasites jumped from monkey to man and became human *P. malariae* (Tazi and Ayala, 2011). Though directions of the cross-species transfer are opposing, the two hypotheses share the common element of "host switching" which implies host specificity of *P. brasilianum* and *P. malariae* to monkey and man, respectively.

Our results allow an alternative view. For the first time, quartan malaria parasites, which are identical to those found in naturally infected primates of Latin America, were detected in naturally infected humans. Thus, our results provide evidence that quartan parasites are able to cross host species boundaries with impunity and that humans and non-human primates—in conditions of close contact—share quartan parasites without host specificity. Moreover, it can be speculated that *P. brasilianum* and *P. malariae* neither are distinct species, as the name suggests, nor are they distinct variants of one species, which became specialized on different hosts after switching, but are rather the same quartan malaria parasite species—an anthropozoonosis—circulating freely between monkeys and humans.

This confirmation of host sharing characteristics has long been pending Coatney, 1971; Escalante et al., 1995 and might give a reason to re-appraise quartan malaria, a largely neglected disease thus far, with the qualities to become an emerging infection.

In 1890, *P. malariae* was the first malaria species to get a scientific name (Collins and Jeffery, 2007). To date, the causative agent of quartan malaria has been largely understudied, mainly because—due to low levels of parasitaemia—in most epidemiological surveys only a few infections were detected (Mueller et al., 2007). Nonetheless, in studies with improved detection limits, *P. malariae* has been demonstrated in all malaria-endemic regions of the world Autino et al., 2012 and therefore—strictly speaking—might be the malaria parasite with the widest geographical distribution.

Furthermore, quartan malaria parasites might represent the best adapted malaria parasites. The adaption of these protozoans to its hosts results in a mainly chronic clinical outcome with many carriers suffering no symptoms. It is well-known that quartan parasites can persist in dormancy for decades in the host without causing symptoms (Collins and Jeffery, 2007). For example, two chimpanzees, Takaboh and

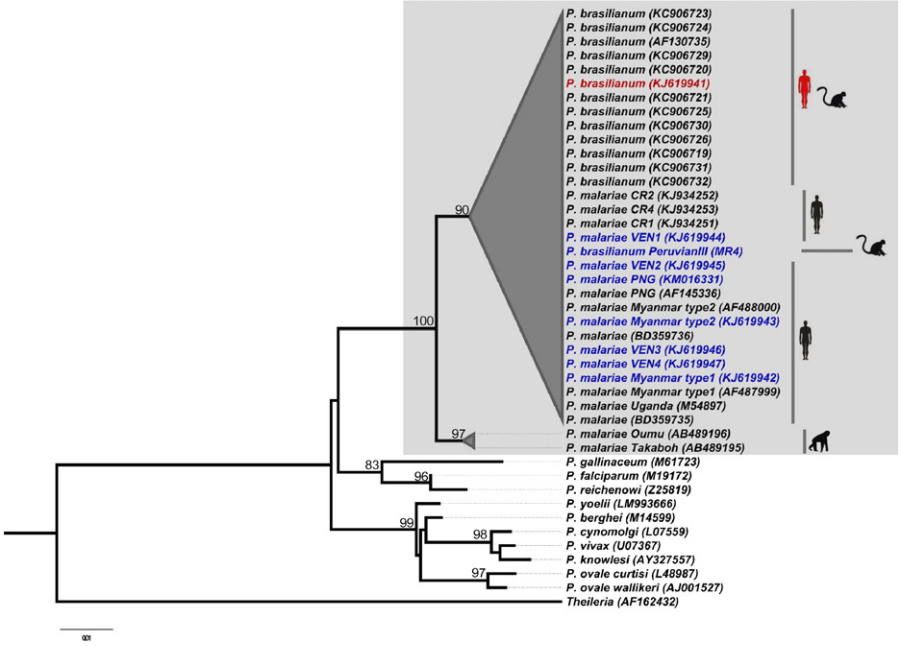

Fig. 1. Neighbor Joining Tree based on 18S gene sequences of *Plasmodium* species. The tree shows that all quartan malaria parasites from humans and monkeys cluster into a monophyletic clade supported by a high bootstrap value of 99%. *P. brasilianum* and *P. malariae* sequences from this study are shown in color (red and blue). Hosts (non-human primate, human) are indicated by graphic symbols beside the taxa names. The sequence highlighted in red was found by Fandeur et al. in Alouatta monkeys, and in this study in humans. The two additional *P. malariae* isolates from human infections from Bangladesh (GenBank Ac. KF906514 & GenBank Ac. KF906514); Fuehrer et al. showed that these two isolates were 100% identical with a *P. malariae*-like isolate from the Chimpanzee Takaboh (GenBank Ac. AB489195).

Table 2
Nucleotide polymorphisms in 18S gene sequences of *P. brasilianum* and *P. malariae* isolates.
The table shows that no distinctive marker for *P. brasilianum* could be identified. The SNP positions given vertically above are numbered according to the nucleotide sequences of *P. brasilianum* (GenBank AF130735). Dots represent identical residues; dashes represent deletions. *P. brasilianum* and *P. malariae* sequences from this study are shown in red and blue, respectively. The numbers in brackets denote the number of identical isolates found in this study. Apart of the four *P. malariae* strains VEN1, VEN2, VEN3, and VEN4, all isolates identified in this study are identical to pre-existing *P. malariae*/*P. brasilianum* sequences.

		Nucleotide positions based on GenBank accession AF130735																													
		3	3	3	3	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	7
		1	3	8	9	1	2	2	4	6	1	5	6	6	6	7	7	7	8	8	9	0	7	7	8	9	9	2
Isolates	GenBank accession	9	8	4	3	9	5	8	1	1	2	0	5	6	8	3	7	8	5	6	6	8	3	9	0	1	6	8
P. brasilianum	AF130735	T	T	T	A	A	G	A	T	C	A	T	A	G	G	C	A	A	T	G	T	A	A	A	T	T	C	C	A
P. brasilianum (n = 12)	KJ619941	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906719	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906720	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906721	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906723	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906724	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906725	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906726	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906729	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906730	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906731	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.
P. brasilianum	KC906732	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	T
P. brasilianum Peruvian III (n = 1) (MR4)	KT266778	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR1	KJ934251	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR2	KJ934252	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR4	KJ934253	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type1	AF487999	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type1 (n = 4)	KJ619942	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type2	AF488000	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type2 (n = 6)	KJ619943	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae PNG	AF145336	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae PNG (n = 1)	KM016331	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN1 (n = 3)	KJ619944	.	.	C	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
P. malariae VEN2 (n = 2)	KJ619945	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN3 (n = 1)	KJ619946	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN4 (n = 4)	KJ619947	.	C	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Uganda-I	M54897	G	.	.	.	.	.	A	.	.	T	A	.	.	T	.	.	.	.	.	.	.	.	C	C	G	A	.
P. malariae	BD359736	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae	BD359735	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Takaboh	AB489195	.	.	.	.	.	.	.	.	.	.	A	A	T	.	T	A	A	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Oumu	AB489196	.	.	.	.	.	.	.	.	.	.	A	A	T	.	T	A	A	A	.	.	.	.	.	.	.	.	.	.	.

Oumu, acquired quartan *P. malariae*-like parasites in the African rain forest when they were babies, and the parasites remained undiscovered until detection 30 years after their first day of confinement in a Japanese zoo (Hayakawa et al., 2009). This is perplexing because "hypnozoites"—as in *P. vivax* and *P. ovale*—have never been discovered or have not been well investigated.

What discriminates quartan malaria parasites from other *Plasmodium* spp. is their expansive reservoir of mammalian hosts. Besides the human

Table 3
Comparison and characterization of the circumsporozoite protein (CSP) central repeat region among quartan malaria species.
The table shows that the otherwise species-specific immunodominant repeat region of the CS protein is the same for all quartan malaria parasites. It consists of the major repeat unit NAAG and the minor repeat unit NDAG. Numbers of the repeat unit can change between isolates; this has been shown for all *Plasmodium* spp. Clones of PCR amplified CSP alleles from three isolates are displayed each representing *P. brasilianum*, *P. malariae* Myanmar strain 1, and *P. malariae* Myanmar strain 2, identified by 18S gene sequencing in this study. African isolates start with a NDAG unit, whereas isolates from Asia and South America (*P. malariae* and *P. brasilianum*) start with NDEG.

Central immunodominant repeat units								
Protein ID	Species (strain)	Origins (authors)	Repeat start	Minor unit	No.	Major unit	No.	Repeat size
KM016332	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016333	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016334	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016337	*P. malariae* (Myanmar type 1)	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016338	*P. malariae* (Myanmar type 1)	Venezuela (this study)	NDEG	NDAG	4	NAAG	49	53
KM016335	*P. malariae* (Myanmar type 2)	Venezuela (this study)	NDEG	NDAG	5	NAAG	50	55
KM016336	*P. malariae* (Myanmar type 2)	Venezuela (this study)	NDEG	NDAG	5	NAAG	51	56
AAA29553	*P. brasilianum*	Unknown (Lal et al., 1988)	NDEG	NDAG	5	NAAG	58	63
AGO33295	*P. brasilianum*	Brazil (Araújo et.al. 2013)	NDEG	NDAG	2	NAAG	21	23
AAA18618	*P. malariae* (China-1 CDC)	China (Qari et.al. 1994)	NDEG	NDAG	7	NAAG	46	53
AAA29557	*P. malariae* (Uganda-1 CDC)	Uganda (Lal et al., 1988)	NDAG	NDAG	6	NAAG	45	51
CAA04809	*P. malariae*	Cameroon (Tahar et al., 1998)	NDAG	NDAG	7	NAAG	44	51
CAA04812	*P. malariae*	Cote d'Ivoire (Tahar et al., 1998)	NDAG	NDAG	6	NAAG	46	52

host, there have been studies showing quartan malaria parasites in several dozens of monkey species. Merely in South America, quartan parasites were described in 35 monkey species (as *P. brasilianum*) (Fandeur et al., 2000). In Asia, monkeys are infected by the quartan *Plasmodium inui* (Coatney et al., 2003), and in the African rain forest, great apes are infected by quartan parasites (as *Plasmodium rodhaini* or *P. malariae*, and *P. malariae*-like) (Ramasamy, 2014). To our knowledge, this is by far the widest host reservoir compared with malaria of other periodicity.

Clinically, quartan malaria is considered as relatively harmless, but sufficient data are lacking to substantiate this assessment. Many investigators assume that *P. malariae* is the causal factor—perhaps with co-factors—for renal pathologies (Ehrich and Eke, 2007). Generally, it can be assumed that the clinical outcome of *P. malariae* infections is misinterpreted (McKenzie et al., 2001). Due to the chronic nature of the infection, capturing the true burden of the disease would require large longitudinal studies to assess the impact of the infection on occupational and social life of an individual.

Globally, the disease burden of quartan malaria is difficult to assess, because data on the incidence of *P. malariae* are faulty. The main reason is that *P. malariae* is principally underdiagnosed, because it thrives with a few hard-to-detect parasites, which are indistinguishable from *P. vivax* on the thick blood smear and which can also be misinterpreted as *P. falciparum*. Therefore, time-consuming reading of a thin smear or molecular methods would be necessary to identify this species. This explains why in the 1980s the Brazilian Health Ministry had "eradicated" *P. malariae* from Brazil by simply switching the official method of diagnosis from thin to thick blood smear (Oliveira-Ferreira et al., 2010).

In areas with marked variation in seasonal climate, *P. malariae* may account for 50% of the malaria episodes during the low-transmission season (Greenwood et al., 1987). As *P. malariae* is commonly found in sympatry with other *Plasmodium* species of humans, better understanding of species-interaction is necessary. Especially the interactions with *P. falciparum* in mixed infections is a controversially discussed topic (Mueller et al., 2007). Recent seroepidemiological and biomolecular surveys indicated that the prevalence of *P. malariae* are underestimated Mueller et al., 2007; Autino et al., 2012 and high prevalences of *P. malariae* have been reported from Africa (Doderer-Lang et al., 2014), Asia (Bharti et al., 2013), and Latin America (Volney et al., 2002). These findings correspond well with results of our pilot study of the Upper Orinoco Metzger et al., 2008 which prompted us to undertake the current investigation.

Initially, we hypothesized that *P. brasilianum* and *P. malariae* might be discernible by 18S gene sequencing since we noticed that the SNP at position 565 was shared by all hitherto published *P. brasilianum* isolates. Consequently, we identified this SNP as a possible distinctive marker. However, when we sequenced the *P. brasilianum* Peruvian III strain (provided by MR4), our hypothesis was rebutted. It was rebutted a second time, when three *P. malariae* isolates from humans in Costa Rica were published during the revision process of this article displaying a G at position 565. It appeared that SNPs were distributed at random, and none was specific for *P. malariae* or *P. brasilianum* (see Table 2).

To further ensure what was found in the 18S sequence, we analyzed the CS protein sequence, which also is used for species distinction, because the amino acid composition of the CSP gene is an adaptation for eliciting species specific antibody response. For example *P. vivax*, *P. falciparum* and *P. knowlesi* differ considerably in the CSP repeat region: *P. vivax*: DRAGGQPAG, *P. falciparum*: NANP, and *P. knowlesi*: GQPQAQGDGANA (Verra and Hughes, 1999). The CS protein sequence was identical between *P. brasilianum* and *P. malariae*, consistent with results by other investigators (see Table 3) (Escalante et al., 1995; Tahar et al., 1998).

So, unlike the zoonotic parasite *P. knowlesi*, which is genetically different from other *Plasmodium* spp. in several typical marker genes such as CSP and 18S genes (see Table 1) (Escalante et al., 1995; Escalante and Ayala, 1994), *P. brasilianum* is indistinguishable from *P. malariae* and infects the same hosts. And other than the two recently distinguished sympatric *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al., 2010), the quartan parasites *P. malariae* and *P. brasilianum* do not segregate into distinct types in all genomic markers used so far (see Fig. 1) (Fandeur et al., 2000; Guimarães et al., 2012; Tanomsing et al., 2007).

It has to be adverted, that logic does not allow demonstration of identity, or non-difference, even if a few whole genome sequences of the parasites were available. Hence, the best approach to show that the parasites are indeed one species would be to do cross-mating experiments. However, if no distinctive markers are known whereby the off-spring can be identified, these experiments are very difficult to design and it is questionable if they are ethically justified.

So far, the evidence of naturally acquired human infections with parasites termed as *P. brasilianum* was a missing piece in the puzzle of the identity of this quartan parasite (Baird, 2009). Based on the results presented here, we conclude that anthropozoonotic transmission of quartan malaria occurs in areas where the habitat of man and monkey overlap. Similar transmission scenarios wit simian participation have been reported in areas outside the Amazonian region, for example in the Atlantic Forest of Brazil (De Pina-Costa et al., 2014).

We wonder if *P. brasilianum* will undergo a nomenclatorial revision, if further research confirms our findings. This would not be the first time that the name of a quartan malaria parasite is revised: In the 1940s, when *P. rodhaini*, the quartan parasite of apes in Africa, was identified to be *P. malariae*, the former name was soon "sinking into synonymy with *P. malariae*" (Coatney et al., 2003) and is not anymore in use today. This was justified just recently: Alignment of the 18S sequences of *P. malariae* from humans in Bangladesh with the parasite sequences of one of the two simian immigrants in the Japanese zoo, Takaboh (GenBank Ac. AB489195), resulted in 100% identity (see also legend of Fig. 1) (Fuehrer et al., 2014).

Malariologists know for some time that simian malaria will play an important role when human malaria eradication is envisaged (Bruce-Chwatt, 1968). As quartan parasites are the only global human malaria parasites successfully infecting multiple mammalian hosts, they could evade control measures that do not account for the animal reservoir. These hidden and possibly emerging parasites could, therefore, represent an important area for future research efforts.

Contributors

WGM, MM, and SVM carried out the primary data collection and supervised field and laboratory work. AL, MK, and PK performed the molecular experiments. BM, ME, SJ, and DJP supervised the laboratory work, facilitated the collaboration and gave overall input. WGM, MM, and AL conceived the study and designed the experiments. WGM and AL did the analysis, drafted and revised the manuscript. All authors interpreted the results, revised and approved the final manuscript.

Declaration of interests

We declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.07.033.

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PARASITOLOGY

Recombinase Polymerase Amplification and Lateral Flow Assay for Ultrasensitive Detection of Low-Density *Plasmodium falciparum* Infection from Controlled Human Malaria Infection Studies and Naturally Acquired Infections

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ABSTRACT Microscopy and rapid diagnostic tests (RDTs) are the main diagnostic tools for malaria but fail to detect low-density parasitemias that are important for maintaining malaria transmission. To complement existing diagnostic methods, an isothermal reverse transcription-recombinase polymerase amplification and lateral flow assay (RT-RPA) was developed. We compared the performance with that of ultrasensitive reverse transcription-quantitative PCR (uRT-qPCR) using nucleic acid extracts from blood samples ($n = 114$) obtained after standardized controlled human malaria infection (CHMI) with *Plasmodium falciparum* sporozoites. As a preliminary investigation, we also sampled asymptomatic individuals ($n = 28$) in an area of malaria endemicity (Lambaréné, Gabon) to validate RT-RPA and assess its performance with unprocessed blood samples (dbRT-RPA). In 114 samples analyzed from CHMI trials, the positive percent agreement to uRT-qPCR was 90% (95% confidence interval [CI], 80 to 96). The negative percent agreement was 100% (95% CI, 92 to 100). The lower limit of detection was 64 parasites/ml. In Gabon, RT-RPA was 100% accurate with asymptomatic volunteers ($n = 28$), while simplified dbRT-RPA showed 89% accuracy. In a subgroup analysis, RT-RPA detected 9/10 RT-qPCR-positive samples, while loop-mediated isothermal amplification (LAMP) detected 2/10. RT-RPA is a reliable diagnostic test for asymptomatic low-density infections. It is particularly useful in settings where uRT-qPCR is difficult to implement.

KEYWORDS CHMI, *Plasmodium falciparum*, diagnostics, elimination, isothermal, mass screen and treat, submicroscopic

Light microscopy (LM) and antigen-detecting rapid diagnostic tests (RDTs) are the primary diagnostic tools for the confirmation and management of suspected clinical malaria (1). Nucleic acid amplification tests (NAATs) based on PCR are commonly used for studies that require detection of low-density malaria infections, below the limit of detection of LM or RDTs, such as in controlled human malaria infection (CHMI) for early treatment decisions (2) and in low-transmission areas for epidemiological research and surveys (1).

In CHMI trials, volunteers are purposely infected either by the bite of infectious

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mosquitoes, direct intravenous injection of *Plasmodium falciparum* sporozoites (PfSPZ), or by injection of blood-stage parasites (3). In both cases, the asexual blood-stage infection starts with an extremely low parasitemia, below the detection threshold of conventional PCR assays or expert microscopists. In sporozoite-induced CHMI, the first-cycle blood-stage parasites can be detected by ultrasensitive quantitative real-time PCR (qPCR) around day 7, when hepatic merozoites are released into the bloodstream (4, 5), while expert microscopists require about 3 cycles of multiplication (~day 11, 100- to 1,000-fold higher parasitemia) before thick blood smears are read as positive (5, 6). The qPCR sensitivity is enhanced by the inclusion of reverse transcriptase (RT-qPCR) and use of a higher blood volume enabling ultrasensitive detection (≤20 parasites/ml) (4, 5).

Sensitive diagnostics are fundamental for transmission intervention campaigns, particularly in areas of low endemicity or regions with declining transmission intensity, where a large proportion of *P. falciparum* infections are asymptomatic with very low parasite densities (7). These "hidden" submicroscopic infections constitute up to one-half of all infections detected by PCR (8). Without proper diagnosis and treatment, these highly prevalent asymptomatic infections represent an important infectious reservoir for malaria transmission (8).

Malaria control and prevention in the most vulnerable and high-risk groups (e.g., pregnant women) include intermittent preventive treatment (IPTp) with sulfadoxine-pyrimethamine (SP) (9). Screening with RDTs and treatment (intermittent screening and treatment in pregnancy [ISTp]) with artemisinin-based combination have been evaluated as an alternative approach to IPTp in areas with SP-resistant parasites (10). Highly sensitive diagnostics will be essential to improve clinical management and outcome of ISTp, since sequestration frequently leads to peripheral parasitemias below the diagnostic thresholds of microscopy and RDTs (10).

In recent years, non-PCR-based NAATs for use in low-resource settings have been developed. Best known are loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA) techniques. Recombinase polymerase amplification (RPA) is one of the most versatile isothermal amplification techniques which works at a fixed temperature between 37°C and 42°C, using minimally prepared samples in less than 20 min (11). Furthermore, RPA can be integrated with lateral flow strips for a quick read out without any complex equipment. These features potentially make RPA the preferred choice for use at the point-of-need, especially in low-resource settings, as a PCR substitute.

In this study, the application of reverse transcription-RPA (RT-RPA) as a robust, highly sensitive, and rapid diagnostic test for *P. falciparum* is described. The approach utilized abundant asexual stage-expressed rRNA and the coding genes (GenBank AL844504 and AL844506) as the template for amplification and the lateral flow strip for the readout. The diagnostic performance was evaluated in healthy volunteers with low-density parasitemia induced by standardized controlled human malaria infection (CHMI) with cryopreserved *P. falciparum* sporozoites (Sanaria PfSPZ challenge) (5, 6, 12, 13). A further simplified direct blood amplification approach was tested in Lambaréné, Gabon, to evaluate the assay in asymptomatic individuals from a setting where malaria is endemic.

MATERIALS AND METHODS

RPA primers and probe design. Nucleotide sequence domains unique to *P. falciparum* were scanned by aligning large-subunit rRNA genes (28S), including those from human and other non-*falciparum* species. Amplification primers and the probe for lateral flow detection by a so-called "sandwich assay" were designed based on the guidelines from TwistDx (TwistAmp DNA amplification kits, combined instruction manual [2016]). The most efficient primer pair specific for *P. falciparum* RT-RPA was determined by testing three forward primers and reverse primers in different combinations analyzed by endpoint capillary electrophoresis (QIAxcel; Qiagen). Specificity of the primers was tested using malaria-naive samples as well as PCR-confirmed non-*falciparum* monospecies infection with *Plasmodium vivax*, *P. malariae*, *P. ovale curtisi*, and *P. ovale wallikeri*. The amplification was confirmed by direct sequencing using an ABI 3130XL DNA sequencer and homology search (BLAST) against NCBI GenBank. After working out the best pair of primers, a probe was designed along with modifications for lateral flow

TABLE 1 Primer and probe sequences

Primer ID[a]	5′→3′ sequence	Length (bp)
RPA_Pfal28S_F1	GGAGTAGAAACTGAAATATGTTTTTACGACAG	32
RPA_Pfal28S_R1_Bio	Biotin-GAAATTGGGAGAAAGATAAGAAACAAGTTTC	31
RPA_Pfal28S_F2	FAM-GTTGTTTTACTTATCCATTTATAGGGAAAT [dSpacer]TATTATGCTTTATCCTTCG-SpC3[b]	50

[a]ID, identifier.
[b]The probe consists of 5′ antigenic label (6-carboxyfluorescein [FAM]), an internal abasic nucleotide analogue (dSpacer), and a polymerase extension blocking group (SpC3) at the 3′ end.

detections. The forward primer (Pfal28S_F1) was an unmodified oligonucleotide, while the reverse primer (Pfal28S_R1-Bio) was labeled with biotin at the 5′ end. The probe (Pfal28S_LF2) consists of an oligonucleotide backbone with a 5′ antigenic label (6-carboxyfluorescein), an internal abasic nucleotide analogue (dSpacer), and a polymerase extension blocking group (SpC3) at the 3′ end. All primers and probes were ordered from Eurofins Genomics (Ebersberg, Germany) and are shown in Table 1.

RT-RPA lateral flow test. RT-RPA was performed using a TwistAmp nfo kit (TwistDx, TANFOO2) with the addition of SensiFAST reverse transcriptase (Bioline). The assay was performed in a total volume of 50 μl containing 420 nM each primer, 120 nM probe, 1× reverse transcriptase, 20 U RNase inhibitor, rehydration buffer, 4 μl template, and 14 mM magnesium acetate (MgAc). The master mix was prepared without the template and magnesium acetate and was distributed into each tube of a 0.2-ml 8-tube strip containing freeze-dried enzyme pellet followed by the addition of 4 μl template into the tubes. Magnesium acetate was pipetted into the tube lids and centrifuged into the reaction mix using a benchtop centrifuge. The reaction tubes were immediately incubated at 40°C for 20 min in a thermoblock-shaker (ThermoMixer Comfort; Eppendorf) with constant shaking at 300 rpm. After 6 min of incubation, the reaction tubes were removed and mixed by inverting 2 to 3 times, centrifuged, and reincubated for another 14 min. External positive- and negative-amplification controls were included in each run. The positive-amplification control consisted of nucleic acids prepared from confirmed *P. falciparum*-infected blood. The no-template control (NTC) consisted of nucleic acids extracted from blood of healthy malaria-naive volunteers. To eliminate carryover contamination, pre- and postamplification steps were performed in a separate room using pipettors for each work. Sterile aerosol protection filter tips (Nerbe plus, Winsen, Germany) were used in all assay setups. Amplification products were detected by nucleic acid lateral flow immunoassays using either HybriDetect 1 (Milenia Biotec, Germany) or PCRD (Abingdon Health, UK). For detection using HybriDetect 1, products were diluted 1 in 20 with 1× phosphate-buffered saline with 0.5% Tween (PBST) in a microcentrifuge tube. Strips were dipped into the diluted product, and the readout was observed within 10 min. Detection with PCRD was performed by diluting the product with the kit buffer (1 in 15), and the readout was observed within 10 min. Interpretation of results was based on a semiquantitative scoring system for the test line: none (no line visible), weak (barely visible), medium (similar to control line), strong (stronger than control line). Absence of the control line was considered an invalid result. Readings were performed by two investigators. In the case where the two readings were not concordant due to a weakly stained test band, the lateral flow assay was repeated with a new dipstick. A smartphone camera was used to capture the test results for electronic data storage and record keeping.

CHMI sample background. Samples for evaluation of the RT-RPA were collected from volunteers enrolled in CHMI trials conducted in Tübingen, Germany (n = 39), and Lambaréné, Gabon (n = 36). Details of the three studies in Tübingen are published elsewhere (5, 6, 13). CHMI samples from Lambaréné included volunteers recruited for a malaria vaccine trial (PACTR201503001038304) published recently (12). All CHMI samples were collected following direct venous inoculation (DVI) of 3,200 aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites (strain NF54) as shown in Table 2. From the three studies conducted in Tübingen, samples were collected from three time points (days 6, 7, and 7.5)

TABLE 2 CHMI samples

Study (no. of specimens)	No. of specimens			Ethical approval
	Aparasitemic[a]	Subpatent parasitemic[b]	Patent parasitemic[c]	
NCT02115516 (38)	17	18	3	The ethics committee of the Eberhard Karls University and the University Clinics of Tübingen
NCT02450578 (16)	12	4	0	The ethics committee of the Eberhard Karls University and the University Clinics of Tübingen
EudraCT-2017-002723-16 (24)	13	11	0	The ethics committee of the Eberhard Karls University and the University Clinics of Tübingen
PACTR201503001038304 (36)	4	17	15	The national ethics committee of Gabon and the Gabonese Ministry of Health

[a]Aparasitemic, uninfected, negative by RT-qPCR and TBS.
[b]Subpatent parasitemic, positive by RT-qPCR only.
[c]Patent parasitemic, positive by both RT-qPCR and TBS.

postinoculation of PfSPZ challenge; the time point of parasite egress from the liver. This time represents the subpatent period of low-density parasitemia in malaria-naive volunteers, approximately 4 days prior to malaria symptoms and microscopic detection of parasites by expert readers (5, 6, 13).

In a subset of samples ($n = 5$), the performance of RT-RPA was also compared to that of the Loopamp Malaria Pf detection kit (lot 49001; Eiken Chemical) at time points corresponding to second and third blood-stage cycles (days 9 and 11). Lastly, the assay was tested on semi-immune volunteers enrolled in a CHMI study conducted in Lambaréné. This included volunteers who became thick-smear positive and those who remained negative until the end of the follow-up period (day 35).

Blood samples from these volunteers were purified and previously tested by a validated RT-qPCR assay during the studies (5). Purification was either performed manually or automated using QIAsymphony SP (Qiagen). For manual extraction, 0.5-ml aliquots of EDTA-anticoagulated blood were processed using a QIAamp DNA blood minikit (catalog number [no.] 51306; Qiagen) and eluted into 100 μl of nuclease-free water (catalog no. 129114; Qiagen). With automation by QIAsymphony, nucleic acids were purified using 0.4 ml blood and eluted in 100 μl elution buffer.

Thick blood smear (TBS) microscopy and RT-qPCR methods have been implemented as the primary diagnostic tools for CHMI studies at the clinical trial platform in Tübingen. The TBS procedures are standardized to allow detection of at least 10 parasites/μl (14). Quantification of parasitemia by RT-qPCR relies on a standard curve obtained from laboratory *P. falciparum* 3D7 culture and is expressed as parasites per milliliter (p/ml) of blood (detailed standard operating procedures available upon request from the corresponding author) (5).

Direct blood RT-RPA. For direct blood RPA, 28 blood samples were collected from asymptomatic, healthy Gabonese children (7 to 12 years) and adults after informed consent was given to be screened for prospective malaria drug/vaccine trials at CERMEL, Lambaréné, in October 2018. Volunteers willing to participate were screened for plasmodial infection with RDTs and microscopy. In parallel, those screening samples were evaluated for the diagnostic accuracy testing of the RPA. Inclusion criteria were provision of consent and being healthy, the exclusion criterion was clinically diagnosed or reported illness. The screening samples were collected in 1.2-ml blood collection tubes (Sarstedt S-Monovette 1.2 ml, K3 EDTA) for parasitological testing. From this, 100-μl blood aliquots were obtained from the study team for RPA. Direct blood RT-RPA was performed as mentioned above except that 4 μl fresh blood instead of purified nucleic acid was used in the reaction. All direct blood assays were performed in Lambaréné, and the test operators were blinded from any laboratory test results. The remaining 96 μl blood was used for purification (QIAamp DNA blood minikit). Both RT-RPA and RT-qPCR were performed using purified nucleic acids for comparison between the three tests.

Malaria LAMP assay. From a subset of CHMI volunteers ($n = 5$), 1 ml heparin-anticoagulated whole blood was collected separately at days 9 and 11 after intravenous injection of *P. falciparum* sporozoites (PfSPZ) for LAMP, because EDTA interferes with the assay's fluorescence readout (calcein). DNA extraction was performed by the boil method as described in the malaria LAMP standard operating procedure (SOP) by FIND's malaria program (ver. AUG2012) and by spin columns (QIAamp DNA blood minikit; Qiagen). Amplification of *P. falciparum* DNA was carried out using the Loopamp Malaria Pf detection kit (lot 49001; Eiken Chemical) following the protocol by FIND's malaria program (ver. AUG2012). For detection and interpretation of results, amplified products were visualized under a UV transilluminator. Calcein contained in the reagent mix emitted green fluorescence under UV light for a positive sample, while no light is emitted in a negative sample. For comparison, the same set of nucleic acid preparations were amplified by RT-RPA.

Data analysis. The estimates of sensitivity and specificity of the assay were reported as positive percent agreement (PPA) and negative percent agreement (NPA), respectively, as the comparator RT-qPCR assay was a nonreference standard. The metrics to assess the diagnostic accuracy of the assay were calculated in R (version 3.5.1) using the epibasix package. The PPA was calculated as true positives/(true positives + false negatives) $\times$ 100 and the NPA as true negatives/(true negatives + false positives) $\times$ 100. The overall percent agreement was calculated as (true positives + true negatives)/(true positives + false negatives + true negatives + false positives) $\times$ 100.

RESULTS

Laboratory validation. We designed primers from *P. falciparum* 28S rRNA genes located on chromosomes 5 and 7. Newly designed primers showed high specificity to *P. falciparum*. No cross-reactivity was observed with four non-*falciparum* species (*P. vivax*, *P. malariae*, *P. ovale curtisi*, and *P. ovale wallikeri*) as well as human DNA controls. Direct sequencing of the RPA amplicon showed 100% sequence similarity to GenBank *P. falciparum* (PF3D7_0726000).

Detection of submicroscopic low-density *P. falciparum*. The analytical sensitivity was first tested on samples from CHMI volunteers at time points 6 to 7.5 days after inoculation with PfSPZ challenge. The median parasitemia during these time points by the reference RT-qPCR was 43 parasites/ml (95% confidence interval [CI], 20 to 76). From the total of 68 samples measured, the diagnostic accuracy was 95.6% compared to the reference. The positive percent agreement (PPA) and the negative percent agreement (NPA) were 88% (95% CI, 70 to 98) and 100% (95% CI, 92 to 100), respectively (Table 3).

TABLE 3 Agreement between RT-RPA and RT-qPCR in detecting ultra-low-density parasitemia

RT-RPA result	RT-qPCR reference (*n*)		PPA[a] (95% CI)	NPA[b] (95% CI)	Median parasite density (no. parasites/ml)
	Positive	Negative			
Positive	23	0	88 (70–98)	100 (92–100)	43
Negative	3	42			

[a]PPA, positive percent agreement.
[b]NPA, negative percent agreement.

Overall performance of RT-RPA with CHMI samples. In total, 114 analyses were performed using nucleic acids prepared from aparasitemic, subpatent parasitemic, and patent parasitemic volunteers across four CHMI studies (in Tübingen and Lambaréné). The overall percent agreement between the reference and the RT-RPA was 93.9%, while the PPA and the NPA were 90% (95% CI, 80 to 96) and 100% (95% CI, 92 to 100), respectively. The kappa estimates of 0.88 suggested an excellent agreement between the tests (Table 4). Diagnoses of RT-RPA with 15 microscopically confirmed volunteers in Lambaréné were all positive (100% agreement). In total, seven volunteers with low-density infections were missed by the RT-RPA (median parasitemia, 24; 95% CI, 6 to 36) as shown in Fig. 1. Based on logistic regression model, the limit of detection in 95% of RT-qPCR positives was 64 parasites per ml (Fig. 1).

Validation of direct blood RT-RPA in asymptomatic subjects. To simplify the assay further, the direct use of blood was first tested in the laboratory using frozen blood from a volunteer with confirmed low-density malaria infection. RPA worked with three EDTA-blood concentrations tested, and 8% blood in the reaction showed the best amplification with minimal inhibition (Fig. 2). Of 28 asymptomatic individuals tested in Lambaréné, nine were positive with the simplified RPA (dbRT-RPA). RT-qPCR later confirmed seven were truly positive and, in addition, showed one false negative by dbRT-RPA. The diagnostic accuracy of dbRT-RPA was 89% with fair to good agreement with the reference test ($\kappa = 0.75$). However, when RPA was performed on purified nucleic acids from the same blood aliquot of 100 μl, the test agreement was 100% with the reference (Table 5).

Comparison of isothermal techniques. LAMP kit for *P. falciparum* detection was originally evaluated during a PfSPZ vaccine trial (5) from five placebo-immunized control volunteers. For the purpose, blood samples were collected at two time points that coincided with peak parasitemia at day 9 (second-cycle blood stage) and day 11 (thick-smear patent period). RT-qPCR showed early blood-stage parasitemia in all five volunteers from day seven. Three became positive by thick smear at day 11, and the remaining became positive at days 12 and 18. By LAMP assay, clear positive amplification of *P. falciparum* DNA was seen only from two thick-smear-positive volunteers at day 11. The estimated parasitemia by thick smear were 4 and 14 parasites/μl. The same sample sets were analyzed with RT-RPA, and Table 6 shows the comparative results. All but one was positive by RT-RPA, showing 90% agreement with the reference RT-qPCR results (Fig. 3).

DISCUSSION

Despite the need for improved diagnostics to enable the shift from malaria control to elimination, investment in research to accelerate the development and delivery of

TABLE 4 Overall performance of RT-RPA compared to that of RT-qPCR for the detection of *Plasmodium falciparum* using purified nucleic acids

RT-RPA result	RT-qPCR reference (*n*)		PPA[a] (95% CI)	NPA[b] (95% CI)	Accuracy (%)	κ value (95% CI)
	Positive	Negative				
Positive	61	0	90 (80–96)	100 (92–100)	94 (88–97)	0.88 (0.79–0.96)
Negative	7	46				

[a]PPA, positive percent agreement.
[b]NPA, negative percent agreement.

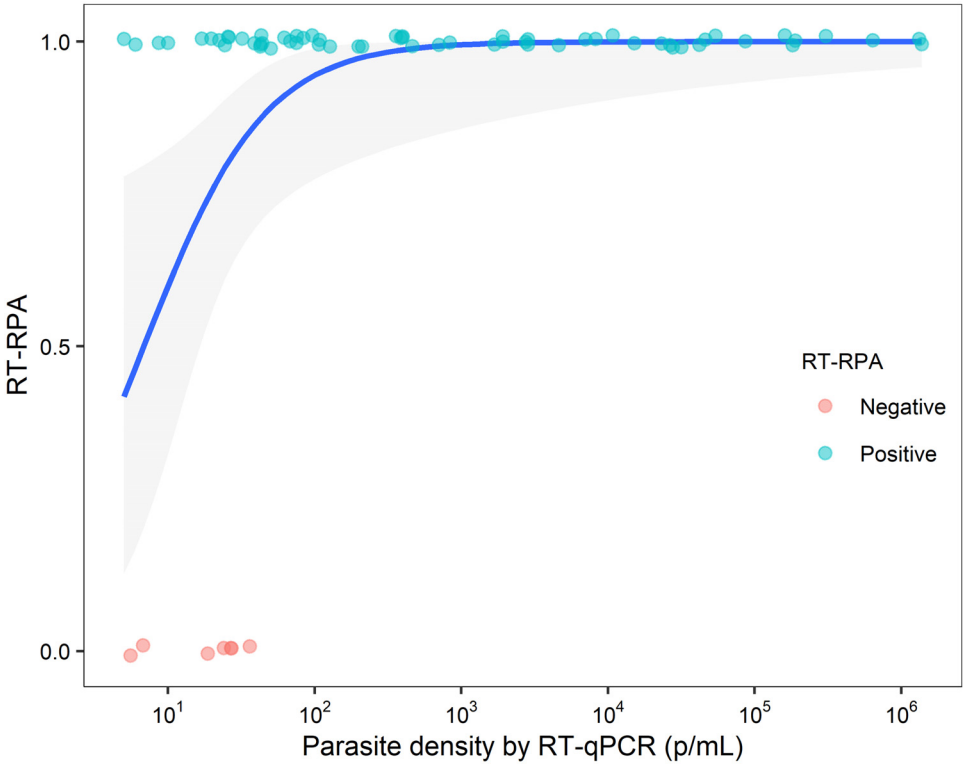

FIG 1 Logit analysis of RT-RPA diagnosis compared to that with RT-qPCR. Logit regression analysis using R on data sets of 114 RT-RPAs. The limit of detection in 95% of RT-qPCR positives was 64 parasites per ml.

new diagnostics is minimal compared to the investment in drugs and vaccine development (15). Even though investments have grown since 2007, diagnostics comprised the smallest portion (2.8%) of all funding allocations available for malaria research and development during the period from 2014 through 2016 (16).

While preventing morbidity and death on the individual level can be achieved with treatment guided by LM and RDTs, malaria elimination requires targeting the infectious reservoir on a population level, which includes "asymptomatic carriers," often with low-density parasitemia (17). The situation is most critical in low-endemicity settings, where such carriers constitute a large proportion of infections and contribute to malaria transmission (8). Current strategies to interrupt transmission, such as mass screening and treatment (MSAT) and focal testing and treatment, rely on the sensitivity of the diagnostics used to screen infected individuals for treatment (18). An effective outcome

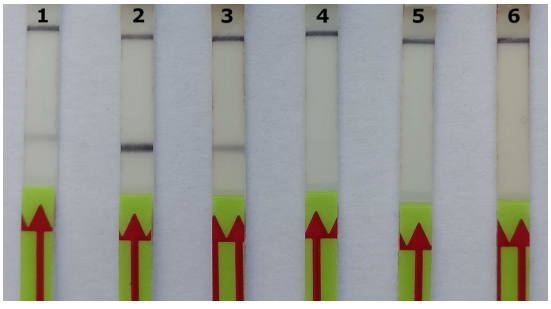

FIG 2 Direct blood RT-RPA optimization with different blood volumes. Lateral flow detection test strips (HybriDetect; Milenia Biotec GmbH). Strip 1, reaction with 4% (vol/vol) malaria-positive blood; strip 2, reaction with 8% (vol/vol) malaria-positive blood; strip 3, reaction with 16% (vol/vol) malaria-positive blood; strip 4, reaction with 4% (vol/vol) malaria-negative blood; strip 5, reaction with 8% (vol/vol) malaria-negative blood; strip 6, reaction with 16% (vol/vol) malaria-negative blood.

TABLE 5 Performance of direct blood RT-RPA for malaria screening test in healthy subjects

Method	Result	RT-qPCR reference (*n*)		PPA[a] (95% CI)	NPA[b] (95% CI)	Accuracy (%)	κ value (95% CI)
		Positive	Negative				
dbRT-RPA	Positive	7	2	88 (47–100)	90 (68–99)	89 (72–98)	0.75 (0.48–1.02)
	Negative	1	18				
RT-RPA	Positive	8	0	100 (63–100)	100 (83–100)	100 (88–100)	1
	Negative	0	20				

[a]PPA, positive percent agreement.
[b]NPA, negative percent agreement.

is modeled with a diagnostics test sensitivity at or below 0.1 parasites/μl (19), a threshold that can be easily achieved with RT-RPA. In the absence of field diagnostics with such a threshold, many of these trials rely on LM or RDTs; the results so far have not proven to influence transmission due to the high proportion of asymptomatic carriers that were not detected (18).

While asymptomatic malaria infection, first observed by Robert Koch in 1900 (20), has recently gained renewed interest in the context of malaria elimination and eradication, little is known about the clinical and long-term health consequences of harboring submicroscopic parasitemia. Generally, this gap in knowledge is attributed to the lack of efficient diagnostic tools and the notion of benefit conferred as "premunition" to superinfection among such carriers, thus impeding the urge to investigate the infection outcome (21). On the contrary, recent studies suggest that chronic asymptomatic infections can have an important impact, at least in high-risk groups, and has been associated with low birth weight and premature births in pregnancy (22). Hence, new sensitive diagnostics may have a role beyond epidemiology and guide treatment decision not only in CHMI trials. Depending on the primary endpoint, detecting and clearing asymptomatic parasitemia is essential to reduce the confounding factors of vaccine efficacy measurement and could also be improved by a quick point-of-need test (23).

Some advances were made in 2017 with the launch of a new-generation ultrasensitive RDT (uRDT) to corroborate screening tools in the field (24). However, its performance in settings of low endemicity with low-density parasitemia is still not good, wherein up to 56% of PCR-detectable *P. falciparum* infections were missed by uRDT (25). The performance of standard RDT as well as uRDT has been tested recently in the framework of CHMI studies for early diagnosis. However, RDTs do not meet the diagnostic sensitivity required to detect early blood-stage parasitemia (<1,000 parasites/ml). The sensitivity of RDTs is similar to that of expert microscopists (limit of detection [LOD] = ~10 parasites/μl) in the CHMI trial platform (26). Therefore, in this study, we used ultrasensitive RT-qPCR as the only comparator to RPA.

LAMP is another diagnostic tool recommended by the WHO Global Malaria Programme for use in low-transmission settings (1). More than 26 malaria LAMP assays have been developed, and two are commercially available as a ready-to-use kit: the

TABLE 6 Performance of RT-RPA, LAMP, and microscopy compared to that of RT-qPCR

Method	Result	RT-qPCR reference (*n*)		PPA[a] (95% CI)
		Positive	Negative	
RT-RPA	Positive	9	0	90 (55–100)
	Negative	1	0	
LAMP	Positive	2	0	20 (3–56)
	Negative	8	0	
TBS[b]	Positive	3	0	30 (7–65)
	Negative	7	0	

[a]PPA, positive percent agreement.
[b]TBS, thick blood smear.

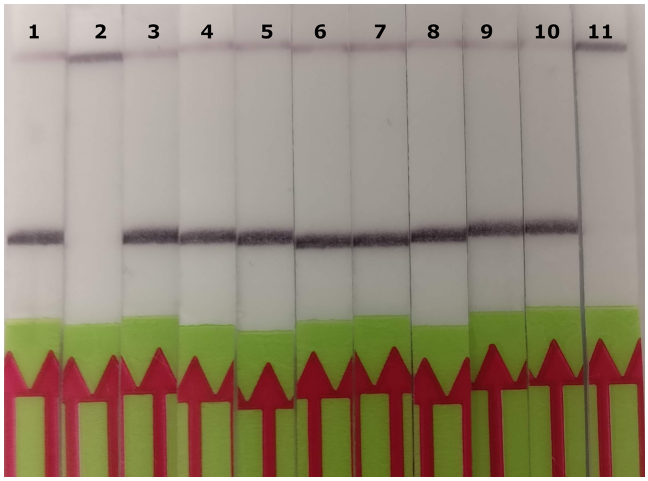

FIG 3 Recombinase polymerase amplification of *P. falciparum* NF54. Strip 1, volunteer 1, day 9 post-CHMI; strip 2, volunteer 2, day 9 post-CHMI; strip 3, volunteer 3, day 9 post-CHMI; strip 4, volunteer 4, day 9 post-CHMI; strip 5, volunteer 5, day 9 post-CHMI; strip 6, volunteer 1, day 11 post-CHMI; strip 7, volunteer 2, day 11 post-CHMI; strip 8, volunteer 3, day 11 post-CHMI; strip 9, volunteer 4, day 11 post-CHMI; strip 10, volunteer 5, day 11 post-CHMI; strip 11, malaria-naive healthy control.

Loopamp MALARIA kit (Eiken Chemical Co.) and the Illumigene malaria LAMP (Meridian Bioscience) (27). Similar to the results from the previous field evaluations in Uganda (28) and Zanzibar (29), our result showed the performance of Loopamp MALARIA Pan/Pf detection kit (Eiken Chemical Co.) is not reliable for pre-elimination settings especially for screening low-density infections. Recently, RT-LAMP with increased diagnostic sensitivity compared to that of commercial LAMP kit has been developed (30). Together with our findings, this corroborates that targeting highly abundant and stable rRNA could greatly benefit malaria diagnosis of low parasite levels for malaria control and elimination efforts.

This is the first study to evaluate the diagnostic performance of RT-RPA for the detection of *P. falciparum* infections. RT-RPA far exceeded the sensitivity of the LAMP kit evaluated in this study. Notably, this study demonstrated that RT-RPA worked using unprocessed blood for the rapid detection of *P. falciparum*, i.e., without any nucleic acid extraction step, although purification of nucleic acids improved the diagnostic sensitivity. Further optimization is needed to improve the direct use of blood for malaria diagnosis as well as determine the diagnostic performance using nucleic acid purification from standard dried blood spots (DBS).

The assay limitations include a reduction in specificity of the lateral flow assay with incubation time. Thus, results should be interpreted immediately (within 10 min), which may be a hindrance to large sample analyses. However, we observed improved specificity by using 1× PBST buffer (with 0.5% Tween) as diluent for lateral flow assays. The other is a residual risk of carryover contamination using the lateral flow strips employed in this study. To detect the RPA product, the amplified product was first diluted and pipetted to a dipstick for analysis. This inevitable step thereby increases the risk of contamination of work surfaces and equipment with abundant amplicons from the reaction tube. Minimizing the chance of contamination is likely to be the major challenge for routine use of the assay with the current lateral flow strips, especially outside the laboratory. As an alternative, the new disposable lateral flow cassette (USTAR01, TwistDx) allows direct detection in a sealed cartridge without the need for dilution and opening of tubes, thus minimizing the chance of cross-contamination, suitable for field use. In addition, with modification in probe design, real-time detection of amplification is possible, e.g., by fluorescent probes and use of a real-time PCR instrument (or a fluorometer at 40°C). Hence, the technique is amenable to nucleic acid quantification and is compatible to standard high-throughput setups when a fully equipped laboratory is available.

In conclusion, this study highlighted steps taken toward the development of a simplified molecular test that shows potential as an effective screening tool for the rapid and accurate detection of submicroscopic *P. falciparum* infections.

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We report no conflicts of interest.

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