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A cross-sectional survey of seroprevalence to *P. falciparum* antigens in children living along the border of Thailand and Myanmar and its lack of correlation with nutritional status and anaemia.

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Abstract

Background: In Thailand, the prevalence of malaria was highest in the northern region and most infected cases were school-age children. This study aimed to determine the seroprevalence of antibodies against malaria antigens among school-age children living on the Thailand-Myanmar border and its correlation with nutritional status and anaemia.

Methods: One-hundred and forty eight children in Tak Province were enrolled. Brief histories were taken, and dried blood spots collected on filter paper were used to determine antibodies to malaria antigens. Weight and height were measured, and body mass index (BMI) was calculated to assess health status.

Results: The mean BMI was 16.2 kg/m^2 (SD 2.6) and 116 (78%) children had normal BMI-for-age. The mean haematocrit was 38 % (SD 3) and 12 (9%) children had anaemia. Seroprevalence of antibodies to *Plasmodium falciparum* schizont extracts, the 19kDa of merozoite surface protein-1 and the apical membrane antigen-1 were 13.5%, 8.1% and 5.4%, respectively. The mean haematocrit and BMI-for-age were not statistically different between children with and without antibodies to malaria antigens.

Conclusion: We report an overall rate of 17.6% of antibodies to malaria antigens in school-age children living in an endemic area. No significant differences in nutritional status or anaemia were found between children who had and did not have the antibodies. The data from this study may contribute to understanding the relationship between health status and specific anti-*P. falciparum* antibody response in an area of low malaria transmission.

Keywords: Malaria, Antibody, Thailand, Children.

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Introduction

Malaria is a parasitic disease caused by protozoa in the Plasmodium Genus. Among the five species that can infect humans, *Plasmodium falciparum* and *P. vivax* are the most common cause of malaria, with *P. falciparum* the deadliest. The asexual intra-erythrocytic stage of the parasite life cycle is the apparent cause of the pathology. Although increased malaria prevention and controls have dramatically reduced malaria cases in many countries, it still caused nearly 600,000 deaths worldwide in 2013 [1]. Among these, 78% of the children were less than 5 years old. Despite a substantial decline in the infection rate in Thailand, malaria is still a public health concern, especially for those living along the Thai-Myanmar and Thai-Cambodia borders [2]. Of approximately 40,000 confirmed cases in 2013, 40% were children between the ages of 10 and 14 years [3].

Naturally acquired immunity that confers protection against clinical malaria is essentially mediated by antibodies against blood-stage malaria antigens [4,5]. Levels of antibodies specific to blood-stage malaria vaccine candidates, namely the 19 kDa of merozoite surface protein-1 ($MSP1_{19}$) and the apical membrane antigen-1 (AMA-1), are associated with protection against

malaria infection in children [6-9]. The mechanisms by which these antibodies confer protection against malaria include inhibition of the proteolytic process of the MSP1 precursor, inhibition of red blood cells invasion by the parasites and inhibition of parasite development in the red blood cells [10-13]. The existence of these antibodies can be used to assess the epidemiological situation and to monitor changes of malaria transmission intensity, which may be valuable for the management of malaria control programs [14-17].

It is perceived that the immune response in children is dependent on nutritional status to some extent [18-20]. Malnourished children were reported having lower prevalence and levels of anti-*P. falciparum* antibodies compared to well-nourished controls, regardless of the intensity of infection [18]. Malnutrition causes stunting and impairs host immunity, leading to increased malaria severity [21]. However, a systemic review of 15 clinical studies revealed that the relationship between malaria-nutritional status remains controversial and requires further investigation [22].

Most published data on nutritional status and acquired immunity to malaria in children are from Africa, where malaria transmission is intense [9,18,21]. Information from areas with lower transmission rates is limited. In the present study, we conducted a cross-sectional survey aimed to determine seroprevalence to crude extract of *P. falciparum*, Pf $MSP1_{19}$ and Pf AMA-1 in children living along the Thai-Myanmar border. Pf $MSP1_{19}$ and PfAMA-1 recombinant proteins were selected for the study of seroprevalence as they are frequently recognized antigens and are associated with protection in children [6-9]. Nutritional status and prevalence of anaemia in children with and without antibodies to malaria antigens were also evaluated.

Methods

Study Population and Blood Collection

A cross-sectional study was conducted in February 2012. Inclusion criteria were primary school children (5-15 years old) living in five Karen villages – Kubo, Chongpae, Talakong, Unite, and Mae Chantha – in Umpang District, Tak Province located on the Thai-Myanmar border. Potential participants were approached during their yearly visit to the Dental Mobile Unit of the Faculty of Dentistry, Chiang Mai University. The Research Ethics Committee at the Research Institute for Health Sciences, Chiang Mai University, approved the study. Written informed consent was obtained from guardians on the day of enrollment, and assent was obtained from each child participant.

Brief histories were taken by interviewing participants and their teachers or parents. History of malaria infection was obtained by self-report, interviewing parents and health records at the health care centres. Weight and height were measured. Body Mass Index (BMI) was calculated from weight (kg) over height squared (m^2). BMI-for age between the 10^{th} to 90^{th} percentiles was defined as normal. Blood from a finger prick was collected into a Na-heparinized, micro-haematocrit tube to assess haematocrit, and onto filter paper to assess antibody levels against malaria antigens. Anaemia was defined as having haematocrit below 34% for children younger than 12 years and 36% for those older than 12. Spots on filter paper were left to dry at ambient temperature and relative humidity for 24 h and then stored in individual, self-sealing, plastic bags that contained self-indicating silica desiccant gel. Packed specimens were kept in a box protected from sunlight until shipped to the laboratory.

Malaria Antigens

Crude *P. falciparum* schizont extract (PfSE) was obtained from continuous *in vitro* culture following standard conditions [23]. Mature schizonts were obtained by gradient centrifugation over 60% Percoll (GE Healthcare Life Science, Buckinghamshire, England), adjusted to a concentration of 1×10^8 schizont-infected red blood cells/ml and exposed to three freeze/thaw cycles to obtain PfSE. $PfMSP1_{19}$ was a gift from A. Holder (National Institute of Medical Research, London, UK). PfAMA-1 was a gift from R.F. Anders (LaTrobe University, Victoria, Australia).

Elution of Antibodies from Blood Spots

Antibodies were eluted from dried blood spots as described previously [24]. In brief, a 2.5 mm diameter disc was cut from filter paper using a leather punch, and was transferred to a 96-well microtitre plate (Nunc, Thermoscientific, Denmark). One hundred and fifty microlitres of 0.05% NaN_3, 0.05% Tween-20 (Ajax Finechem) in phosphate buffer saline (PBS) was added into each well and the plates were left on a rocking shaker overnight at room temperature. This gives a concentration of eluted antibodies equivalent to approximately 1:200 of original serum [24].

Enzyme-Linked Immunosorbent Assay (ELISA)

The antibody levels against malaria antigens were determined by ELISA, as described previously [25]. Briefly, Maxisorb immunoplates (Nunc, Thermoscientific, Denmark) were coated with antigens (at a concentration equivalent to 10^5 iRBC/ml for PfSE, 0.5 µg/ml for $MSP1_{19}$ and 1 µg/ml for AMA-1) in bicarnonate buffer (pH 9.6) overnight at 4°C. The plates were washed 4 times with 0.05% Tween-20 PBS and wells were blocked with 200 µl of 1% non-fat milk powder in PBS for 1 h at 37°C; supernatants were discarded. 10 µL of eluted blood were added into individual wells containing 40 µl of 0.05% Tween-20, 1% non-fat milk powder in PBS, giving a final concentration equivalent to 1:1000. After incubating for one hour, the plates were washed and 50 µl of 1:2000 anti-human IgG horseradish peroxidase conjugate (Invitrogen, Camarillo, CA, USA) was added and incubated for 1 h

at 37°C. After 4 washes, 50 µl of *o*-phenylenediamine substrate (Sigma) solution was added, and plates were incubated at room temperature (RT) for another 20 min. Twenty five microlitres of 2 N sulphuric acid was added to each well and absorbance was then read at 492 nm on a Spectra MR plate reader (Dynex Technology, Chantilly, VA, USA). Antibody levels were calculated from a standard curve (derived by serial dilution of a pool of high titer sera collected from *P. falciparum* infected patients, which was given an arbitrary value of 1,000 units/ml of anti-PfSE Abs) on each plate, as described previously [26]. Negative control was a pool of sera collected from healthy individuals who lived in Chiang Mai city, where malaria transmission was eliminated more than 30 years ago (Suwonkerd W; The Ministry of Public Health; personal communication).

Detection of Malaria Parasites from Dried Blood Spots

Dried blood spots collected on filter papers were checked for the presence of *P. falciparum* and *P. vivax* by nested PCR. Template DNA was isolated using the simple Chelex boiling method and subjected to nested PCR as described previously [27,28].

Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Corporation, Armonk, New York). Seroprevalence of anti-malaria antibodies, prevalence of anaemia, and nutritional status were reported as a number (percentage). Categorical variables were tested using a chi-squared or Fisher's exact test, as appropriate. P-value<0.05 (two-tailed) was considered as statistically significant.

Results

Demographic Data

The study enrolled 148 children; 75 (51%) were male. Their mean age was 9.6 years (SD 2.7). All were schoolchildren attending either kindergarten or primary school in the villages. Their mean weight and height was 26.4 kg (SD10.2) and 125 cm (SD15), respectively. The mean body mass index was 16.2 kg/m^2 (SD 2.6). Twenty three (16%) and 45 (30%) of the children had weight-for-age (WFA) and height-for age (HFA)<-2.0 SD, respectively. Of the 141 children whose haematocrit was assessed, the mean haematocrit was 38% (SD3). Twelve (9%) of the children had anaemia.

Prevalence of Anti-Malaria Antigens Antibodies and Malaria Infection

Seventy-two children (49%) reported at least one prior episode of malaria infection. Among these, relative to the date of blood collection, 15 (10%) had malaria symptoms within the past 6 months, 31 (21%) 6-12 months ago, and 26 (18%) over 12 months ago. For antibody detection, samples were calculated from the positive and negative reference sera as described previously, and were considered seropositive if the antibody responses to PfSE, PfMSP1$_{19}$ and PfAMA-1 were higher than 0, 0 and 100 arbitrary units per ml, respectively [25,26]. Of the 148 children, 26 (17.6%) were positive to at least one *P. falciparum* antigen, as determined by ELISA. The frequencies of antibody responses to crude PfSE, MSP1$_{19}$ and AMA-1 were 20 (13.5%), 12 (8.1%) and 8 (5.4%), respectively. Among the 26 seropositive children, 3 (11.5%) had antibodies to all three antigens, 5 (19.2%) had antibodies to PfSE and MSP1$_{19}$, 3 (11.5%) had antibodies to PfSE and AMA-1, 9 (34.6%) had antibodies to PfSE only, 4 (15.4%) had antibodies to MSP1$_{19}$ only and 2 (7.7%) had antibodies to AMA-1 only, respectively (Table 1). The levels and pattern of antibody responses to malaria antigens in the 26 seropositive children are shown in Figures 1 and 2, respectively.

Of the 26 children who had antibodies to at least one antigen, 17 (65%) self-reported a history of malaria infection. The median time of their last reported malaria infection was 9 months ago (IQR: 7-20). At the time of blood collection, one child was found positive for *P. falciparum* and one for *P. vivax* as determined by PCR. The antibody levels to PfSE, PfMSP1$_{19}$ and PfAMA-1 were 27.9, 61.2 and 148.9 arbitrary units/ml in *P. falciparum*-infected child and 13.1, 167.3 and 20.5 arbitrary units/ml in *P. vivax*-infected child,

Table 1. *Comparison of characteristics between children with and without antibody to P. falciparum antigens*

	All children	Children with antibodies	Children without antibodies	p-value
Number of participants	148	26	122	
Male sex	75 (51)	16 (62)	59 (48)	0.222
Age (years)	9.6 (2.7)	10.5 (2.6)	9.4 (2.6)	0.059
Weight (kilograms, kg)	26.4 (10.2)	29.6 (10.7)	25.7 (10.0)	0.076
Weight <-2.0SD	23 (16)	3 (12)	20 (16)	0.354
Height (centimeters, cm)	125 (15)	132 (13)	124 (15)	0.012
Height <-2.0SD	45 (30)	5 (19)	40 (33)	0.107
Body mass index (kg/m^2)	16.2 (2.6)	16.5 (2.6)	16.2 (2.6)	0.617
Haematocrit (%)	38 (3)	38 (2)	38 (3)	0.413
Anaemia	12/141 (9)	1/25 (4)	11/116 (9)	0.693
Self-reported a history of malaria infection	72 (49)	17 (65)	55 (45)	0.060
Elapsed time since their last malaria episode (months)	9 (7-20)	9 (8-32)	8 (7-20)	0.219

Data in mean (SD), number (%), or median (IQR), as appropriate.

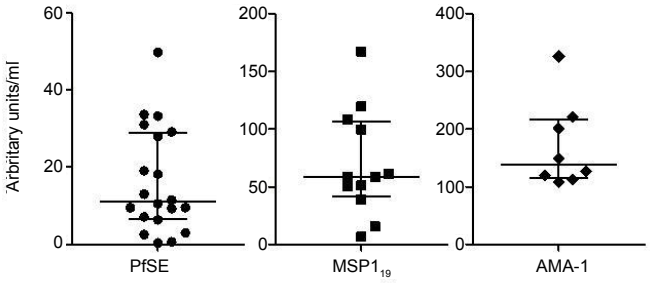

Figure 1. *Antibody levels in seropositive children*

Sera eluted from dried blood spots were tested for antibodies against PfSE, MSP119 and AMA-1 by ELISA. Samples were calculated from the positive and negative reference sera, and were considered seropositive if the antibody responses to PfSE, PfMSP119 and PfAMA-1 were higher than 0, 0 and 100 arbitrary units per ml, respectively. Each dot represents the sample that gave positive value to the antigens. The median (25th, 75th percentile) antibody levels against PfSE, MSP-119 and AMA-1 were 11.1 (6.6, 28.9), 58.5 (41.9, 106.4) and 138.1 (114.7, 216.6) arbitrary units/ml, respectively. Wide horizontal bars represent the medians and narrow bars represent 25%-75% interquartile ranges.

Subjects	PfSE	MSP1$_{19}$	AMA-1
1	x[a]	x	x
2	x	x	x
3	x	x	x
4	x	x	
5	x	x	
6	x	x	
7	x	x	
8	x	x	
9	x		x
10	x		x
11	x		x
12	x		
13	x		
14	x		
15	x		
16	x		
17	x		
18	x		
19	x		
20	x		
21		x	
22		x	
23		x	
24		x	
25			x
26			x

Figure 2. *Patterns of antibody responses to malaria antigens in 26 seropositive children*

respectively.

Comparison of Clinical Characteristics between Malaria Antigen-Seropositive and Seronegative Children

No statistically significant differences in age, weight, height, body mass index, haematocrit, numbers of children with anaemia, numbers of children self-reporting a history of malaria infection and the duration of time since last malaria infection existed between children with and without antibodies to *P. falciparum* antigen (Table 2).

Table 2. *Antibody response to specific malaria antigens among 148 study participants*

Antibody response to specific malaria antigens	History of clinical malaria	No history of clinical malaria
Number of participants	72 (49)	76 (51)
No response	55 (76)	67 (88)
1 antigen		
anti-PfSE	7 (10)	2 (3)
MSP1$_{19}$	3 (4)	2 (3)
AMA-1	2 (3)	1 (1)
2 antigens		
anti-PfSE and MSP1$_{19}$	2 (3)	2 (3)
anti-PfSE and AMA-1	2 (3)	0
All 3 antigens	1 (1)	2 (3)
Data in number (%)		

Discussion

The overall seroprevalence of the antibody to *P. falciparum* antigens in this study was 17.6%. No significant difference in the characteristics of children with and without malarial antibodies existed. The proportion of children with detectable *P. falciparum* antigens was quite low, despite a history of malaria. This might be explained by a poor antibody response in children with malnutrition. Even though protective immunity against malaria is claimed to be incomplete, antibodies to blood stage malaria might inhibit merozoite invasion into RBCs, block adherence of infected red blood cells to endothelial cells, and enhance phagocytic activity of macrophages [10-13]. Reports have shown that B cell responses to malaria can sustain for years after infection [25,29]; this encourages the development of a malaria vaccine capable of inducing long-lasting protection, at least against the severe fatal form of malaria. Seroprevalence studies may provide helpful baseline data on previous exposure relevant for subsequent vaccine development trials.

Unlike widely established data from African children, far fewer studies have reported on the prevalence of antibodies to *P. falciparum* antigens in children living in ecologically similar areas in Southeast Asia. Our cross-sectional survey found an overall prevalence of antibodies to malaria antigens of 17.6%. Different patterns of antibody responses (Figure 2) suggest that children also have heterogeneity of such responses, as seen in adults [25]. However, the prevalence of antibodies reported in this study may be lower than the actual B cell responses to malaria. Our previous study shows that some Thai adults, whose time since last detected malaria infection was known, had no detectable circulating memory B cells and/or antibodies, although a significant proportion effectively maintained their B cell memory responses

[25]. In addition, some of these individuals had circulating memory B cells capable of producing antibodies upon *in vitro* stimulation, but no plasma antibodies to malaria antigens could be detected. Similarly, several subjects had memory B cells below the limit of detection, but stable frequencies of antigen-specific antibodies were observed. These suggest that memory B cells and antibody-secreting plasma cells are independently regulated and, therefore, serum antibody response alone may not fully represent the humoral immune response to malaria parasites.

Anaemia in individuals infected with malaria could result from the destruction of parasitized erythrocytes, shortened survival of unparasitized erythrocyte, and bone marrow dyserythropoiesis [30]. The prevalence of anaemia by haematocrit criteria observed in this study was 9%, which was comparable to that reported in a 2004 survey of school-age children in 10 provinces in Thailand [31]. Neither a history of clinical malaria or being seropositive to blood stage malaria antigens was associated with anaemia in this population. A study in Ghana, in which 75-82% of children (infant to 9 years old) were infected with malaria, reported that 64% of these children had anaemia [32]. A complete hematologic discovery after an episode of acute malarial infection was reported to be within 6 weeks in most cases; however it might be longer in cases with pre-existing anaemia prior to infection [33]. However, we know that anaemia in school-age children, especially in rural areas, can occur from various causes, including iron and nutritional deficiency, hookworm infestation, thalassemia, and other illnesses. The low prevalence of anaemia in the study villages on the Thai-Myanmar border might be explained by good surveillance systems and ongoing school health educational programs, including nutritional support and iron supplementation.

We found 16% and 30% of Karen children in the study villages had WFA and HFA<-2.0 SD, respectively, which were far more than that reported by the Thai Ministry of Public Health among children 6-18 years old [34]. Malaria and other infections in children can decrease food intake due to poor appetite, impair nutrient absorption, cause direct nutrient losses by concomitant gastrointestinal symptoms, increase metabolic requirements or catabolic losses and impair transport of nutrients to target tissues. However, the prevalence of anti-PfSE Ab in the group with poor nutritional status was not statistically different from the group of children with normal growth parameters. It was conceivable that growth impairment in this population was affected by many possible associated factors, including inadequate food intake and common parasitic infestation [35,36].

While primary prevention of disease is one of the important goal in the field of community medicine and public health, identification of a persistent malaria antibody as a gateway to developing effective malaria vaccine remains an issue of interest to many research scientists. Most current malaria-related studies in South East Asia focus on the treatment of resistant strain/complicated cases and development of a diagnostic technique that is more sensitive than conventional microscopic examination.

The strength of the present study is that it was the first serological survey in Thai children using dried blood spots. We have demonstrated a simple and convenient method for collecting blood samples that provides good antibody recovery after transferring to the laboratory. It was inexpensive and offered minimal risk to study participants, as only a very small volume of blood is needed. Studies in northern Tanzania and South Africa have reported that dried blood spots were particularly useful for isolating parasite DNA in mapping the spread of drug resistance in malarial parasites [37,38].

Nevertheless, this study had some limitations. First, this cross-sectional survey provided anaemia and health status at a single point in time, rather than capturing the data at the time of malaria infection. Therefore, the data could not indicate anaemia and health status at the time of infection. Although we found one *P. falciparum*- and one *P. vivax*-infected children, the numbers were too small to look for associations between malaria infection and anaemia and health status, or with antibody responses. Second, we could not always confirm the accuracy of self- or family-member-reported malaria infection, as the medical records of some cases were not available. Third, for growth parameters, we could not calculate a z-score for any of the children, because a precise date of birth was not documented, even in the school registry, as most children were born at home without a birth certificate. Longitudinal studies are warranted to elucidate the precise association of health status at the time of infection and antibody responses to malaria.

Poor nutritional status in children might result in impaired antibody responses to malaria infection. We report the seroprevalence to blood stage *P. falciparum* antigens in children living in a low-malaria-transmission setting and its lack of association with either nutritional status or anaemia. Our data may provide baseline data that could be helpful for future malaria control and vaccine development.

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