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

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Abstract

Background

China has been certified as a malaria-free country by World Health Organization (WHO) in June 2021, yet the pressure of preventing the dissemination of imported malaria persists and thus calling for continued effort of timely detection and management. To compensate for the risk of missed diagnosis of using peripheral blood alone, medical institutions in Yunnan Province launched bone marrow puncture to confirm malaria *Plasmodium* infection more accurately.

Methods

Patients with a recent history of travelling to malaria-endemic areas outside of China, who were excluded from microbial infections such as tuberculosis and exhibited persistent abnormalities, including hepatosplenomegaly and decreased platelet pressure and the negative for *Plasmodium* lactate dehydrogenase (*p*LDH) antigen in the peripheral blood, were enrolled in the study. The cases ware conducted the bone marrow aspirate from anterior superior iliac spine to screen for Plasmodium infection in bone marrow fluid. Then the *p*LDH genes of the infected strains from cases ware amplified by conducted nested PCR. The amplification products were sequenced, and the B-cell epitopes and the oligomeric spatial structures of the *p*LDH peptide chains were analyzed.

Results

Bone marrow puncture was performed on 5 eligible subjects in total. The *Plasmodium* were found in the bone marrow fluid from all cases, which included two patients with *p*LDH-negative and three patients with *p*LDH-positive in peripheral blood detected by malaria rapid tests (RDTs). Two *p*LDH negative cases under malaria RDTs were diagnosed as *Plasmodium vivax* infection, with the proportion of ring stage, large trophozoites, schizonts, and stage III-V gametocytes reaching 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 determinants along *p*LDH peptide chains of the infected strains in the five cases. Of them, the four sequences of 63^{th} ~70^{th} aa, 86^{th} ~96^{th} aa, 198^{th} ~207^{th} aa, 287^{th} ~295^{th} were commonly found on *p*LDH peptide chains from *Plasmodium vivax*, *Plasmodium falciparum* and *Plasmodium malariea* strains. Of note, the sequence of “211-EEVEGIFDR-220" was only detected in *Plasmodium vivax* strain, whereas the sequence of “207-LISDAE-213” was unique for *Plasmodium falciparum* strain. The spatial location of both two sequences were proximal to the “fusion region" of antigenic epitopes on the surface of *p*LDH tetramer.

Conclusion

Examination of the *Plasmodium* infection in Bone marrow puncture fluid could make up for the missed diagnosis of malaria that solely relies on peripheral blood examination. For patients who travelled to malaria-endemic areas without common pathogenic microbial infections, yet with hepatosplenomegaly, granulocytic myelosuppression, decreased platelet counts, low level of hemoglobin and suspicious *p*LDH antigen results, bone marrow puncture can be applied to confirm the diagnosis of malaria more accurately.

Background

In spite of the risk of invasive detection, bone marrow puncture has played a vital role in the etiological diagnosis of malaria in people returning to Europe from malarial-endemic areas with unexplained fevers since the 1930s and 1940s [1-2]. It 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 inaccuracy in the confirmation of malaria infection that relies on 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 aspirate and peripheral blood of 174 Mozambican children, reporting that the positive rate of *Plasmodium* under 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 □to □) gametocytes of *Plasmodium falciparum* was 4.8-5-fold higher in bone marrow aspirate [7] than in peripheral blood. Brito et al [8] reported 2-3-fold higher in the comparison between bone marrow aspirate 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 the 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 reservoir of *Plasmodium 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 suggested the enriched expression of CD71 [10-11], CD44 [10, 7], EBL (the erythrocyte binding-like protein) and Rh (reticulocyte binding-like protein) [12], which are also the binding receptors for *Plasmodium*, on the surface of stage I and II naïve reticulocytes. Among them, CD71 is the adhesion molecule for *Plasmodium vivax* trophozoites to invade reticulocytes. As the expression of CD71 peaked in stage-I reticulocytes, *Plasmodium vivax* trophozoites mostly invade stage-I reticulocytes. Although CD71 level was the lowest on the surface of stage-III reticulocytes, stage-III reticulocytes are the only reticulocytes that can penetrate through the sinusoidal capillary lumen to enter the circulating peripheral blood [10-11]. Hence, 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. This also justifies the lower concentration of *Plasmodium* in peripheral blood than in bone marrow, hence the difference in sensitivity of detection.

In view of such finding, Pinho et al [13] pointed out that the *Plasmodium* existing in the hematopoietic natural niche resulted in these pathological changes such as marrow depression [14-17], osteoporosis [18-19], and allergy [20], the feature of locally enriched *Plasmodium* could be applied as the cornerstone for optimizing the laboratory diagnosis and treatment of malaria cases.

Although the number of malaria cases diagnosed in Yunnan is still among the highest rank in China in recent years, the mortality rate is the lowest in the country [23]. This is mostly ascribed to for the high capability of accurate diagnosis in the grassroot hospitals [24], the prompt implementation of bone marrow puncture in malaria-designated hospitals, the reduced misdiagnosis of febrile diseases, as well as the improved etiological treatment. In order to further improve the capacity of treating severe malaria and imported malaria cases, and to gradually standardize the protocol of bone marrow puncture in practice, the current study analyzes and summarizes the cases in our hospital for the diagnosis of suspected malaria infection, and the results are elaborated as follows.

Materials And Methods

Exposure to malaria and suspected malaria episodes in endemic areas

All 5 cases returned to Yunnan from African countries within 2 to 36 days (median= 27 days). They exhibited fever, headache, discomfort, with body temperatures reaching above 39°C during hospitalization. Their Hematocrit (HCT) and platelet pressure (PCT) were at low levels, and 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 indicate that the five patients were in a state of hemolytic anemia, compromised function of coagulation and strong immune emergency. Two cases of them 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, and *p*LDH antigen was not detected in two cases (Table 2).

All the patients tested negative for pathogenic microbial infections (such as HIV and Mycobacterium tuberculosis). The possibility of common microbial infections could be excluded.

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

Cases	Age	Gender	Infection from	Time since infection source (d)	Temperature on admission (°C)	PCV [a]	PCT [b]	hs-CRP [e] (mg/L)	ATL [d] (U/L)	IBIL [c] (μmol/L)
Case 1	33	Male	Congo	27	39	0.37	0.06	153.81	45	10.1
Case 2	26	Male	Guinea	31	40	0.47	0.11	130.74	10	8.8
Case 3	40	Male	Cameroon	36	40	0.42	0.11	144.01	19	5.6
Case 4	46	Male	Guinea	21	39	0.21	0.11	32.46	29	4.7
Case 5	30	Male	Congo	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 value of CR is 1.71-11.97 μmol/L.

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

Cases	Splenomegaly (thickness*length) (mm)	Fever (d)	Chills (d)	Profuse sweating (d)	Headache (d)	Fatigue (d)	Tea-colored Urine (d)	Monocyte ($\times 10^9$/L)	Microscopic parasitemia	RDT (*p*LDH)
Case 1	6*4	22	20	20	23	24	23	0.15 ~0.21	Positive	Non-falciparum
Case 2	0	7	7	7	8	9	2	0.64~1.02	Negative	Negative
Case 3	1*0	6	6	6	6	10	8	0.33~ 0.53	Negative	Negative
Case 4	20*0	12	12	12	12	20	20	0.68~0.55	Negative	Falciparum
Case 5	11*8	3	3	3	5	5	2	0.95~0.26	Negative	Non-falciparum

Antimalarial treatment

According to the Chinese Diagnostic Criteria for Malaria (WS269-2015) [27], people that exhibit one or more clinical indicators, including coma, severe anemia (hemoglobin < 5 g/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 severe malaria, otherwise are 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) [28]. For both vivax malaria and malariae malaria patients, the total oral 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. In addition, for benign tertian malaria patients, primaquine should be used to eradicate the hypnozoites on the first day of chloroquine administration or when the clinical symptoms had improved slightly (total dose = 180 mg, 22.5 mg once a day for 8 days in a row).

Analysis of the B-cell epitope in *p*LDH antigen between different Plasmodium

Venous blood was collected from the five subjects before antimalarial treatment on day 0 for preparing the filter paper dried blood samples to analyze *Plasmodium* molecular composition. *p*LDH gene was amplified by performing nested PCR for infected strains of *Plasmodium vivax*, *Plasmodium falciparum*, and *Plasmodium malariae* in the five cases, according to the protocol clarified in the literature [28]. The amplification products of *p*LDH genes were sequenced and the sequences were compared with the available referent sequences of *Plasmodium vivax* (NC_009917.1), *Plasmodium falciparum* (NC_004331.3), and *Plasmodium malariae* (LT594500.1) on NCBI (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). If the coverage (Query cover) > 90% and similarity (Identifies) > 95%, the respective *p*LDH 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, and then translated into *p*LDH peptide chains. The modus of "B Cell Epitope Prediction" of the online software IEDB (http: //www.iedb.org/) was used to predict B cell antigenic determinants of *p*LDH peptide chain. The spatial conformation of *p*LDH 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 aspirate fluid showed that the volume of erythrocytes with *Plasmodium* parasites in case 1 and case 5 were not distended and slightly reduced, and exhibited a typical stripe-shaped large trophozoite morphology,

and that the various stages of *Plasmodium*, including ring, large trophozoite, schizonts, and round gametophytes could be detected (Figure 1). Therefore, the two cases were identified as *Plasmodium 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 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 gametophytes (1.5%, 6/200), respectively. Hence, the two cases could be diagnosed as Plasmodium 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 was identified as *Plasmodium falciparum* infection.

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

Cases	Species	Density (parasites/ul)	No.	Stages				
				R	T	S	G (Ⅰ-Ⅳ)	G (Ⅴ)
				No. (%)	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) Ⅰ-Ⅳ: Stage Ⅰ-Ⅳ gametocytes, stage Ⅴ gametocytes.

A melioration 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 (Figure 2). In addition to chloroquine, artemether and artesunate were added to control the clinical malarial episodes of case 1.

The changes of 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 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 increases were not high enough to reach the normal range, indicating that the indexs of erythrocyte lineage were hindered to return to normal within a short period of time even after the cause of malaria was removed.

Leukocytes and neutrophils of the granulocyte lineage also showed parallel changes, showing 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 2.55-4.41 (×109/L). Although leukocyte count and neutrophil count of case 4 were within the normal range throughout the course (leukocytes: 5.79-7.33 ×109/L), neutrophils: 2.38-3.819 ×109/L), both two indicators decreased after antimalarial treatment, with the magnitude of -30.2% and -35.3%, respectively. The decreasing patterns were present 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 recovered to normal after the treatment, with the counts ranging from 146 to 455 (×109/L) (Figure 2). Platelet counts of the five cases rose by 466%, 378%, 252%, 168%, and 35%, respectively, indicating that megakaryocytes' function of platelet production has been restored swiftly after the treatment of *Plasmodium* infection

B-cell epitope clustering of *p*LDH antigen in the *Plasmodium* strains

Four to five B-cell active antigenic regions were present in the primary peptide chains of *Plasmodium* *p*LDH 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 common, 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 *p*LDH 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 *p*LDH peptide chain for different *Plasmodium* species (Table 4).

Table 4 Polymorphism of *p*LDH gene and prediction of B cell epitopes

Plasmodium	No. epitopes	B-cell epitope		
		Completely homologous sequence	[a]Most homologous sequence	Unique sequence
Plasmodium vivax	*5*	85-PGKSDKEWNRD-96 197-IPLQEFINNK-207 286-EQVIELQLN-295	62-GSN**S**[b]Y**D**[c]DL-70	211-EEVEGIFDR-220
Plasmodium falciparum	*4*		62-GSN**T**[b]Y**D**[c]DL-70	207-LISDAE-213
Plasmodium 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 *p*LDH peptide chain is S, it belongs to the sequence of *Plasmodium vivax* and *Plasmodium malariae*, while when it is T, it belongs to the sequence of *Plasmodium falciparum*. c: When the 68thaa of the *p*LDH peptide chain is D, it belongs to the sequence of *Plasmodium vivax* and *Plasmodium falciparum*, when it is E, it belongs to the sequence of *Plasmodium malariae*.

*p*LDH peptide chains of *Plasmodium varix* and *Plasmodium falciparum* (*pv*LDH and *pf*LDH) exhibited specific B-cell antigenic active regions of "211-EEVEGIFDR-220" and "207-LISDAE-213", respectively (Table 4). *p*LDH antigen chains of all five malaria cases showed an oligomeric spatial conformation with four subunits (Figure 3A-E). The short peptides of the five epitope clusters 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 among *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium falciparum*. However, in the proximity of the fusion region composed of two active short peptides (198th ~207th aa and 287th ~295th aa), antigenic determinants of “211-EEVEGIFDR-220” and “207-LISDAE-213” were detected in *pv*LDH and *pf*LDH, respectively (Figure 3B, C and D).

No additional antigenic determinants were found in *pm*LDH (Figure 3A, E). Therefore, the antigenic determinants of 211-EEVEGIFDR-220 and 207-LISDAE-213 could be used in the differential diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* infection by using RDT.

Discussion

In recent years, *Plasmodium* infection within the bone marrow aspirate is merely supplementary for the clinical diagnosis of other diseases that requires the testing of the bone marrow [4-6]. However, bone marrow aspirate could have heavier burden of *Plasmodium vivax* infection than peripheral blood [10-11], which only produces a lower density and deficient symptom of parasitemia. Hence, the examination of *Plasmodium* infection using peripheral blood alone may result in missed diagnosis of malarial infection. In comparison with bone marrow puncture, peripheral blood screening for *Plasmodium* infection has been reported to cause a rate of 11.4% (4/35) missed diagnosis in Pakistan [5].

In the current study, neither *Plasmodium* nor *p*LDH antigens were detected in peripheral blood of the two cases of *Plasmodium vivax* infection, yet late-stage large trophozoite and stage-IV gametophytes with intact cell structure were detected in bone marrow aspirate [5,22]. Such finding provides a solid etiological basis for the diagnosis of *Plasmodium 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 *Plasmodium vivax* showed potent proliferation and accumulation in the bone marrow puncture fluid.

For *Plasmodium malariae* infection cases with positive results of *p*LDH antigen detection in peripheral blood, bone marrow puncture for *Plasmodium* was still performed to exclude the possible false-positive rate in RDT test. In recent years, false-positive results for *p*LDH 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, our hospital explores the method of bone marrow aspirate examination for malaria pathogens. Such a clinical practice provides yet another empirical reference for 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 [14, 5]. The persistent parasitemia after malarial infection would in turn trigger myelosuppression [16] and leading to heterogeneity hematological abnormalities [30]. PCV shows a slow yet continuous decreasing trend during the first 3 days of *Plasmodium falciparum* infection [30-31], and the symptom of anemia was relieved on day 17 after treatment. For patients with cerebral malaria, the decreasing extent of hemoglobin was inversely related to the severity of parasitemia [31]. 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. The red blood cell (RBC) count and hemoglobin levels of cases 1 and 4 were still below the normal range on day 13 and 16 (Figure 1). These unfavorable indicators may be ascribed to the lack of supplementary chalybeate therapy during course of treatment, hence less conducive to the accumulation of red blood cell counts by the bone marrow puncture [14, 16].

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 [32], which indicated that neutrophil counts would increase at the onset of malaria infection and then decrease, reflecting the process of inflammatory response and anti-inflammatory response. Platelet count was the indicator most 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 [33] and hence the persistent decrease of platelet counts [34-35]. Consistent with the observations of previous research, we found that platelet counts increased by several-fold, and thus returned to the normal range after the treatment [35-36]. 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 initiation of bone marrow puncture for the diagnosis of malaria: For patients with atypical manifestations, especially those who exhibit pancytopenia and febrile neutropenia [37], more invasive screening test, such as bone marrow puncture, could be performed to exclude malaria infection [38-39]. However, bone marrow puncture should be avoided for patients with acute attack of malaria, who exhibit clinical symptoms and more pronounced pancytopenia in the peripheral blood, due to the concern of unjustified procedure [40,5]. In summary, the appropriate candidates for bone marrow puncture of *Plasmodium* testing are those with a traveling history to malaria- endemic areas, exhibition of fever, anemia, thrombocytopenia and splenomegaly, undetected *Plasmodium* in the peripheral blood, suspicious antigen test results, as well as the exclusion of other microbial infections, such as bacteria and mycoplasma.

Keluskar et al [41] suggested that *p*LDH, as a target antigen for malaria immune-diagnosis, has the stable antigenic determinant clusters, in which the genetic polymorphisms of *pf*LDH and *pv*LDH are the same. Huang et al [29] confirmed the high homology of *p*LDH gene in four human *Plasmodium* species, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. Moreover, *p*LDH antigen could only be detected during the life cycle of *Plasmodium* [42], and the amount of *p*LDH antigen is proportional to the density of *Plasmodium* [19,43]. The RDT products based on *p*LDH antigen show high sensitivity in the detection of *Plasmodium* infection, especially in extravascular tissues and organs [19,21]. Evidence showed that the false-negative rate of *Pf*HRP2-based RDTs reaches as high as 65% (11/17) and is even close to 100% (12/12), as opposed to the detection of *p*LDH antigen [44-45].

However, in the current study, *pv*LDH antigen was not detected in the peripheral blood of two cases, even though *Plasmodium vivax* had been detected in the bone marrow puncture fluid. As the role of *p*LDH antigen as an indicator of *Plasmodium vivax* infection could not be clarified in the cases [19,43], we speculated that this could be attributable to the low specificity of *pv*LDH-based RDT products. The B-cell epitope of the *p*LDH peptide chains of the five cases showed that expect for the common sequences of "85-PGKSDKEWNRD-96", "197-IPLQEFINNK-207 " and "286-EQVIELQLN-295" in *pv*LDH and *pm*LDH, the epitope of "211-EEVEGIFDR-220" is adjacent to the fusion region composed of "197-IPLQEFINNK-207" and "286-EQVIELQLN-295" (Figure 3). Of note, the presence of this very antigenic determinant in the vicinity of the "fusion region" could determine the ability of RDTs to detect the difference between *pv*LDH and *pm*LDH. 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 outcome for *pv*LDH. This also suggests that multiple RDT products that can capture different antigenic determinants of *p*LDH should be used in the screening of *Plasmodium* infection.

Conclusions

In summary, examination of *Plasmodium* in bone marrow puncture fluid is a promise tool to compensate for the missed diagnosis of malaria based on peripheral blood examination alone. The appropriate candidates for bone marrow puncture are people who have travelled to malaria-endemic region, with exhibition of fever, anemia, thrombocytopenia and splenomegaly, undetected *Plasmodium* in the peripheral blood, suspicious antigen test results. The other microbial infections, such as bacteria and mycoplasma, have been ruled out.

Abbreviations

*p*LDH: *Plasmodium* lactate dehydrogenase

*pv*LDH: *Plasmodium vivax* lactate dehydrogenase

*pf*LDH: *Plasmodium faciparum* lactate dehydrogenase

*pm*LDH: *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

RDT: Rapid diagnosis test

Declarations

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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 as co-corresponding author. All authors read and approved the final manuscript.

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Not applicable.

Ethics approval and consent to participate

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 were collected 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. 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. he patients also consented to their illness being published.

Consent for publication

All authors provided their consent for the publication of this report.

Competing interests

The authors declare no conflicted interest regarding the publication of the present manuscript.

Availability of data and materials

Not applicable.

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Figures

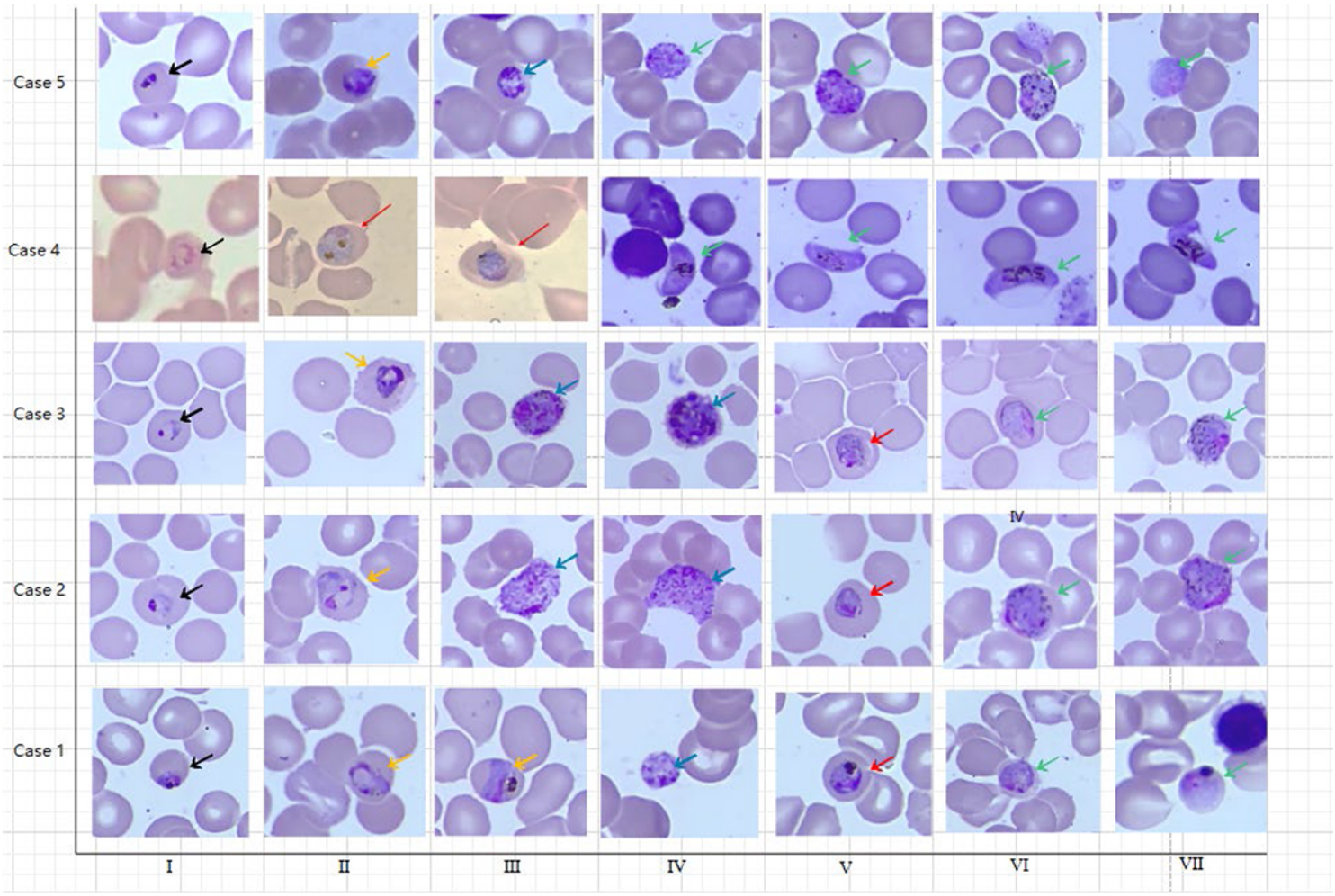

Figure 1

***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 is the schizonts stage, Red arrow is the stage Ⅴ-Ⅵ gametocytes, Green arrow is the Ⅵ-Ⅶ stage gametocytes.

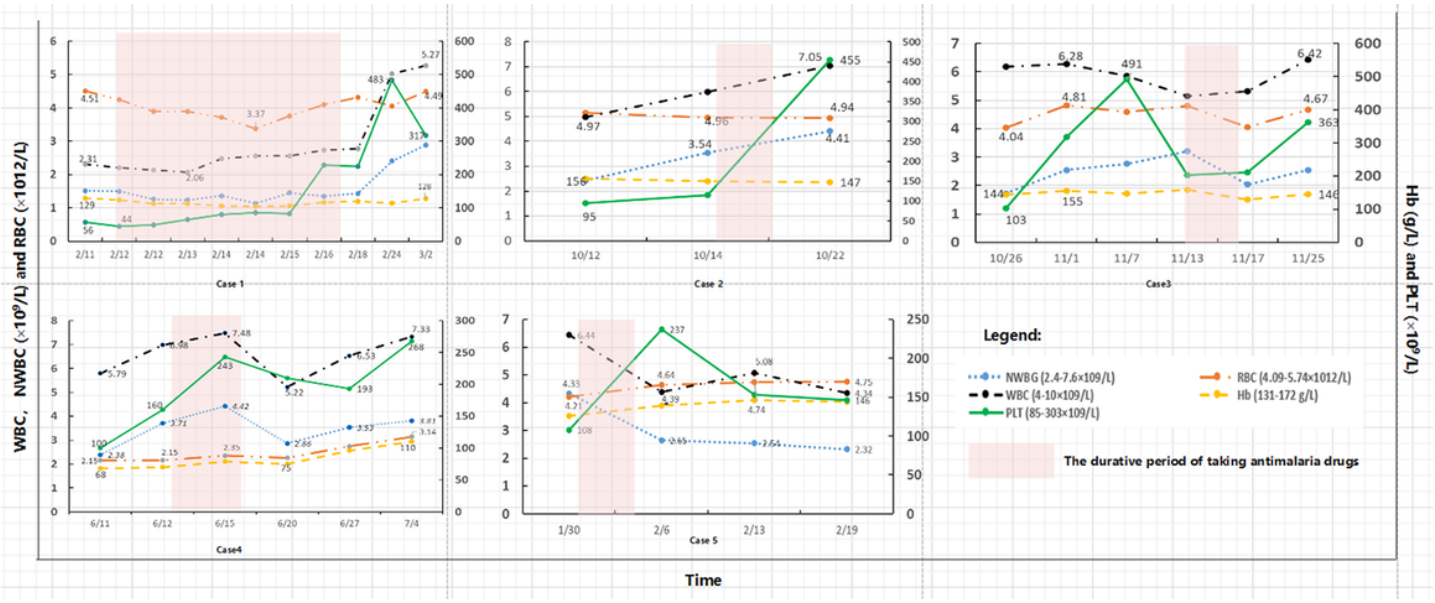

Figure 2

A melioration of myelosuppression in 5 patients after antimalarial treatment.

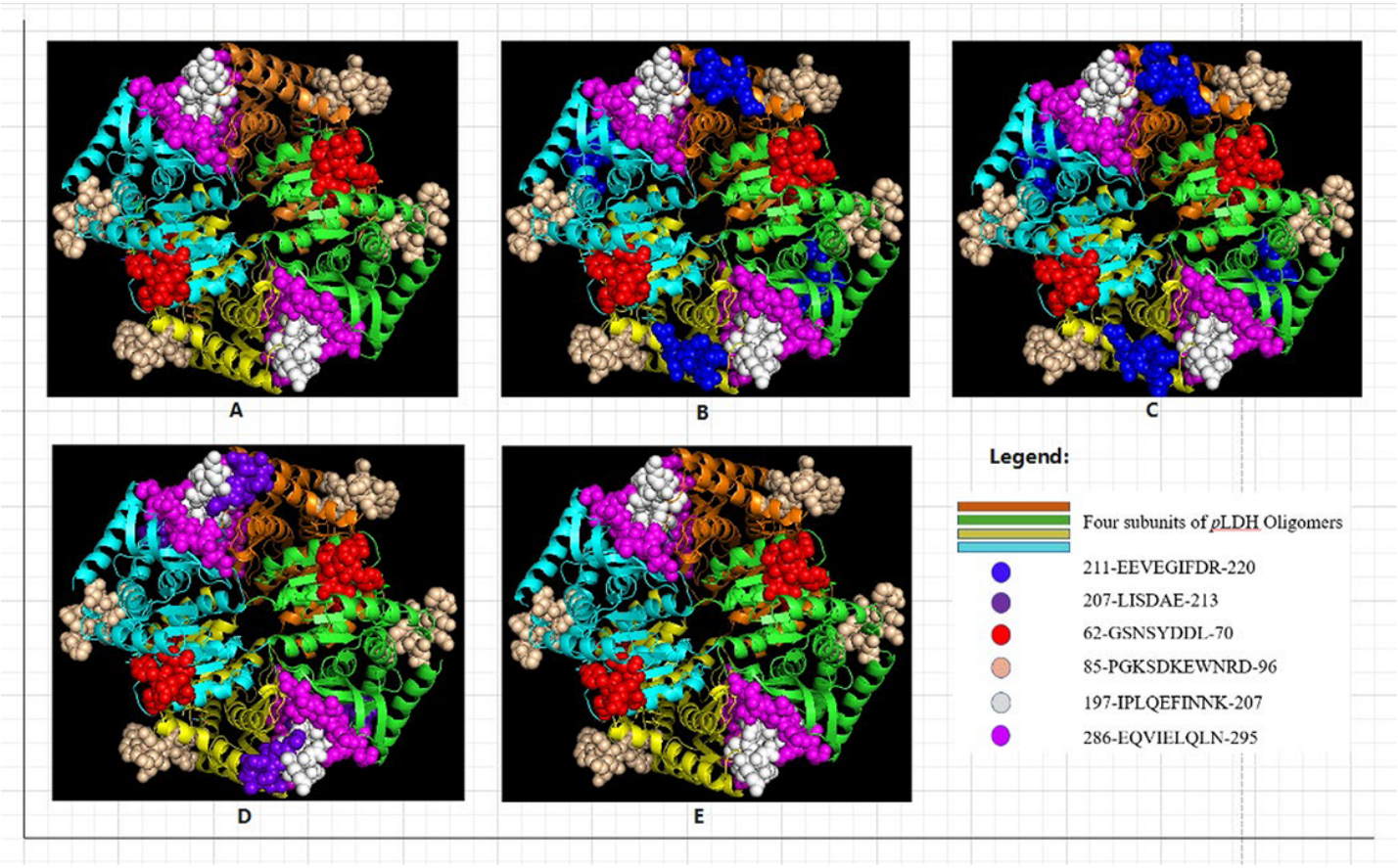

Figure 3

Spatial structures of *Plasmodium* pLDH peptide chain and distribution of B-cell antigenic determinants of five strains

Supplementary Files

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