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Examination of plasmodium parasites in bone marrow puncture fluid for the diagnosis of imported-malaria in patients from the Yunnan Province, China

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Abstract

Background: *Some suspected malaria individuals who have returned to Yunnan from Africa have been delayed antimalarial treatment due to not detection out Plasmodium infection in their peripheral blood.*

Methods: *The fever patients with a history of exposure to malaria endemic areas and suspected malaria episodes accepted the detection of the Plasmodium infection in bone marrow fluid by microscopy and gene testing.*

Results: *Plasmodium in bone marrow fluid were found in all of five patients, including four patients with Plasmodium negative and one patient with Plasmodium falciparum positive in peripheral blood. The proportion of found ring stage, large trophozoites, schizonts, and stage III-V gametocytes accounted for 28.3%, 38.3%, 4.8%, 11.5%, 16.5% and 0.8%, respectively. The erythrocyte count and hemoglobin concentration of the five cases post-treatment merely increased to the lower end of the normal range. Platelet count returned to the normal range, increasing by 466%, 378%, 252%, 168% and 35%, respectively. There were four to five B-cell antigenic epitopes along pLDH peptide chains of the infected strains in the five cases. Of note, the sequence of "211-EEVEGIFDR-220" was only detected in P. vivax strain, whereas the sequence of "207-LISDAE-213" was unique for P. falciparum strain.*

Conclusion: *Examination of the Plasmodium in bone marrow puncture fluid could make up for the missed diagnosis of malaria that solely relies on peripheral blood.*

Keywords: Bone Marrow Aspirate Fluid, Detection, Plasmodium, Etiological Diagnosis, Imported-Malaria, Anti-Malaria Treatment, Yunnan Province

Abbreviations: YPMDRL: Yunnan Province Malaria Diagnosis Reference Laboratory; KTPH: Kunming Third People's Hospital; pLDH: Plasmodium Lactate Dehydrogenase; pvLDH: Plasmodium Vivax Lactate Dehydrogenase; pfLDH: Plasmodium Faciparum Lactate Dehydrogenase; pmLDH: Plasmodium Malariae Lactate Dehydrogenase; EBL: The Erythrocyte Binding-Like Protein; Rh: Reticulocyte Binding-Like Protein; BM: Bone Marrow; PCV: Hematocrit; PCT: Platelet Hematocrit; ALT: Alanine Aminotransferase; IBIL: Indirect Bilirubin; CRP: High Sensitivity C-Reactive Protein; NE: Neutrophilicgranulocyte; RBC: Red blood cell; WBC: White Blood Cell; Hb: Hemoglobin; PLT: Platelet; RDT: Rapid Diagnosis Test.

Introduction

In spite of the risk of invasive detection, bone marrow puncture has played a vital role in the etiological diagnosis of malaria for suspected malaria cases returning to Europe and America from malarial-endemic areas and showing unexplained fevers since the 1930s and 1940s [1,2]. The application of bone marrow puncture fluid examination has contributed greatly to the diagnosis of pancytopenia [3], thereby reducing the risk of malaria infection on immune-incompetent people and salvaging the lives of patients. In 1942, Levine et al. [1] performed sternum piercing examination on 11 highly-suspected malaria patients returning to Australia from malaria-endemic areas, reporting Plasmodium infection in the puncture fluid of 7 patients. This procedure compensated for the low accuracy in the confirmation of malaria infection by using

blood film examination. Gandapur et al. [4] detected Plasmodium infection in the bone marrow fluid of 1.3% (26/1966) of Gambians and 0.13% (35/25867) of Pakistani [5]. Aguilar et al [6] detected malaria parasites in both bone marrow fluid and peripheral blood of 174 Mozambican children, reporting that the positive rate of Plasmodium in bone marrow aspirate was significantly higher than that in peripheral blood (14.4%v.s.5.2%, $p<0.05$), and that the percentage of early and mid-stage (stage Ito IV) gametocytes of P. falciparumm was 4.8-5-fold higher in bone marrow fluid [7] than in peripheral blood. Brito et al [8] reported 2-3-fold higher sensitivity in the comparison between bone marrow fluid and peripheral blood. In conclusion, bone marrow puncture is more sensitive than peripheral blood in the detection of Plasmodium, possibly amounting to 2~10-fold higher in terms of sensitivity [1,9].

De Niz et al. [8] applied biofluorescence imaging on animal model to detect the distribution of malaria parasites, reporting that liver and spleen, which are also part of the human reticuloendothelial system, are the main reservoir of P. vivax gametocytes and lysosomes, and that the highest concentration of gametocytes was observed in the spleen and bone marrow. This finding could be attributable to the fact that bone marrow is the main developmental site of reticulocytes and that Plasmodium targets the reticulocytes for invasion. Converging evidence revealed the highly enriched expression of CD71 [10,11], CD44 [7,10], EBL (the erythrocyte binding-like protein) and Rh (reticulocyte binding-like protein) [12], the binding receptors for Plasmodium, on the surface of stage I and II naïve reticulocytes. Among them, CD71 is the adhesion molecule for P. vivax merozoites to invade reticulocytes. As the expression of CD71 peaked in stage-I reticulocytes, P. vivax merozoites mostly invade stage-I reticulocytes. Although CD71 level was the lowest on the surface of stage-III reticulocytes, stage-III reticulocytes are the only group of reticulocytes that can penetrate through the sinusoidal capillary lumen to enter the circulating peripheral blood [10,11]. Even though the invasion of Plasmodium into reticulocytes could be detected in the peripheral blood, the proportion is much lower than that of bone marrow fluid. This finding also justifies the lower concentration of Plasmodium in peripheral blood than in bone marrow fluid, hence the difference sensitivity of detection between the two methods.

Plasmodium parasites constantly hided and accumulated in hematopoietic natural niche, on the one hand, probable resulting in the pathological changes such as marrow depression [13-16], and osteoporosis after constantly undergoing formation by osteoblasts (OBs) and resorption by osteoclasts [17,18]. On the other hand, the feature of locally enriched Plasmodium could be applied as the cornerstone for the optimizing of laboratory diagnosis and even clinical treatment of malaria cases [8,19,20].

Although the number of malaria cases diagnosed in Yunnan is still the highest in China in recent years, the mortality rate is the lowest in the country [21]. This is mostly ascribed to for the high capability of accurate diagnosis in the grassroot hospitals [22], thanks to the prompt implementation of bone marrow puncture in malaria-designated hospitals, the reduced misdiagnosis of febrile diseases, and the improved etiological treatment. In order to strengthen the capacity of the treatment of severe malaria cases and imported malaria cases, and to gradually standardize the protocol of bone marrow puncture in clinical practice, the current study analyzes and summarizes the cases in our hospital for the diagnosis of suspected malaria infection.

Materials and Methods

Exposure to Malaria and Suspected Malaria Episodes in Endemic Areas

All included 5 cases returned to Yunnan from African countries within 2 to 36 days (median=27 days) after staying in African countries for more than three months. The patients exhibited fever, headache, discomfort, with body temperatures reaching above 39°C during hospitalization. Their Hematocrit (HCT) and platelet pressure (PCT) were at low levels, yet the level of hypersensitive C-reactive protein (hs-CRP) was substantially elevated. Two of them reported higher levels of IBIL and alanine aminotransferase (ALT) (Table 1). These characteristics indicate that the five patients were in a state of hemolytic anemia, with compromised function of coagulation and strong immune emergency. Two cases showed various degrees of impaired hepatic function.

Typical symptoms, such as fever, chills, sweating, headache, muscle aches and tea-colored urine, persisted in these five patients after undergoing antibacterial symptomatic treatments. Their spleen sizes were enlarged to varying degrees. During the administration course, Plasmodium parasite was not found in the peripheral blood of four cases, nor was pLDH antigen detected in two cases by using malaria rapid diagnosis test (RDT, W056-C, One Step Malaria HRP2/pLDH (P.f/P.v) Tests). Except that case 1 was unable to clearly tell the intake which antimalaria drug, other cases had irregularly taken artesunate and quinine during staying in Africa (Table 2).

All the patients tested negative for common viruses and microorganism infections (such as HIV and Mycobacterium tuberculosis), hence the possibility of common microbial infections could be excluded.

Microscopic examination of bone marrow fluid for Plasmodium

To rule out the possibility of Plasmodium infection, bone marrow puncture was performed on the enrolled patients. Bone marrow fluid was collected from the anterior iliac spine to make the thin blood smear, which was dried naturally, fixed with methanol, and stained with 10% Giemsa staining for 30 min in a step-wise manner. The morphology of Plasmodium in the thin blood smear was observed under the microscope (1000×magnification). The features identification of Plasmodium at different developmental stages was clarified in the literature according to the protocol (Additional file 1) [23,24].

Cases	Age	Gender	Infection from	Time of returning to Yunnan after leaving Africa (d)	Temperature on admission (℃)	PCV[a]	PCT[b]	hs-CRP[c] (mg/L)	ATL[d] (U/L)	IBIL[e] (μmol/L)
Case 1	33	Male	Congo, Kolwezi	27	39	0.37	0.06	153.81	45	10.1
Case 2	26	Male	Guinea, Mongomo	31	40	0.47	0.11	130.74	10	8.8
Case 3	40	Male	Cameroon, Sangmelima	36	40	0.42	0.11	144.01	19	5.6
Case 4	46	Male	Guinea, Malabo	21	39	0.21	0.11	32.46	29	4.7
Case 5	30	Male	Congo, Lubumbashi	2	39	0.37	0.12	110.01	97	29.4
Note: a: The normal value of PCV is 38-50.8 %; b: The normal value of PCT is 0.11-0.28; c: The normal value of hs-CRP is 0-6 mg/L; d: The normal value of ATL is 8-40 U/L; e: The normal values of IBIL is 1.71-11.97 μmoL/L.										

Table 1: Medical indicators of infectious patients returning to Yunnan Province from Africa.

Cases	Splenomegaly (thickness*length) (mm)	Fever (d)	Chills (d)	Profuse sweating (d)	Headache (d)	Tea-colored Urine (d)	Monocyte ($\times10^9$/L)	Microscopic parasitemia[a]	RDT (*p*LDH)	Taken antimalarial drugs during Africa
Case 1	6*4	22	20	20	23	23	0.15 ~0.21	Positive	Non-falciparum	Can't tell clearly
Case 2	0	7	7	7	8	2	0.64~1.02	Negative	Negative	Irregular Artesunate
Case 3	1*0	6	6	6	6	8	0.33~ 0.53	Negative	Negative	Irregular Artesunate
Case 4	20*0	12	12	12	12	20	0.68~0.55	Negative	Falciparum	Irregular Artesunate
Case 5	11*8	3	3	3	5	2	0.95~0.26	Negative	Non-falciparum	Irregular Quinine
Note: a: The density of parasitemia measured in the peripheral blood is 26097 parasites/ul close to the time when the bone marrow aspirate.										

Table 2: Vital signs and symptoms of imported malaria cases during the acute attack.

Antimalarial Treatment

According to the Chinese Diagnostic Criteria for Malaria (WS269-2015) [25], the suspected patients that exhibit one or more clinical indicators, including coma, severe anemia (hemoglobin<5g/dL, hematocrit<15%), acute renal failure (serum creatinine concentration >265 μmol/L), pulmonary edema or acute respiratory distress syndrome, hypoglycemia (blood glucose <2.2 mmol/L or<40 mg/ dL), circulatory failure or shock (systolic blood pressure<70 mmHg in adults and<50 mmHg in children), metabolic acidosis (plasma bicarbonate<15 mmol/L), are eligible for the treatment of severe malaria, otherwise they can be treated as non-severe malaria.

Subsequently, antimalarial treatment was administered to confirmed cases according to the Regulations for application of antimalarials (WS/T 485-2016) [26]. For both vivax malaria and malariae malaria patients, the total dose of chloroquine was 1200 mg: 600 mg on day 1 (dosed) and 300 mg day 2 and day 3. If the symptoms of malaria were not relieved at the end of the treatment course, artemether (total dose=293 mg) and artesunate (total dose=1296 mg) could be supplemented. For non-severe falciparum malaria cases, a total dose of 8 tablets of dihydroartemisinin-piperaquine should be performed (One table in the morning and one in the evening). The treatment lasted for 2 days. For the treatment of benign tertian malaria patients, primaquine should be used to eradicate hypnozoites on the first day of chloroquine administration or after the patients showed slightly improved clinical symptoms (total dose=180 mg, 22.5 mg once a day for 8 days in a row).

Analysis of the B-cell Epitopes in pLDH Antigen between Different Plasmodium

Bone marrow puncture fluid was collected from the five subjects before antimalarial treatment on day 0 for preparing the filter paper dried blood samples to identify the Plasmodium spp. and analyze Plasmodium molecular composition. The mono-infection of Plasmodium spp was identified by detection of Plasmodium

18S rRNA gene [22,27] in Yunnan Province Malaria Diagnosis Reference Laboratory (YNMDRL) (Additional file 2). pLDH gene was amplified by performing nested PCR for infected strains of *P. vivax, P. falciparumm*, *and P. malariae* in the five cases, according to the protocol clarified in the literature [28]. The amplification products of pLDH genes were sequenced; and the sequences were compared with the available referent sequences of *P. vivax* (NC_009917.1), *P. falciparumm* (NC_004331.3), and *P. malariae* (LT594500.1) on NCBI (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). If the coverage (Query cover) is greater than 90% and similarity (Identifies) is greater than 95%, the respective pLDH sequence could be confirmed as the corresponding the Plasmodium species sequence.

Furthermore, MEGA5.04 software was used to identify the missense mutations and synonymous mutations of each sequence, which were then translated into pLDH peptide chains. The modus of "B Cell Epitopes Prediction" of the online software IEDB (http: //www.iedb.org/) was used to predict B cell antigenic epitopes of pLDH peptide chain. The spatial conformation of pLDH peptide chain was analyzed by using PyMOL 2.3.2 software.

Results

Morphologic Features of Plasmodium in Bone Marrow Fluid

Microscopic examination of Plasmodium in the bone marrow puncture fluid showed that the volume of erythrocytes with Plasmodium parasites in case 1 and case 5 were not distended but slightly reduced. The Plasmodium exhibited a typical stripe-shaped large trophozoite morphology, and could be mostly detected at various stages, including ring, large trophozoite, schizonts, and round gametophytes (Figure 1). Therefore, the two cases were identified as P. malariae infection, with large trophozoites occupying the largest proportion (38.3%, 153/200) (Table 3). The volume of erythrocytes with Plasmodium parasites of case 2 and case 3 was consistently distended. The ring-infected and large trophozoites-infected erythrocytes showed the feature spine-like protrusion. While various stages of Plasmodium parasites were found (Figure 1), the largest and smallest proportions were large trophozoites (44.8%, 179/200) and stage V gametocytes (1.5%, 6/200), respectively. Hence, the two cases could be diagnosed as P. vivax infection (Table 3). The size of erythrocytes with Plasmodium parasites of case 4 remained normal. The Plasmodium parasites covers various stages, such as ring, large trophozoites, and crescent-shaped gametocytes (Figure 1), and the largest proportion was stage V gametocytes (24.5%, 49/200) (Table 3). Hence, case 4 could be reasonably identified as *P. falciparumm* infection.

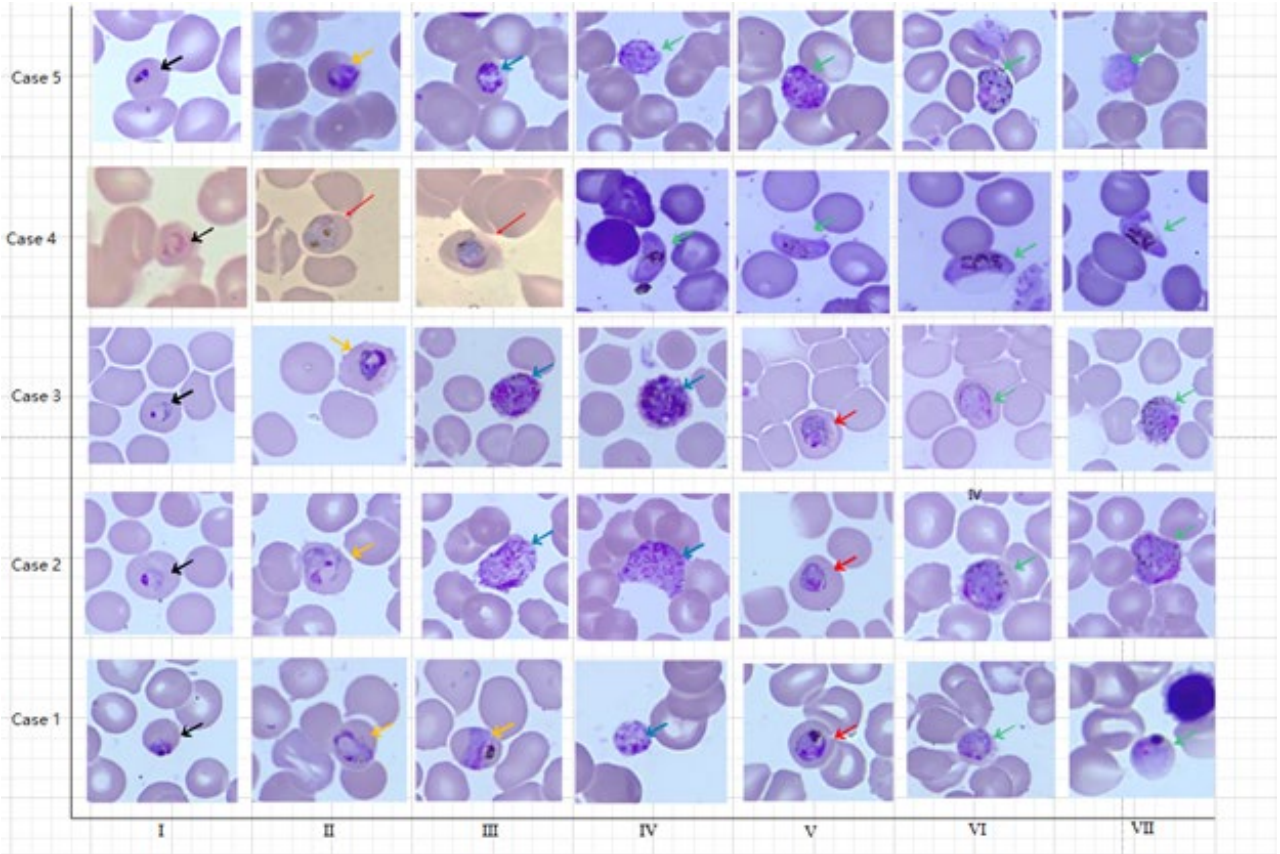

Figure 1: Plasmodium stages of five malaria acute attack patients in Yunnan Province. (1) The nucleus and the cytoplasm of Plasmodium were stained as red and blue, respectively. (2) Black arrow is the ring stage; Yellow arrow is the trophozoite stage; Blue arrow refers to the schizonts stage; Red arrow is the stage II-III gametocytes; Green arrow indicates the IV-V stage gametocytes.

Cases	Species	Density (parasites/ul)	No	Stages				
				R	T	S	G (III-IV) No. (%)	G (V) No. (%)
				No. (%)	No. (%)	No. (%)		
Case 1	*P. malariae*	560	200	38 (19.0)	60 (30.0)	19 (9.5)	83 (41.5)	0
Case 5	*P. malariae*	80	200	75 (37.5)	93 (46.5)	0	29 (14.5)	3 (1.5)
Total	--	600	400	113 (28.3)	153 (38.3)	19 (4.8)	112 (28.0)	3 (0.8)
Case 2	*P. vivax*	160	200	31 (15.5)	114 (57.0)	11 (5.5)	40 (20.0)	4 (2.0)
Case 3	*P. vivax*	440	200	45 (22.5)	65 (32.5)	37 (18.5)	41 (21.5)	2 (1.0)
Total	--	640	400	76 (19.0)	179 (44.8)	48 (12.0)	68 (17.0)	6 (1.5)
Case 4	*P. falciparum*	120	100	0	2 (2.0)	0	9 (9.0)	89 (89.0)

Note: (1) R, T, S and G are the abbreviations of Ring, Trophozoites, Schizontes, and Gameocytes, respectively; (2) III-IV: Stage III-IV gametocytes; stage V gametocytes.

Table 3: Constituent ratio of different Plasmodium parasites in this group patients.

A Change of Myelosuppression after Antimalarial Treatment

As none of the five patients exhibited any of the following unfavorable clinical manifestations, such as coma, severe anemia, acute renal failure, pulmonary edema or acute respiratory distress syndrome, hypoglycemia, circulatory failure or shock, or metabolic acidosis, they were diagnosed as non-severe malaria and received antimalarial treatment in due course (Figure 2). In addition to chloroquine, artemether and artesunate were added to control the clinical malarial episodes of case 1.

The hemogram test results of five patients before, during and after antimalarial treatment are shown in Figure 2. Red blood cell count (×109/L) and hemoglobin concentration (g/L) showed the pattern of parallel changes and remained roughly stable throughout the treatment course, only reaching the lower end of normal range by the end of treatment. Although Red blood cell count increased significantly to 110×109/L and hemoglobin concentration was elevated to 3.81 g/L after antimalarial treatment (Figure 1), the increasing extent was not high enough to reach the normal range, thus indicating that the restoration of the indexes of erythrocyte lineage were hindered within a short period even after the cause of malaria was removed.

Leukocytes and neutrophils of the granulocyte lineage also showed parallel changes, exhibiting an upward trend and fluctuation afterwards in Case 3 and case 5. After antimalarial treatment, the leukocyte counts of cases 1, 2 and 3 returned to the normal range (5.27-7.05×109/L), and neutrophil count was within the range of 2.55-4.41 (×109/L). Although leukocyte count and neutrophil count of case 4 were within the normal range throughout the treatment course (leukocytes:5.79-7.33×109/L), neutrophils:2.38-3.819×109/L), both two indicators decreased with the magnitude of -30.2% and -35.3% after antimalarial treatment, respectively. The decreasing patterns were observed in case 5, and the indexes were only close to the low end of normal range after the recovery (Figure 2).

Pre-treatment platelet counts in case 1 and case 4 decreased by 35.1% and 20.0%, respectively, yet rebounded to normal after the treatment (Figure 2). Platelet counts of the five cases rose by 466%, 378%, 252%, 168%, and 35%, respectively, indicating that the function of megakaryocytes to produce platelets has been restored swiftly after the treatment of Plasmodium infection

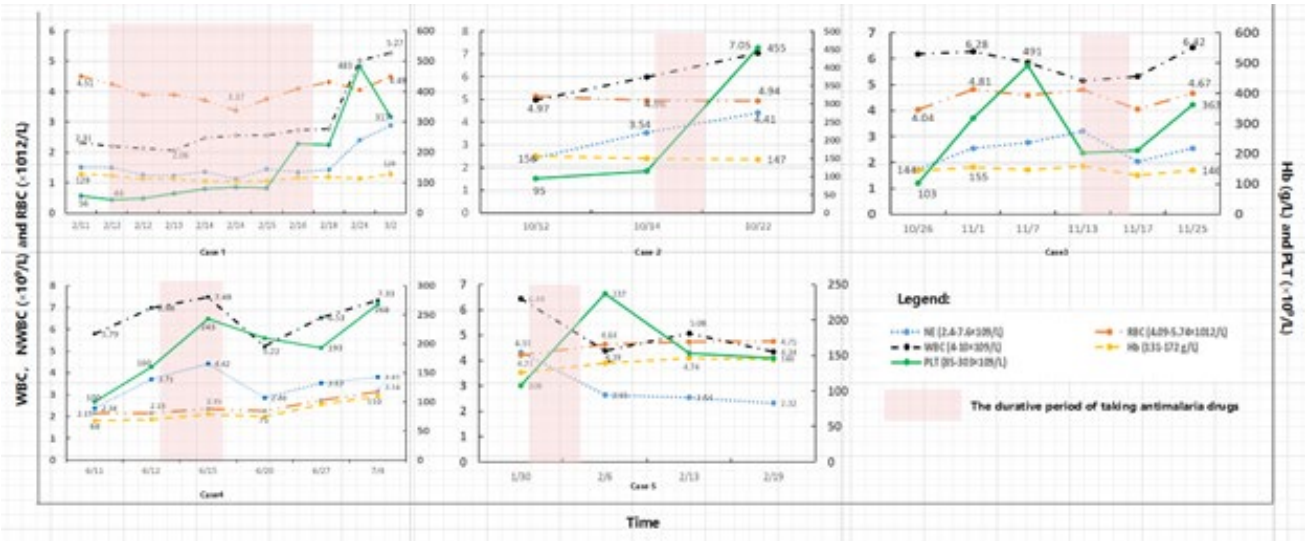

Figure 2: Changes of myelosuppression in 5 patients after antimalarial treatment.

Plasmodium	No. epitopes	B-cell epitope		
		Completely homologous sequence	[a]Most homologous sequence	Unique sequence
P. vivax	*5*	85-PGKSDKEWNRD-96 197-IPLQEFINNK-207 286-EQVIELQLN-295	62-GSN**S**[b]Y**D**[c]DL-70	211-EEVEGIFDR-220
P. falciparumm	*4*		62-GSN**T**[b]Y**D**[c]DL-70	207-LISDAE-213
P. malariae	*4*		62-GSN**S**[b]Y**E**[c]DL-70	--

Note: a: There are two or less amino acid differences in B-cell epitopes among four species of human Plasmodium. Underline and bold indicate substituted amino acids between non-homologous sequences. b: When the 66thaa of the pLDH peptide chain is S, it belongs to the sequence of P. vivax and P. malariae, while when it is T, it belongs to the sequence of P. falciparumm. c: When the 68thaa of the pLDH peptide chain is D, it belongs to the sequence of P. vivax and P. falciparumm, when it is E, it belongs to the sequence of P. malariae.

Table 4: Polymorphism of pLDH gene and prediction of B cell epitopes.

B-cell Epitopes of pLDH Antigen in the Plasmodium Strains for Malaria RDTs

Four to five B-cell active antigenic regions were present in the primary peptide chains of Plasmodium pLDH in five cases (Table 3). Among them, the peptide chains of 63th ~70th aa, 86th ~96th aa, 198th ~207th aa and 287th ~295th aa were commonly found, with an activity score of up to 0.43. The sequences of "85-PGKSDKEWNRD-96","197-IPLQEFINNK-207",and "286-EQVIELQLN-295" were commonly distributed in the pLDH peptide chains of all five strains (Table 3). Moreover, variations at 66th aa and 68th aa were determined in the active region (63th aa to 70th aa) of pLDH peptide chain for different Plasmodium species (Table 4).

pLDH peptide chains of Plasmodium varix and *P. falciparumm* (pvLDH and pfLDH) exhibited specific B-cell antigenic active regions of "211-EEVEGIFDR-220" and "207-LISDAE-213", respectively (Table 4). pLDH antigen chains of all five malaria cases showed an oligomeric spatial conformation with four subunits (Additional file 3, Figure 1A-E). The short peptides of the five epitopes in Table 4 were commonly distributed on the oligomeric surface. Among them, the spatial conformation and regional distribution of the active regions of 63th ~70th aa and 86th ~96th aa were mostly the same across *P. malariae, P. vivax and P. falciparumm*. However, in the proximity of the fusion region composed of two active short peptides (198th ~207th aa and 287th ~295th aa), antigenic epitopes of "211-EEVEGIFDR-220" and "207-LISDAE-213" were detected in pvLDH and pfLDH, respectively (Additional file 3, Figure 1B, C and D).

No additional antigenic epitopes were found in pmLDH (Additional file 3, Figure 1A, E). Therefore, the antigenic epitopes of 211-EEVEGIFDR-220 and 207-LISDAE-213 could be used in the differential diagnosis of P. vivax and P. falciparumm infection by using RDT.

Discussion

The examination of bone marrow fluid for Plasmodium infection is mostly applied as supplementary for the clinical diagnosis of other diseases that requires the testing of the bone marrow [4-6]. Essentially, bone marrow puncture could play a more vital role in the diagnosis of P. vivax infection than peripheral blood examination [10,11], by virtue of the production of a lower density and deficient symptom of parasitemia. In comparison with bone marrow puncture, peripheral blood screening for Plasmodium infection has been reported to cause a false-negative rate of 11.4% (4/35) in Pakistan [5].

In the current study, neither Plasmodium nor pLDH antigens were detected in peripheral blood of the two cases of P. vivax infection, yet late-stage large trophozoite and stage-IV gametophytes with intact cell structure were detected in bone marrow aspirate [5,20] and the erythrocytes infected by P. vivax had not still exhibited the Schuffner' dots, which consistent with the previous features observed in peripheral blood (Additional file 4). Such finding provides a solid etiological basis for the diagnosis of *P. vivax* by using bone marrow puncture. The surface of erythrocytes is parasitized by ring-form trophozoites and late-stage trophozoites with abundant spine-like protrusions, indicating the persistent invasion of Plasmodium into reticulocytes with abundant expression of CD71 receptors residing in bone marrow [10]. The P. vivax showed potent capacities of proliferation and accumulation in the bone marrow puncture fluid.

For P. malariae infection cases with positive results of pLDH antigen in peripheral blood, bone marrow puncture could still be performed to exclude the possible false-positive rate in RDT test. In recent years, false-positive results for pLDH antigens have been reported in patients with rheumatoid arthritis from time to time in Yunnan Province. While the other health institutions or hospitals can only conduct malaria infection screening in febrile patients through epidemiological investigation or antimalarial medication, KTPH explores the method of bone marrow aspirate examination to identify the malaria pathogens. Such a clinical practice contributes to more accurate diagnosis of suspected Plasmodium infection in Yunnan Province.

Bone marrow infection may be a common symptom for acute severe malaria and chronic malaria [5,13]. The persistent parasitemia after malarial infection would in turn trigger myelosuppression [15] and leading to heterogeneity hematological abnormalities [29]. PCV shows a slow yet continuous decreasing trend during the first 3 days of P. falciparumm infection [29,30], and the symptom of anemia was relieved on day 17 post-treatment. For patients with cerebral malaria, the decreasing extent of hemoglobin was inversely related to the severity of parasitemia [30]. The changes in erythrocyte lineage indicators were similar in these cases. For three cases, the decreasing PCV was not rescued to the normal range until the end of treatment. Red blood cell (RBC) count and hemoglobin levels of cases 1 and 4 were still below the normal range on day 13 and 16 post-treatment (Figure 1). These unfavorable indicators may be ascribed to the lack of supplementary chalybeate therapy, rendering it unconducive to the accumulation of red blood cell counts by the bone marrow puncture [13,15].

In contrast, leukocyte and neutrophil counts did not deviate markedly from the normal range in any of the five cases. Even though case 1 and case 2 exhibited higher counts of leukocyte and neutrophil (Figure 1), the change of neutrophil changes in most cases (3/5) was still in line with the pattern of Abdalla et al [31], which indicated that neutrophil counts would increase at the onset of malaria infection and would then decrease, which reflects the process of inflammatory response and anti-inflammatory response. Platelet count was the indicator most affected by malaria infection and the subsequent clearance of Plasmodium. The exhibition of splenomegaly before antimalarial treatment suggested that platelets were redistributed between the peripheral blood and the hematopoietic system [32] and therefore the platelet counts decrease persistently [33,34]. Consistent with the observations of previous research, it was found that platelet counts increased by several-fold, and yet returned to the normal range after the treatment [34,35]. This suggests that Plasmodium infection may contribute to the recovery of platelet production by megakaryocytes and that platelet count is a sensitive indicator of malaria infection and the effective antimalarial treatment.

The hemogram blood test results of patients with Plasmodium infection in the bone marrow concur with the consensus regarding the application of bone marrow puncture for the diagnosis of malaria: For patients with atypical manifestations, especially those who exhibit pancytopenia and febrile neutropenia [36], invasive screening test, such as bone marrow puncture, could be performed to exclude malaria infection [37,38]. However, bone marrow puncture should not be performed on patients with acute attack of malaria, and exhibit clinical symptoms and more pronounced pancytopenia in the peripheral blood, due to the concern of unjustified procedure [5,39]. In summary, the appropriate candidates for bone marrow puncture of Plasmodium testing are those with a traveling history to malaria- endemic areas, with exhibition of fever, anemia, thrombocytopenia and splenomegaly, undetected Plasmodium in the peripheral blood, suspicious antigen test results. Other microbial infections, such as bacteria and mycoplasma, should also be excluded.

Keluskar et al. [40] suggested that pLDH, as a target antigen for malaria immune-diagnosis, has the stable antigenic epitopes, in which pfLDH and pvLDH share the same genetic polymorphism. Huang et al [28] confirmed the high homology of pLDH gene in four human Plasmodium species, including P. falciparumm, *P. vivax, P. malariae, and Plasmodium ovale*. Moreover, pLDH antigen could only be detected during the life cycle of Plasmodium [41], and the amount of pLDH antigen is proportional to the density of Plasmodium [18,42]. The RDT products based on pLDH antigen show high sensitivity in the detection of Plasmodium infection, especially in extravascular tissues and organs [18,19]. Accumulating evidence showed that the false-negative rate of PfHRP2-based RDTs was as high as 65% (11/17) and was even close to 100% (12/12), as opposed to the detection of pLDH antigen [43,44].

However, in the current study, pvLDH antigen was not detected in the peripheral blood of two cases, even though P. vivax had been detected in the bone marrow puncture fluid. The role of pLDH antigen, as an indicator of P. vivax infection, could not be clarified in the cases [18,42], and this could be attributable to the low specificity of pvLDH-based RDT products. The B-cell epitopes of the pLDH peptide chains of the five cases showed that aside from the common sequences in pvLDH and pmLDH "85-PGKSDKEWNRD-96", "197-IPLQEFINNK-207"and"286-EQVIELQLN-295", the epitopes of "211-EEVEGIFDR-220" is adjacent to the

fusion region composed of "197-IPLQEFINNK-207" and "286-EQVIELQLN-295" (Additional file 3). Of note, the presence of this antigenic epitopes in the vicinity of the "fusion region" could largely affect the sensitivity of RDTs to tell the difference between pvLDH and pmLDH. It is assumed that the "fusion region" might be the specific binding site for the monoclonal antibody of RDTs used in this study. The "fusion zone" or the interfered "double-antibody sandwich" immune-reaction incorrectly indicated the false-negative detection result for pvLDH. Moreover, multiple RDT products that can capture different antigenic epitopes of pLDH should be used in the screening of Plasmodium infection to ensure accuracy and specificity.

Conclusion

In summary, the examination of Plasmodium in bone marrow puncture fluid is a promise tool to compensate for the omissive diagnosis of malaria based on peripheral blood examination alone. The unknown etiology fever patients with travelling history to malaria-endemic region, and exhibition of anemia, thrombocytopenia and splenomegaly, undetected Plasmodium in the peripheral blood are applicable for bone marrow puncture examination after ruling out with other microbial infections, such as bacteria and mycoplasma in the first place.

Authors' Contributions

YH was responsible for the study design, and wrote the contents of clinical diagnosis and treatment in the manuscript; YD wrote the main manuscript, and was responsible for the coordinated the project, carrying out the genetic testing and statistical analysis; YW was responsible for clinical observation, carrying out bone marrow puncture and as co-first author; GL was responsible for making pathological slides of bone marrow puncture fluid and examination of Plasmodium under light microscope; HH, JX, YL, YL and YZ performed the collection of blood samples and implementation of doctor's advice; HH assisted YD in writing the manuscript. All authors read and approved the final manuscript.

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Institution Review Board Statement

These patients were admitted to hospital suffering from persistent fever after living in malaria endemic areas abroad. The study approved both by Kunming Third People's Hospital and by the Yunnan Institute of Parasitic Diseases. The clinical samples, which were only the remnant blood and bone marrow fluid for diagnosis and treatment fever patients, were reused under the following ethical guidelines in the approved protocols: 2019 Yunnan Ethics Auditing No. 5 and from Yunnan Institute of Parasitic Diseases and Ethical Committee.

Informed Consent Statement

The patients were fully informed on the aims of the study and signed an informed consent agreement after understanding the risks of bone marrow puncture. The patients also consented to their illness being published. A waiver of informed consent was approved by Kunming Third People's Hospital.

Data Availability of Data and Materials

The datasets used and/or analysisd during the current study available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare no conflicted interest.

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