Prevalence of *Plasmodium* spp. in Symptomatic BaAka Pygmies Inhabiting the Rural Dzanga Sangha Region, Central African Republic

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Abstract

Background: Malaria remains a diagnostic and therapeutic challenge in many endemic regions of sub-Saharan Africa. The objective of the study was to assess the prevalence of *Plasmodium* spp. in BaAka Pygmies with clinical symptoms of malaria, inhabitants of the rural Dzanga Sangha region in Central African Republic. Additionally, the research aimed to define the percentage distribution of infections caused by other than *P. falciparum* species in order to assess the need for diversification of malaria treatment protocols in the region of Central Africa.

Material and methods: The study was conducted during dry and rainy season in 2018 and involved a group of 540 symptomatic BaAka Pygmies, patients of both sexes, aged 1–75 years old. Two diagnostic methods for detecting *Plasmodium* in the bloodstream were used: RDTs targeting HRP2–protein specific for *P. falciparum* and PCR assays aimed at detecting *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* species.

Results: Only 40.5% of symptomatic patients tested with RDTs for *P. falciparum* infections were positive. While molecular tests (PCR) confirmed *P. falciparum* in 94.8% of the samples and also revealed the genetic material of *P. malariae* (11.1%), *P. ovale* (9.8%), and *P. vivax* (0.7%). BaAka Pygmies aged <5 years old dominated in patients with positive results; the common clinical symptoms reported by the sick individuals were fever, shivers and fatigue.

Conclusions: The presented study suggest the need for introducing more accurate diagnostic methods for the diagnosis of malaria and the revision of malaria treatment protocols. The assessment of the Pfhrp2/Pfhrp3 deletions is necessary for evaluating malaria epidemiology in Central Africa.

Background

Malaria, a vector-borne parasitic disease, is a major health issue in sub-Saharan Africa. In humans it is caused by five different species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. The disease is transmitted from a bite of malaria-infected female *Anopheles* mosquito, which introduces the invasive forms of *Plasmodium* (sporozoites) into the human host's bloodstream. It may also be transmitted through transfusion of blood containing *Plasmodium* trophozoites or schizonts, or by the vertical route, i.e. from the infected mother to the fetus. *Plasmodium* life cycle is divided into several distinct stages taking place in the red blood cells and the liver.

Malaria is endemic in 95 countries and affects approximately 3 billion people globally. In 2018, the World Health Organization (WHO) reported of 228 million malaria cases, of which 93% were reported in Africa. In the same year, there were an estimated 405,000 deaths from malaria globally, 94% of the deaths in Africa [1, 2]. The most common pathoetiological malaria species responsible for a vast majority of all cases worldwide are *P. falciparum* and *P. vivax* (80–95%). The WHO reports have pointed to the predominance of *P. falciparum* infections in Africa [1]. Therefore, in line with the WHO recommendations, malaria control on this continent is primarily based on the use of rapid diagnostic tests (RDTs) targeting the HRP2–protein found exclusively in *P. falciparum*. The results obtained from RDTs, however, do not

reflect the actual number of malaria cases in the region. The exact rates of malaria caused by other than *P. falciparum* species in sub-Saharan Africa are unknown or underestimated. The important consequence of this fact is the need for introducing a different drug protocol for the treatment of other than *P. falciparum* infections.

Patients infected with *P. vivax* or *P. ovale* require an extended course of antimalarial treatment. In *P. vivax* and *P. ovale* infections the predominant proportion of merozoites is released into the bloodstream, while a certain number invades other hepatocytes (parenchymal cells in the liver) entering a dormant phase known as the hypnozoite. Hypnozoites can reactivate and undergo erythrocytic schizogony at a later time resulting in the relapse of malarial symptoms after several weeks or even years from the primary infection; therefore the treatment of *P. vivax* and *P. ovale* requires the use of 8-aminoquinoline (primaquine) targeting the liver stage schizonts and preventing a relapse [1].

The diagnosis of malaria is based on the detection of *Plasmodium* parasites in the bloodstream. Currently, RDTs targeting *Plasmodium* antigen or enzymes are used as the major diagnostic tool, especially for screening purposes. In Central African Republic (CAR), RDTs detecting histidine rich protein 2 (HRP2-protein) of *P. falciparum* have been distributed free of charge. However, in cases of low parasitemia or when malaria is caused by other than *P. falciparum* species, RDTs are likely to give a negative result. The gold standard, unavailable in the most remote areas of sub-Saharan Africa, are light microscopy and molecular biology methods, mostly polymerase chain reaction (PCR).

According to the WHO, the number of malaria cases in CAR, the country inhabited by 4.8 million people, was estimated at 1.8 million and the number of deaths due to malaria at 4,800 (383,000 cases and 3,689 deaths were laboratory-confirmed). All of the reported malaria cases, and malaria deaths, were reported as *P. falciparum* infections, even though the tests for *P. vivax* or *P. ovale* were rarely being conducted [3]. The vast majority of patients in the CAR received the artemisin-combined therapy (ACT) which requires the administration of six doses of arthemeter-lumefantrine over three consecutive days. The majority of patients in the rural regions of CAR, including hunters-gatherers BaAka Pygmies inhabiting the local forests, obtain medical care at the lowest level medical facilities or mobile health units run by non-governmental organizations (NGOs). The most common health problems among BaAka Pygmies are fevers of unknown origin (FUO), which are commonly diagnosed as malaria. About 50% of malaria cases are confirmed with *P. falciparum*-specific RDTs, but the remaining group of patients with a negative result of RDTs do not receive any anti-malarial treatment despite the presence of malarial symptoms: recurring fever, shivers, dehydration, abdominal pain, diarrhea and vomiting. If left untreated, malaria can be fatal, especially in children < 5 years old, pregnant women and patients with comorbidities.

The objective of this study was to assess the prevalence of *Plasmodium* spp. in BaAka Pygmies with clinical symptoms of malaria, inhabitants of the rural Dzanga Sangha region in Central African Republic (CAR). Additionally, the research aimed to define the percentage distribution of infections caused by different than *P. falciparum* species in order to assess the need for diversification of malaria treatment protocols in the region of Central Africa.

Material And Methods

The study was conducted during dry and rainy season in 2018 and involved a group of 540 BaAka Pygmies with clinical symptoms of malaria, patients of both sexes, aged 1–75 years old, living in the rural areas within 50 km distance from Monasao village in the south-western parts of the CAR (Fig. 1.). Two diagnostic methods for detecting *Plasmodium* in the bloodstream were used. During the first stage, immunochromatographic RDTs targeting *P. falciparum*-specific HRP2–protein (CareStart Malaria Pf/HRP2/Ag, Access Bio; SD Malaria Ag Pf/05FK50/, Bioline Standard Diagnostics, Inc.) were performed. The tests were carried out immediately after collecting 0.5 ml of whole blood from the participants enrolled in the study; the samples were collected by venopuncture. The next stage of the study was the application of blood samples onto the FTA Micro Cards (Whatman™); the biological material was then transported to Poland (under the agreement with the Ministry of Science and Research, CAR). The specimens were analyzed at the Institute of Maritime and Tropical Medicine, Department of Tropical Parasitology of the Medical University of Gdańsk, Poland with the PCR method (detecting four species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*).

Patients included in the study were Pygmies presenting with malaria clinical symptoms: fever, shivers, signs of dehydration, abdominal pain, diarrhea, vomiting. On behalf of the pediatric patients, the consent to participate in the study was given by their parents/guardians. Excluding criteria of the study were anti-malarial treatment received in the past 28 days and difficulties in venopuncture procedure.

Immunochromatographic rapid diagnostic test (targeting HRP2-protein of *P. falciparum*)

RDTs are based on the immune reaction. A drop of venous blood (5 µl, taken from the test tube with a calibrated capillary attached to the test kit), placed in the appropriate well on the test cassette, moves along the nitrocellulose membrane (chromatographic paper) using capillary effects. After placing the drop of blood in the round well, 2–3 drops of assay diluent (depending on the manufacturer of the test) are put in the second well situated closer to the edge of the test cassette which is part of the test kit. The test pad is pre-coated with specific antibodies., most often in a complex with colloidal gold. After the blood sample is absorbed by the membrane, *P. falciparum* HRP2 antigen binds to the antibody-colloidal gold complex (if the test is positive). The reaction leads to the formation of antigen-antibody complexes which the test is able to detect. The interpretation of the test will depend on the presence or absence of colour bands in the control and test areas (the presence of the color line in the test area confirms the presence of antigens in the blood sample). The appearance of bands in both areas - the control and the result area indicates a positive result. The appearance of the band only in the control area indicates a negative result. The control band should appear for all results, if not, the result is considered as invalid, it should not be interpreted and a new test kit should be used. The interpretation of the test is possible after 15 minutes. The sensitivity of the RDTs is 88–90% (depending on the manufacturer) [4].

PCR (polymerase chain reaction)

PCR is the method of choice (its sensitivity is 100%) and is used to confirm the diagnosis of malaria, especially in doubtful cases or in mixed infections with two or more different species of *Plasmodium*. Venous blood (300 µl) is applied onto a filtered paper FTA Micro Card (Whatman™), which is then stored in a foil barrier bag until the PCR test can be performed in a molecular biology laboratory. The structure and composition of FTA blotting paper enable automatic DNA isolation on its surface. Therefore, FTA cards are ready for molecular testing immediately after a blood sample has been applied onto the card.. The blotting paper can be stored for a long period (up to several years) at room temperature, maintaining its diagnostic properties. The advantages of using FTA filtered paper include: simple collection and safe storage (nucleic acids are automatically preserved without the need for refrigeration), fast purification (nucleic acids are purified on FTA cards by a simple three-step procedure, all in a single test tube at room temperature; DNA is immobilized on the matrix and ready for PCR or other amplification techniques), automation (by using reusable punches, cutting out a fragment which is the template in PCR). Storing DNA on FTA filtered papers reduces the need for freezing the samples (thus reducing storage costs). DNA retained on the filter paper is ready for purification and analysis after 30 minutes. It only requires washing in specific reagents and TE buffer, after which the disk is ready for molecular analysis. One field of blotting paper is capable of storing up to 125 µl of whole blood.

Identification *Plasmodium* species and their differentiation with molecular biology methods

To confirm the *Plasmodium* DNA in the tested sample and differentiate the species responsible for malaria symptoms, the multiplex nested PCR reaction based on fragments of the SSU rRNA gene is used. In the first step of the reaction, a gene fragment common to all *Plasmodium* species is amplified, while in the second step, species-specific fragments are amplified: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*. PCR reactions were performed on the GeneAmp PCR System 9700 Thermal Cycler apparatus (Applied Biosystems, USA) and the PCR products, after electrophoretic separation, were analyzed on the Gel Doc-It Imaging System apparatus (UVP, USA).

Sequencing

In order to confirm the results obtained by PCR methods, the products of this reaction were sequenced. The obtained DNA sequences were compared with the sequences available in the Gene Bank. The chain termination sequencing method (Sanger method) uses the properties of dideoxynucleotides, i.e. nucleotides that do not have a hydroxyl group (and only hydrogen in the 2' and 3' positions of the sugar). The attachment of a dideoxynucleotide to the newly synthesized DNA inhibits strand elongation, due to the lack of a hydroxyl group at the 3' position of the pentose necessary for the formation of the phosphodiester bond. Dideoxynucleotides are fluorescently labeled. The detector distinguishes between the individual fluorescent markers and the DNA sequence which is read directly as the electrophoresis bands move along one lane in the polyacrylamide gel. The obtained sequences were analyzed using specialist software, e.g. GeneStudio™ Professional (GeneStudio, Inc., USA) and Chromas DNA sequencing software.

Statistics

All statistical calculations were performed using the StatSoft statistical package STATISTICA Inc. (2014) (data analysis software system) version 12.0. www.statsoft.com and an Excel spreadsheet. Quantitative variables were characterized by the arithmetic mean, standard deviation, median, minimum and maximum value (range), and 95%CI (confidence interval). The variables of the qualitative type were presented in terms of counts and percentages (percentage). Chi-square tests of independence were used for qualitative variables (respectively using Yates correction for cell counts below 10, checking Cochran conditions and Fisher's exact test). Cohen's kappa and Scott's pi coefficients inter-rater reliability were used to assess the compliance of RDT and PCR methods. In all calculations, the level of significance was set at $p = 0.05$.

Results

The study involved 540 BaAka Pygmies (328 females and 212 males) presenting with clinical signs of malaria; *P. falciparum*-specific RDTs were positive in 40.5% of the subjects. While PCR tests performed on the same blood samples confirmed *P. falciparum* infections in 94.8% of the study group; in addition, PCR tests revealed infections with species other than *P. falciparum*(Pf), i.e. *P. malariae*(Pm), *P. ovale*(Po) and *P. vivax*(Pv), which were not detected by RDTs targeting solely HRP2-protein specific for *P. falciparum* (Table 1).

Table 1
Detection rates of *Plasmodium* species by RDTs and PCR in the group of BaAka Pygmies with clinical symptoms of malaria (n = 540)

Method <i>Plasmodium</i> species	CareStart Malaria Pf /HRP2/Ag test	Molecular biology (PCR)
<i>P. falciparum</i>	40.5%	94.8%
<i>P. vivax</i>	0%	0.7%
<i>P. ovale</i>	0%	9.8%
<i>P. malariae</i>	0%	11.1%

The test results were not consistent between the methods used (RDTs and PCR). Cohen's kappa coefficient inter-rater reliability (0.03/0.02) was close to zero, i.e. at the random level.

In turn, Scott's pi coefficient inter-rater reliability (-0.34/-0.28) took a negative value, i.e. lower than at the random level (Table 2).

Table 2
Comparison of RDTs and PCR results in BaAka Pygmies with clinical symptoms of malaria
(n = 540)

Coefficient inter-rater reliability	Females (n = 328)	Males (n = 212)	Total (n = 540)
Cohen's kappa	0.03	0.02	0.03
Scott's pi	-0.34	-0.28	-0.31

Table 3 presents the demographic data (age, gender, body weight) of the BaAka Pygmies tested with RDTs including their body temperature and of the time between symptoms onset and medical consultation. A majority of subjects with positive tests results were individuals < 5 years old, with body temperature < 38.0 °C, and symptoms lasting for up to 7 days.

Table 3
Demographic and clinical data of BaAka Pygmies tested with RDTs (n = 540)

Demographic and clinical data	RDT (–) negative (n = 321)	RDT (+) positive (n = 219)	Total (n = 540)	P-value
sex				0.0070 ¹
female	210 (65.4%)	118 (53.9%)	328 (60.7%)	
male	111 (34.6%)	101 (46.1%)	212 (39.3%)	
age (years)				0.0001 ²
< 5	81 (25.2%)	142 (64.8%)	223 (41.3%)	
5–18	58 (18.1%)	33 (15.1%)	91 (16.9%)	
> 18	182 (56.7%)	44 (20.1%)	226 (41.9%)	
body weight (kg)				0.0001 ²
< 10	58 (18.1%)	96 (43.8%)	154 (28.5%)	
10–40	154 (48.0%)	98 (44.7%)	252 (46.7%)	
> 40	109 (34.0%)	25 (11.4%)	134 (24.8%)	
body temperature (°C)				0.0001 ²
< 38.0	209 (65.1%)	110 (50.2%)	319 (59.1%)	
38.0–39.0	84 (26.2%)	52 (23.7%)	136 (25.2%)	
> 39.0	28 (8.7%)	57 (26.0%)	85 (15.7%)	
duration symptoms (days)				0.0001 ²
1–7	244 (76.0%)	198 (90.4%)	442 (81.9%)	
8–28	35 (10.9%)	8 (3.7%)	43 (8.0%)	
> 28	42 (13.1%)	13 (5.9%)	55 (10.2%)	
¹ Chi-square, ² U Mann–Whitney				

Patients with positive results of the RDTs were more likely to present with fever, fatigue, and shivers (Table 4).

Table 4
BaAka Pygmies with clinical symptoms of malaria tested with RDTs (n = 540)

Symptoms of malaria	RDT (–) negative (n = 321)	RDT (+) positive (n = 219)	Total (n = 540)	P-value
fever	231 (72.0%)	182 (83.1%)	413 (76.5%)	0.0027 ¹
shivers	228 (71.0%)	96 (43.8%)	324 (60.0%)	0.0001 ¹
headache	141 (43.9%)	41 (18.7%)	182 (33.7%)	0.0001 ¹
dizziness	50 (15.6%)	16 (7.3%)	66 (12.2%)	0.0040 ¹
generalized pain	172 (53.6%)	60 (27.4%)	232 (43.0%)	0.0001 ¹
arthralgia and myalgia	174 (54.2%)	47 (21.5%)	221 (40.9%)	0.0001 ¹
weakness	154 (48.0%)	113 (51.6%)	267 (49.4%)	0.4083 ¹
vomiting	72 (22.4%)	77 (35.2%)	149 (27.6%)	0.0012 ¹
diarrhea	78 (24.3%)	87 (39.7%)	165 (30.6%)	0.0001 ¹
abdominal pain	87 (27.1%)	41 (18.7%)	128 (23.7%)	0.0245 ¹
loss of appetite	87 (27.1%)	70 (32.0%)	157 (29.1%)	0.2220 ¹
¹ Chi-square				
A majority of patients with positive results of the PCR tests (as in the case of positive RDTs) were aged < 5 years old, with body temperature < 38.0°C, and symptoms lasting for up to 7 days (Table 5).				

Table 5
Demographic and clinical data of BaAka Pygmies tested with PCR (n = 540)

Demographic and clinical data	PCR <i>Pf/Pv/Po/Pm</i> ² (-) negative (n = 24)	PCR <i>Pf/Pv/Po/Pm</i> (+) positive (n = 516)	P-value
sex			0.0586 ¹
female	19 (79.2%)	309 (59.9%)	
male	5 (20.8%)	207 (40.1%)	
age (years)			0.4055 ¹
< 5	7 (29.2%)	216 (41.9%)	
5–18	4 (16.7%)	87 (16.9%)	
> 18	13 (54.2%)	213 (41.3%)	
body weight (kg)			0.9189 ¹
< 10	6 (25.0%)	148 (28.7%)	
10–40	12 (50.0%)	240 (46.5%)	
> 40	6 (25.0%)	128 (24.8%)	
body temperature (°C)			0.5709 ¹
< 38.0	16 (66.7%)	303 (58.7%)	
38.0–39.0	6 (25.0%)	130 (25.2%)	
> 39.0	2 (8.3%)	83 (16.1%)	
duration symptoms (days)			0.7433 ¹
1–7	20 (83.3%)	422 (81.8%)	
8–28	1 (4.2%)	42 (8.1%)	
> 28	3 (12.5%)	52 (10.1%)	
¹ Chi-square			
² <i>Pf</i> - <i>P. falciparum</i> ; <i>Pv</i> - <i>P. vivax</i> ; <i>Po</i> - <i>P. ovale</i> ; <i>Pm</i> - <i>P. malariae</i>			

BaAka Pygmies with positive PCR test results were more likely to present with fever, shivers, and fatigue (Table 6).

Table 6
BaAka Pygmies with clinical symptoms of malaria tested with PCR (n = 540)

Symptoms of malaria	PCR <i>Pf/Pv/Po/Pm</i> (–) negative (n = 24)	PCR <i>Pf/Pv/Po/Pm</i> (+) positive (n = 516)	P-value
fever	17 (70.8%)	396 (76.7%)	0.5045 ¹
shivers	16 (66.7%)	308 (59.7%)	0.4952 ¹
headache	8 (33.3%)	174 (33.7%)	0.9687 ¹
dizziness	2 (8.3%)	64 (12.4%)	0.5518 ¹
generalized pain	10 (41.7%)	222 (43.0%)	0.8956 ¹
arthralgia and myalgia	11 (45.8%)	210 (40.7%)	0.6169 ¹
weakness	13 (54.2%)	254 (49.2%)	0.6360 ¹
vomiting	3 (12.5%)	146 (28.3%)	0.0906 ¹
diarrhea	7 (29.2%)	158 (30.6%)	0.8799 ¹
abdominal pain	5 (20.8%)	123 (23.8%)	0.7352 ¹
loss of appetite	12 (50.0%)	145 (28.1%)	0.0209 ¹
¹ Chi–square			

Discussion

According to the WHO data, *Plasmodium falciparum* is responsible for 99.7% of all malaria cases in sub-Saharan Africa [1] and as much as 100% malaria cases in the CAR [3].

The results of the present study suggest otherwise.

In 2018, the WHO reported of 1,367,986 suspected malaria cases in the CAR, of which 972,119 (71.1%) were confirmed as *Plasmodium falciparum* malaria. Infections caused by *Plasmodium* other than *falciparum* or mixed infections were not found [1]. The WHO reports that samples were examined by light microscopy or RDTs. A total of 117,267 malaria cases were not confirmed by any diagnostic method [1]. If, however, the molecular biology PCR methods had been used, the results could have been surprising.

The data on the prevalence of different species of malaria in the Central African Republic (CAR), collected in independent researches is insufficient to define the epidemiological situation in this country. Decades of conflicts and political instability disrupted research field in many directions. The results of another

studies which had been conducted in Africa have also indicated that the rates of infections with other *Plasmodium* species are higher than the WHO suggests.

More accurate assessment of malaria epidemiology in CAR could be obtained by analyzing the data gathered in the neighboring countries with similar climate conditions. The overall malaria prevalence in Cameroon is 29% [5]. In the group of children aged 6 months up to 5 years old malaria was reported in 30% in 2011 but there were differences within urban and rural zones with malaria prevalence at the level 20.6% and 37.1% respectively [6]. The eastern region of Cameroon, i.e. the area with the largest CAR refugee population (due to recurrent ethnic and political conflicts) is considered the main cause of high malaria transmission in Cameroon [7], suggesting a higher malaria prevalence rate in the much poorer CAR. The studies which were conducted in Cameroon using molecular biology methods, confirmed the presence of four species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* [7–10], with a definite predominance of *P. falciparum*, responsible for 95% of infections [7]. *P. vivax* was found in 5.6% patients (which constituted 38.6% of all PCR positive samples) [9], *P. ovale* and *P. malariae* were found in a very low percentage, as a co-infection predominantly [11]. These results were consistent with the results obtained by the authors of the present research task. The data published by Kwenti et al. proves unequivocally that *P. falciparum* is the unique cause of malaria in Cameroon [12], which is a conclusion contrary to the results of the above-mentioned studies.

Another study on the prevalence of malaria in the neighboring region was carried out by Tsumori et al. [13] in the Republic of Congo, adjoining to the north with the CAR and it involved patients from both urban and rural areas presenting with fever. The study demonstrated that malaria infection rates were found to be different between the methods used. Microscopic examination revealed that 37% of the subjects living in urban areas were infected with malaria, whereas PCR tests were positive in 42% of the sample. In the group coming from rural areas, RDTs were positive in 59% cases and PCR tests in 72% of the subjects [14]. The studies conducted in the Republic of Congo indicate that *P. falciparum* was responsible for a vast majority of malaria cases in the region [13, 15, 16], but cases caused by *P. vivax* [17], *P. ovale* and *P. malariae* have also been reported and should not be neglected [13].

According to the results of the present study conducted in Dzanga Sangha, *P. falciparum* was responsible for 94.8% of all malaria cases; however, the percentage distribution of other *Plasmodium* species was significantly higher (*P. malariae* 11.1%, *P. ovale* 9.8%, *P. vivax* 0.7%) than the 0.3% reported in the 2019 WHO report. This fact is important in the context of the treatment of malaria patients and highlights the necessity to introduce more accurate diagnostic methods and to revise malaria treatment protocols (introduction of a drug regimen targeting the latent forms of *Plasmodium* residing in the liver).

In a routine malaria diagnostics in rural areas in CAR the role of PCR is none, but in the context of the epidemiological studies is crucial. The PCR was essential to establish the presence of the different *Plasmodium* species and was a comparative method for RDTs false-negative sensitivity and try to find the factors responsible for it.

A study by Berzosa et al. [18] conducted in Equatorial Guinea confirmed the presence of *P. falciparum* alone in 69.2% of all RDT positive samples, and in 27,8% with other than *P. falciparum* species as a co-infection, whereas PCR tests were positive for *P. falciparum* in 97% cases and in 1,9% PCR tests also revealed infections with *P. malariae*, *P. ovale* and *P. vivax*. A study which was conducted in Senegal and was based on microscopic examination of blood samples taken from study participants demonstrated that apart from *P. falciparum* (45.1%) infections, *P. ovale* and *P. malariae* infections were also detected (together, they accounted for 5.6% malaria cases) [19]. The epidemiology of malaria and prevalence of different species of *Plasmodium* in CAR can be similar to these described in the surrounding countries mentioned above.

Until 2010, patients with suspected malaria were administered the recommended treatment but were rarely offered diagnostic tests, which was largely due to limited testing capabilities in the region. Since 2010, however, owing to the WHO support, and its recommendations for pre-treatment diagnosis of malaria in every symptomatic patient, RDTs and microscopic examinations have become more common [20]. RDTs in particular are now widely used in the region, and their sales increased from 46 million in 2008 to 320 million in 2013 globally [4].

In the rural areas of Africa, such as Dzanga Sangha in the south-western parts of the CAR, RDTs are often the only available diagnostic method for malaria detection. Unfortunately, the number of RDTs distributed by non-governmental organizations (NGOs), as part of the global malaria program is often insufficient. The author's (EB-S) observations made during the study period have led to the conclusion that malaria diagnosis and treatment protocols applicable in the CAR leave much to be desired. As an example, a vast majority of medical interventions in the Dzanga Sangha region are performed by people without a medical background; the use of expired medications and RDTs or random drug use is common. Additionally, medications and RDTs are rarely stored properly and a large number of patients purchases medications at local street markets.

The results of the research task conducted in the group of BaAka Pygmies have demonstrated significant differences between the results of RDTs and PCR tests. Therefore the question should be raised on the accuracy and usefulness of RDTs in a situation, where the initiation of anti-malaria treatment depends entirely on the interpretation of the RDTs results, regardless of the patient's condition or the severity of clinical signs and symptoms. On the other hand, the decision to administer anti-malarial treatment on the basis of non-specific symptoms, which may be the manifestation of many other diseases, e.g. bacterial infections of the respiratory and urinary tract or cosmopolitan and tropical diseases of viral etiology, is not always justified and may lead to overdiagnosis of malaria [21, 22]. Primary healthcare providers in sub-Saharan Africa often prescribe anti-malarial treatment in a combination with other antibiotics to treat all kinds of health problems a patient may have, without performing the basic diagnostic tests [21, 23].

There are four main possible causes of false-negativity of the RDTs. Lower sensitivity of RDTs (estimated at 88–90% by their manufacturer) compared to PCR tests is attributable to several factors. One of the limitations of an RDT is its detection limit which has been estimated at between 200 and 2000–5000

parasites/ μ l, which corresponds to 200 infected erythrocytes per microliter [4]. In cases of very low parasitemia, RDT is likely to give a false negative result. This conclusion is also supported by the work of Djallé et al. conducted in Central African Republic where the sensitivity of three different RDTs was proved to correlate with an intensity of the parasitemia in the blood sample. The sensitivity was 95% in a high parasitemia level (> 500 parasites/ μ l), but in low parasitemia (100 parasites/ μ l) the sensitivity was lower than 70% [24]. Many researchers have addressed the issue of malaria parasite transmission on the sensitivity of RDTs. The authors agree, that in areas with low malaria transmission, where low parasitemia $\leq 200/\mu$ l is more common, the sensitivity of RDTs is lower than in areas with high transmission rates [25–30]. This applies to approximately half of *P. falciparum* infections worldwide [31].

Upon comparison of patients with a positive vs. patients with a negative RDT result we concluded that patients aged < 5 , weighing up to 10 kg were more likely to have a positive RDT result (64.8% vs. 25.2%). The conclusion is consistent with the WHO data [1], according to which malaria is most prevalent in children < 5 years old. The results were quite the opposite in the group of adults aged over 18 years old (20.1% vs. 56.7%).

In the group of patients with an RDT-positive result, there were significantly more patients with body temperature $> 39^{\circ}\text{C}$ (26.0% vs 8.7%). It can be assumed that these patients had a higher level parasitemia with *P. falciparum* and therefore the HRP2–specific RDT was positive in their case [24, 32], or that the elevated body temperature in the youngest subjects could have been a result of their hyper-reaction to infection with *P. falciparum* given their lack of immunity, (with age, people living in endemic areas acquire immunity to *P. falciparum* malaria naturally) [19, 33–35]. Premunition or otherwise a partial immunity observed in the asymptomatic or poor-symptomatic individuals with the detectable presence of malaria parasites in the blood, inhabiting endemic areas is gained with repeated contacts/infections with *Plasmodium* spp. It is considered as a protective factor against the severe manifestation of the disease [36]. Another cause of the false-negative RDTs might be deletion of Pfhrp2 gene which results in the lack of the antigen HRP2-protein which is a target for the RDTs HRP2.

A high percentage of infections caused by *P. falciparum* parasites with a Pfhrp2 gene deletion has been reported from sub-Saharan Africa, in the Indian subcontinent and in some countries of South America (Peru). Pfhrp2 gene is responsible for the production of HRP2 protein, which is an antigen detected by the *P. falciparum*-specific RDTs. The rate of such infections has been estimated at approximately 5% in sub-Saharan Africa [37–39], up to 40% in Peru [40, 41] and even over 80% in Eritrea depending on the region [42]. As suggested by the WHO, the use of RDTs targeting HRP2 protein for the diagnosis of malaria is questionable when the percentage of *P. falciparum* species with a Pfhrp2 deletion is over 5% in a given area [43, 44]. The threshold of 5% was assumed to have an important impact on public health, and the number of undetected cases was lower than when tests targeting other less sensitive *P. falciparum* antigens were used [45]. There are no data available on the prevalence of species with Pfhrp2 deletion in the Central African Republic; however, in the neighboring Democratic Republic of Congo, the rates of *P. falciparum* species with the Pfhrp2 gene deletion have been estimated at 6.4% [46], the results may be similar in other countries of the region, including the CAR. The authors of the present research task did

not undertake the analysis of exon 2 for the assessment of the Pfhrp2/3 deletion. Further studies to evaluate the prevalence of the Pfhrp2/3 gene deletion seem necessary in the context of determining the usefulness of RDTs targeting HRP2 protein in Central Africa.

In the study by Mensah–Addai et al. which was carried out in Ghana, the issue of genetic variability of exon 2 of the Pfhrp2 gene, which may result in lower sensitivity of RDTs using the *P. falciparum* HRP2 protein antigen was raised [13].

Furthermore the existence of anti-HRP2 antibodies forming the immune-complexes with HRP2 antigen in *P. falciparum* infected individuals may be another cause of the false-negative RDTs results [47].

Amoah et al. tested blood samples taken from patients living in Rwanda who presented with clinical symptoms of malaria using three methods: RDTs, microscopy examination and PCR; of all RDT-negative samples 24.4% tested positive when examined by light microscopy and 44.1% were positive when PCR tests were used [48]. The differences between the results obtained by using different methods were less significant in comparison to the results of the present study, yet, they clearly show that a large proportion of RDTs are false-negatives. In Gambia, where the epidemiological situation has improved significantly over the past twenty years, owing to the introduction of malaria control measures [26, 27], Mwesigwa et al. screened a group of symptomatic and asymptomatic patients using two diagnostic methods: RDTs and PCR. The results of the study demonstrated that 62% of the RDT-negative samples were found to be positive when PCR method was used [49].

In order to reduce the number of false negative RDT results, in countries with a high prevalence of *P. falciparum* species with a Pfhrp2 deletion or regions with high rates of malaria parasites other than *P. falciparum*, RDTs detecting pLDH are additionally used [32] (they are, however, less sensitive in cases of low parasitemia ≥ 200 –1000 parasites / μ l than RDTs directed against HRP2 protein [19] or aldolase). Both enzymes are produced by all malaria species found in humans [43] and have a short half-life of 2–4 days. Therefore, they can be found in the blood of individuals with an active malaria infection [50]. Nevertheless, the WHO recommends restricting the use of RDTs that detect both HRP2 and pLDH proteins to regions where infections by one species of malaria are expected [51], presumably for a cost-effective reasons. According to the author's observations, RDTs other than those targeting HRP2 specific for *P. falciparum* were not available in the Dzanga Sangha region.

In recent years, there has been a lot of debate on the usefulness of RDTs in malaria diagnosis and control. In some countries, microscopic examination of thick blood films remains the gold standard for diagnostics of malaria [52, 53], in other countries PCR tests are the recommended detection method in the epidemiological studies or research [32, 49], in others both methods are in use [13, 25, 48, 54]. It is difficult to decide which of these methods are optimal and should be recommended for malaria diagnosis.

Conclusions

Malaria diagnosis is an important element of effective treatment, and thus limiting the transmission and ultimately elimination of the disease in the world. In developing countries in sub-Saharan Africa, RDTs provide a good, though imperfect, alternative to clinical symptoms-based diagnosis. The presented study of BaAka Pygmies, showing a significant disproportion of RDTs and PCR results, requires that the clinical manifestation should be an important component of the final diagnosis. It is worth considering whether, due to the presence of over 5% of infections with *Plasmodium* species other than *P. falciparum*, the use of RDTs detecting pLDH and/or aldolase would not contribute to greater diagnostic efficiency. In the future, the prevalence of Pfhrp2/Pfhrp3 gene deletions in Central Africa should be assessed, and thus the utility of RDTs based on the detection of *P. falciparum* HRP2 antigen.

Abbreviations

CAR: Central African Republic; WHO: World Health Organization; PCR: polymerase chain reaction; RDT: rapid diagnostic test; HRP2: histidine rich protein 2; ACT: artemisinin-combined therapy; NGO: non-governmental organization; FUO: fever of unknown origin; LLIN: long-lasting insecticidal net.

Declarations

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Availability of data and materials

The datasets supporting the conclusions are included within the article. Raw data used for analysis of the study are available from the corresponding author on reasonable request.

Authors' contribution

EB-S and KK designed the study, EB-S and AL executed the study, EB-S and KK analysed the data and wrote the first draft of the manuscript, All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research task entitled *The assessment of the prevalence Plasmodium infections in sub-Saharan Africa in symptomatic BaAka Pygmies inhabiting the rural Dzanga Sangha region in the Central African Republic* was approved by the Bioethics Committee at the Military Institute of Medicine (resolution no. 137/WIM/2018).

The research task entitled *Evaluation de la survenue d'infections à Plasmodium spp dans la population de Pygmées BaAka des zones forestières de la République Centrafricaine* was approved by the Ministry of the Research and Innovative Technologies in the Central African Republic (resolution no. 176/MERSIT/DIRCAB/CB.18).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Dzanga Sangha Complex of Protected Areas Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.