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PERSISTENCE OF MALARIAL ANTIGEN FOLLOWING ANTIMALARIAL CHEMOTHERAPY IN KOLKATA, WEST BENGAL

Tropical Medicine

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ABSTRACT

Early diagnosis and complete treatment is one of the important aspects of malaria elimination programme worldwide. In many areas the diagnosis is based on detection of malarial antigen using commercially available rapid diagnostic kits. The problem remains with persistence of antigen following parasite clearance by proper treatment. The present work was undertaken to study the pattern of persistent antigen of *P. vivax* and *P. falciparum* following antimalarial treatment. A total of 300 microscopically positive mono-infected with *P. vivax* (160) and *P. vivax* (140) patients were recruited, treated with antimalarial drugs and followed up on day 3, 7, 14, 21 and 28 for persistent parasites and antigen. *P. vivax* specific pLDH antigen was disappeared from peripheral blood within 14 days post treatment period. *P. falciparum* specific HRP-2 antigen was persisted even after 28 days of treatment. Depending only on antigen based diagnosis, attention should be paid before treatment, particularly in areas with high malaria transmission.

KEYWORDS

P. vivax, P. falciparum, HRP2, PLDH, Antigen

INTRODUCTION:

Malaria still remains as major public health problem throughout the world with an estimated 229 million cases and 409000 deaths [1]. The World Health Organization (WHO) launched Global Technical Strategy for malaria 2016-2030 in 2015 with the vision of a World free of malaria by 2030 [2]. Similarly, India developed the National Framework for Malaria Elimination in India 2016 - 2030 in line with the WHO Global Technical Strategy for Malaria 2016–2030 (GTS) and the Asia Pacific Leaders Malaria Alliance Malaria Elimination Roadmap in 2016 [3]. The goals of the National Framework for Malaria Elimination in India 2016–2030 are - eliminate malaria (zero indigenous cases) throughout the entire country by 2030; and maintain malaria–free status in areas where malaria transmission has been interrupted and prevent re-introduction of malaria.The main interventions of this frame work are early detection and treatment of all malaria cases by means of active and/or passive case detection to prevent onward transmission and implementation of effective vector control measures [3].

Proper diagnosis of malaria cases is the first and foremost important steps for the prompt and complete treatment of malaria cases. Several methods are being used for the diagnosis of malaria such as blood smear examination by microscopy, detection of antigen by rapid diagnostic test (RDT) kit, PCR detection, etc. Though microscopy remains the gold standards for detection of malaria [4], RDTs are now being in use in the remote health settings and at field level for active and passive case detection where microscopy is not possible. Different types of RDT kits are now available in the markets and most of them are designed to detect a single parasite antigen, the histidine-rich protein 2 (HRP2) or the Plasmodial lactate dehydrogenase (pLDH), while others are designed to detect both antigens in a single test.

Histidine-rich protein 2 (HRP2) is a unique protein produced exclusively by *P. falciparum*. It is a water-soluble protein produced by trophozoites and young (but not mature) gametocytes of *P. falciparum* [5]. The amino acid sequence of HRP2 contains histidine (37%), and repeats of histidine plus alanine which cover almost 85% of its sequence. HRP2 is exported by the parasite into the RBC cytosol [5]. As parasites rupture from the host cell, RBC cytosolic components, including HRP2, are released into the blood stream. As HRP2 protein is produced only by the *P. falciparum* species so, detection of this protein can therefore confirm only *P. falciparum* infections. HRP2-based RDTs show very high sensitivity and specificity [6]. However, the performance of HRP2-based RDTs is poor in the field, particularly in terms of specificity, due to the slow elimination of the HRP2 antigen from the blood stream. This persistence of the HRP2 antigen means that patients remain to test positive with HRP2-based RDTs long after the successful treatment and complete elimination of the parasites from the patients. During this period, these RDTs cannot distinguish between pr-existing antigen and new infection. This is well documented in the areas with high malaria transmission where patients frequently experience repeated malaria infections and treatments [7, 8, 9].

Plasmodial lactate dehydrogenase (pLDH) is an essential enzyme of *Plasmodium* parasite which is very crucial for survival becauseit helps in anaerobic glycolysis. It is produced at high levels during the blood stage such as asexual and sexual stages (gametocytes) of malaria parasites [10, 11]. pLDH has been widely examined as a promising diagnostic antigen and has been adopted in many RDTs for malaria because the level of pLDH in the blood is directly correlated to the parasitaemia level. Initially, pLDH based RDTs are used to detect pLDH from all four *Plasmodium* species that infect humans and they can distinguish *P. falciparum* from the non-falciparum species, but cannot distinguish between *P. vivax*, *P. ovale* and *P. malariae*. But recently several RDTs have been developed based on the *P. vivax* specific pLDH which can detect *P. vivax* from other human malaria species.Their main advantage is that pLDH is rapidly eliminated from the blood stream after treatment, and therefore pLDH-based RDTs returns a negative result within a few days after treatment. Moreover, few studies have showed that genetic variations of pLDH have no effect on the diagnostic performance of pLDH-based RDTs [12, 13, 14].

Presently several kinds of dual RDTs are available in the market which

are based on *P. falciparum* specific HRP2 and *P. vivax* specific pLDH antigen. These RDTs are now widely used for diagnosis of malaria either alone or along with peripheral blood smear examination by microscopy because it does not require any specific expertise and it gives results very quickly (within 10-15 minutes). The main limitations of use of RDTs are false negative and false positive results. The reasons behind the false negative results are the very low level of parasitemia (5-15 parasite per µL of blood) [15] and deletion in the target antigen gene such as HRP2 [16]. One of the main reasons of false positive results is the persistence of malaria antigens in the blood stream after recent parasite clearance through anti-malarial medication. The persistence of malaria antigen in blood stream depends on several factors such as type of anti-malarial treatment the patient receives [17], parasite density at the time of anti-malarial medication [18], and age of theindividual in malaria endemic areas.

HRP2 antigen may persist in blood circulation up to several weeks after clearance of parasites and effective anti-malarial treatment [19, 20, 21, 22, 23, 24]. Unlike the pfHRP2-based tests, pLDH-based tests can detect all human-related *Plasmodium* species, and pLDH antigen is rapidly cleared from the blood after successful treatment [24, 25, 26, 27]. So, understanding the duration of positivity after successful parasite clearance is also critical for accurate diagnosis of malaria. Reports on persistence of malaria antigen after clearance of parasite and complete treat is very rare. Therefore, the present work was designed to determine the duration of time required for clearance of *P. falciparum* specific HRP2 and *P. vivax* specific pLDH antigen from the blood circulation among microscopically positive mono-infected *P. falciparum* and *P. vivax* cases after completion of anti-malarial treatment.

MATERIALS AND METHODS

Study site and design

The study was carried out at the Malaria Clinic attached to the Department of Protozoology, Calcutta Schoolof Tropical Medicine, Kolkata, India from May, 2017 to April, 2018, where the annual parasite index was 3.72 in 2016. In Kolkata, malaria transmission is seasonal (July-December). *P falciparum* and *P vivax* are the two predominant species, with an incidence of almost 1:1.

The study was an observational study for determination of time duration required for clearance of *P. falciparum* specific HRP2 and *P. vivax* specific pLDH antigen from the blood circulation among microscopically positive mono-infected *P. falciparum* and *P. vivax* cases after completion of anti-malarial treatment. The study protocol was approved by the Institutional Ethics Committee of the Calcutta School of Tropical Medicine.

Screening and enrolment of patients: All the febrile patients from the surrounding areas attendingthe Malaria Clinic of the Calcutta School of TropicalMedicine were screened for malarial parasite by examining Giemsa stained thick and thin peripheral blood smears (PBS) and dual RDTs (SD Bioline One step Malaria HRP2 (Pf) and pLDH (Pv) antigen rapid test kit). All patients with confirmed *P. falciparum* and *P. vivax* mono-infectionwere explained about the study protocol and requested toparticipate in the study. Those who fulfilled the following inclusion criteria were enrolled afterobtaining written, informed consent.

The inclusion criteria are microscopically confirmed *P. falciparum* and *P. vivax* mono infection, with fever (axillarytemperature $\geq 37.5^{0}$C) or history of fever in preceding 24 hours, and no history of malaria or anti-malaria treatment within last one months, no signs and symptoms of complicated malaria.

Treatmentand follow-up: All the study participants were treated as per the Guidelines for Diagnosis and Treatment of malaria in India, 2011. Confirmed *P. falciparum mono-infected* patients were treated with Artemisinin Combination Therapy (ACTs) i.e., AS+SP combination (artesunateat 4 mg/kg body weight once daily for 3 days and a single dose of SP (25/1.25 mg base/kg body weight) on day 0) and a single dose of primaquine at a dose of 0.75 mg/kg body weight on day 1. Whereas, confirmed *P. vivax* mono-infected cases were treated with chloroquine in full therapeutic dose of 25 mg/kg body weight divided over a period of three days and primaquine at a dose of 0.25 mg/kg body weight daily for 14 days.

The day, the patient was enrolled and received the first dose of medicine was designated day 0. After enrolment all patients were given a follow-up schedule for attending the clinic on days 3, 7, and every subsequent seven days till became negative parasitologically and antigenically. During this follow-up period, study participants were screened for malaria parasite and antigen by microscopic PBS examination and dual RDTs (SDBioline One step Malaria HRP2 (Pf) and pLDH (Pv) antigen rapid test kit).

Laboratory Methods:

Microscopic blood examination and parasite count: Thick and thin blood smear slides were collected from each study participants on day of recruitment and follow-up days. Collected PBS were stained with Giemsa stain and examined by expert microscopist for presence malaria parasites. Parasite counts were done on Giemsa-stained thick films and the number of parasites per 200 WBC was counted.Assuming a WBC count to be 8000/µL, parasitaemia was calculated and expressed as per µL of blood. A thick smear was diagnosed as negative on initial review if no parasites were seen in 100 oil immersion fields and all positive and negative slides were cross checked by a second microscopist.

Rapid diagnostic test: All study participants were also tested by dual RDT kit (SDBioline One step Malaria HRP2 (Pf) and pLDH (Pv) antigen rapid test kit) for detection of malarial antigen. The RDTs are rapid, qualitative immunochromatographic test for the differential detection of HRP2 antigen specific to *P. falciparum* and pLDH antigen specific to *P. vivax* in human whole blood. All RDTs were performed as per manufacturer instruction.

DATA ANALYSIS: The recorded observations of all study variables were double entered in MS Excel and validated. The data analysis and calculation of basic statistics was carried out by using Minitab version 17. The association of different factors and persistence of antigen after complete treatment were analysed by chi square test by using Minitab version 17.

RESULTS:

Base line characteristics of the study participants:

During the study period, a total of 1,544 patients with fever were screened for malaria parasites. After microscopic and RDTs examination it was found that among the tested individuals, 279 were positive for *P. falciparum* and 438 were positive for *P. vivax*, while 11 had mixed infections. Among the confirmed mono-infected *P. falciparum*-positive cases, 140 subjects were consented and enrolled in the study, while 160 mono-infected *P. vivax* patients were participated in the study. The base line characteristics of the study patients were summarized in **Table 1**. The mean age, temperature and parasite count of the *P. falciparum* positive patients at D0 was 33.01 years (95% CI: 30.58 – 35.45), 37.86 °C (95%CI: 37.68 – 37.98) and 4081.36 (95%CI: 3458.3 – 4704.4). While, at D0 the mean age, temperature and parasite count of the *P. vivax* positive patients was 29.8 years (95% CI: 27.59 – 32.02), 37.92°C (95%CI: 37.86 – 38.05) and 5234.58 (95%CI: 4166.9 – 6302.2).

Table 1: Base line characteristics of study patients

Characteristics	Pf (n = 140)	Pv (n = 160)
Sex (no., %) Male Female	123 (87.86) 17 (12.14)	145 (90.62) 15 (9.38)
Age category (no., %) < 14 yr > 14 yr	17 (12.14) 123 (87.86)	21 (13.13) 139 (86.87)
Age (yr) Mean Range SD 95% CI	33.01 12 – 65 ± 14.73 30.58 – 35.45	29.8 12 – 69 ± 14.21 27.59 – 32.02
Temperature (°C) Mean Range SD 95% CI	37.86 37.5 – 38.3 ± 0.17 37.68 – 37.98	37.92 37.6 – 39.1 ± 0.41 37.86 – 38.05
Parasite count (no/µl) Mean Range SD 95% CI	4081.36 240 – 21800 ± 3761.1 3458.3 – 4704.4	5234.58 160 – 36840 ± 6890.3 4166.9 – 6302.2

Treatment and follow-up: All *P. falciparum* mono-infected study patients were treated with AS+SP combination at a dose of artesunateat

4 mg/kg body weight once daily for 3 days and a single dose of SP (25/1.25 mg base/kg body weight) on day 0) and a single dose of primaquine at a dose of 0.75 mg/kg body weight on day 1. Whereas, all *P. vivax* mono-infected cases were treated with chloroquine at a dose of 25 mg/kg body weight divided over a period of three days and primaquine at a dose of 0.25 mg/kg body weight daily for 14 days.After enrolment all study patients were followed up clinically, parasitologically and antigenically on day 3, 7, 14 and every subsequent seven days till became negative in all respect. During the follow-up it was observed that clinical sign and symptoms were subsidised within 3-7 day of post treatment whereas all the study participants became negative parasitologically within 3 days of given treatment except 1 (0.33%) *P. falciparum* cases in which parasites were detected till day 3 and parasite count was 40 µL of blood.

Persistence of HRP2 and pLDH antigen in the blood circulation: The follow up of the study participant revealed that 4 (2.86%) *P. falciparum* infected participants were positive for HRP2 antigen till day 28 though they became negative parasitologically on or before day 3. It was also found that 118 (84.29%), 73 (52.14%), 43 (30.71%) and 17 (12.14%) individuals were found positive for HRP2 antigen on day 3, 7, 14, and 21 respectively (**Table 2, Figure 1**). Whereas, it was observed that *P. vivax* specific pLDH antigen was found in the blood circulation till day 14 and must be cleared from before day 21 of post treatment period (**Table 2, Figure 1**). pLDH antigen test was found positive by RDTs among 81 (50.63%), 14 (8.75%), and 6 (3.75%) *P. vivax* infected individuals on day 3, 7, and 14 respectively (**Table 2**).

Table 2: Association between malaria parasite species and duration RDT positivity

Factors		Duration of RDT positivity						X^2, p value
		D 0	D 3	D 7	D 14	D 21	D 28	
Parasite species	Pf (n = 140)	140	118	73	43	17	4	X^2 = 72.83, p <0.0001
	Pv (n = 160)	160	81	14	6	0	0	

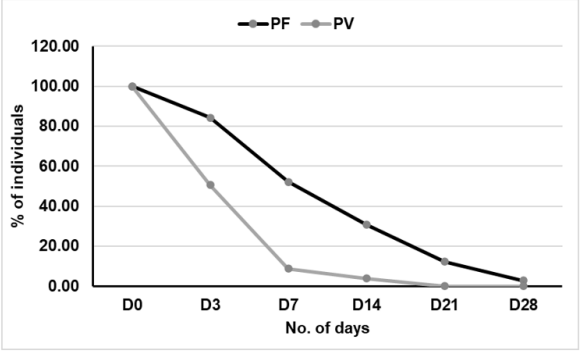

Figure 1: Pattern of persistence of antigenemia in *P. falciparum* and *P. vivax* patients

Association between persistence of malaria antigen and different factors: The *P. falciparum* specific HRP2 antigen persisted in the blood stream till day 28 of post treatment and clearance of parasite, whereas *P. vivax* specific pLDH antigen was present till day 14. So, a significant difference was noted between the duration of persistence of malaria antigen in the blood stream *P. falciparum* and *P. vivax* (X2 = 72.83,p <0.0001) (**Table 2**). But no significant difference was observed between the duration of persistence of *P. falciparum* specific HRP2 antigen among age groups (<14 yrs and > 14 yrs), sex (male and female) and parasite count on day 0 (<2000 and >2000) (X2 <4.41,p >0.05) (**Table 3, Figure 2, 3, 4**). Similarly, no significant difference was observed between the duration of persistence of *P. vivax* specific pLDH antigen among age groups (<14 yrs and > 14 yrs), sex (male and female) and parasite count on day 0 (<2000 and >2000) (X2 <3.45, p >0.05) (**Table 4, Figure 5, 6, 7**).

Table 3: Association between different factors and duration of persistence of P. falciparum specific HRP2 antigen

Factors		Duration of RDT positivity						X^2, p value
		D 0	D 3	D 7	D 14	D 21	D 28	
Age group	<14 yr (n = 17)	17	12	8	1	1	0	X^2 = 4.41, p = 0.4915
	>14 yr (n = 123)	123	106	65	42	16	4	
Parasite count	PC<2000 (n = 58)	58	49	32	17	9	3	X^2 = 2.81, p =0.7298
	PC>2000 (n = 82)	82	69	41	26	8	1	
Sex	Female (n = 17)	17	12	9	5	0	0	X^2 = 3.04, p =0.6943
	Male (n = 123)	123	106	64	38	17	4	

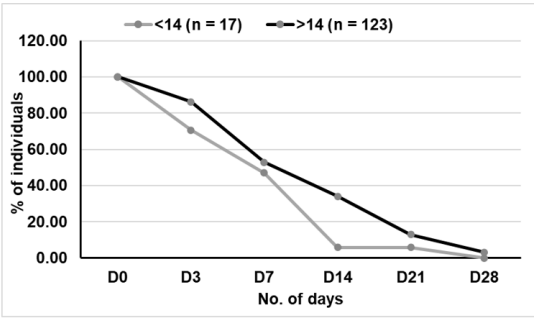

Figure 2: Pattern of persistence of HRP2 antigen in different age group

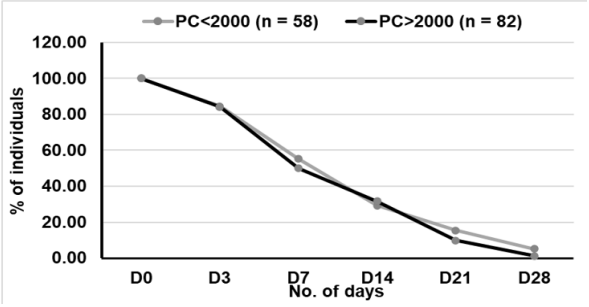

Figure 3: Pattern of persistence of HRP2 antigen in different parasite count

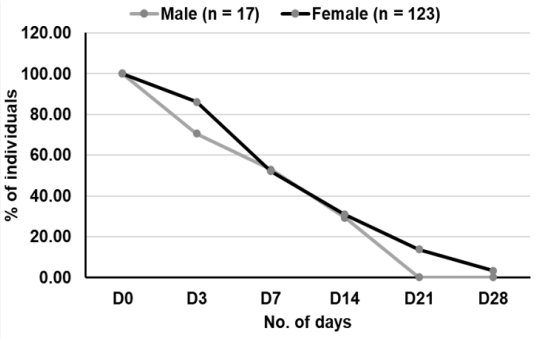

Figure 4: Pattern of persistence of HRP2 antigen in different sex

Table 4: Association between different factors and duration of persistence of *P. vivax* specific pLDH antigen

Factors		Duration of RDT positivity						X^2, p value
		D 0	D 3	D 7	D 14	D 21	D28	
Age group	<14 yr (n = 21)	21	6	1	1	0	0	X^2 = 2.19, p = 0.5334
	>14 yr (n = 139)	139	75	13	5	0	0	
Parasite count	PC<2000 (n = 57)	57	29	7	4	0	0	X^2 = 3.45, p = 0.3272
	PC>2000 (n = 103)	103	52	7	2	0	0	
Sex	Female (n = 15)	15	5	2	0	0	0	X^2 = 1.89, p = 0.5947
	Male (n = 145)	145	76	12	6	0	0	

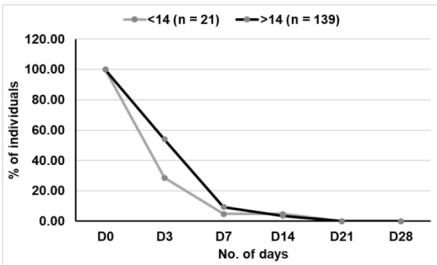

Figure 5: Pattern of persistence of pLDH antigen in different age group

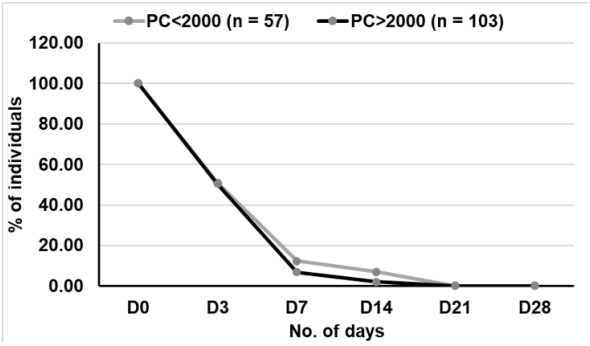

Figure 6: Pattern of persistence of pLDH antigen in different parasite count

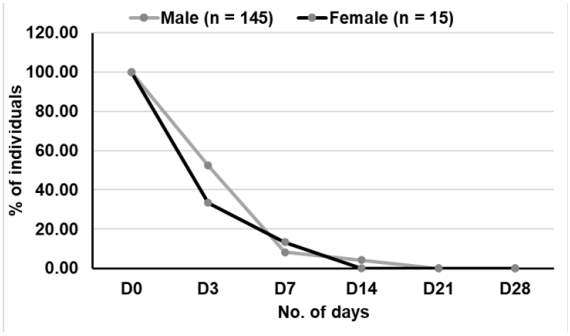

Figure 7: Pattern of persistence of pLDH antigen in different sex

DISCUSSION:

Malaria case management constitutes a vital component of the malaria control strategies [28] in which proper diagnosis plays an important role. This is more relevant in countries where more than one species are prevalent like India with *P. falciparum* and *P. vivax* having equal distribution [29]. Proper identification of species is also very essential for treatment where different drug combinations are in use for different parasitic infections [30]. Microscopy is still considered as gold standard for malaria diagnosis [4], but the method is prone to misdiagnoses especially in cases of mixed infections and wrong diagnosis in case of small rings of *P. vivax* [31]. Several new methods have been developed such as antigen-based detection by RDTs and more accurate molecular diagnosis by PCR methods [32]. Presently RDTs are now being used widely for diagnosis of malaria in the remote health settings and at field level for active and passive cases detection where microscopy is not possible because it doesn't require any particular expertise, easy to perform the test and generate result very quickly. But one of the main limitations of RDTs is false positive results due to the persistence of malaria antigens in the blood stream after recent parasite clearance through anti-malaria lmedication. So, understanding the duration of positivity after successful parasite clearance is also critical for accurate diagnosis of malaria.

In the present study, we have screened a total of 300 mono-infected malaria cases (140 *P. falciparum* and 160 *P. vivax*) for persistent antigenemia after antimalarial therapy to day 28. Clinical evaluation, microscopy, and RDT antigen testing were performed on days 3, 7, 14, 21 and 28 after antimalarial chemotherapy. All the recruited patients showed a positive result in RDTs. Among the *P. falciparum* cases HRP2 antigen was persisted in the blood stream till 28 days in 2.86% cases after anti-malaria treatment and even clearance of parasite. Our finding was in line with the findings reported by Grandesso et al., 2016 and Swarthout et al., 2007 [33, 8]. In contrast shorter persistence of HRP2 antigen (no longer than 21 days) was reported by WHO, 2014, Gerstl et al., 2010 and Njama-Meya et al., 2013 [34, 35, 36]. Among the *P. vivax* cases pLDH antigen was persisted in the blood stream up to day 14 in 3.75% cases which is in contrast with study finding of Njama-Meya et al., 2013 [36] but similar to the findings of Grandesso et al., 2016 [33].

The causes of long persistent HRP2 antigen in *P. falciparum* might be due to existence of viable asexual stages below the detection limit of microscopy, delayed clearance of circulating antigen (free or in antigen-antibody complex) [37], rheumatoid factor [21], and detection of circulating sexual stages [38, 37].

In the present study, a significant difference in the duration of persistence of malaria antigen was noted between HRP2 of *P. falciparum* and pLDH of *P. vivax* ($X2 = 72.83$, $p < 0.0001$). But no significant difference was observed between the duration of persistence of HRP2 and pLDH antigen among age groups (<14 yrs and > 14 yrs), sex (male and female) and parasite count on day 0 (<2000 and >2000) ($X2 < 3.45$, $p > 0.05$). But in other studies, it was found that the action of antimalarial treatment on the parasites may also influence the persistence of HRP2 and the persistent HRP2 antigenemia has also been suggested to be associated with gametocytemia [37].

So, in areas with high transmission of malaria, caution should be taken before treatment of any patient diagnosed by detecting antigen using RDTs only. In cases with history of *P. vivax* infection within 14 days and 28 days of *P. falciparum*, parasitemia should be confirmed by microscopy before any antimalarial treatment.

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Conflict of interest: We have no conflict of interest

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