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A more appropriate white blood cell count for estimating malaria parasite density in *Plasmodium vivax* patients in northeastern Myanmar

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Abstract

The conventional method of estimating parasite densities employs an assumption of 8000 white blood cells (WBCs)/ μ l. However, due to leucopenia in malaria patients, this number appears to overestimate parasite densities. In this study, we assessed the accuracy of parasite density estimated using this assumed WBC count in eastern Myanmar, where *Plasmodium vivax* has become increasingly prevalent. From 256 patients with uncomplicated *P. vivax* malaria, we estimated parasite density and counted WBCs by using an automated blood cell counter. It was found that WBC counts were not significantly different between patients of different gender, axillary temperature, and body mass index levels, whereas they were significantly different between age groups of patients and the time points of measurement. The median parasite densities calculated with the actual WBC counts (1,903/ μ l) and the assumed WBC count of 8,000/ μ l (2,570/ μ l) were significantly different. We demonstrated that using the assumed WBC count of

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8000 cells/ μl to estimate parasite densities of *P. vivax* malaria patients in this area would lead to an overestimation. For *P. vivax* patients aged five years and older, an assumed WBC count of 5,500/ μl best estimated parasite densities. This study provides more realistic assumed WBC counts for estimating parasite densities in *P. vivax* patients from low-endemicity areas of Southeast Asia.

Graphical Abstract

Keywords

malaria parasite; white blood cell count; parasite density; parasite species; age

Introduction

Malaria continues to be a leading cause of morbidity and mortality worldwide, with more than 200 million cases of disease annually. In 2014, it is estimated that malaria infection results in 438,000 deaths (WHO, 2015). In the Greater Mekong Subregion (GMS) of Southeast Asia, the tropical and subtropical climate is highly conducive to malaria transmission (Cui et al., 2012). Despite that several countries in this region are moving toward malaria elimination, malaria remains highly prevalent along international borders, where hill tribes and ethnic minorities reside. In the border regions, political, economic and geographic factors hinder efforts to eliminate malaria. Increased control efforts, better diagnosis and treatment, and efficient management of drug resistance are needed.

Estimating malaria parasite density (number of parasites per μl of blood) is necessary for disease management, clinical trials and drug efficacy studies (WHO, 2010). White blood cell (WBC) counts have been a useful surrogate for direct scoring of parasite load under field conditions. Complete blood counts, particularly WBC counts, can be performed with automated hematological analyzers, or manually using stains, a microscope and a cell counting chamber and counters. However, in most field settings, it is not practical to score WBC values of malaria patients by automated blood cell counters. To allow for estimation of parasite densities in areas lacking infrastructure required for scoring actual WBC counts,

assumed WBC values have been used. The World Health Organization (WHO) recommended the use of an assumed WBC value of 8,000/ μ l (WHO, 2010), which was based on a study with a Nigerian population with a large proportion of children younger than five years (Dowling and Shute, 1966). Yet, recent studies found that estimating malaria parasite densities using this WBC value yielded results that were inconsistent with the actual observed malaria parasite density of patients in some areas (Alves-Junior et al., 2014; Jeremiah and Uko, 2007; McKenzie et al., 2005; Rishikesh et al., 2015). Furthermore, whereas most studies conducted so far focused on *P. falciparum* patients, studies conducted in areas co-endemic for *P. vivax* and *P. falciparum* revealed considerable differences in WBC counts between patients infected with these two parasite species (Alves-Junior et al., 2014; McKenzie et al., 2005). Since the abundance of WBCs is influenced by the host immune status (Berens-Riha et al., 2014), and may vary in people of different races, ages, nutritional conditions, and pathogen infections, the baseline values of leukocytes in patients may differ significantly between populations and geographical regions (Bain, 1996). Therefore, it has been suggested that the best solution for estimating parasite densities is to use different WBC counts for different geographical regions, age groups and sub-populations (e.g., pregnant women), and epidemiological settings (Adu-Gyasi et al., 2012; Alves-Junior et al., 2014; Haggaz et al., 2014).

Here we assessed the accuracy of WHO recommended WBC count for estimating malaria parasite density in an area of the GMS along the China-Myanmar border. *Plasmodium vivax* is the predominant parasite species and the study population consists primarily of peoples of the Kachin (Jingpo) ethnicity (Li et al., 2013). By quantifying WBCs using an automated hematological analyzer, we found that the assumed WBC count of 8,000 cells/ μ l significantly overestimated parasite densities, particularly in patients $\leq$ 5 years. We further established that a WBC count of 5,500 provides a significantly closer estimation of the parasite density in this region among patients of five years and older.

2. Materials and methods

2.1. Study site and participants

The study was carried out in two settlements for internally displaced people and four villages near Laiza city in northeastern Myanmar along the China-Myanmar border. A total of 256 patients of mostly Kachin (Jingpo) ethnicity, 1 to 59 years old, with uncomplicated *P. vivax* infections were recruited from April 2014 to October 2015. Pregnant women, those suffering severe malnutrition, and patients found to harbor other infectious diseases were excluded. Demographic information of patients was obtained using a questionnaire during admission of patients. Axillary temperature of the participants was measured at the time of admission. Their body weight and heights were measured. Body mass index (BMI) was calculated using the formula: [weight (kg)/height squared (m^2)]. Informed consent/assent was obtained from enrolled patients/guardians and this study was approved by the Kachin bureau of public health and institutional review board of Kunming Medical University (#KMC2012-01).

2.2. Blood sample collection and analysis

Malaria diagnosis was performed by light microscopy of thin and thick Giemsa-stained blood smears from finger prick blood. A venous blood sample (approximately 0.5–2 ml) was drawn from each patient into an EDTA-filled tube for WBC counting by using an automated hematological analyzer (TEK-II Mini, China) on day 0 and day 7. Blood was analyzed within 30 min of collection. Daily internal quality controls and scheduled external quality assessments were performed following the instructions from the manufacturer. *P. vivax* malaria patients were treated with chloroquine and primaquine per local drug policy.

2.3. Estimation of parasite density

Plasmodium parasites were counted against 200 WBCs (or 500 WBCs when the initial number of parasites was less than 99) on thick blood films. Parasite densities were first recorded as the average value of parasites per 200 leukocytes counted by two trained microscopists. For numbers differing by >20% (and negative slides), a senior microscopist provided an additional evaluation to reconcile the divergent results. Parasite densities (parasite/μl of whole blood) were then calculated as: (Number of parasites counted/WBC counts) × WBC count/μl. Calculation of parasite densities was based on either assumed WBC counts (ranging from 4,000 to 11,000 WBCs/μl of blood) or actual WBC counts from the automated WBC counter.

2.4. Statistical analysis

Data was analyzed and visualized using Graphpad Prism (version 6.0). Since the WBC counts had a skewed distribution, we assessed parameters that could influence the WBC counts using several non-parametric tests. Kruskal-Wallis test was used for comparison among multiple age groups and BMI data, whereas Dunn's multiple comparison test was used for between-group comparisons among multiple age groups. Mann-Whitney *U* test was used for comparing two groups of gender, stratified BMI data, and two axillary temperature categories (38.0 vs >38.0 °C). The Wilcoxon matched-pairs signed rank test was used for comparison of WBC counts between the two detection time points, and between parasite densities calculated by actual WBC counts and estimated by assumed WBC counts. To evaluate the best assumed WBC for the estimating of parasite densities, Bland-Altman plot analysis was carried out after logarithmic transformation of parasite densities.

3. Results

We recruited a total of 256 *P. vivax* patients in northeast Myanmar to assess the validity and accuracy of determining parasite density using the assumed WBC count of 8,000/μl. Among these patients, fever, chills, headache and weakness were the most common symptoms. Automated blood count revealed that 51 (19.92%) and 12 (4.69%) patients had leucopenia (<4,000/μl) and leukocytosis (>10,000/μl), respectively.

Automated blood counts were stratified by patient age, gender, axillary temperature, and BMI (Table 1). WBC counts were not significantly different between patients of different genders, presence (>38.0°C) and absence of fever (38.0°C), and the two BMI groups (Table 1). In contrast, there was a significant difference in WBC counts between different

age groups. Specifically, WBC counts from children younger than five years were significantly different from those of other groups ($P = 0.001$ and $P = 0.0002$ compared with the 5–14 and 15 years groups, respectively; Dunn's multiple comparison test). In addition, WBC counts were also significantly different between the two time points of measurement, with WBC counts performed on day 7 being significantly higher than those on day 0 ($P = 0.0163$, Wilcoxon matched-pairs signed rank test). It is noteworthy that all *P. vivax* patients enrolled were parasite-negative on day 7 based on microscopic examinations of finger-prick blood smears.

The median parasite density determined by automated WBC counting was 1,903/ μ l (interquartile range: 752–3,579/ μ l). To determine which assumed WBC count best estimates parasite density, we used assumed WBC counts of 8,000, 7,000, 6,000, 5,500, 5,000 and 4,000/ μ l, and predicted a median parasite density of 2,570, 2,249, 1,928, 1,767, 1,607 and 1,285/ μ l, respectively (Table 2). The parasite densities estimated by assumed WBC counts of 8,000 and 7,000 cells/ μ l were significantly higher than the densities calculated by the actual WBC counts, whereas assumed WBC counts of 5,000 and 4,000 cells/ μ l significantly underestimated parasite densities. The parasite density estimated using the assumed WBC counts of 5,500 ($P = 0.2315$) and 6,000 cells/ μ l ($P = 0.0814$) were not statistically significantly different from that calculated using the WBC counts determined by the automated counter (Table 2). Comparison using the Bland-Altman plot showed that parasite density estimated using the assumed WBC count of 5,500 cells/ μ l was most approximate to that calculated by using the actual WBC count (Figure 1).

The WBC count of 8,000/ μ l recommended by WHO was based on a study of a population in Africa, which consisted of almost 50% children under the age of five (Dowling and Shute, 1966). To determine whether this number is suitable for children of <5 years in our study group, we made similar comparison between parasite densities calculated using assumed and actual WBC counts. This analysis showed that parasite densities estimated using assumed WBC counts of 7,000 and 8,000 were not significantly different from that calculated from the actual WBC count (Table 3) ($P > 0.05$, Wilcoxon matched-pairs signed rank test). For patients five years and older, only parasite densities estimated from assumed WBC value of 5,500 ($P = 0.4967$) cells/ μ l was not significantly different from that calculated using the actual WBC counts (Table 3).

4. Discussion

Estimation of blood stage malaria parasite densities by microscopy conventionally adopts an assumed constant WBC count of 8000 cells/ μ l in peripheral blood. In this study we further evaluated the suitability of this number for estimating malaria parasite density in an endemic area of Southeast Asia. After stratifying the WBC data by potential factors (age, gender, fever, and time of measurement), we identified that age and the time of measurement had a substantial influence on WBC counts.

One major finding of this study is that using the assumed WBC count of 8000 cells/ μ l for estimating parasite density significantly exaggerated the parasite density in this region. Our study further suggested that the proportions of different age groups in a study population are

probably the most important factor influencing WBC counts and estimation of parasite densities. We found that an assumed WBC count of 5,500/ μ l best approximated the parasite density in our entire patient population, especially for patients five years of age and older. For children younger than five years, a WBC count of 8,000/ μ l was appropriate, though the small number of patients in this age group (26) prevented a more vigorous analysis. Nonetheless, this notion has been repeatedly observed in similar studies conducted in other malaria-endemic regions (Table 4). Of particular notice is that despite regional difference, the assumed WBC count of 5,500 cells/ μ l from this study coincided well with the proposed WBC count in a region of the Brazilian Amazon, where the majority (92.6%) of the study subjects was adults (Alves-Junior et al., 2014). An assumed WBC count of 5,000 cells/ μ l for patients five years and older matched well with a recent finding in south-western India, where all patients were adults and infected with *P. vivax* only (Rishikesh et al., 2015). In patient populations of exclusively or mostly children under five years, it has been documented that the recommended WBC count 8,000 cells/ μ l either was appropriate (Laman et al., 2014) or in a rare case underestimated parasite densities (Adu-Gyasi et al., 2012). In contrast, in populations consisting of mostly adults, this assumed WBC value would lead to significant overestimates of parasite densities (Alves-Junior et al., 2014; Haggaz et al., 2014; Jeremiah and Uko, 2007; McKenzie et al., 2005; Rodriguez-Morales et al., 2005; Tangpukdee et al., 2008). Taken together, the WHO recommend WBC value of 8000 cells/ μ l, based on a study cohort that was nearly 50% under the age of five, may have inflated the WBC counts in a population consisting of mostly adults (Dowling and Shute, 1966).

The time when WBCs are counted during the course of the disease or after treatment appears to also impact the outcome of the WBC counts. On day 7 after treatment, there was a recovery of WBC counts and this finding is largely consistent with those from other malarious regions. For example, in Papua New Guinea WBC counts in children younger than five years are dependent on the time of examination relative to the onset of illness (Laman et al., 2014). Similarly, WBC counts in Thai adults of both *P. falciparum* and *P. vivax* malaria quickly recovered from a significantly lower than normal level at the time of admission to the normal level within a week after treatment (Tangpukdee et al., 2008). The reduced WBC counts and in some cases leucopenia during an initial infection need to be taken into account when parasite densities are estimated based on assumed WBC numbers.

In areas of co-existence of different parasite species, WBC counts in patients of different malaria parasites have been compared. It has been documented in both Southeast Asia and South America that *P. falciparum* patients tended to have lower WBC counts than *P. vivax* patients (McKenzie et al., 2005; Tangpukdee et al., 2008). However, some studies did not find differences in WBC counts between patients of the two parasite species (Jadhav et al., 2003; Reiley and Barrett, 1971). In addition, the opposite was also noticed in a study conducted in South America, where lower WBC counts and leucopenia were more common among *P. vivax* patients than in *P. falciparum* patients (Rodriguez-Morales et al., 2005). *P. vivax* and *P. falciparum* are both endemic in our study region, where the former is the predominant species. Future studies should be conducted to make a comparison between the two species.

In conclusion, our results showed that in a malaria hypoendemic region of Southeast Asia a WBC count of 5,500 cells/ μ L is most appropriate for estimating malaria parasite densities of *P. vivax* malaria patients 5 years of age and older. These findings may have implications for other populations, as the influence of age on actual values may be greater than heretofore recognized. Though parasite density estimation is not needed for routine treatment of malaria cases diagnosed using either rapid diagnostic kits or microscopy, accurate parasite counts are more critical for clinical management of severe, hospitalized cases and for research purposes. Therefore, based on the consistent findings from multiple sites, WHO should consider modifying the current recommendation of using a WBC count of 8,000 cells/ μ L for estimating malaria parasite density.

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Highlights

We compare *Plasmodium vivax* parasite density using assumed and actual WBC counts.

The WHO recommended WBC count of 8000/ μ l blood overestimates parasite density.

A WBC count of 5500/ μ l most closely estimates parasite density in patients $\geq$ 5 years.

Fig. 1.

A comparison of different parasite densities of the entire study population estimated using assumed WBC counts (A. 5,500 cells/ μ l; B. 6,000 cells/ μ l) and automated WBC counts by Bland-Altman plot after logarithmic transformation of parasite densities.

Table 1
Comparison of white blood cell (WBC) counts between different malaria patient groups

Groups	N	WBC/ μ L Median	WBC/ μ L Mean (SD)	P
Age (years)	<5	7,750	7,719 (2,536)	0.0003 ^{a*}
	5–14	5,800	5,868 (2,200)	0.0010 ^b (vs <5 group)
	15	5,400	5,571 (2,025)	0.0002 ^b (vs <5 group)
Gender	Female	6,000	6,060 (2,349)	0.4501 ^c
	Male	5,800	5,828 (2,151)	
Axillary temperature (°C)	38.0	5,600	5,800 (2,102)	0.3223 ^c
	>38.0	6,050	6,023 (2,331)	
Measurement time	Day 0	5,500	5,767 (2,076)	0.0163 ^d
	Day 7	5,600	6,562 (3,365)	
Body mass index	<18.5	6,000	6,109 (2,414)	0.1255 ^c
	18.5	5,400	5,537 (1,927)	

* P value for the entire age groups;
^a Kruskal-Wallis test;
^b Dunn's multiple comparison test;
^c Mann-Whitney test; and
^d Wilcoxon matched-pairs signed rank test. SD, standard deviation.

Table 2

Comparison between parasite densities (parasites/ μ l) calculated using actual WBC count and those estimated using assumed WBC counts

WBC count/ μ l	Median	Range (min-max)	IQR	Mean $\pm$ SD	P*
Actual count	1,903	30~44,493	752~3,579	3,196 $\pm$ 4,881	--
8000	2,570	40~48,100	1,055~5,320	4,362 $\pm$ 5,801	<0.0001
7000	2,249	35~42,088	923~4,655	3,817 $\pm$ 5,076	<0.0001
6000	1,928	30~36,075	791~3,990	3,272 $\pm$ 4,351	0.2315
5500	1,767	28~33,069	725~3,658	2,999 $\pm$ 3,988	0.0814
5000	1,607	25~30,063	660~3,325	2,727 $\pm$ 3,626	<0.0001
4000	1,285	20~24,050	528~2,660	2,181 $\pm$ 2,901	<0.0001

* P: Wilcoxon matched-pairs signed rank test between the median parasite density derived from an assumed WBC count (4,000–8,000) and that from an automated WBC counter. IQR, interquartile range; SD, standard deviation.

Table 3

Comparison of different parasite densities between estimated by assumed and automated WBC counts in different age groups.

Assumed WBC/ μ l	<5 years		5 years	
	Median Parasite density (/ μ l)	<i>P</i> *	Median Parasite density (/ μ l)	<i>P</i> *
11,000	5,308	< 0.0001	3,479	< 0.0001
10,000	4,825	0.0001	3,163	< 0.0001
9,000	4,343	0.0072	2,847	< 0.0001
8,000	3,860	0.7454	2,530	< 0.0001
7,000	3,378	0.1574	2,214	< 0.0001
6,000	2,895	0.0010	1,898	0.0117
5,500	2,654	0.0003	1,740	0.7353
5,000	2,413	0.0001	1,582	0.0016
4,000	1,930	< 0.0001	1,265	< 0.0001
Automated count	3,630	-	1,760	-

* **P**: Wilcoxon matched-pairs signed rank test between the median parasite density derived from an assumed WBC count (4,000–11,000) and that from an automated WBC counter. The most appropriate WBC counts for the two age groups were highlighted in bold.

Table 4

Summary of studies for comparison between parasite densities estimated using actual and assumed (8,000 cells/ μ l) WBC counts

Study location	N	Age distribution (%)				Parasite species	Parasite densities estimated using WBC count of 8,000/ μ l	Recommended WBC count (cells/ μ l)	Reference
		<5	5–14	15					
Nigeria	231	48.9	31.6	19.5		<i>P. falciparum</i>	Recommended	8,000	(Dowling and Shute, 1966)
PNG	168	100	-	-		<i>P. falciparum/P. vivax</i>	Appropriate	8,000	(Laman et al., 2014)
Ghana	5,902	100	-	-		<i>P. falciparum</i>	Underestimated	10,000	(Adu-Gyasi et al., 2012)
Sudan	98	-	-	100		<i>P. falciparum</i>	Overestimated	-	(Haggaz et al., 2014)
Nigeria	240	41.3	58.7	-		<i>P. falciparum</i>	Overestimated	-	(Jeremiah and Uko, 2007)
Thailand, Peru	4,697	?	?	>69.0		<i>P. falciparum/P. vivax</i>	Overestimated	-	(McKenzie et al., 2005)
Brazil	403	?	?	92.6		<i>P. vivax/P. falciparum</i>	Overestimated	5,500	(Alves-Junior et al., 2014)
India	284	-	-	100		<i>P. vivax</i>	Overestimated	5,000–5,500	(Rishikesh et al. 2015)
East Myanmar	256	10.2	50.4	39.5		<i>P. vivax</i>	Overestimated (for 5 years)	5,500	This study